UNIVERSITAT DE BARCELONA

Desenvolupament de processos per a la síntesi de pèptids d'interès terapèutic a escala industrial

Macarena Duran Corbera

Tesi doctoral

Programa de Doctorat en Química Orgànica

Desenvolupament de processos per a la síntesi de pèptids d'interès terapèutic a escala industrial

Macarena Duran Corbera

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Secció de Química Orgànica

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Agraïments

El treball experimental d'aquesta tesi doctoral ha estat realitzat entre novembre de 2016 i febrer de 2020 al departament de química orgànica de la facultat de Química de la Universitat de Barcelona i al departament de I+D d'Esteve Química S.A. sota la direcció del Dr. Ernesto Nicolás Galindo i el Dr. Jaume Farràs Soler. M'agradaria donar-los les gràcies pels consells, ajuda i temps dedicat. També voldria agrair la col·laboració i ajuda rebuda pel Dr. Ramón Berenguer Maimó i la Dra. Marta Racamonde Villanueva durant les meves estades a l'empresa.

A continuació m'agradaria donar les gràcies a Esteve Química S.A. pel finançament prestat i, sobretot per la confiança dipositada i la formació rebuda durant aquests anys. M'agradaria agrair als companys d'Esteve Química per l'acollida i ajuda rebuda. En especial, m'agradaria donar les gràcies a la Carol. Retrobar-nos i tenir-te al meu costat durant aquests mesos ha sigut una de les millors coses que m'han passat durant aquesta època. Gràcies pels moments viscuts i pels que vindran.

També m'agradaria agrair a tots els companys del departament de Química Orgànica que he anat coneixent al llarg d'aquests tres anys. En especial, m'agradaria mencionar a l'Stuart i la Marina, els meus incondicionals. Gràcies per totes les xerrades (químiques o no) i pels nostres esmorzars i berenars tan divertits. Sense vosaltres aquests anys no haurien tingut sentit. Ariadna, Alejandro, Javi, Héctor, Roberto, Anna, Tomeu, Roser, Marta... gràcies a tots pels dinars, sopars, viatges i calçotades. Compartir anècdotes amb vosaltres ha sigut molt especial. Finalment, m'agradaria dedicar un trosset d'aquests agraïments a la Colla de Químics Orgànics (Marina, Stuart, Héctor, Tomeu i Anna) que va començar amb l'organització d'un paint ball i que va continuar organitzant grans esdeveniment dins el departament com ara acampades, PopQuizes, calçotades, sopars de Nadal... Organitzar tot això a vegades resultava ser una mica estressant però tots els riures i aventures viscudes en cada activitat organitzada feien que coordinar-ho tot valgués la pena. Amb la mà al cor m'agradaria dir-vos que gràcies per deixar-me formar part d'aquesta colla i sobretot, que formar-ne part ha estat una de les millors experiències d'aquests anys.

Dins del departament, m'agradaria mencionar als estudiants de grau i màster que han passat pel laboratori: Júlia, Josep, Aleix, Raquel, Alexia i Oriol. M'agradaria destacar la gran amistat que va néixer durant el primer any del doctorat amb la Júlia, la meva primera estudiant. Ets fantàstica i coincidir amb tu un altre cop a les instal·lacions d'Esteve uns anys més tard va ser increïble. Gràcies per aquesta amistat tan pura.

Aquest any de pandèmia he tingut l'oportunitat de poder participar en un projecte Covid liderat per la Dra. Miriam Royo. Durant aquests mesos he tingut el plaer de compartir laboratori amb el Kamil, en Dani i la Ginevra. Tots ells m'han ajudat a seguir endavant amb els seus consells al llargs d'aquesta època en la que jo no veia un final. Així que també m'agradaria donar-los les gràcies. El disseny de la portada no hagués estat possible sense l'ajuda del Kamil. Així que moltes gràcies pels càlculs i les hores invertides.

Mencionar a les meves companyes de pis Carla i Marina. Gràcies per la paciència i per fer més fàcils aquests últims sis mesos. La meva petita família de Barcelona.

Agrair a la meva família pel suport i per la paciència durant els moments més difícils de la tesi i sobretot, per acompanyar-me durant aquesta experiència. No hi ha suficients paraules

per poder expressar tot l'agraïment i estima que us tinc. Sense el vostre suport incondicional tot això no hagués estat possible. Us estimo infinit.

Finalment, m'agradaria dedicar aquesta tesi doctoral a les meves àvies Carmen i Maria Purificació. Perdre-us durant el aquest trajecte va ser especialment difícil per a mi però sé que m'heu acompanyat i que m'heu donat força des d'allà on esteu. Us trobo molt a faltar.

Abbreviations and acronyms

A

Aa	Amino acid
ACN	Acetonitrile
APIs	Active pharmaceutical ingredients

B

Boc	*tert*-Butyloxycarbonyl protecting group
BOP	Benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate
Bzl	Benzyl protecting group
BPH	Benign prostatic hyperplasia

C

CIC	*N*-cyclohexyl-*N'*-isopropylcarbodiimide
COSY	Homonuclear correlation spectroscopy
CS	Convergent synthesis
2-CTC	2-Chlorotritylchloride resin

D

δ	Chemical shift
DBF	Dibenzofulvene
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	*N,N'*-Dicylcohexylcarbodiimide
DCU	*N,N'*-Dicyclohexylurea
DCM	Dichloromethane
DIC	*N,N'*-Diisopropylcarbodiimide
DIPEA	*N,N*-Diisopropylethylamine
DKP	Diketopiperazine
DMAP	4-Dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide

E

ε	Extinction coefficient
EDC	*N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide
EDT	1,2-Ethanedithiol
EMEA	European medicine agency
ESI	Electrospray

F

FDA	United States food and drug administration
Fmoc	9-Fluorenylmethyloxycarbonyl protecting group

FT	Fexapotide triflutate

H

HATU	1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
HBTU	3-[Bis(dimethylamino)methyliumyl]-3*H*-benzotriazol-1-oxide hexafluorophosphate
HCTU	*O*-(1*H*-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HF	Hydrofluoric acid
HMBC	Heteronuclear multiple bond correlation spectroscopy
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	1-Hydroxybenzotriazole
HP-APIs	High-potency active pharmaceutical ingredients
HPLC	High performance liquid chromatography
HPLC-MS	High performance liquid chromatography-mass spectrometry
HSQC	Heteronuclear single quantum spectroscopy

J

J	Coupling constant

L

λ	wavelenght
LUTS	Lower urinary tract symptoms

M

MALDI	Matrix-assisted laser desorption/ionisation
MeOH	Methanol
MTBE	Methyl *t*-butyl ether
2-MeTHF	2-Methyltetrahydrofuran

N

NMP	1-Methylpyrrolidin-2-one
NMR	Nuclear magnetic resonance

O

o/n	Overnight

P

Pbf	2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl protecting group
PyAOP	7-Azabenzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate
PyBOP	Benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate

R

R&D	Research and development
RT	Recombinant technology

S

SPS	Solution-phase peptide synthesis
SPPS	Solid-phase synthesis

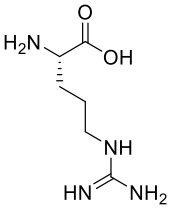
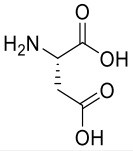
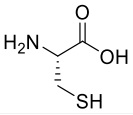
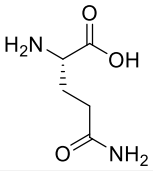
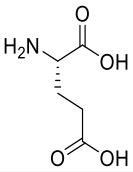
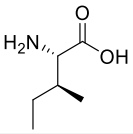
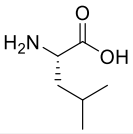
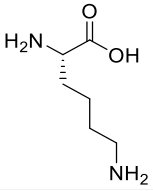
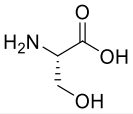
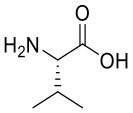
T

^{t}Bu	*tert*-butyl protecting group
TCEP	Tris(2-carboxyethyl)phosphine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TIPS	Triisopropylsilane
TLC	Thin layer chromatography
TOCSY	Total correlated spectroscopy
Trt	Trityl protecting group

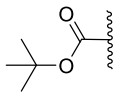
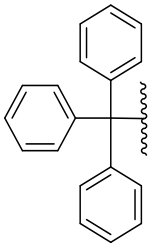
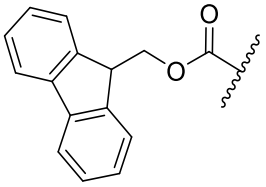
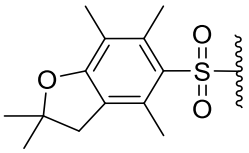
U

UV	Ultraviolet

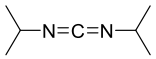
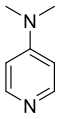
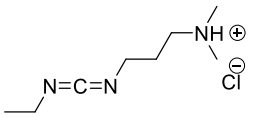
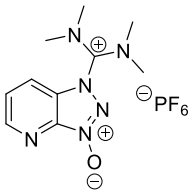
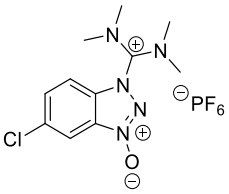
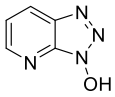
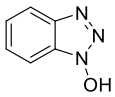
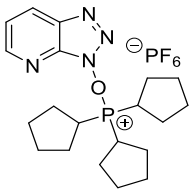
Amino acids

Amino acid	Code	Structure
L-Arginine	Arg, R	
L-Aspartic acid	Asp, D	
L-Cysteine	Cys, C	
L-Glutamine	Gln, Q	
L-Glutamic acid	Glu, E	
L-Isoleucine	Ile, I	
L-Leucine	Leu, L	
L-Lysine	Lys, K	
L-Serine	Ser, S	
L-Valine	Val, V	

Side-chain protecting groups

Protecting group	Symbol	Structure
tert-Butyloxycarbonyl	Boc	
tert-Butyl	^{t}Bu	
Trityl	Trt	
9-Fluorenylmethyloxycarbonyl	Fmoc	
2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl	Pbf	

Coupling reagents and additives

Coupling reagent	Symbol	Structure
N,N'-Diisopropylcarbodiimide	DIC	
4-Dimethylaminopyridine	DMAP	
N-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride	EDC·HCl	
1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate	HATU	
O-(1*H*-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate	HCTU	
1-Hydroxy-7-azabenzotriazole	HOAt	
1-Hydroxybenzotriazole	HOBt	
7-Azabenzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate	PyAOP	

Intermediates and its numeration

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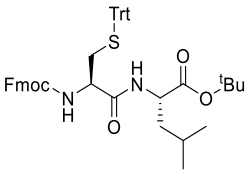
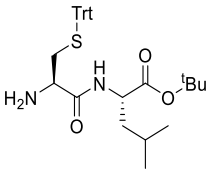
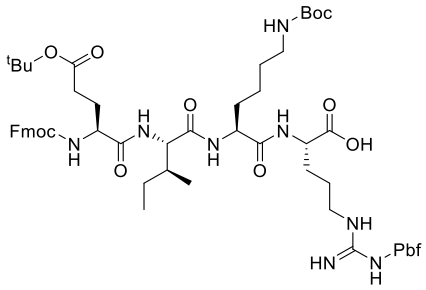
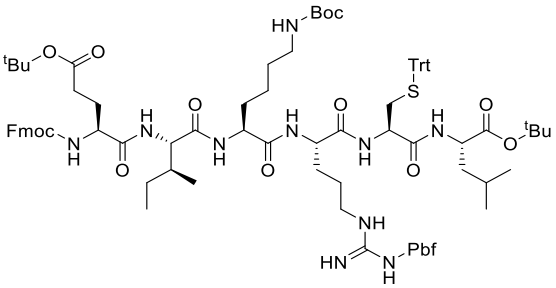
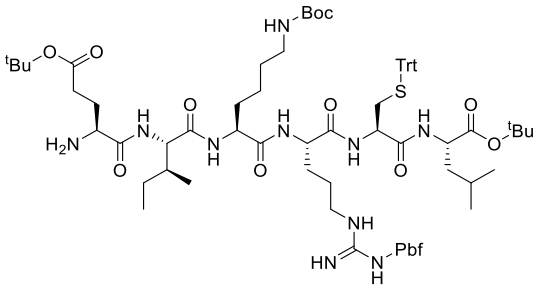
5	
11	
6	
12	
13	

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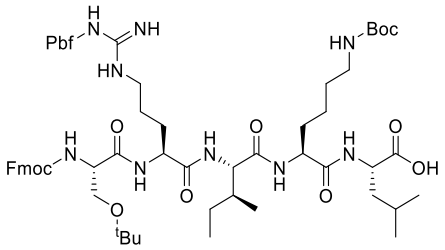
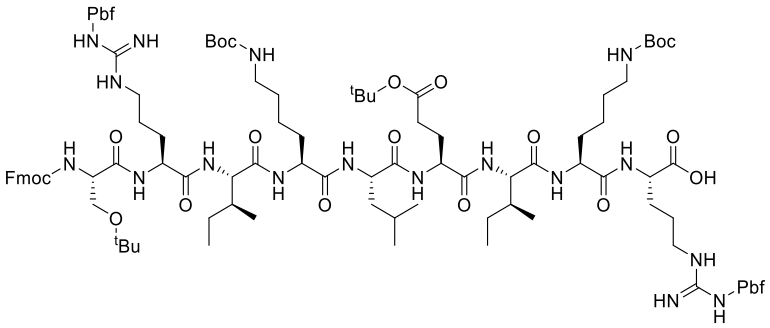
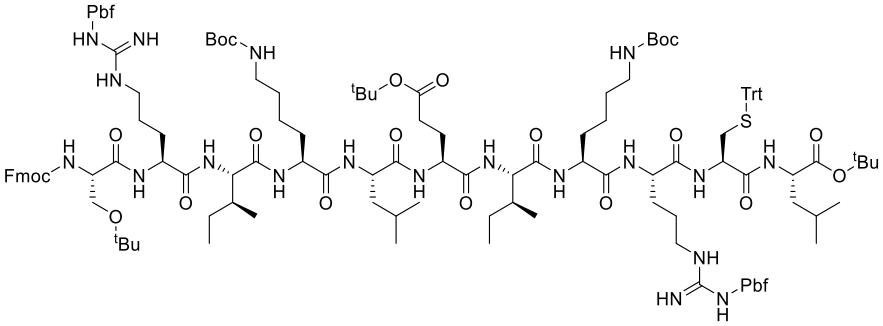
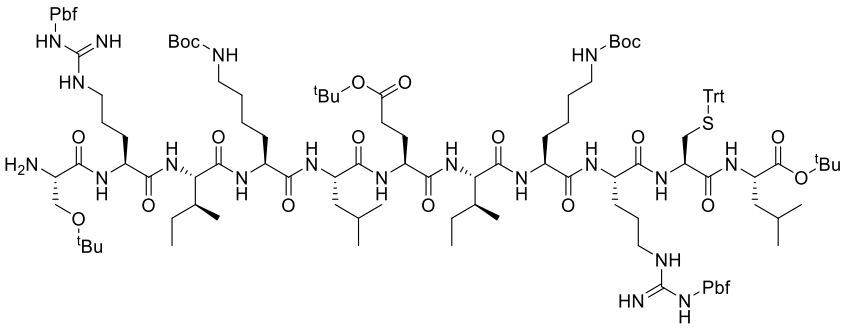
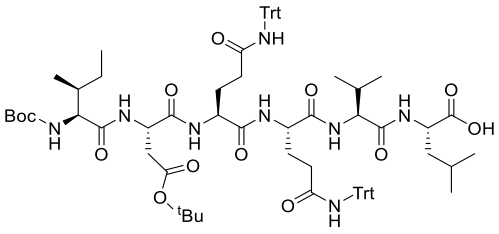
7	
28	
14	
15	
8	

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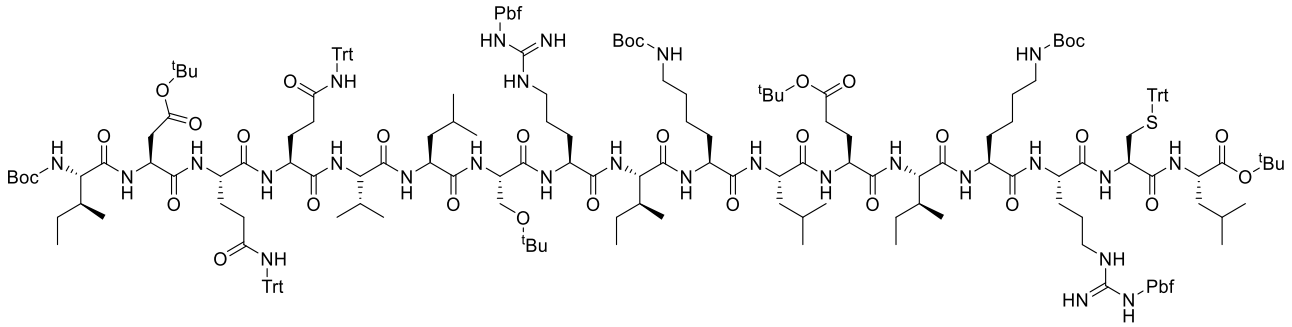
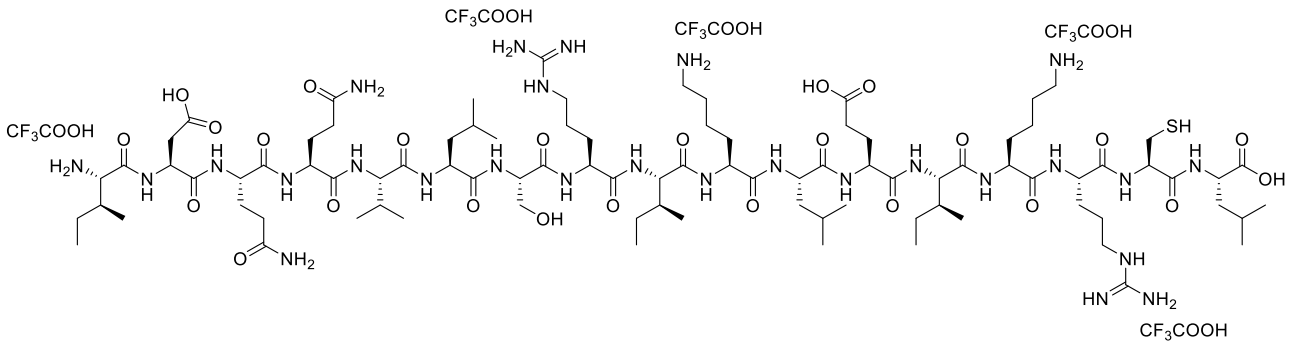
16	
1	

Contents

INTRODUCTION

CHAPTER 1: APPLICATION OF THE LINEAR APPROACH TO THE SYNTHESIS OF FEXAPOTIDE

APPLICATION OF THE CONVERGENT APPROACH FOR THE SYNTHESIS OF FEXAPOTIDE

CHAPTER 2: SYNTHESIS OF FEXAPOTIDE THROUGH A FOUR-FRAGMENT CONVERGENT APPROACH

CHAPTER 3: SYNTHESIS OF FEXAPOTIDE THROUGH A THREE-FRAGMENT CONVERGENT APPROACH

CHAPTER 3

CHAPTER 4

Introduction

It is well known that investigations in the biomedical field have shown that peptide-macromolecular interactions modulate and control the main biological processes such as memory, response to stress, pain, immune response, kidney and cardiovascular function and many others. Development of peptide drug candidates that can target the receptors that regulate these biological functions is one of the main concerns in biology and medicine.[1]

In the past, peptides were considered to be poor drug candidates because of their low oral bioavailability and their susceptibility of being rapidly metabolised by proteolytic enzymes of the digestive system and blood plasma.[2,3] Nonetheless, peptides also present many advantages over small organic molecules that led to a revival of interest of these molecules as potential active pharmaceutical ingredients (APIs).[4,5] The first advantage is that peptides represent one of the smallest functional parts of a protein. Thus, they present greater efficiency, selectivity and specificity to molecular structures than small organic molecules.[4,6] The second advantage is that peptides have less systemic toxicity as the degradation products of peptides are amino acids and hence, drug-drug interactions are minimised.[4,7] Third, because of their short half-life, few peptide drugs accumulate in tissues thus decreasing the risk of complications as a consequence of their metabolites.[1,4] Finally, most therapeutic peptides are receptor agonist and generally, small amounts of these peptide drugs are sufficient to stimulate the targeted receptor.[4,8]

Peptides can be obtained from different approaches but recombinant DNA technology, chemical synthesis and enzymatic technology are the most popular ones. The size of the target molecule determines which technology is more suitable for its manufacture (Table 1).[9]

Chemical synthesis is widely used for the industrial production of peptides as more than 35 peptide drugs are currently prepared using this synthetic approach.[2,3,10,11] The main advantage of this approach is that it permits the incorporation of unnatural amino acids or synthetic probes that cannot be introduced with the recombinant DNA and enzymatic techniques.[3,12] Moreover, the scaling up is easier and the synthesis of the peptide is faster when compared to the other methodologies. Chemical synthesis of peptides can be achieved either by solution-phase synthesis (SPS) or by solid-phase peptide synthesis (SPPS).[3]

SPS was first described in 1953 by du Vigneaud, who synthesised oxytocin, a well-known natural peptide.[13] The process consists on the selection, preparation and isolation of certain building blocks which can be then purified to be coupled in solution to properly construct the desired sequence. The main advantage of this strategy is the economic savings due to the use of less-expensive raw materials. On the other hand, isolation, characterisation and purification of the intermediates lead to a long timeframe, which is the main limitation.[3]

SPPS was first described by Merrifield in 1963.[14] It is based on the attachment of the first amino acid into an insoluble solid support followed by repetitive cycles (protecting group removal and coupling) to complete the sequential assembly of the amino acid residues onto the resin. After the peptide chain elongation, the peptide is released from the solid support.[3,15] In this strategy, the non-reacted residue and coupling reagents are easily removed by filtration and washing of the resin. Isolation and purification of intermediates are not necessary, which leads to an easier and faster process when compared to the SPS approach. Nonetheless, the main drawbacks of this strategy are the need of more expensive reagents and the utilisation of larger quantities of raw materials to achieve complete reactions which yield to an increase of the cost of the overall process. Moreover, quantitative monitoring of the reaction progress is difficult because the growing peptide is anchored to the polymeric support. This becomes an important weakness when the work-scale is modified from laboratory-scale to industrial-scale.[3]

Convergent strategies were designed to overcome the main disadvantages of both SPS and SPPS. It consists of condensing peptide fragments prepared by SPPS either in solution or in a polymeric support.

Table 1. Approaches to the synthesis of peptides.

	SPS	SPPS	Convergent synthesis	Enzymatic synthesis	Recombinant DNA
Scale	Gram to kilogram to ton	Milligram to gram to kilogram	Gram to kilogram to ton	Gram to kilogram to ton	Gram to kilogram to ton
Peptide length	Short peptides	Medium to long peptides	Long peptides	Short peptides	Long peptides
Timeframe	Long	Short	Short	Long	Long
Cost	Relatively expensive	Expensive	Expensive	Relatively inexpensive	Inexpensive

A major advance for peptides as potential APIs was the discovery of T-20 (Enfuvirtide, Fuzeon®), a first-in-class membrane fusion inhibitor for the treatment of HIV. It was the first synthetic peptide that was produced on a large industrial scale.[10] Commercialisation of Enfuvirtide demonstrated that peptides can be produced on a multi-tone scale by chemical synthesis with a cost-effective process.[16] Table 2 summarises other approved peptide pharmaceuticals and the preparation strategy used for its manufacture.

It is worth mentioning that pharmaceutical companies are everyday under a rise in economic pressure because of two main factors. To start, public authorities demand lower healthcare cost through reducing the price of drugs leading to an increment on the use of generics. Second, regulatory authorities are more demanding in terms of drug efficacy, quality and safety which has led to an increase of pharmaceutical R&D cost.[4,17]

Moreover, 38% of the drug candidates under development are abandoned in phase I of clinical trials because of toxicity, the 63% that reach phase II are rejected for lack of efficacy or poor bioavailability, the 45% of the remaining drug candidates fail in phase III, and the 23% that pass the clinical trials are not approved by the United States Food and Drug Administration (FDA) or the European Medicine Agency (EMEA).[4,18]

Even so, in 2006 more than 40 peptides were on the world pharmaceutical market. About 136 peptides were in the clinical phase and a large number in preclinical stages. Nowadays, several peptide drugs are in the last-sate of development and the growing research in the peptide field demonstrates that there is a continuously rising interest for these molecules as APIs. In 2017, 46 new peptide drugs were approved by the FDA.[19] This foreshadows a promising future for the production of innovative synthetic peptide-based drugs.

Therapeutic application of peptides is very wide and can be used to treat several disorders and diseases such as allergy, asthma, arthritis, cardiovascular diseases, cancer, diabetes, gastrointestinal dysfunction, growth disorders, impotence, incontinence, obesity and pain, among others.[7] The present work is focused on the optimisation and synthesis of Fexapotide triflutate (FT), a peptide that is in phase III of clinical trials, to industrially produce it as a generic drug. FT is a new therapeutic peptide with selective pro-apoptotic properties to treat benign prostatic hyperplasia (BPH).

Table 2. Some approved and commercial peptide pharmaceuticals.

Peptide	Length	Strategy
Oxytocin	9	SPS
Atosiban	9	SPS
Cetrorelix	10	SPS
Buserelin	9	SPPS
Deslorelin	9	SPPS
Gonadorelin	10	SPS, SPPS
Goserelin	10	SPPS
Leuprolide	9	SPPS
Triptorelin	10	SPPS
Desmopressin	9	SPS, SPPS
Lypressin	9	SPS
Pitressin	9	SPS
Terlipressin	12	SPS, SPPS
Cyclosporine	11	RT
Parathyroid hormone (PTH)	34	SPS
Exenatide	39	SPPS
Somatostatin	14	SPS, SPPS
Lanreotide	8	SPPS
Octreotide	8	SPS
Thymosin α-1	28	SPPS
Bivalirudin	20	SPS
Liraglutide	32	SPPS
Vasopressin	12	CS
Salmon calcitonin	32	SPS, SPPS
Enfuvirtide	36	CS
Teriparatide	34	RT
Abarelix	10	SPPS

SPS: Solution-phase synthesis; SPPS: Solid-phase peptide synthesis; CS: Convergent synthesis; RT: Recombinant technology

Benign prostatic hyperplasia

BPH is the non-malignant growth of the prostate,[20] characterised by a proliferation of both stromal and epithelial cells of the prostate in the transitional zone surrounding the urethra[21] and commonly affects older men. Figure 1 shows the anatomy of the prostate, which was first described in 1968 by McNeal. He proposed that the prostate has four distinct regions: the non-glandular anterior fibromuscular zone (or stroma) and the glandular peripheral, central and transitional zones.[22] McNeal also introduced the anatomical concept of the transitional zone as the principal site of BPH. Figure 1 also includes histological slides of both normal prostate and BPH tissue.

BPH anatomically compresses the urethra and this results in lower urinary tract symptoms (LUTS), which include symptoms of difficult voiding (hesitancy, straining, weak stream and sensation of incomplete emptying) and irritable voiding (frequency, urgency and incontinence).[23]

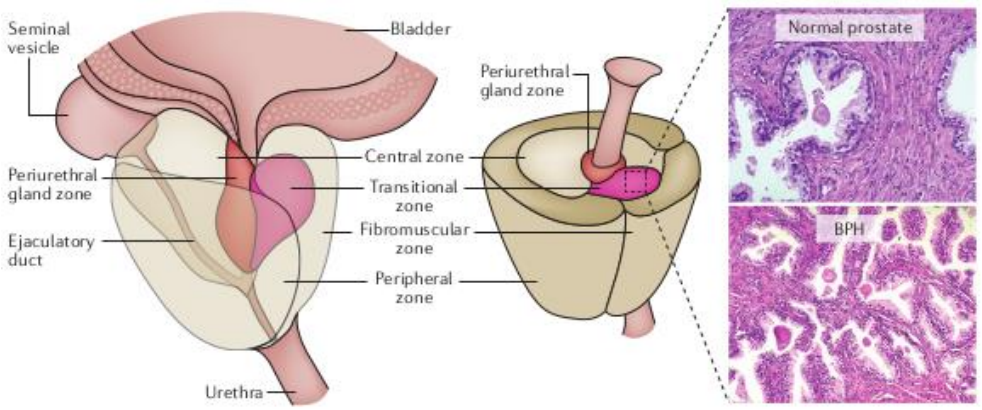


Figure 1. Zonal anatomy of the prostate (from ref. 21).

Epidemiology

Approximately 50 % of men above 50 years of age will have pathological evidence of BPH, going up to an 80 % as men reach their eighth decade of life.[24]

Differences in geographical prevalence for prostate diseases such as BPH have been documented but the reasons for these differences are poorly understood. Nonetheless, these differences could be attributed to genetic factors and/or environmental factors. For example, men from Southeast Asia have significantly smaller prostate volumes than men from western countries.[25]

Pathogenesis

The exact mechanism for the development of BPH is unknown. Nonetheless, metabolic, hormonal and inflammatory mechanisms have been proposed. Basic understanding of the hypothesised metabolic and hormonal mechanisms for BPH is important since the medical treatments for this disease are directed to these pathways.[23]

Metabolic mechanism

This mechanism is based on the observation that cardiovascular risk factors in men are associated with BPH and LUTS (Lower Urinary Tract Symptoms). Preclinical models have shown various mediators of tissue ischemia caused by atherosclerosis. Hypoxic tissue conditions have been revealed to cause cultured human prostatic stromal cells to produce higher levels of growth factors, which leads to prostate epithelial hyperplasia.[26] Components of metabolic syndrome (hypertension, glucose intolerance, obesity and insulin resistance) have also been demonstrated to cause over activation of the autonomic nervous system, which may contribute to LUTS and BPH as a result of alpha-adrenoceptor-mediated smooth muscle contraction of the bladder neck and prostate, as well as to the promotion of the prostate growth.[27,28]

Hormonal mechanism

Testosterone and dihydrotestosterone (DHT) play an important role in the development of the normal prostate as well as BPH and prostate cancer. Testosterone is produced by the testes and adrenal glands and diffuses into stromal and epithelial cells of the prostate. Testosterone and its derivatives interact with the androgen receptor (AR), which upon

binding translocates to the nucleus and binds to the androgen response element (ARE), promoting the expression of genes encoding various growth factors (keratinocyte growth factor (KGF), epidermal growth factor (EGF) and insulin growth factors (IGFs)). In stromal cells, testosterone is converted to DHT by the enzyme 5-alpha reductase, which then acts in an autocrine manner to promote stromal proliferation.[21] However, serum and tissue concentrations of testosterone and DHT are not clearly elevated in men with BPH when compared to normal controls.[29] Figure 2 summarises the role of testosterone in stromal and epithelial cells of the prostate and its role in BHP.

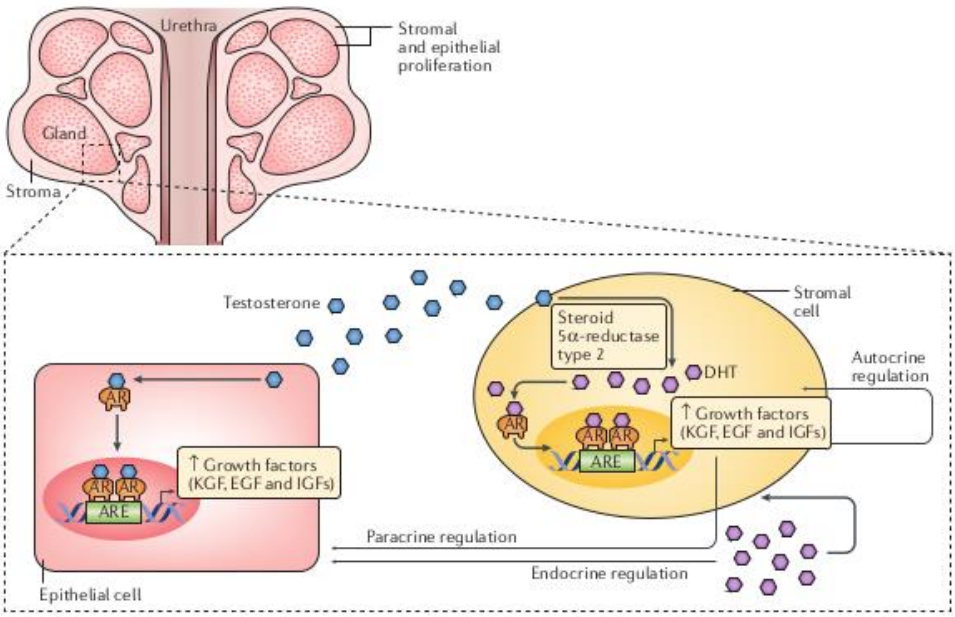


Figure 2. Role of testosterone in stromal and epithelial cells of the prostate (from ref. 21).

Some investigatons have suggested that the increasing estrogen-to-androgen ratio that occurs with normal aging may also play a role in the pathogenesis of BPH.[30] Figure 3 shows how BPH is probably developed due to an imbalance between mechanisms that regulate cell death and cell proliferation. Growth factors such as KGF, EGF and IGFs, which are AR target genes, are probably involved in transforming growth factor-β (TGFβ), which is negatively regulated by androgens.

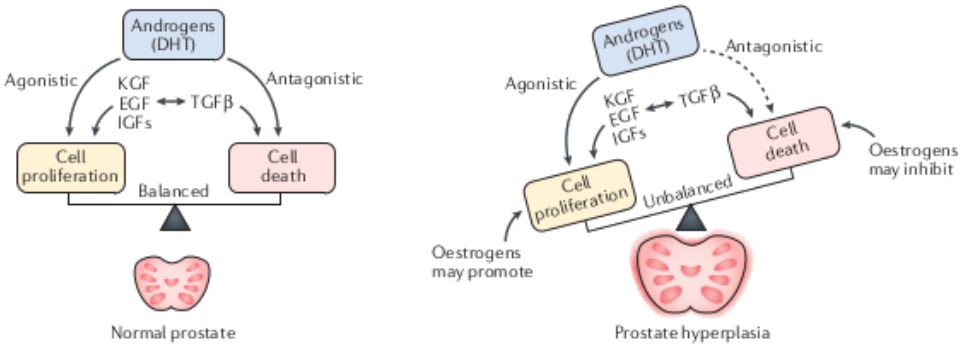


Figure 3. Molecular control of prostate growth (from ref. 21).

Inflammatory mechanism

Chronic inflammation may also contribute to the development of BPH. Preclinical models have demonstrated that pro-inflammatory stimulators cause the growth of prostatic epithelial cells.[31] Moreover, the chronic inflammation associated with BPH is believed to be a result of autoimmune activation against prostatic tissue, which drives to cytokine production, resulting in prostate stromal growth.[32]

Diagnosis

When men over 50 years old present lower urinary tract symptoms, it is important to distinguish LUTS resulting from BPH from the symptoms caused from other pathological processes. Although BPH is the most common cause of LUTS in men over 50 years, other medical problems such as congestive heart failure, diabetes and neurological disorders may cause these symptoms.[23]

Physical examination should be performed, particularly focusing on the urinary tract. The suprapubic region and the penis should be examined for signs of bladder distension, phimosis or abnormal penile lesions which could be the cause for LUTS.[33] Neurological examination should also be performed to exclude a possible neurological disorder that might cause urinary symptoms.[21] Finally, digital rectal examination (DRE) should be performed on patients presenting LUTS. DRE allows the estimation of the prostate volume and can also asses the shape of the prostate and allows the identification of firm and hard areas or nodules which might be a consequence of prostate cancer.[34]

Laboratory analysis should also be performed. Analysis such as urinalysis, measurement of postvoid residual urine (PVR) volume, uroflowmetry and prostate-specific antigen (PSA) test should be performed. The level of PSA has been shown to reflect the prostate volume.[35] However, PSA testing might lead to confusing results since this test is used in prostate cancer diagnosis. Depending on the results, there is a possibility of a need of a biopsy.[21]

Treatment

Treatment options for men with LUTS might start with a watchful waiting strategy. This strategy is used with patients with mild symptoms and no complicating factors and it is based on advice about lifestyle changes that can help to improve or reduce the symptoms. For patients with complicating factors such as urine retention, bladder stones or renal impairment, a more aggressive approach is needed. Determination of the prostate volume might be useful before selecting the medical strategy (treatment or surgery).[21,23]

Medical therapy

Medical therapy is used to improve the symptoms and to lower the risk of progression. There are many pharmaceutical options:

Alpha blockers

Alpha-adrenergic antagonists, also known as alpha-blockers, diminish LUTS by relaxing the smooth muscle in the prostate and bladder neck.[27] Alpha-1A-adrenergic receptors predominate in the lower urinary tract and as a result, alpha-1A-selective alpha blockers such as tamsulosin and silodosin have reduced systemic side effects when compared to non-selective alpha blockers (alfuzosin, doxazosin, prazosin, terazosin). Nonetheless, the main

side effect of alpha-1A-selective alpha blockers is retrograde ejaculation. Serum PSA is not affected by alpha blockers as their mechanism of action does not affect the prostate volume.[23]

5-Alpha reductase inhibitors

5-alpha reductase inhibitors (5-ARI) such as finasteride and dutasteride, block the conversion of testosterone to DHT by the inhibition of type I (peripheral) and/or type II 5-alpha reductase. Reduction of DHT levels leads to a 20 – 25 % reduction of the prostate volume and about a 50 % decrease in serum PSA after one year.[23]

Phosphodiesterase type 5 inhibitors

The nitric oxide-cyclic GMP (cGMP) pathway is involved in smooth muscle contraction. Phosphodiesterase type 5 (PDE5) negatively regulates smooth muscle contraction and thus, inhibition of PDE5 yields to the relaxation of smooth muscle in the bladder neck, urethra and prostate.[36] PDE5 inhibition probably increases tissue perfusion, modulates autonomic nervous system and inhibits the prostatic inhibition process leading to an improvement in voiding symptoms.[37] Currently, tadalafil is the only PDE5 inhibitor that it is approved by the FDA for the treatment of BPH.

Combination therapy

Combination of the above-described medications is usually performed because it results useful. For example, combination of dutasteride and tamsulosin has been shown to not only improve voiding symptoms but also results in a significant reduction in the risk of disease progression.[38]

Surgery

Surgical intervention for LUTS caused by BPH should be considered for patients who have not experienced improvements with medical therapy or for those who have complications from bladder obstruction due to BPH. Surgical intervention may be considered as the initial treatment for patients with moderate to severe LUTS.[23]

Transurethral resection of the prostate (TURP) is the most common surgical intervention for BPH due to significant symptom improvement have been demonstrated.[39,40,41] The success of TURP is long-lasting with less than 1 % of risk per year of requiring to repeat the process. Other surgical interventions such as transurethral incision of the prostate (TUIP), prostatectomy and transurethral laser vaporization, ablation and enucleation are performed and are effective but not as durable as TURP.[42,43,44,45]

Fexapotide triflutate for the treatment of BPH

Fexapotide triflutate (FT) is a new therapeutic peptide (phase III of clinical trials) with selective pro-apoptotic properties. This drug is formulated in phosphate buffered saline (PBS) at physiologic pH 7.4, stored frozen and defrosted before its use. It is administered by injection directly into the transitional zone of the prostate and it is believed to induce focal cell loss in prostate tissue through apoptosis.[46]

FT-treated cells *in vivo* and *in vitro* show high levels of standard apoptotic cell markers such as caspases and annexin V.[47] It simulates the caspase pathway, tumour necrosis factor pathways and B-cell lymphoma (BCL) pathways in prostate glandular epithelial cells.

Moreover, FT selectively causes loss of cell membrane integrity, mitochondrial metabolic arrest, depletion of RNA, DNA lysis and aggregation, cell fragmentation and cell loss leading to decompression of the urethral lumen.[48]

FT does not circulate outside the prostate after intraprostatic administration. The pharmacokinetic studies in patients and in animals showed no detectable plasma level after FT administration into the prostate and therefore, FT is highly selective for prostate glandular epithelium and has no contact with other tissues outside the prostate.[46,47,49] Moreover, no side effects have been found that are not found in placebo administrations. Finally, FT treatment involves prophylactic antibiotic administration to prevent infectious complications of the transrectal injection.[48,50]

This therapeutic approach is analogous to the treatments for BPH that remove or ablate prostate tissue by surgical procedures or that reduce the prostate size by androgen suppression.[46]

To sum up, there is still a need for BPH treatments with less side effects and better safety profiles. Large long-term prospective randomised US studies of FT have shown statistically significant long-term improvement in BPH symptoms. Based on a total of > 1700 patient treatment including FT and placebo to date since 2002, FT has shown to be well tolerated with an excellent safety profile. Moreover, it only requires a few minutes to administer, and a catheter or anaesthesia is not required. As consequence, FT injection represents a novel, first-in-class BPH treatment modality.[48]

Synthesis of Fexapotide triflutate

The chemical structure of FT (**1**) is shown in figure 4. Chronologically, the experimental work of this thesis was mainly carried out in two stages. In the first one, we explored the synthesis of Fexapotide at low scale in our laboratory at the University of Barcelona. In this stage, a linear solid phase synthesis and two different convergent approaches were evaluated. When a potentially suitable strategy was envisaged, the experimental work moved to the laboratories of a pharmaceutical company that were more adequately equipped to work in a secure way. Thus, in this second stage of the thesis, special security protocols implemented in the company laboratories had to be followed for the manipulation of the peptide as what is called a high-potency active pharmaceutical ingredient (HP-API). Working with APIs that have high potency and cytotoxicity present several challenges for pharmaceutical companies such as handling, containment and cost. Therefore, solid state handling of the advanced precursors and the Fexapotide itself had to be carried out in an isolator.

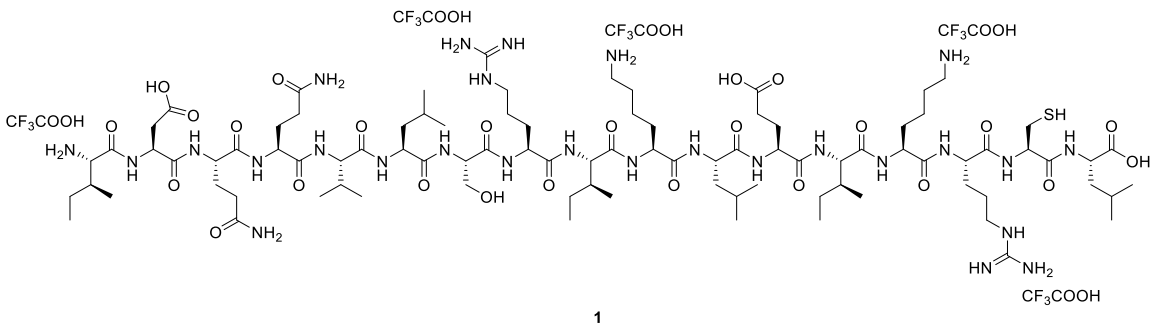


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Figure 4. Chemical structure of **1**.

According to the work context and the objectives that are pursued, some remarks have to be given about the results that are reported. As mentioned before, the main goal of the project is to design a synthetic methodology to prepare a therapeutic peptide suitable for pilot plant scale-up. When working under such conditions, the development of the process involves the synthesis of intermediates following experimental protocols that should fulfil the requirements of the company. In this sense, the purity of the products and how this purity has to be achieved in terms of low cost and compatibility with the technical issues that have to meet the experimental work when adapted to a pilot-plant environment is a special concern. Thus, highly productive reactions together with precipitation or crystallization protocols for purification are pursued instead of, for example, the use of preparative chromatographic techniques that are in general, more expensive, less efficient and hard to scale up. Considering this framework, HPLC chromatographic purities (given in percentages of the observed peaks areas) together with MS analysis were used throughout the experimental work of the thesis to monitor the synthetic processes and to characterise products contained in the crudes resulting from reactions as well as the solids arising from the precipitations that were carried out for purification purposes. In consequence, isolation of products using chromatographic techniques was barely used.

Objectives

The two main objectives of the present thesis were:

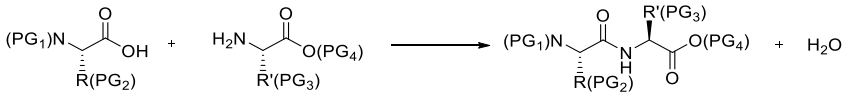
1. Development and optimisation of a robust synthetic strategy for the synthesis of the desired peptide target. This strategy must be scalable and accomplish all the regulatory requirements, delivered by the pharmaceutical company. For this reason, the manufacture process, cost and impurities emerging from the synthetic process had to be studied carefully.

2. Development of new reaction monitoring methods for the solid-phase peptide synthesis since the existing methodologies for the reaction monitoring are usually carried out once the reaction has ended and thus, *in situ* modifications cannot be performed. From the pharmaceutical company's point of view, the existing methodologies are not quantitative enough nor accessible to perform an efficient in-process control of the involved reactions. For this reason, several methodologies were studied to monitor the different solid-phase synthetic steps.

Chapter 1: Application of the linear approach to the synthesis of Fexapotide

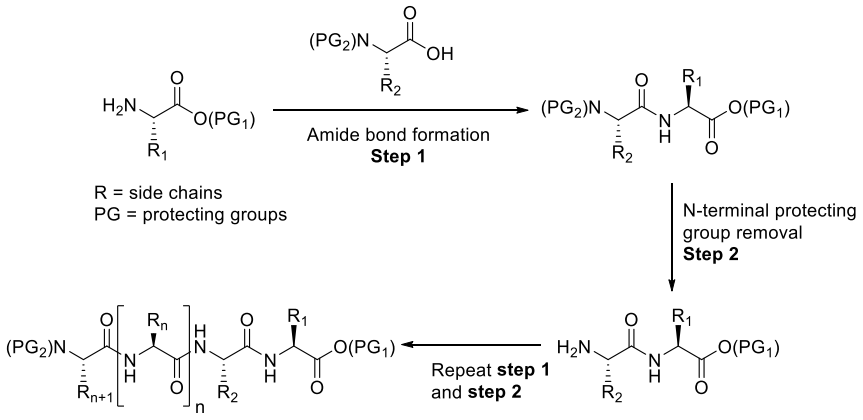
1.1. Solid-phase peptide synthesis: an introduction

Hofmeister[51] and Emil Fischer[52] first described that the structure that best represents a protein or a peptide is a chain of amino acids covalently linked by amide bonds between the α-carboxyl group end from one amino acid with the α-amino group from another amino acid (scheme 1.1). From a synthetic point of view, formation of the amide bond requires the suitable protection of the amino acids (α-carboxyl and α-amino groups in general as well as the side chains of trifunctional amino acids in particular) in order to avoid side reactions between the different functional groups that are not involved in the amide bond formation.[53]



Scheme 1.1. Amide bond formation between amino acids.

As it has been mentioned before, the chemical synthesis of a peptide was first described in 1953 by du Vigneaud, who synthesised oxytocin, a well-known natural peptide, in solution.[13] The general procedure for the synthesis of peptides in solution is shown in scheme 1.2.



Scheme 1.2. General procedure for solution-phase synthesis of peptides.

The first step consists on the formation of an amide bond between the α-carboxyl group end from one amino acid with the α-amino group from another amino acid. Then, removal of the N-terminal protecting group of the protected dipeptide is required to continue with the synthesis. This cycle is repeated as many times as needed to obtain the desired protected peptide that leads to the desired product when the chain is fully deprotected. The main advantage of the solution-phase synthesis of peptides is the economic savings due to the use of less-expensive raw materials. On the other hand, isolation, characterisation and purification of the intermediates lead to a long timeframe, which is the main limitation of this approach.[3]

SPPS was first described by Robert B. Merrifield.[54] This novel synthetic approach consisted on attaching the first amino acid of the peptide sequence to a polymeric support by a covalent bond, generally ester or amide, followed by the addition of the consecutive amino acids in a stepwise manner until obtaining the desired peptide sequence. Finally, the peptide is deprotected and removed from the solid support.

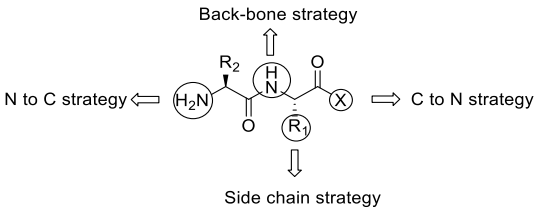
Peptides have four different functional groups that can be used for attachment to the polymeric support (scheme 1.3):

1. C-terminal function (C→N strategy): This methodology is based on anchoring the amino acid through the C-terminal function. Thus, the elongation of the peptide chain is through the N-terminal end. It is the most used strategy for the solid-phase synthesis of peptides.

2. N-terminal function (N→C strategy): For this strategy, the N-terminal moiety is covalently bond to the solid support. Thus, the elongation of the peptide chain is through the C-terminal end. The main advantage of this methodology is the possibility of synthesising C-terminal modified peptides that have potential application in therapeutics. The main drawback is that two side reactions can occur as a consequence of having the carboxylic-activated species anchored to the resin: 5(4*H*)-oxazolone and DKP formation, which leads to epimerisation of the α-residue and capping of the growing peptide chain, respectively.[55]

3. Amide backbone[56,57]: The attachment of the back-bone amide nitrogen to an appropriate support offers a simple way to prepare peptides with a wide variety of C-terminal functionalities. Thus, it is generally used when manipulation of the C-terminal function is required.

4. Side chain[58]: Growing of the peptide chain can either be performed through the C→N or N→C direction. This methodology allows the preparation of modified peptides by the manipulation of the C-terminal function and on-resin cyclisation.

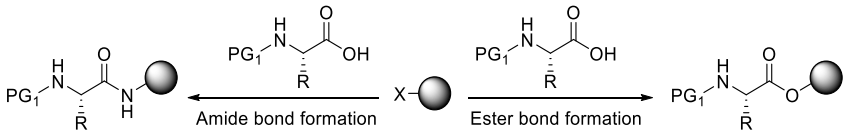


Scheme 1.3. Strategies for attaching a peptide to the polymeric support.

SPPS presents many advantages but the most significant is that the peptide is covalently bound to an insoluble resin during the synthesis, which allows the easy separation of the growing peptide chain from any by-products or unused excess of reagents just by washing the resin with the appropriate solvents. This is a major advantage when compared to the solution phase method because the latter requires harder purification steps such as chromatography and/or recrystallization, resulting in a decrease of the overall yield.[54] The main disadvantages of SPPS are the cost of the reagents and the difficult scaling up of the process.

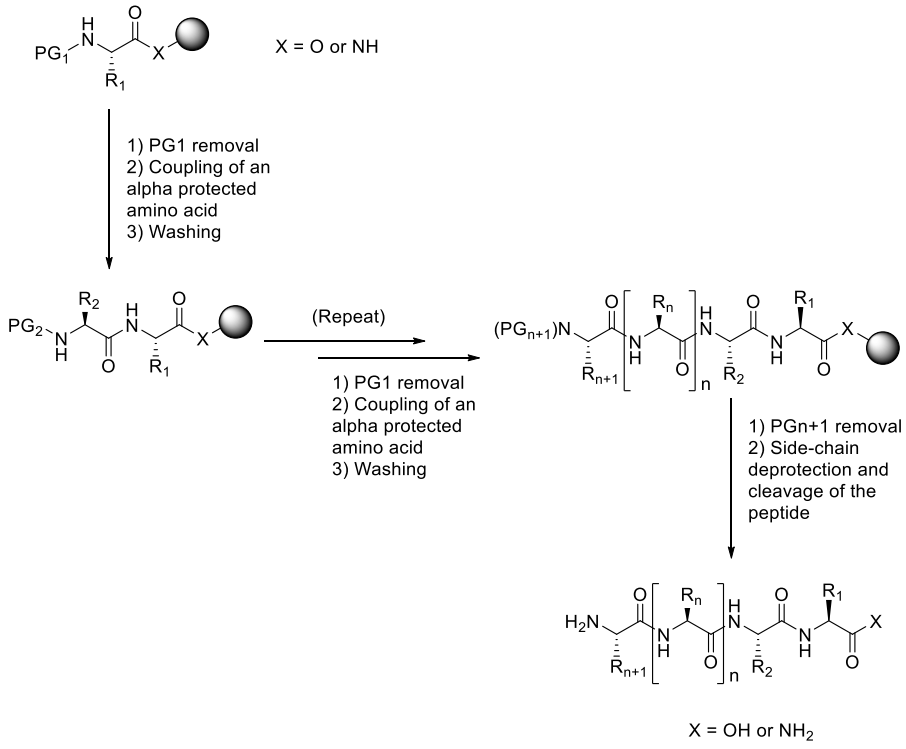
1.1.1. The concept

In the C→N strategy, the initial step in SPPS is the attachment of the first conveniently protected amino acid (α-amino group and side chain in the case of trifunctional amino acids) to the solid support through an ester or amide bond (scheme 1.4).



Scheme 1.4. Attachment of an amino acid through an amide/ester bond.

Then, removal of the α-amino protecting group of the first amino acid is performed followed by the addition of the next protected amino acid in the presence of a coupling reagent, which leads to the formation of an amide bond. This step is repeated as many times as necessary to obtain the desired peptide chain that is fully deprotected and cleaved from the resin at the end of the synthesis (scheme 1.5).



Scheme 1.5. General SPPS procedure.

1.1.1.1. Polymeric support

1.1.1.1.1. Solid supports

The solid support in which the synthesis has to be carried out must accomplish some features such as stability to a range of temperatures, different solvents and reagent conditions; high swelling so that reagents can access to the active sites; homogeneity of bead size and biocompatibility if used in biochemical assays.[59]

There are three types of solid supports commonly used in SPPS: polystyrene (PS) resins, polyethylene glycol-polystyrene resins (PEG-PS®) and hydrophilic PEG-based resins (figure 1.1).

1. *PS resins*. Polystyrene based polymers are the most widely used solid support for the synthesis of peptides because of their good swelling properties and suitable level of

functionalisation.[54] Polystyrene is crosslinked (in general with 1% of divinylbenzene) for insolubility purposes. It swells well in nonpolar solvents such as DCM or toluene but can also be used with polar solvents such as DMF, tetrahydrofuran (THF) or dioxane. PS resins have some limitations for the synthesis of highly hydrophobic peptides. Thus, more hydrophilic supports reporting a better performance should be used in these cases.

2. *PEG-PS resins*. These supports present amphiphilic properties as a consequence of having a hydrophobic PS core and a hydrophilic surface of polyethylene glycol (PEG) chains that allow good levels of solvation in both polar and nonpolar solvents. PEG-PS supports were developed by Zalipsky, Albericio and Barany[60] (PEG-PS®); and Bayer and Rapp[61,62] (Tentagel®). These resins are commonly used when polar solvents are needed or when distancing of the peptide chains from the resin core is required.

3. *Hydrophilic PEG-based resins*. More hydrophilic PEG resins with small amounts of PS, polyamide and acrylate with polymerisable vinyl groups such as Poly(ethylene glycol)-poly(acrylamide) resin[63] (PEGA) and cross-linked ethoxylate acrylate resin[64] (CLEAR) were developed to improve the swelling of the polymeric support. These resins are very polar and thus, present an exceptional swelling in water. Moreover, they possess a flexible structure enabling the access for macromolecules such as enzymes. Nonetheless, low mechanical stability makes handling difficult.

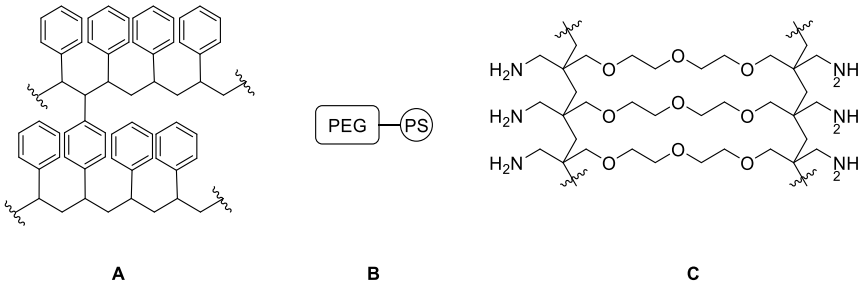


Figure 1.1. (A) PS supports. (B) PEG-PS supports. (C) PEG-based supports.

1.1.1.1.2. Linkers

A linker is a bifunctional molecule that modifies the polymeric support to facilitate the attachment of the peptide as well as its cleavage from the resin. Linkers can be classified into two types: integral and non-integral.[65] In the first type, the linker/handle forms part of the solid support, being an example of this type the 2-chlorotritylchloride resin (2-CTC, figure 1.2 (A)). In the case of non-integral linkers/handles, the linker is a bifunctional molecule that is attached to the solid support usually through an ether bond such as the [4-(hydroxymethyl)-phenoxymethyl]polystyrene (Wang resin, figure 1.2 (B)) or through an amide bond.[66]

The handle has to afford a linkage to the solid support stable to all synthetic processes, including the final step in which the target compound is going to be cleaved from the solid support. Sometimes this bond is not totally stable and the linker can be lost from the solid support.[67,68] This happens when Boc/Bzl protection strategy is used with linkers such as *p*-methylbenzhydrylamine resin (MBHA, figure 1.2 (C)),[69] a polymeric support used for preparing peptides with an amide at the C-terminal end. Similar problems are encountered with the Wang resin, backbone amide linker (BAL) or Rink-type resins. An example of side reaction that may occur during the cleavage step under acidic conditions is the incorporation

of the *p*-hydroxybenzyl moiety cleaved from the Wang resin into the N of the C-terminal amide of a peptide.[70] To overcome these problems, more stable resins and suitable acidic cocktails have been developed.[71,72]

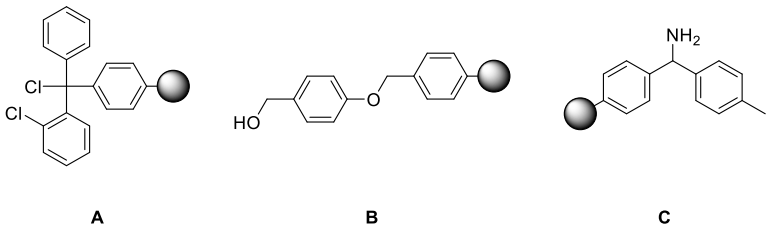


Figure 1.2. (A) 2-CTC resin. (B) Wang resin. (C) MBHA resin.

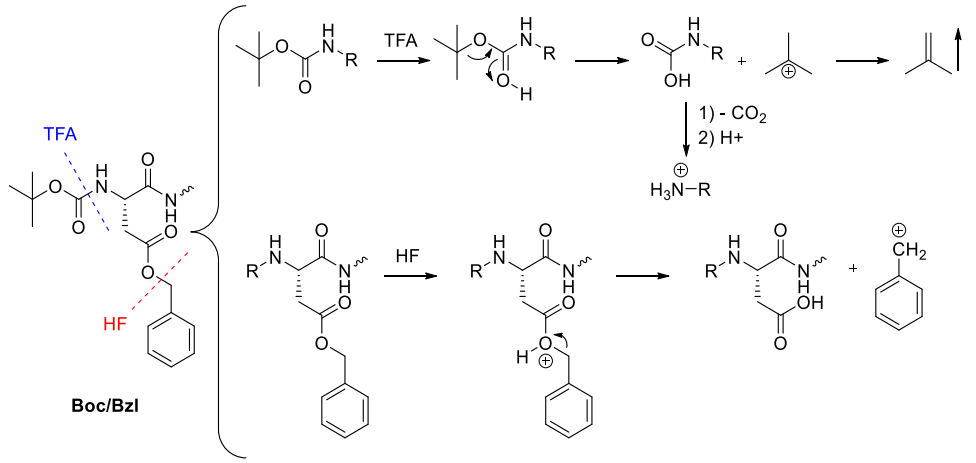
1.1.1.2. Protection strategies

Amino acids present a large variety of functional groups. They present three main functional moieties: the α-amino function, the α-carboxyl function and the side chain which is specific to each amino acid. For this reason, protection strategies are necessary in order to avoid secondary reactions.

The protection strategies commonly used in peptide synthesis are Boc/Bzl (*tert*-butyloxycarbonyl groups to protect the α-amino acid and benzyl like groups for the side chains of the amino acids) and Fmoc/^{t}Bu (9-fluorenylmethyloxycarbonyl for the α-amino protection and *tert*-butyl type groups for the side chains). The α-amino protecting group is temporary as it is continuously being removed before forming an amide bond. Nonetheless, the side chain protecting groups must have a semi-permanent behaviour to avoid undesired reactions during the amide bond formation. Thus, the protection strategy must be at least in some way orthogonal. For this reason, it is very important to choose an appropriate protection strategy before starting the synthesis of a peptide.

1.1.1.2.1. Boc/Bzl strategy

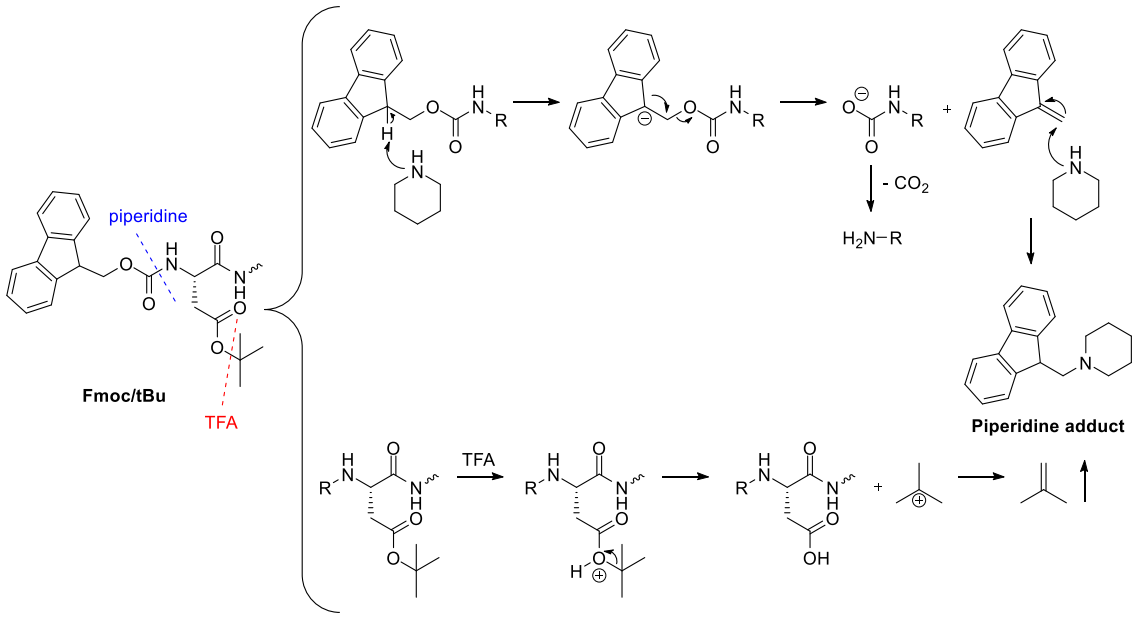
In the Boc/Bzl protection scheme, Boc protecting groups are used to temporally protect the α-amino group of the amino acids while benzyl-based protecting groups provide a semi-permanent protection of side chains during peptide elongation. Boc and benzyl-based protecting groups are both acid labile; therefore Boc/Bzl cannot be considered a fully orthogonal protection scheme. Thus, the Boc group is removed under mild acidic conditions (50% trifluoroacetic acid (TFA) in dichloromethane (DCM)) while benzyl-based protection groups require strong acids such as hydrofluoric acid (HF) to remove them (scheme 1.6).



Scheme 1.6. Mechanism of the protecting groups removal for the Boc/Bzl strategy.

1.1.1.2.2. Fmoc/^{t}Bu strategy

In the Fmoc/^{t}Bu protection scheme, the Fmoc protecting group is used to temporally protect the α-amino group of amino acids while ^{t}Bu-based protecting groups provide a semi-permanent protection of side chains during the elongation of the peptide. The Fmoc group is stable to acids, but not to bases such as piperidine. Thus, conditions usually used for Fmoc group removal are piperidine/*N*,*N*-dimethylformamide (DMF) (20:80, v/v). As ^{t}Bu-based protecting groups are removed with TFA, this strategy follows a true orthogonal protection scheme (scheme 1.7).

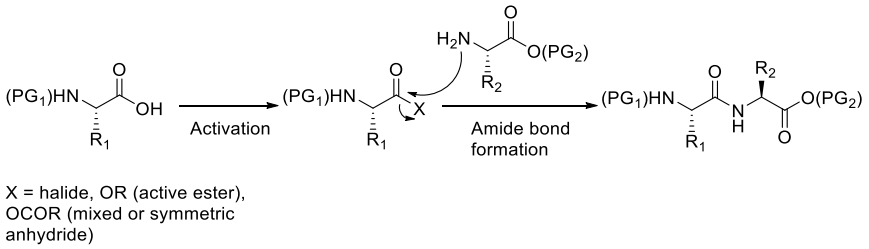


Scheme 1.7. Mechanism of the protecting groups removal for the Fmoc/^{t}Bu strategy.

1.1.1.3. Coupling reagents

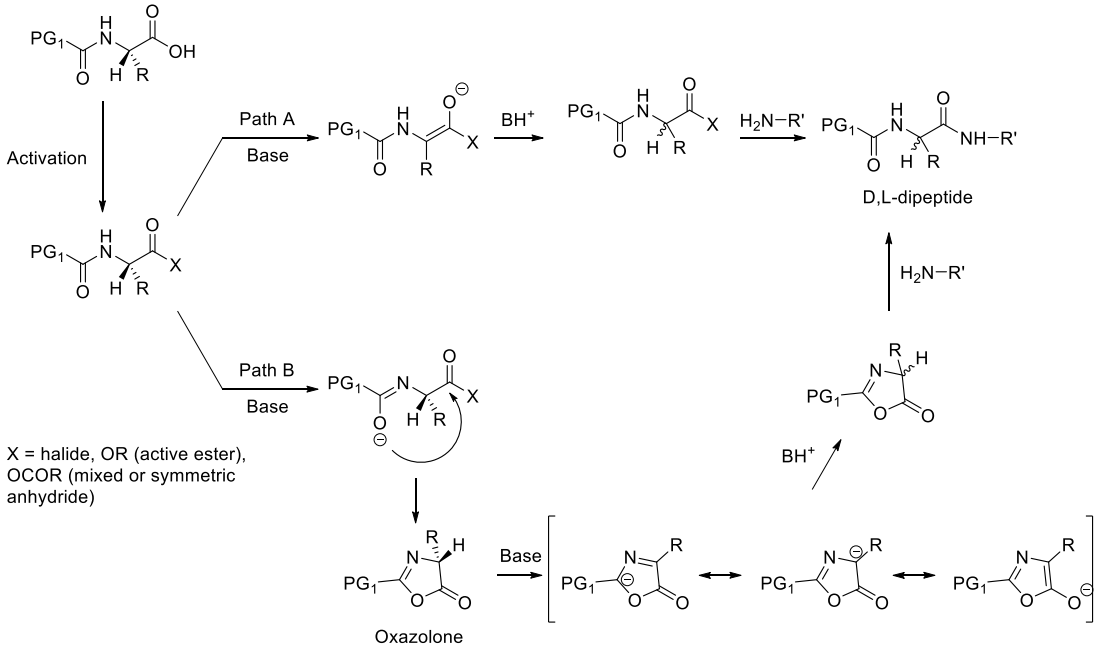
The reaction between two amino acid residues to form an amide or peptide bond can also be referred as coupling reaction. This reaction involves the attack by the amino group of one amino acid to carboxyl group of another amino acid. For this reaction to occur, the latter must have been previously activated by the introduction of an electron-withdrawing group. The activated form of the amino acid can be a stable derivative such as an active ester; a

relatively stable intermediate such an acyl halide or an anhydride (mixed or symmetrical) that may or may not be isolated; or a transient unstable intermediate which is neither isolable nor detectable because immediately undergoes aminolysis to form the peptide bond (scheme 1.8).[73]



Scheme 1.8. Peptide bond formation.

Thus, activation of the carboxylic group is essential for the reaction to occur. However, during the activation step, the loss of chiral integrity of the amino acid that is activated can take place. Two different pathways have been recognised for the loss of chirality, both base catalysed. The first path occurs via direct enolisation (scheme 1.9, path A) while the other occurs through the formation of a 5(4H)-oxazolone (scheme 1.9, path B).[73,74,75]

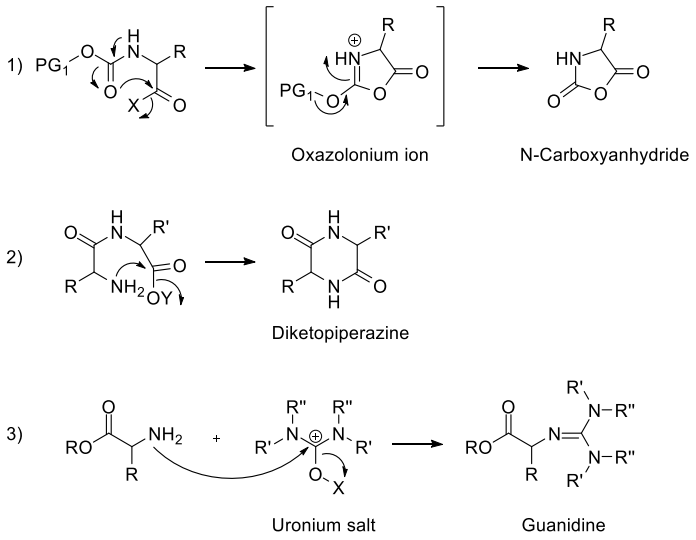


Scheme 1.9. Mechanism of epimerisation. Path A: direct enolisation; Path B: oxazolone formation.

Some reaction parameters can be controlled to avoid epimerisation during the peptide-coupling reactions. In this sense, the use of an appropriate N-protecting group plays a crucial role. Carbamate protecting groups diminish the formation of oxazolones while groups containing electron-withdrawing moieties (like acyl groups) are more likely to suffer direct enolisation.[76-77] Another key factor is the basicity of the tertiary amines that might be used for the coupling reaction. Sterically hindered amines reduce the possibility of proton abstraction.[78]

Other side reactions may occur during the coupling step (scheme 1.10). First, the formation of N-carboxyanhydrides can take place when the α-amino protecting group is a carbamate. Second, diketopiperazine (DKP) formation before anchoring the third residue of the

peptide-chain.[79] This two side reactions are more prone to appear when a good leaving group is present in the C-terminal function. Finally, when coupling reagents such as aminium and uronium salts are used, a guanidine by-product may be generated when the coupling reagent directly reacts with the amine function.[80] Guanidilation is favoured when an excess of coupling reagent is used or when the preactivation rate of the carboxylic acid is slow.

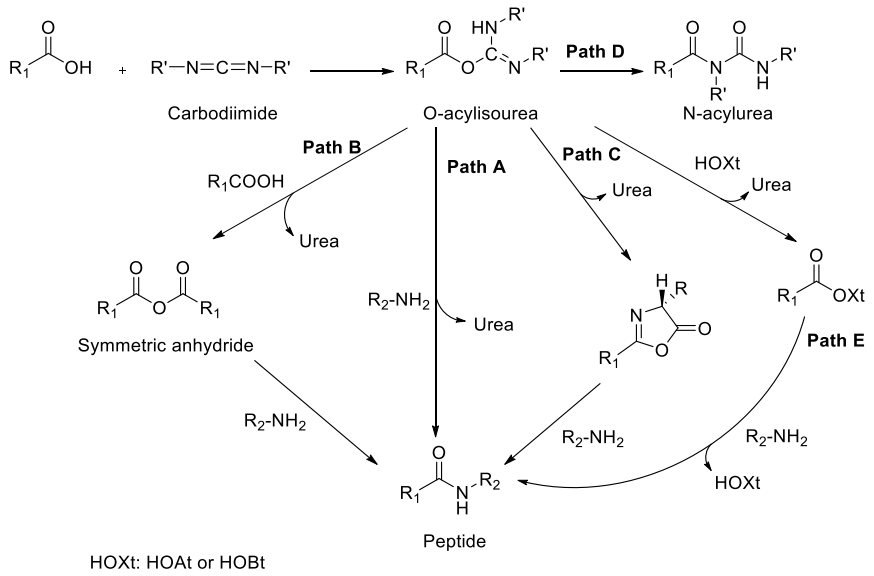


Scheme 1.10. Coupling side reactions.

1.1.1.3.1. Carbodiimides

Carbodiimides are the coupling reagent generally used to form peptide bonds. This reagent contains two nitrogen atoms that present a weak basic behaviour that activates the reaction between the carbodimiide and the carboxylic acid of an N-α protected amino acid generating an activated species known as *O*-acylisourea (scheme 1.11, path A).[73,81,82,83,84,85]

The *O*-acylisourea form of an amino acid or peptide is one of the most reactive species and rapidly undergoes aminolysis in the presence of a C-α protected amino acid leading to the formation of the peptide bond (scheme 1.11, path A). However, the presence of an excess of the N-α protected amino acid can yield the symmetrical anhydride as a consequence of the reaction between the carboxylic acid and the *O*-acylisourea (scheme 1.11, path B). This symmetrical anhydride is then aminolised to give the peptide. Another reaction that the *O*-acylisourea may undergo is a cyclisation to obtain an oxazolone that is less reactive against aminolysis and may yield to epimerisation (scheme 1.11, path C).[73,86] Finally, another undesired reaction that may occur is the irreversible N→O-acyl rearrangement of the *O*-acylisourea to give the stable *N*-acylurea (scheme 1.11, path D). This side reaction consumes starting material and is very fast in polar solvents such as DMF but slow in DCM.[73,87]



Scheme 1.11. Carbodiimide-mediated pathways for peptide bond formation.

Additives are used to overcome the above-mentioned side reactions. They generate the corresponding active ester of the additive which is less reactive towards racemisation than the *O*-acylisourea and increment the efficiency of peptide bond formation. 1-Hydroxybenzotriazole (HOBt) and 1-hydroxy-7-azabenzotriazole (HOAt) are the most common additives used for the carbodiimide-mediated reactions (figure 1.3). Both reagents suppress the formation of *N*-acylurea by protonating the *O*-acylisourea and thus preventing the intramolecular rearrangement (scheme 1.11, path E).[86,88]

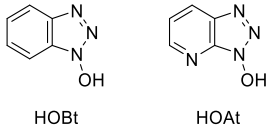


Figure 1.3. Most common additives used in carbodiimide-mediated reactions.

N,N'-Dicylcohexylcarbodiimide (DCC) has been the most used carbodiimide. However, the *N,N'*-dicyclohexylurea (DCU) that is formed during the coupling reaction is very difficult to remove due to solubility problems. For this reason, DCC has been substituted by other carbodiimides such as *N,N'*-diisopropylcarbodiimide (DIC), *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-cyclohexyl-*N'*-isopropylcarbodiimide (CIC) (figure 1.4).[89] The ureas that are formed from these three carbodiimides are highly soluble and are therefore more suitable for solution-phase and solid-phase synthesis.

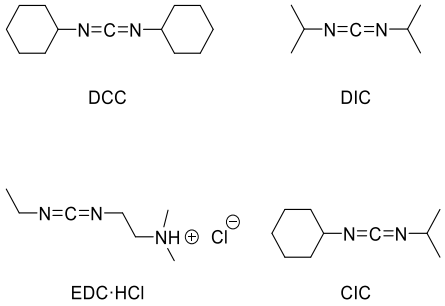


Figure 1.4. Carbodiimide coupling reagents.

1.1.1.3.2. Phosphonium and aminium/uronium salts

Phosphonium salts as coupling reagents (figure 1.5) were first described by Kenner and his co-workers.[90] These salts react with the carboxylate form of the carboxylic acid and thus, at least one equivalent of base is necessary for deprotonation (scheme 1.12). Phosphonium salts are very reactive and immediately react with carboxylate anions even at low temperature. The mechanism aroused controversy because Castro and Dormoy[91,92] suggested initially that the symmetrical anhydride is formed first, which was supported by Hudson[93] with kinetic studies. Nonetheless, years later, Coste and Campagne[94] reported that this species are highly unstable and undergo conversion to the active ester of HOXt (HOAt or HOBt). Couplings with these reagents are usually performed with an excess of base, normally two molar equivalents of *N,N*-diisopropylethylamine (DIPEA), and in the presence of one equivalent of HOBt or HOAt.

Aminium salts (also called uronium salts) contain a carbocationic group instead of the phosphonium group (figure 1.5). The mechanism of action of aminium salts is similar to the one described for phosphonium salts and thus, these reagents are used under similar conditions (scheme 1.12). However, the aminium moiety can directly react with the free amine of the amino acid that is going to be coupled, yielding to a guanidine by-product unsuitable for further couplings.[80] Phosphonium salts do not present this side reaction.

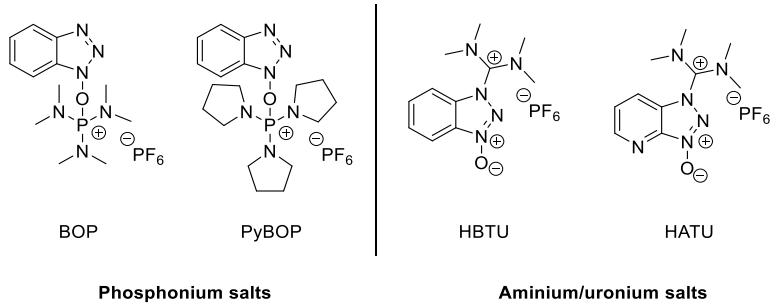
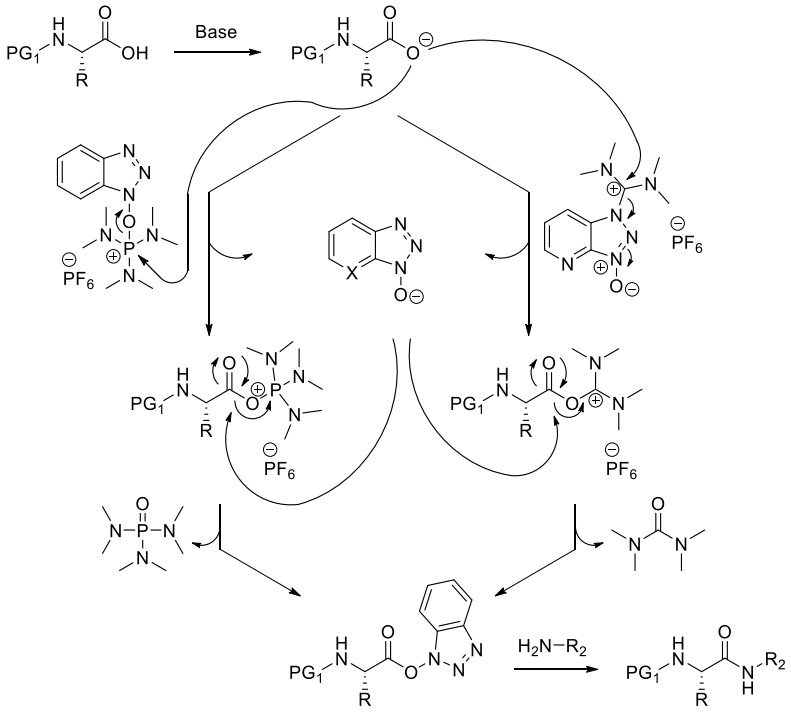


Figure 1.5. Examples of phosphonium and aminium/uronium salts.



Scheme 1.12. Phosphonium/aminium salt-mediated amide formation mechanism.

1.1.1.4. Cleavage of the peptide from the resin

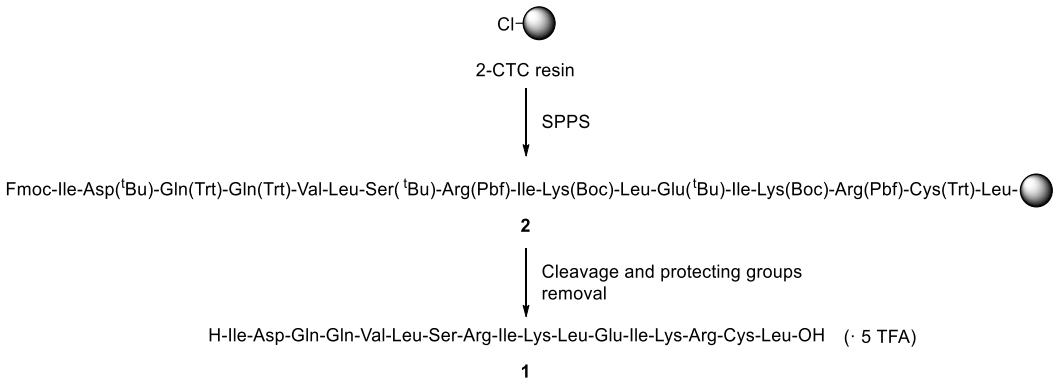
In the Boc/Bzl strategy, anhydrous HF is used to remove the side chain protecting groups and to release the peptide from the resin.[95] HF cleavage has become a reliable technique in the laboratory scale, but it has to be carried out in a special HF-resistant system (Teflon and Kel-F) due to the high corrosive character of the acid.[96] Thus, economic costs of using HF are high.

In the Fmoc/^{t}Bu strategy, TFA, a relatively weak acid, is used for the simultaneous cleavage of the side-chain protecting groups and the peptide-resin bond. The acidolytic cleavage generates highly reactive carbocation species and scavengers have to be used in order to minimise side reactions produced by these species with sensitive amino acids such as cysteine (Cys), methionine (Met), serine (Ser), threonine (Thr), tryptophan (Trp) and tyrosine (Tyr). 1,2-Ethanedithiol (EDT) and dithioerythritol (DTE) are usually used as scavengers. Moreover, these reagents avoid the formation of disulfide bonds in Cys containing peptides as reductants. Another common scavenger is triisopropyl silane (TIPS) that acts as a hydride donor under acidic conditions.[97]

1.2 Synthetic strategy

The first synthetic approach that was assayed to prepare Fexapotide (**1**) was the traditional linear solid-phase peptide synthesis.

As mentioned above, this synthetic approach consists of attaching amino acids in a stepwise manner until obtaining the desired peptide sequence. Further cleavage of the peptide from the solid support with concomitant amino acid side-chain deprotection affords the desired product (scheme 1.13).



Scheme 1.13. Linear synthesis of **1**.

The linear synthesis was performed using the Fmoc/^{t}Bu strategy since this strategy follows a true orthogonal protection scheme and milder conditions are required to remove the N-terminal and side-chain protecting groups when compared to the Boc/Bzl strategy.

In our case, the research was focused on a peptide containing residues of Leu, Cys, Arg, Lys, Ile, Glu, Ser, Gln and Asp, side-chain protected with pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl (Pbf) for the protection of the side chain of Arg, Boc for the protection of Lys, ^{t}Bu for the side chain protection of Glu, Ser and Asp, and trityl (Trt) for the protection of Cys and Gln. All these protecting groups are acid labile (figure 1.6).

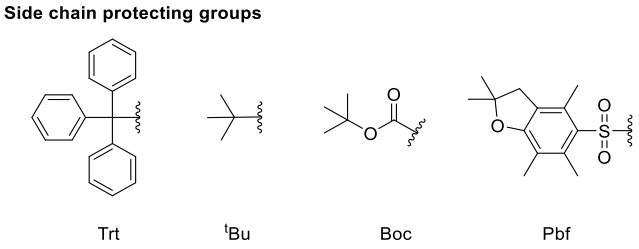
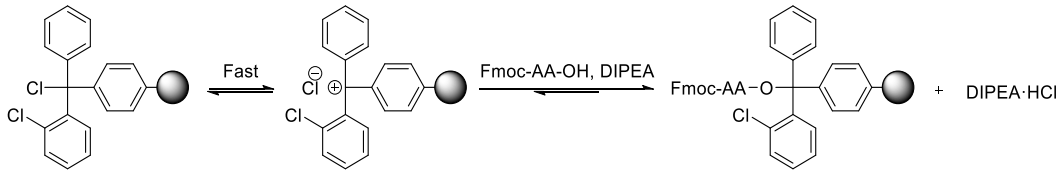


Figure 1.6. Protecting groups compatible with the Fmoc/^{t}Bu strategy used in the present project.

The 2-CTC resin was the polymeric support chosen for the linear solid-phase synthesis of the target peptide. This resin is generally used since it minimises the most common side reactions in peptide synthesis such as DKP formation and epimerisation during the incorporation of the first amino acid.[98] The position of the chlorine in the trityl group makes this polymeric support to be hindered thus avoiding the DKP formation during the preparation of peptides.[99,100] The degree of racemisation occurring with the 2-CTC resin was found to be less than 0.05 % because the esterification reaction that takes place does not need electrophilic activation. Hence, the reaction proceeds with low levels of epimerisation (scheme 1.14).[101]

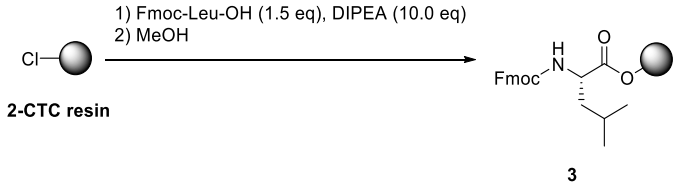


Scheme 1.14. Reaction of the polymeric 2-CTC with Fmoc amino acids.

1.3 Linear solid-phase synthesis of Fexapotide

1.3.1. Incorporation of the first amino acid

The coupling of commercially available Fmoc-Leu-OH to the polymeric support (f = 1.1 mmols/g) was performed through a S_N1 reaction between the carboxylate form of the Fmoc-protected amino acid and the chlorotrityl group of the resin to form an ester bond (scheme 1.15). The chlorotrityl non-reacted active sites of the resin were capped with the addition of MeOH (0.8 mL/g of resin) after 1.5 h of reaction in order to avoid the addition of further amino acids into the polymeric support that would yield to deletion peptides.



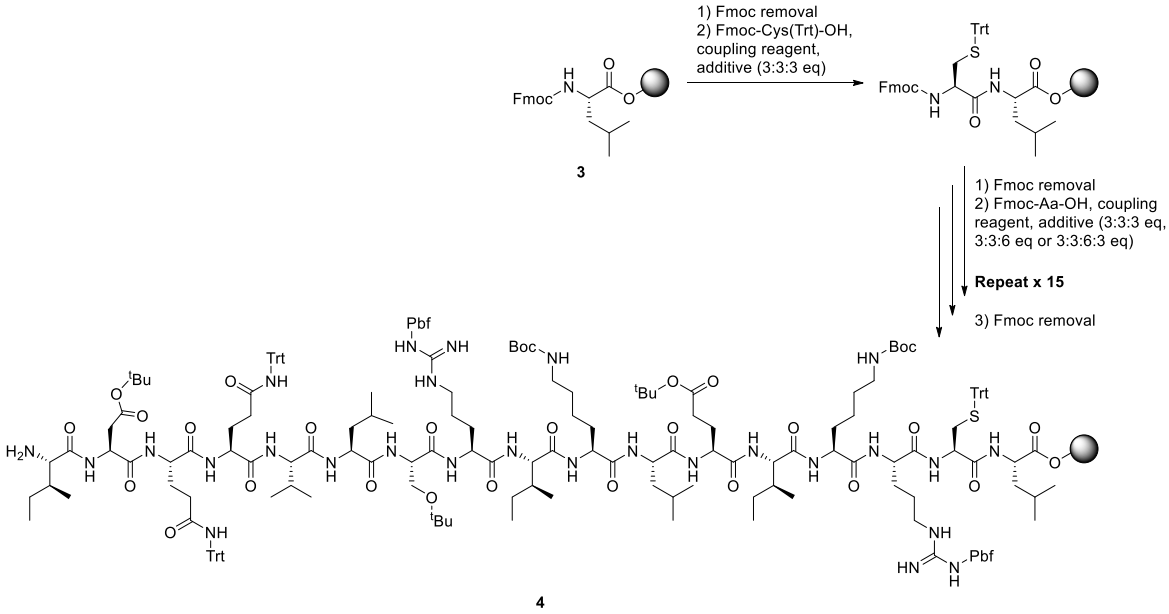
Scheme 1.15. Incorporation of Fmoc-Leu-OH to the 2-CTC resin.

The amount of amino acid that was incorporated to the resin was determined by UV quantification at 301 nm of the piperidine adduct formed after removing the Fmoc group (see scheme 1.7). The substitution value obtained in in the aminoacyl-resin for the synthesis of **1** was of 0.29 mmol/g.

1.3.2. Peptide chain elongation

Our first approach to the synthesis relied on the use of the conventional and economic DIC and Oxyma (3:3 eq) as coupling agent and additive. Unfortunately, our first attempts showed that many coupling steps were problematic and required stronger coupling systems such as HATU/DIPEA (3:6 eq) and HATU/HOBt or HOAt/DIPEA (3:3:6 eq).

Scheme 1.16 and table 1.1 summarise the final approach to the linear synthesis of the peptidyl-resin **4**. The coupling reactions were monitored with the ninhydrin test and recoupling steps were performed when the test was positive (blue colour).



Scheme 1.16. On-resin peptide chain elongation.

Table 1.1. Number of couplings needed for the preparation of **4**.

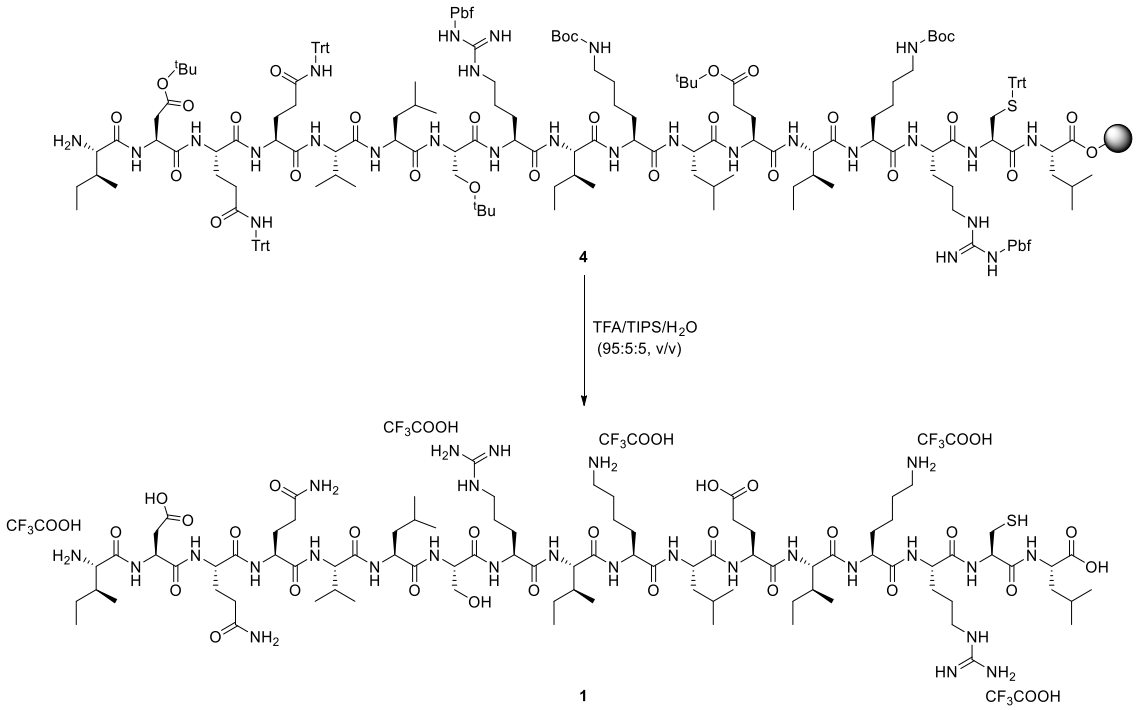
Coupling	Amino acid	Number of couplings
1	Fmoc-Cys(Trt)-OH	2[a]
2	Fmoc-Arg(Pbf)-OH	3[a]
3	Fmoc-Lys(Boc)	3[a]+2[b]
4	Fmoc-Ile-OH	5[b]
5	Fmoc-Glu(^{t}Bu)-OH	2[b]
6	Fmoc-Leu-OH	2[c]
7	Fmoc-Lys(Boc)	2[c]
8	Fmoc-Ile-OH	3[c]
9	Fmoc-Arg(Pbf)-OH	2[b]+1[d]
10	Fmoc-Ser(^{t}Bu)-OH	2[d]
11	Fmoc-Leu-OH	1[c]
12	Fmoc-Val-OH	1[c]
13	Fmoc-Gln(Trt)-OH	1[c]
14	Fmoc-Gln(Trt)-OH	1[c]
15	Fmoc-Asp(^{t}Bu)-OH	1[c]
16	Fmoc-Ile(^{t}Bu)-OH	1[c]

(a) DIC/Oxyma system.
(b) HATU/DIPEA system.
(c) HATU/HOAt/DIPEA system.
(d) DIC/HOAt system.

Some couplings proved to be very difficult and recouplings were required even using the HATU/DIPEA/HOAt system (entries 6 to 8). However, this system proved to be efficient from the eleventh coupling and no recouplings were needed in these cases.

1.3.3. Cleavage of the peptide from the resin

The peptide chain was deprotected and removed from the resin by using the acidolytic mixture TFA/TIPS/H_2O (90:5:5, v/v) for 1.5 h (scheme 1.17).



Scheme 1.17. Full deprotection and removal of **1** from the resin.

After the work-up, 71.0 mg of crude peptide (batch 1A) were obtained as a white solid (arising from 100 mg of the starting 2-CTC resin and a loading of 0.29 mmol/g after the incorporation of the first amino acid). The HPLC chromatogram and the MALDI-TOF spectra revealed that the peptide crude contained the desired Fexapotide (**1**) (figure 1.7, B) but the chromatographic purity of the peptide was only a 6 %. Among other impurities, several partially protected peptides were found in the mixture, such as peptides containing a ^{t}Bu protecting group (4 %), a Pbf protecting group (4 %), a Pbf and a ^{t}Bu protecting group (17 %) and two Pbf protecting groups (10 %) (figure 1.7, A).

This result suggested that the reaction time of the acidolytic treatment performed to the peptidyl-resin was not enough to achieve the full deprotection of the peptide chain.

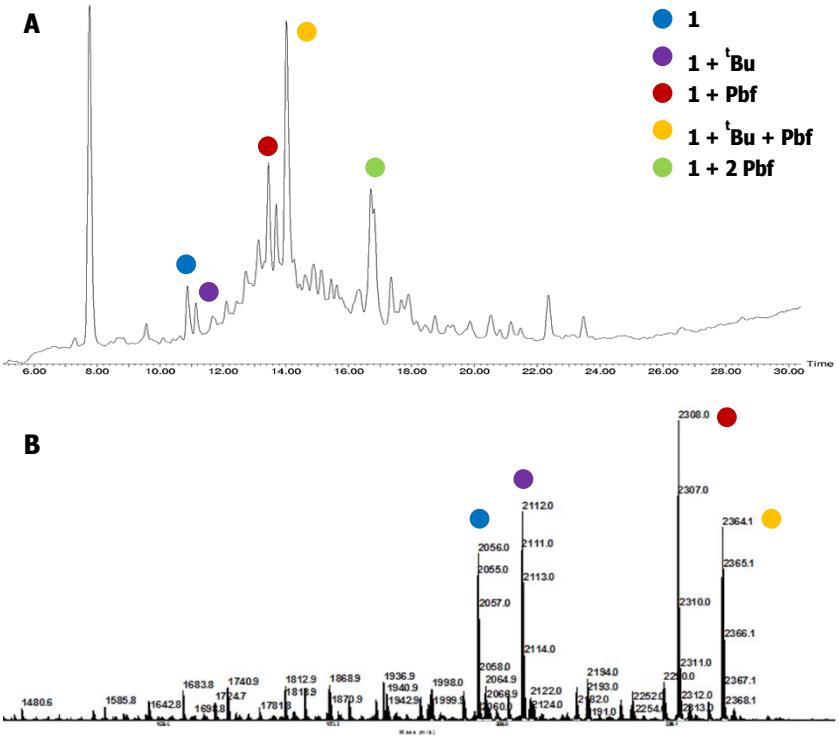


Figure 1.7. HPLC chromatogram (A) and MALDI-TOF spectra (B) of the acidolytic crude (batch 1A).

1.4. Conclusions

The use of the linear approach to synthesize Fexapotide on solid phase revealed potential difficulties that had to be faced when trying to scale up the process following this strategy. Thus, this preliminary assay showed the need of recouplings for some amino acids using conventional and economic coupling agents such as DIC, or even using more efficient coupling agents such as HATU. Fexapotide is a medium size peptide containing several β-branched amino acids and trifunctional amino acids that usually are protected with bulky groups that could difficult couplings. In a laboratory scale, when a few mg of the peptide are needed, the use of even more efficient coupling agents and a higher number of recouplings would be probably enough to get the target product after a chromatographic purification. However, when a multigram or even kilogram scale is required in an industry framework, this strategy becomes a serious drawback from the economic and environmental points of view.

Application of the convergent approach for the synthesis of Fexapotide

Convergent synthesis: an introduction

Solution-phase and solid-phase have proven to be effective for the synthesis of peptides. However, each approach has its limitations. On one hand, solution-phase synthesis requires time and effort to develop of the procedure. Therefore, this technique should not be considered if rapid production of the product is needed for early-phase clinical studies to confirm the pharmaceutical potential of the product. On the other hand, while solid-phase synthesis is used during the early stages of the development of a peptide, the scale-up of the methodology is generally difficult.[11]

Nonetheless, with the introduction of new acid sensitive resins such as 2-CTC[58] and 2-methoxyl-4-alkoxybenzyl alcohol (SASRIN)[102] resins, it is now possible to synthesise protected peptides and fragments that can be released from the resin without removing any side chain protecting group. This has introduced the possibility of a hybrid approach, also known as convergent synthesis. This strategy allows the production of complex peptide sequences by the synthesis of short protected peptide fragments of the target peptide using the solid-phase approach and then, the assembly of these fragments either by solution or solid-phase methods.[3] Finally, removal of the side-chain protecting groups and the cleavage of the peptide from the resin affords the desired peptide (scheme 1).[10] This new strategy is promising for the commercial-scale production of large peptides.

Fragment selection is crucial if a convergent approach is going to be used and some factors must be taken in consideration to achieve good purities for fragment condensation. Preferably, the fragments should contain Gly or Pro residues at the C-terminal since the risk of epimerisation on those residues is avoided or minimised. If this is not possible, the C-terminal residue should be an amino acid that is less prone to epimerise such as Ala or Arg.[53]

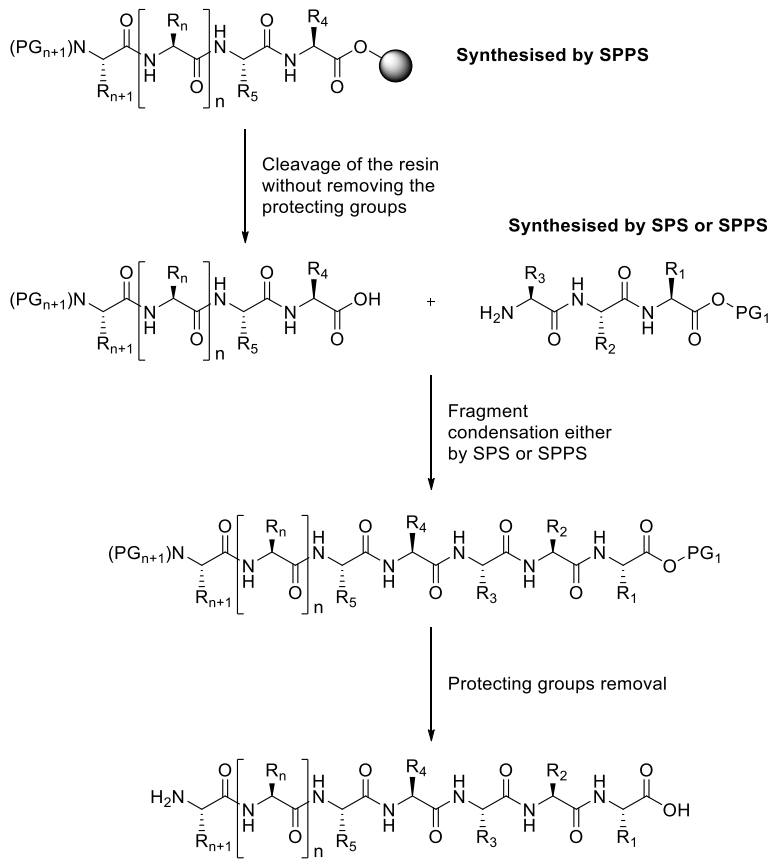
It is worth mentioning that isolation and purification of the protected fragments at this step by chromatographic techniques is difficult due to solubility problems. However, these fragments can be purified from the low-molecular weight by-products by precipitation.[3]

Protection strategies

For large-scale solution synthesis, the most frequently used α-amino protecting groups are the Boc and benzyloxycarbonyl (Z) groups because of the volatile by-products generated during the deprotection step.[53] Nonetheless, the Boc/Bzl protection strategy cannot be considered a full orthogonal protection scheme and the removal conditions for the Boc group and benzyl-based protection groups require strong acids such as 50% TFA in DCM in the case of the former and HF to remove the latter one. The Fmoc group is less attractive because of the lack of volatility and reactivity of the dibenzofulvene (DBF) by-product but follows a true orthogonal protection scheme. For the carboxyl function, the acid labile benzyl or *t*-butyl esters are the most suitable carboxyl protecting groups.[11] Finally, side-chain protecting groups should be chosen in such a way that can be removed simultaneously with the N-terminal and C-terminal protecting groups.

Solid-phase synthesis of protected fragments

Preparing the protected fragments by SPPS requires selectively cleave the protected peptide from the polymeric support without removing the side chain protecting groups. To perform that, the Boc/Bzl strategy can be combined with nucleophile, base-labile, palladium-labile and photo-labile polymeric supports. On the other hand, Fmoc/^{t}Bu strategy is compatible



Scheme 1. General methodology for the convergent synthesis of peptides.

with dilute-acid-labile, palladium-labile and photo-labile linkers. This side chain-polymeric support orthogonality allows the synthesis of N-terminal and side chain protected fragments.[103]

For the Fmoc/^{t}Bu strategy, the most favorable resin linkers for the synthesis of protected peptides are the ones that afford a highly acid-labile peptide-to-resin bond. The scission of protected peptides from these resins is achieved with dilute acid solutions such as 1 % (v/v) TFA in DCM, conditions under which ^{t}Bu-based protecting groups are not affected. Resins compatible with these cleavage conditions are the SASRIN and the 2-CTC resins.[12]

Coupling of fragments

Coupling of protected peptide fragments in a solid support

The first fragment can be incorporated either by a stepwise solid-phase synthesis or by incorporating a fragment previously synthesised, purified and characterized. The second approach would yield to a purer final product but attaching protected peptide fragments onto a polymeric support is sometimes difficult and leads to low yields.[104,105]

Coupling a protected peptide fragment is more challenging than coupling an amino acid. First, the risk of epimerisation at the C-terminal residue depends on the nature of the N-α group of this residue (see scheme 1.10, chapter 1). Thus, carbamate protecting groups (as in the case of Fmoc amino acids) are less prone to induce the formation of the oxazolone intermediate when compared to amides (protected fragments) due to the electronic withdrawing properties of the carbamate group that destabilises the anion that would be

formed by proton abstraction.[53] Second, large excess of the protected peptide that is being coupled should be used under solid-phase conditions to achieve quantitative results, which is a drawback from the economic point of view. Finally, the poor solubility of protected peptide fragments drives to use dilute solutions. The combination of these factors together with the difficulty of making a peptide bond between two large molecules leads to long time reactions and sometimes, low yields.[105]

Coupling methods for protected peptides fragments were first carried out via the use of azides and oxidation-reduction methods but were then replaced by carbodiimides (DCC, DIC and EDC) in the presence of additives such as HOBt to minimise epimerisation. Developments in coupling reagents allowed the use of reagents such as BOP, PyBOP, HBTU, PyAOP and HATU. For these reagents, it is important to consider the nature of the base that has to be used for the coupling reaction. In this sense, DIPEA and N-methylmorpholine (NMM) are among the most common bases in order to minimise side reactions.[78] Lower levels of epimerisation have been demonstrated with the use of non-polar solvents. However, the solubility of the protected peptide fragment limits the use of certain solvents.[106]

Coupling of protected peptide fragments in solution

An alternative to overcome the main drawbacks of coupling protected peptide fragments into a solid support is to perform the condensation in solution. The first difference between the two strategies is the need of protection of the C-terminal end of the first fragment when using the solution strategy to avoid side reactions in the presence of coupling reagents. Benzyl or *t*-butyl esters are the most common protecting groups for the C-terminal end when using the Fmoc protection on the N-terminal end.[11]

As said before, during the coupling reaction between segments, the risk of racemisation is increased due to the oxazolone formation. Nonetheless, several methods diminishing the epimerisation risk have been proposed such as carbodiimides, phosphonium and aminium/uronium salts. The presence of an additive such as HOBt or HOAt is recommended when using these coupling reagents. An alternative to these coupling reagents is the mixed anhydride method using isobutyl chloroformate with the occasional presence of additives.[107] The azide method is less frequent due to safety concerns even though low racemisation levels are associated with it.[108]

Nonetheless, the main disadvantage of this strategy is that harder purification steps such as chromatography and/or recrystallization are needed, resulting in a decrease of the overall yield.[54]

Removal of the protecting groups

Concentrated TFA (95 % TFA) solution is generally used to perform full removal of the protecting groups. As mentioned before, during this reaction, highly reactive carbocations are generated and must be trapped with scavengers such as TIPS and EDT to avoid side reactions. The fully deprotected peptide is finally isolated by precipitating it with an aprotic solvent such as diethyl ether (Et_2O) or methyl *t*-butyl ether (MTBE). The crude peptide can be stored as a dry powder or dissolved in an appropriate solvent for purification purposes.

Scale-up considerations

When a peptide synthesis has to be scaled-up, the final goal is to develop a procedure that has to be efficient, cheap and fast. However, it also must accomplish the requirements of the regulatory authorities.

In a multistep process like the synthesis of a peptide, the number of steps of the synthetic route should be kept at minimum to increase the total yield. Moreover, evaluation of the overall economics of the process should be considered. This leads to the discussion of whether to use a convergent or linear strategy.

When deciding the strategy, some considerations should be taken. One of the advantages of the convergent strategy is that the synthesis of smaller fragments might shorten the production time because of the possibility of using parallel production equipment. Second, the use of large excess of amino acid and coupling reagents to complete solid-phase reactions is economically not viable when performing the large-scale synthesis of a peptide. Finally, the chemicals and solvents used in an industrial process must not only be cheap and commercially available but also must be safe for humans and minimise the environmental impact. In a fully SPPS, high volumes of solvent such as DMF or DCM have to be used. For these reasons, the convergent synthesis following a combination of solid-phase and solution-phase is usually the best alternative for the synthesis of industrial peptides.[11]

Chapter 2: Synthesis of Fexapotide through a four-fragment convergent approach

2.1. Synthetic strategy

In general, when the synthesis of a product has to be scaled-up in an industrial environment, the main goal is to develop a procedure that has to be efficient and economical in terms of costs and time. Moreover, it must accomplish the requirements of the regulatory authorities. Concerning to peptides, the solid-phase synthesis is generally used in the development of synthetic protocols for short to medium size peptides, but the scale-up of the process can be difficult for long peptides. Therefore, the convergent approach that combines solid-phase and solution-phase has become the strategy of choice for the synthesis of peptides in an industrial scale.

Taking into account the disappointing results obtained in the linear solid-phase synthesis of Fexapotide, the research was focused on the convergent approach. This approach consists of the solid-phase synthesis of protected fragments that all together represent the sequence of the desired peptide, and further assembly of these fragments in solution to yield the full protected peptide chain. The last step consists of removing the side-chain protecting groups to afford the desired target peptide.

Fragment selection is crucial if a convergent approach is going to be used and some factors must be taken into consideration.

- The C-terminal of the fragment on which the rest of fragments are going to be coupled, has to be protected to avoid side reactions during fragment condensations. For this reason, the first fragment has to be prepared in solution and should be a short sequence of the target peptide in order to minimise the number of synthetic steps.

- The fragments synthesised by SPPS should not contain racemisable amino acids such as cysteine or histidine at the C-terminal. When the first amino acid is linked to the polymeric support through an ester bond, racemisation can take place due to the increased lability of the α-hydrogen of the amino acid provoked by the inductive effect of the ester group.[11,109,110] This problem is minimised when the peptide chain is linked through an amide bond to the polymeric support.

- The C-terminal residue of the fragments synthesised by SPPS should be an amino acid that is less prone to epimerise during fragment condensation such as Ala or Arg.[53]

- Peptide fragments no longer than 5 amino acids are prefered.[11]

Considering these guidelines, the desired target peptide **1** was divided into fragments **5**, **6**, **7** and **8** (figure 2.1).

As shown in scheme 2.1, fragment **5** was synthesised in solution-phase while the remaining ones were synthesised on a solid support. These fragments were then condensed in solution-phase to afford the desired full protected peptide **16**, which led to **1** after removing the protecting groups.

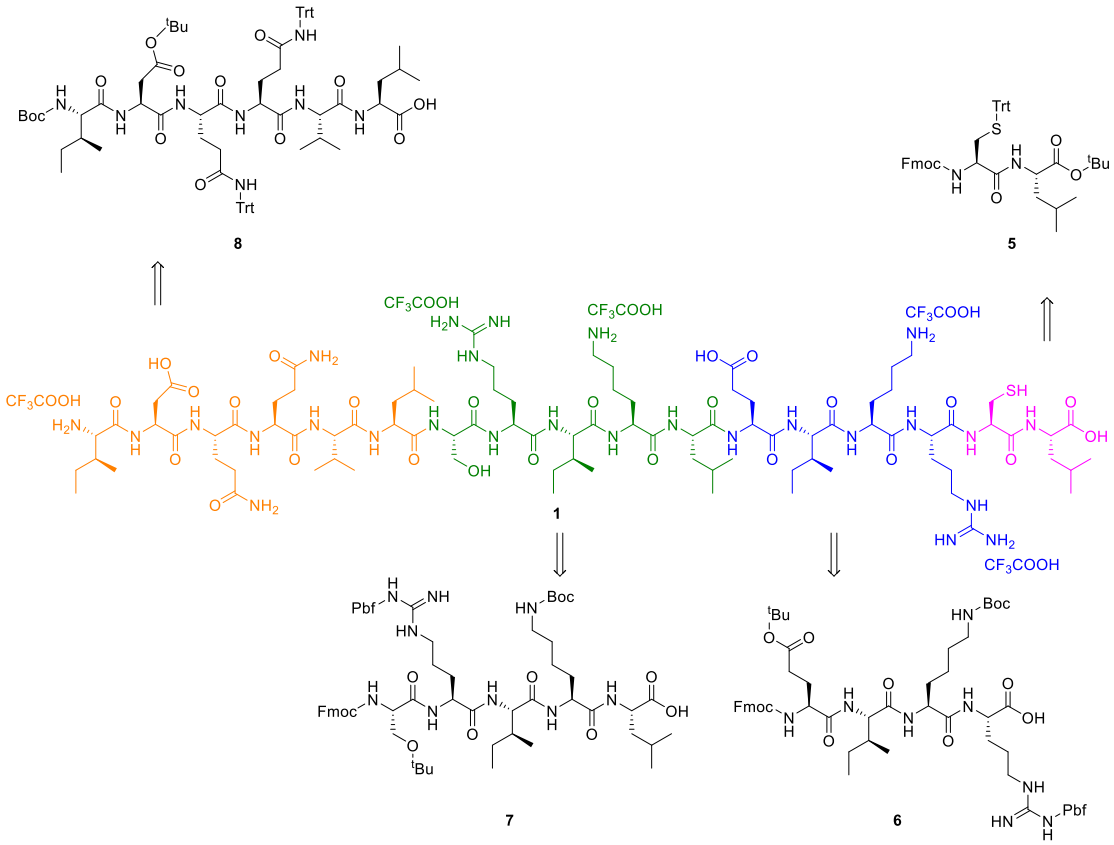
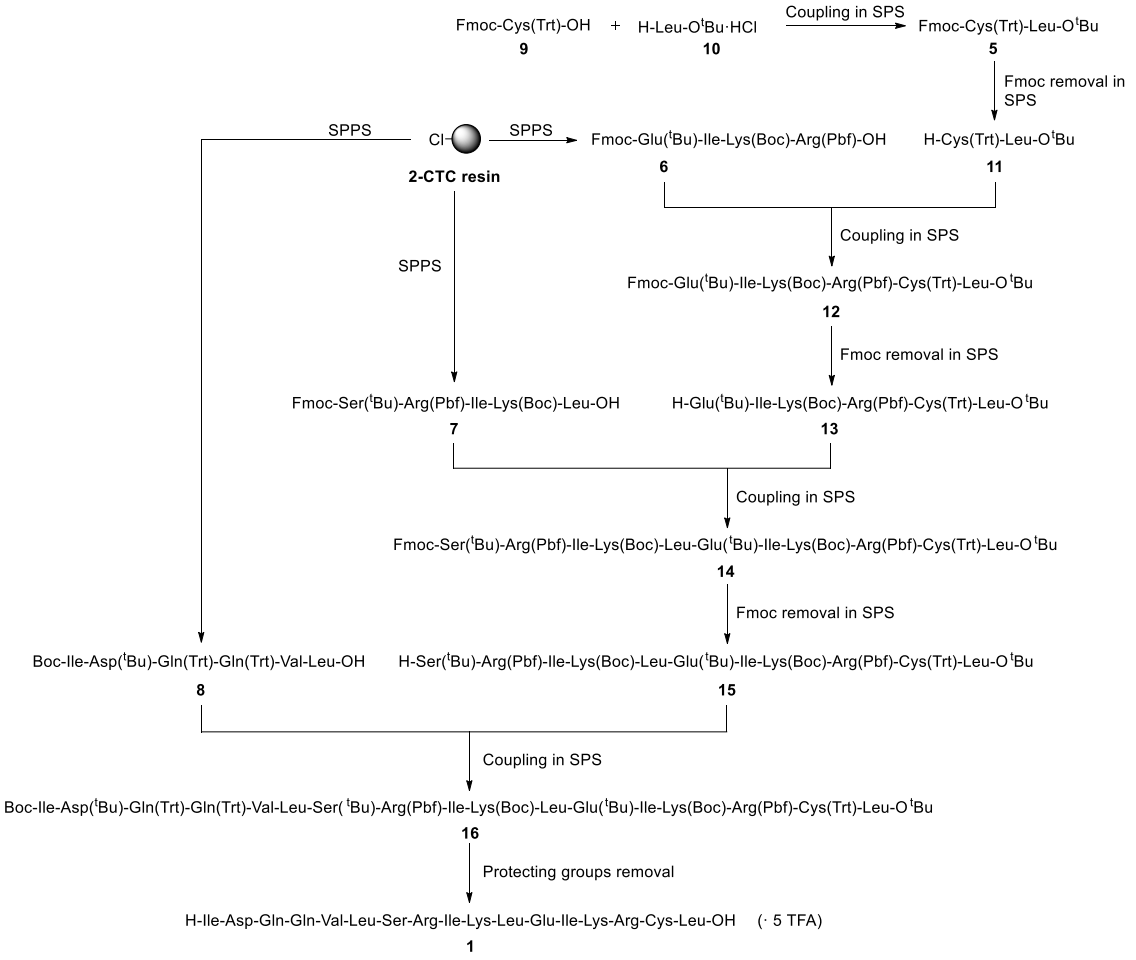


Figure 2.1. Fragmentation of the desired peptide **1** to follow a convergent approach.

An alternative approach would have been to couple the amino acid derivative NH_2-Leu-O^{t}Bu to a five residue fragment with a Cys residue at the C-terminal end. However, a risk of racemisation had to be considered during activation of the fragment through the carboxylic acid of the Cys residue prior to form the amide bond. It general, racemisation occurs through the oxazolone intermediate (section 1.1.1.3, chapter 1), but it has been described that the loss of Cys configuration takes place mainly by α-hydrogen abstraction of the activated species.[11,109,110] Consequently, fragments **6**, **7** and **8**, have Arg and Leu residues in the C-terminal, which in general are less prone to racemise.[11,109,110]

In the present chapter, the solid-phase synthesis of the protected peptide fragments and their condensation in solution to afford the target product **1** will be discussed. All products and intermediates were analyzed by HPLC or HPLC-MS and chromatographic purity was determined. In this work we make a heavy use of the "chromatographic purity" concept, that is, the percentage that the area of a given peak represents over the cumulated area of all the peaks present in the chromatogram. This is only an "apparent" purity; to get the real purity, it will be necessary to obtain pure samples of the target compound and impurities to calibrate the instrument, which is impractical in most cases. When the "chromatographic purity" is high, the error is small, and it is a good parameter to evaluate the outcome of a reaction during the development process.

In those cases, as "real" purities are unknow, it is not possible to calculate the "real" yield. We use the term "crude" yield to refer to the yield calculated assuming "pure" reagents and products. When their "chromatographic purities" are high, the error of the "crude" yield is small and, again, it is a good, practical and easy-to-use parameter to qualitatively express the outcome of a reaction during the development stages.



Scheme 2.1. First approach to the convergent synthesis of Fexapotide (**1**).

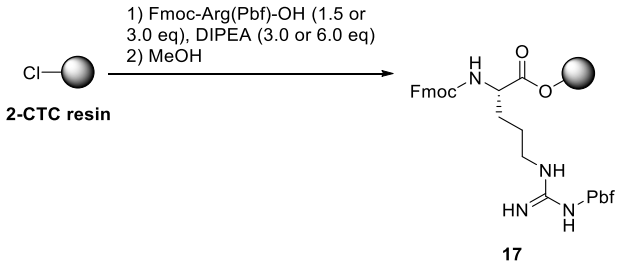
2.2. Solid-phase synthesis of the protected peptide fragments

The 2-CTC resin was considered the most suitable polymeric support to synthesise the protected fragments. Thus, while the common polymeric supports used in SPPS require strong acidic conditions to release the unprotected peptide from the resin, the 2-CTC resin enables its removal under very mild conditions such as 1-2 % of TFA or 25 % of hexafluoroisopropanol (HFIP), which allows the obtention of fully protected peptide fragments.[98] Moreover, as mentioned before, the 2-CTC resin minimises epimerisation of the first amino acid and DKP formation.

2.2.1. Synthesis of the tetrapeptide fragment 6

2.2.1.1. Incorporation of the first amino acid

The coupling of commercially available Fmoc-Arg(Pbf)-OH to the polymeric support was performed under similar conditions to those described in section 1.3.1 of chapter 1 (scheme 2.2). The non-reacted active sites of the resin were capped with the addition of an excess of MeOH after 1.5 h or 2 h of reaction.



Scheme 2.2. Coupling of Fmoc-Arg(Pbf)-OH to the 2-CTC resin.

The amount of amino acid that was coupled to the resin was determined by the UV quantification at 301 nm of the piperidine adduct formed after the removal of the Fmoc group. Table 2.1 summarise the substitution values obtained in the different syntheses of aminoacyl-resin **17** using different scales and reaction times that were performed to obtain the intermediate **6**.

Table 2.1. Substitutions obtained for the coupling of the first amino acid on the 2-CTC resin in the different syntheses of **6**.

	2-CTC resin [g]	**Reaction time** [h]	**Substitution** [mmol/g]
Batch 17A	1.99	2.0	0.64[a]
Batch 17B	10.6	1.5	0.42[b]
Batch 17C	11.1	1.5	0.57[b]

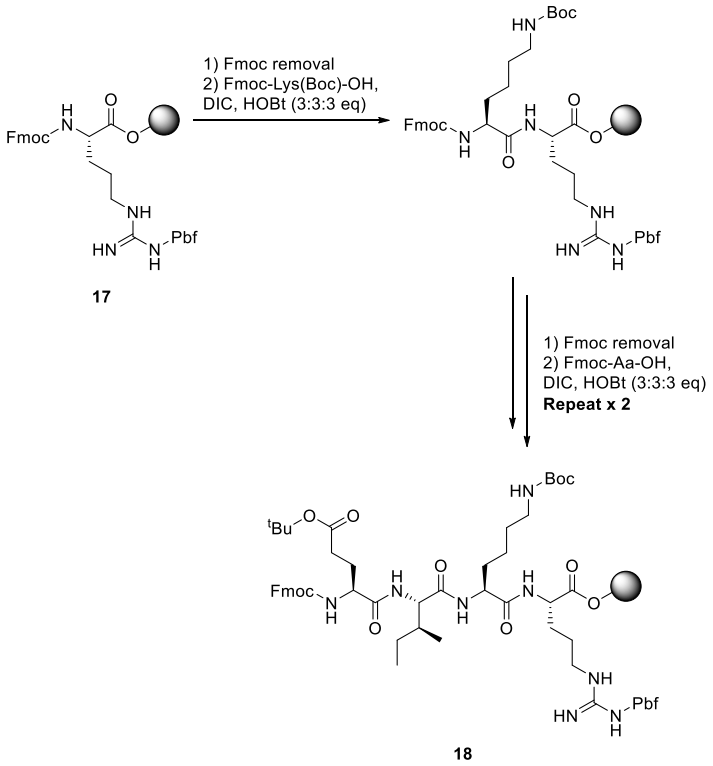
(a) Calculated following the protocol described in section 5.5.3 (Experimental Section).
(b) Calculated following the protocol described in section 5.5.4 (Experimental Section).

In general, commercially available 2-CTC resins are highly functionalized for peptide synthesis purposes, but lower functionalisations are usually required in order to avoid potential difficulties during growing the peptide chain on the solid support. Thus, suitable loadings are obtained controlling the reaction time for the coupling of the first amino acid.

In the particular case of **6**, a functionalisation below 0.6 mmol·g^{-1} was achieved after 1.5 h of reaction, which was considered suitable to continue the synthesis.

2.2.1.2. Peptide chain elongation

Three independent syntheses of **6** were carried out using the batches of aminoacyl-resin described in table 2.1. The coupling reactions were performed using the Fmoc-protected amino acid with the standard DIC/HOBt coupling system (3:3:3 eq) and were monitored with the ninhydrin test. The Fmoc protecting group was removed by using a 20 % piperidine in DMF (scheme 2.3). It is interesting to note that no recouplings were needed in any case under the conditions that were used, in sharp contrast with the results obtained in the linear approach where several couplings were required for these amino acid derivatives (section 1.3.2). In this sense, Fmoc-Cys(Trt)-OH and Fmoc-Arg(Pbf)-OH, the second and third amino acids of the target peptide sequence, already proved to be difficult to couple in the linear approach. Altogether, these results confirmed the potential usefulness that could have a convergent approach considering the two first residues of the sequence, Leu and Cys, as a protected dipeptide prepared on solution.



Scheme 2.3. Peptide chain elongation to obtain the peptidyl-resin **18**.

After the addition of the last amino acid residue, the peptidyl-resins coming from batches 17B and 17C, that were carried out at ~10 g scale, were washed, dried under vacuum and weighed as an in-process control (see table2.2) Unexpected results were obtained in both cases. Taking in consideration the initial substitution value of the aminoacyl-resin coming from batch 17B, a mass increment of only 19% was determined in that peptidyl-resin. More surprisingly, no mass increment was observed in the peptidyl-resin coming from the batch 17C, which indicated that unexpected premature capping of the resin and/or premature loss of peptide chains during the syntheses could have occurred. Unfortunately, ninhydrin tests after deprotection steps were not carried out and the premature cleavage of peptide chains from the resin could not be confirmed.

The explanation of why peptide chains were prematurely lost is yet unknown, but the very high sensitivity of 2-CTC resin to acidic environments might be the most probable cause of this loss. It is worth mentioning that these syntheses might took few days to be completed so it was unavoidable to leave the washed resins in the reactor *overnight* before continuing with the subsequent coupling steps next day. We speculate that during this period, traces of acid present in the mixture could lead to the premature detachment of a significant amount of peptide chains. Nevertheless, peptide chain loss levels such as those found in synthesis 17C are surprisingly high and hard to be accounted for.

We also observed similar behaviour in the preparation of fragments **7** and **8**. In order to minimise this problem, we decided that when a long period of time is required between couplings, the peptidyl-2-CTC resins should be stored without protection at the N-terminal end of the chain, in order to allow amino groups to trap any acid traces present on the enviroment. We have tested this approach in the syntheses of fragments **28** and **8** (see chapter 3).

Table 2.2. On-resin mass increment obtained in the different batches of **6**.

	2-CTC resin [g]	**Substitution** [mmol/g]	**Experimental mass increment of 18**[d] [g]	**Expected mass increment of 18** [g]
Batch 18B[a]	10.6	0.42[c]	1.40	7.21
Batch 18C[b]	11.1	0.57[c]	-	7.43

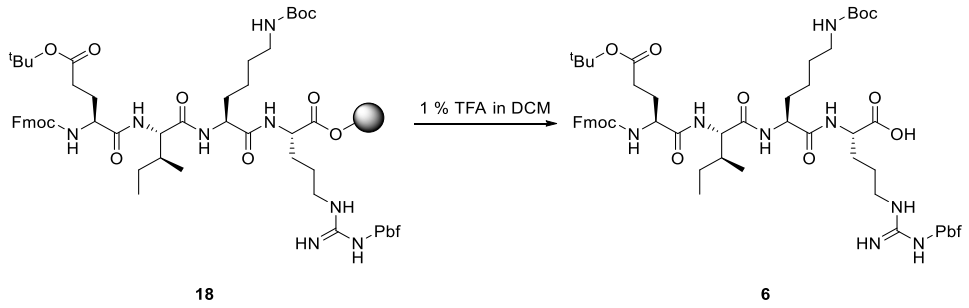
(a) Peptidyl-resin from batch 17B (table 2.1).
(b) Peptidyl-resin from batch 17C (table 2.1).
(c) Calculated following the protocol described in section 5.5.4 (Experimental Section).
(d) Estimated by washing, drying under vacuum and weighing the peptidyl-resins after the addition of the last amino acid residue.

2.2.1.3. Cleavage of the protected peptide from the resin

The peptidyl-resin coming from batch 17C was discarded due to the yield problems described in the previous section. The protected peptide was released from the peptidyl-resins coming from batches 17A and 17B by using a solution of 1 % TFA in DCM (scheme 2.4). All the peptidyl-resin coming from batch 17A was treated with 1 % TFA in DCM and afforded 2.52 g (96 %) of crude peptide with a chromatographic purity of 96 %. In the case of the peptidyl-resin from batch 17B, treatment of 3.92 g of the peptidyl-resin afforded 0.96 g of crude peptide with a chromatographic purity of 98 % (figure 2.2).

It must be pointed out that the work-up methodology was different depending on the synthesis. In the case of the peptidyl-resin coming from batch 17A, the filtrates were collected in a round-bottom flask containing Et_2O and the peptide was obtained when the suspension was transferred into a conical centrifuge tube and centrifuged. For the peptidyl-resin coming from batch 17B, the filtrates were collected in a round-bottom flask containing Et_2O and the peptide was obtained after removing TFA under reduced pressure.

The results obtained indicate that precipitation and TFA removal under vacuum could be considered good work-up alternatives in terms of the chromatographic purity of the product.



Scheme 2.4. Removal of **6** from the resin.

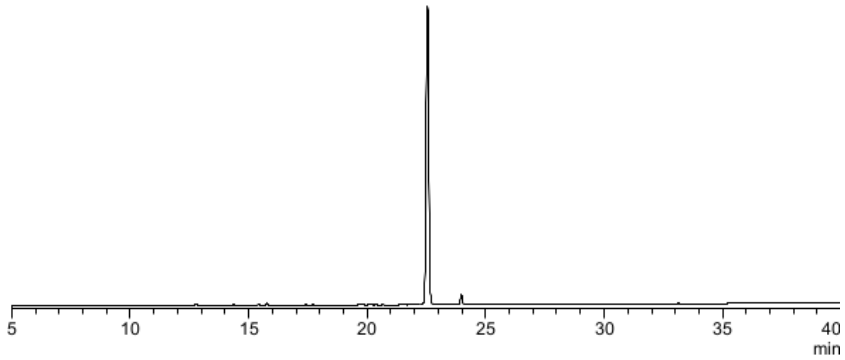
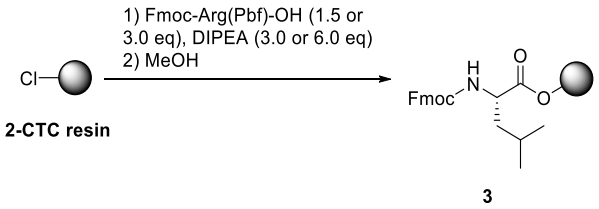


Figure 2.2. HPLC chromatogram of **6** obtained from the peptidyl-resin from batch 18B.

2.2.2. Synthesis of the pentapeptide fragment 7

2.2.2.1. Incorporation of the first amino acid

The coupling of the commercially available Fmoc-Leu-OH to the polymeric support was performed under conditions similar those used in section 2.2.1.1 (scheme 2.5).



Scheme 2.5. Coupling of Fmoc-Leu-OH to the 2-CTC resin.

The amount of amino acid that was incorporated to the resin was determined by the UV quantification at 301 nm of the piperidine adduct formed after the removal of the Fmoc group. Table 2.3 summarises the substitution values obtained in the different syntheses of aminoacyl-resin **3** using different scales and reaction times.

Table 2.3. Substitutions obtained for the coupling of the first amino acid on the 2-CTC resin in the different syntheses of **7**.

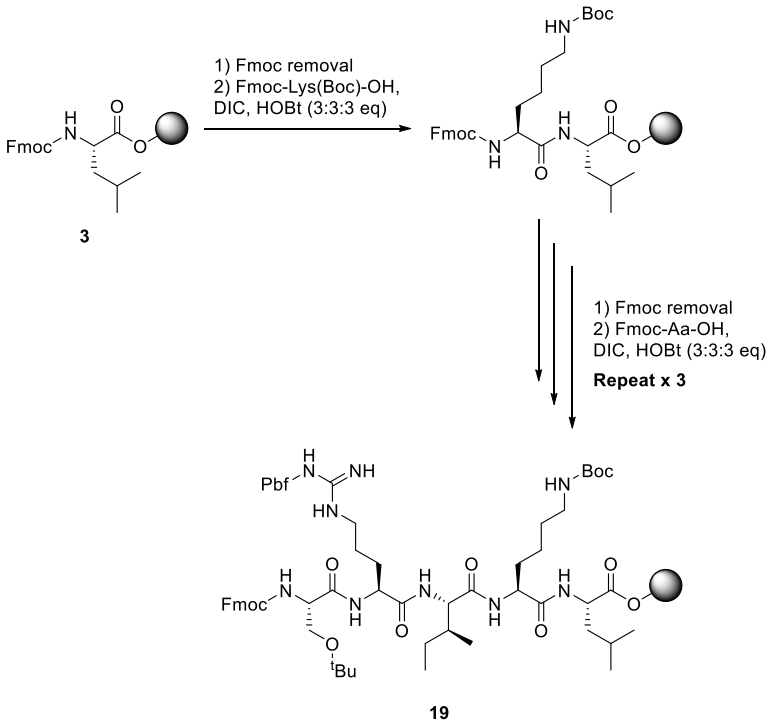
	2-CTC resin [g]	**Reaction time** [h]	**Substitution** [mmol/g]
Batch 3A	1.05	2.0	0.79[a]
Batch 3B	10.4	1.5	0.83[b]

(a) Calculated following the protocol described in section 5.5.3 (Experimental Section).
(b) Calculated following the protocol described in section 5.5.4 (Experimental Section).

The substitution values obtained for the incorporation of the first amino acid of **7** were slightly higher to those obtained for the incorporation of the first amino acid of **6** (section 2.2.1.1) even though the reaction conditions were similar. These differences might be a consequence of the amino acid residue that was coupled. In the particular case of **7**, the amino acid residue was Leu while in the case of **6**, the amino acid residue was Arg, which was side-chain protected with Pbf, a bulky protecting group that might lead to lower substitution values.

2.2.2.2. Peptide chain elongation

Two independent syntheses of **7** were performed using the batches of aminoacyl-resin described in table 2.3. The coupling reactions were carried out using the Fmoc-protected amino acid with the standard DIC/HOBt coupling system (3:3:3 eq). The Fmoc protecting group was removed by using a 20 % piperidine in DMF (scheme 2.6).



Scheme 2.6. Peptide chain elongation to obtain the peptidyl-resin **19**.

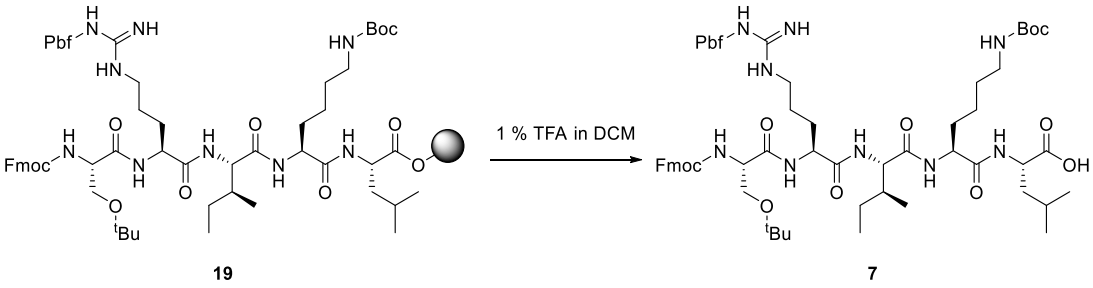
Recoupling steps were needed for some of the amino acids in the peptidyl-resin coming from batch 3A while batch 3B proceeded efficiently and no recouplings were needed (table 2.4). These differences might be a consequence of the scale of the synthesis. The peptidyl resin from batch 3A was done at the scale of 1.05 g of resin, using a polypropylene syringe with manually stirring. On the other hand, the scale of batch 3B was of 10.35 g of resin and was performed in a reactor vessel of 150 mL with mechanical stirring, which could probably increase the efficiency of the coupling reactions that resulted in a reduction of the number of the required coupling-steps.

Table 2.4. Number of couplings needed per amino acid for the syntheses of **7**.

		Number of couplings	
Entry	**Amino acid**	**Batch 3A**	**Batch 3B**
1	Fmoc-Lys(Boc)-OH	2	1
2	Fmoc-Ile-OH	1	1
3	Fmoc-Arg(Pbf)-OH	2	1
4	Fmoc-Ser(^{t}Bu)-OH	1	1

2.2.2.3. Cleavage of the protected peptide from the resin

The protected peptide was released from the peptidyl-resin coming from batches 3A and 3B by using a solution of 1 % TFA in DCM (scheme 2.7). As mentioned in section 2.2.1.3, the work-up methodology consisted of collecting the filtrates in a round-bottom flask containing Et_2O and the peptide was obtained after removing TFA under reduced pressure.



Scheme 2.7. Removal of **7** from the resin.

The whole peptidyl-resin from batch 3A was treated with the acidolytic mixture and afforded 1.45 g of crude peptide with a chromatographic purity of 94 %. In the particular case of this batch, the previously experimented premature loss of peptide chains was not detected since the expected amount of **7** was of 1.43 g.

On the other hand, the peptidyl-resin coming from batch 3B was dried under vacuum after the addition of the last amino acid of the sequence. A final mass increment of 6.60 g (5.30 mmol, 45 %) was observed. Considering that the expected mass increment on the resin was of 14.49 g, this result suggested again a premature loss of peptide chains during the synthesis when the scale was raised to 10 g of resin. After the 1 % TFA in DCM treatment of the peptidyl-resin, 6.70 g (46 %) of crude peptide were obtained as a white solid with a 98 % of chromatographic purity (figure 2.3).

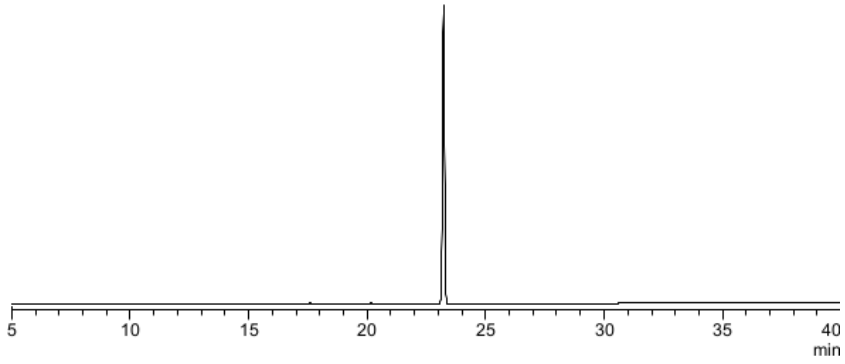
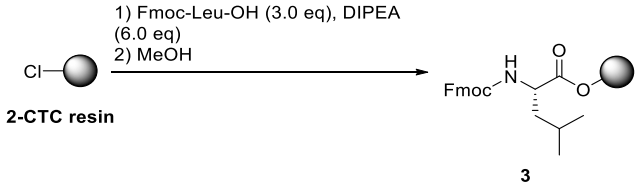


Figure 2.3. HPLC chromatogram of **7** obtained from the peptidyl-resin from batch 3B.

2.2.3. Synthesis of the hexapeptide fragment 8

2.2.3.1. Incorporation of the first amino acid

Addition of the commercially available Fmoc-Leu-OH to the polymeric support was performed under conditions similar to those used in section 2.2.1.1 (scheme 2.8).



Scheme 2.8. Incorporation of Fmoc-Leu-OH to the 2-CTC resin.

The amount of amino acid that has been incorporated to the resin was determined by the UV quantification at 301 nm of the piperidine adduct formed after the removal of the Fmoc group. Table 2.5 summarises the substitution values obtained in the different syntheses of aminoacyl-resin **3** using different scales and reaction times to obtain the intermediate **8**. Different substitution values were obtained for the two batches even though the incorporation of the first amino acid was carried out under similar conditions. As mentioned before, 2-CTC resins are very sensitive to experimental conditions, which must be controlled very carefully in order to achieve reproducible results.

Table 2.5. Substitutions obtained for the coupling of the first amino acid on the 2-CTC resin in the different syntheses of **8**.

	2-CTC resin [g]	**Reaction time** [h]	**Substitution** [mmol/g]
Batch 3C	10.1	1.5	0.79[a]
Batch 3D	10.2	1.5	0.56[a]

(a) Calculated following the protocol described in section 5.5.4 (Experimental Section).

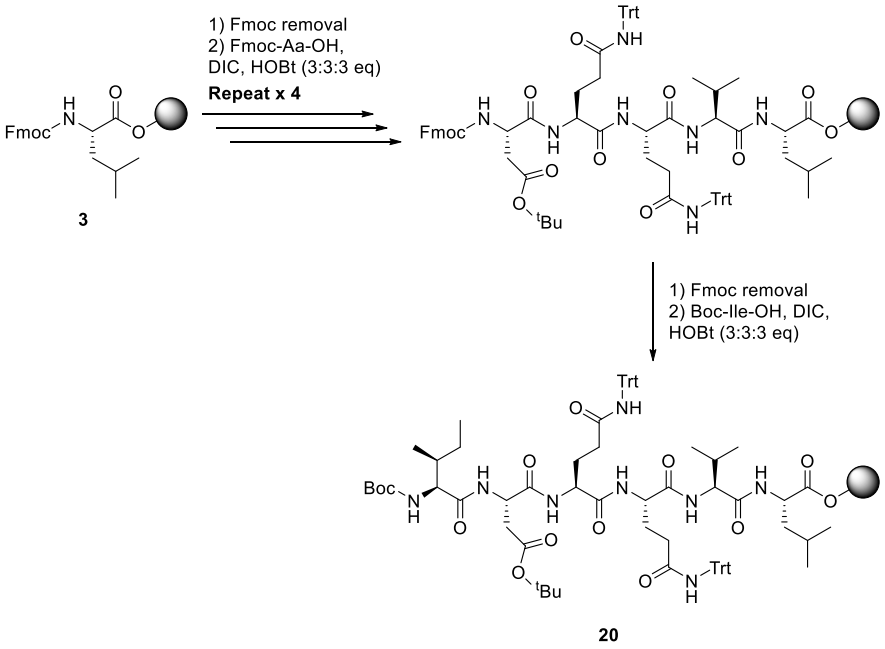
2.2.3.2. Peptide chain elongation

Two independent syntheses of **8** were carried out using the batches of aminoacyl-resin described in table 2.5. The coupling reactions were performed using the Fmoc-protected amino acid with the standard DIC/HOBt coupling system (3:3:3 eq). The Fmoc protecting group was removed by using a 20 % piperidine in DMF (scheme 2.9).

Recoupling of the fourth amino acid residue (entry 3, table 2.6) was needed in the peptidyl-resin coming from batch 3D while the peptidyl-resin from batch 3C proceeded efficiently and no recouplings were needed.

After the addition of the last amino acid residue, the peptidyl-resins from batches 3C and 3D were washed, dried under vacuum and weighed. A final mass increment of 2.14 g was observed in the peptidyl resin from batch 3C. Considering that the expected mass increment on the resin was of 15.04 g, this suggests again a premature loss of peptide chains during the synthesis. On the other hand, a final mass increment of 9.68 g was observed for the peptidyl-resin from batch 3D. The expected mass increment for this batch was of 9.74 g which means that premature loss of peptide chains during this synthesis was not experienced.

The explanation of why peptide chains were prematurely lost is yet unknown. Nonetheless, it seems that the scale of the synthesis has an impact on the premature loss of peptide-chains. Generally, these losses were experienced when the work scale was around 10 g of resin.



Scheme 2.9. Peptide chain elongation to obtain the peptidyl-resin **20**.

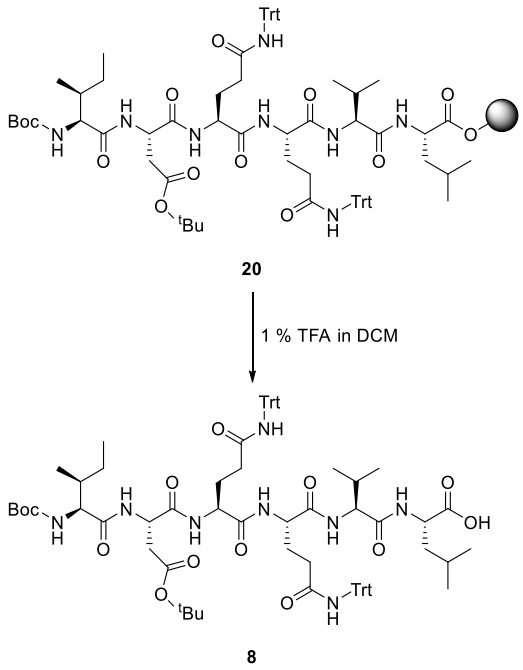
Table 2.6. Number of couplings needed per amino acid for the syntheses of **8**.

		Number of couplings	
Entry	**Amino acid**	**Batch 3C**	**Batch 3D**
1	Fmoc-Val-OH	1	1
2	Fmoc-Gln(Trt)-OH	1	1
3	Fmoc-Gln(Trt)-OH	1	2
4	Fmoc-Asp(^{t}Bu)-OH	1	1
5	Boc-Ile-OH	1	1

2.2.3.3. Cleavage of the protected peptide from the resin

The protected peptide was released from the peptidyl-resin coming from batches 3C and 3D by using a solution of 1 % TFA in DCM (scheme 2.10). As mentioned before, the work-up methodology for both syntheses consisted of collecting the filtrates in a round-bottom flask containing Et_2O and the peptide was obtained after removing TFA under reduced pressure.

After performing the cleavage of 4.23 g of the peptidyl-resin from batch 3C, 0.74 g of crude peptide were obtained as a white solid with a 73 % of chromatographic purity (figure 2.4). This chromatographic purity was considerably lower to the ones obtained in the other protected peptides. On the other hand, 64.5 mg of crude peptide were obtained as a white solid with a 91 % of chromatographic purity after the cleavage of 0.127 g of the peptidyl-resin coming from batch 3D (figure 2.4).



Scheme 2.10. Release of **8** from the resin.

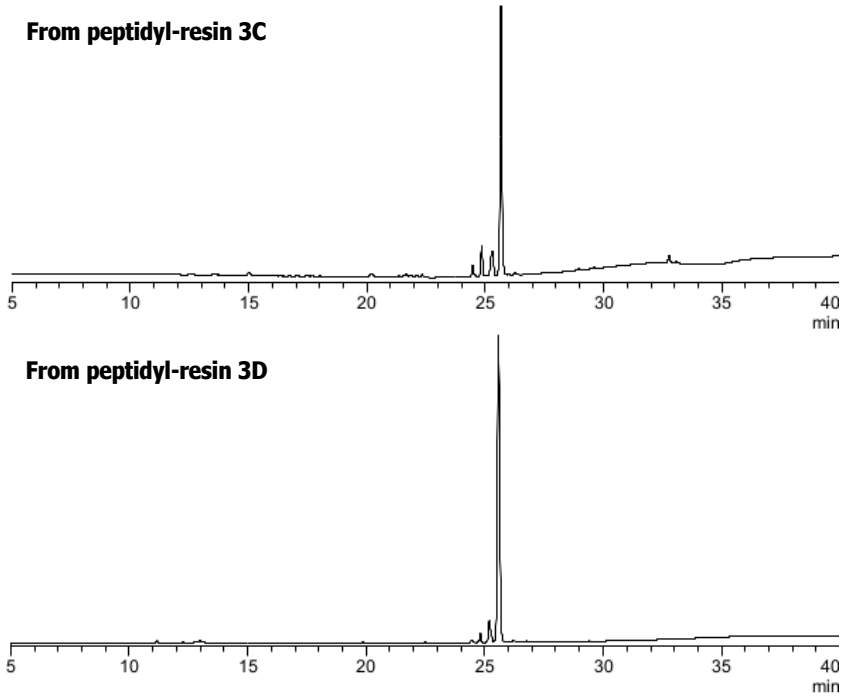
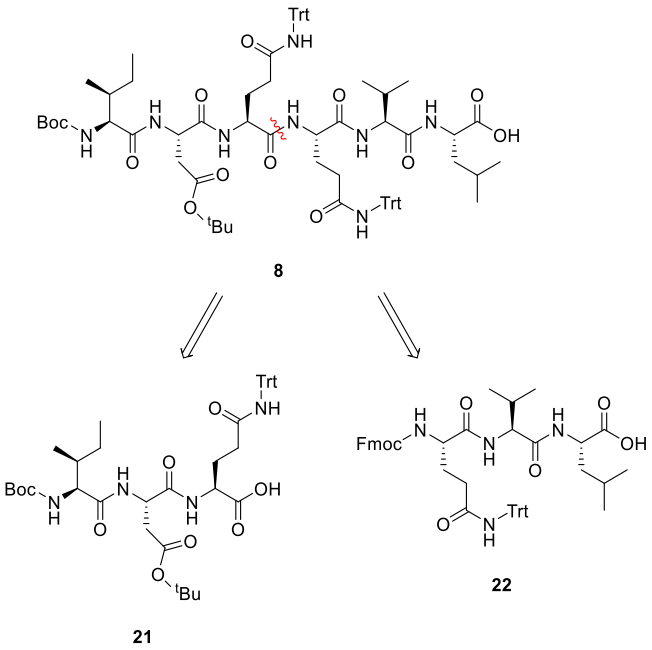


Figure 2.4. HPLC chromatograms of **8**.

The chromatograms of figure 2.4 show some impurities at retention times of 24-25 min, which are lower than the retention time of **8**, and with m/z values difficult to explain. The presence of these peaks led us think in the possibility of side reactions that occurred during the syntheses. In order to get insight into that, the peptide sequence **8** was split off in two three-amino acid fragments (scheme 2.11).



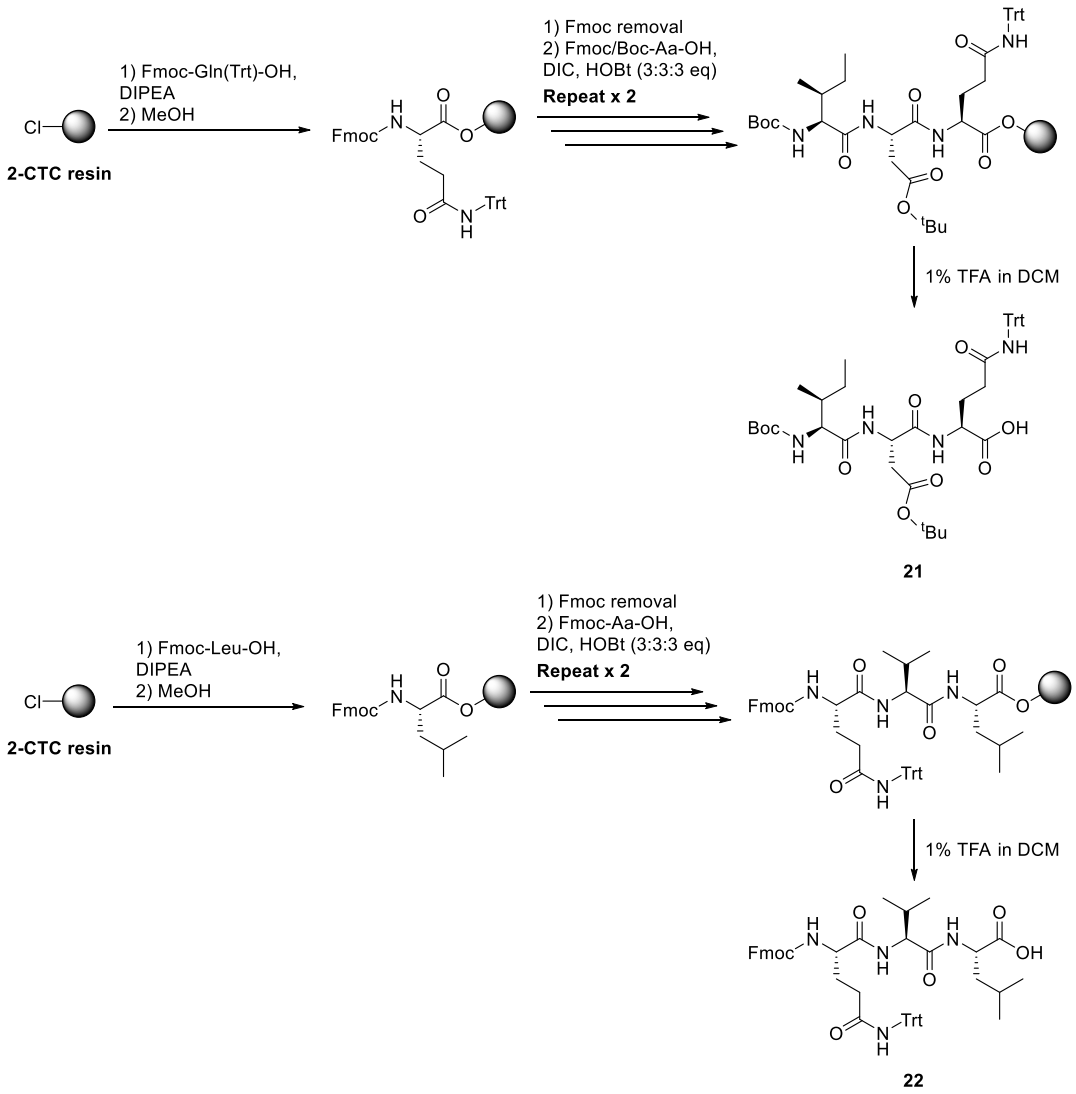
Scheme 2.11. Fragmentation of the protected **8** into protected tripeptides **21** and **22**.

The corresponding protected tripeptides, **21** and **22**, were synthesised following the coupling conditions used for the preparation of the peptidyl-resins coming from batches 3C and 3D (scheme 2.12).

The HPLC chromatograms of the tripeptide **21** and tripeptide **22** showed that the former presents an impurity profile similar to those obtained in the peptide crudes resulting from the peptidyl-resins from batches 3C and 3D (figure 2.5).

At this point, it was decided to analyse by HPLC and NMR of the commercial amino acid derivatives that were used for the synthesis of the tripeptide **21**. The HPLC analysis and the NMR spectrum of Boc-Ile-OH showed some small impurities (figure 2.6).

For this reason, a small-scale synthesis of **8** using a new batch of this amino acid derivative, whose purity was previously verified by NMR was carried out under similar conditions to those mentioned in sections 2.2.3.1 and 2.2.3.2; in this case, the target peptide **8** was obtained with a chromatographic purity of 97 % (figure 2.7). These results suggest that the origin of the by-products shown in figure 2.4 came most probably from the impurities detected in the amino acid derivative Boc-Ile-OH.



Scheme 2.12. Synthesis of **21** and **22**.

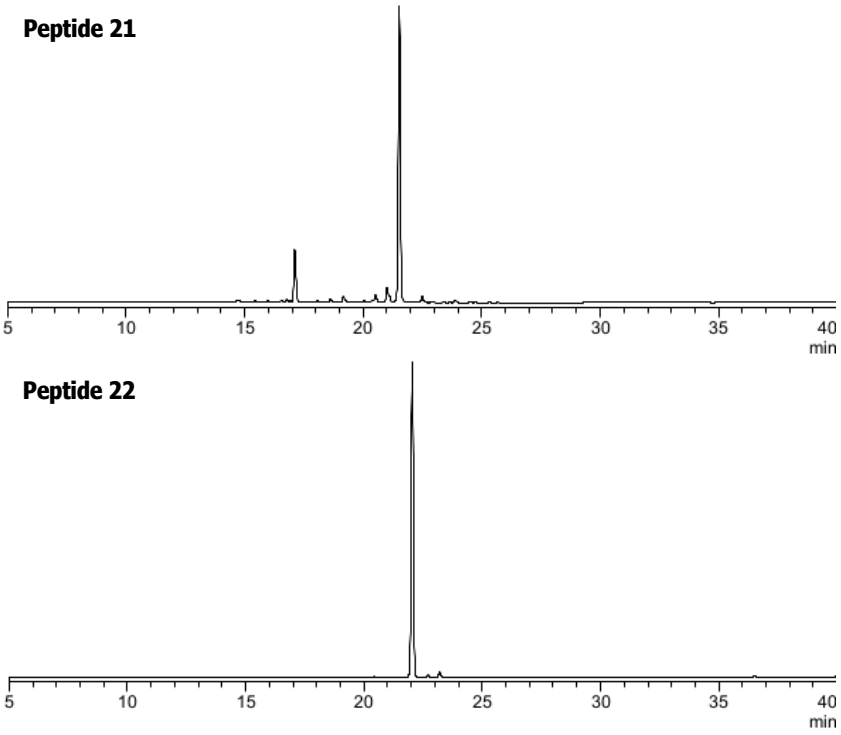


Figure 2.5. HPLC chromatograms of the crude peptides **21** and **22**.

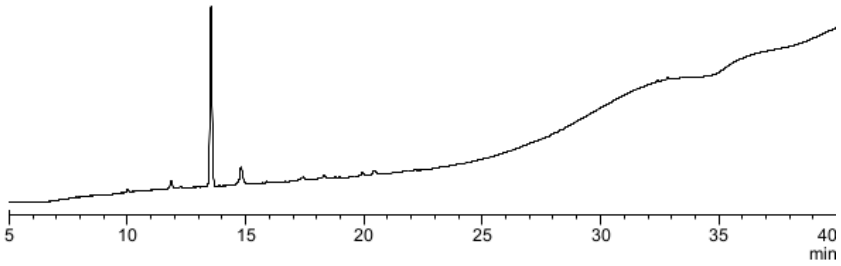


Figure 2.6. HPLC chromatogram of the amino acid derivative Boc-Ile-OH.

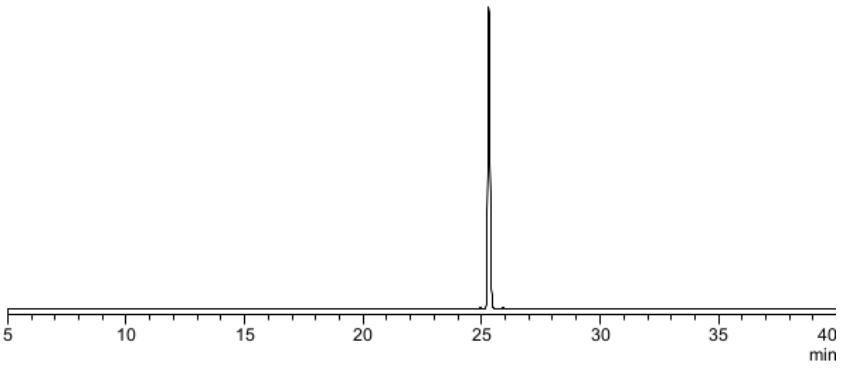


Figure 2.7. HPLC chromatogram of the crude peptides **8** using a new batch of amino acid derivative.

2.3. Condensation of the protected peptide fragments in solution

2.3.1. Optimisation of the synthesis of the dipeptide 5

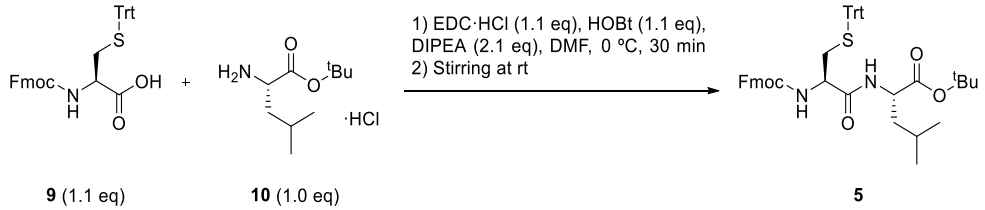
As mentioned in the introduction, the preparation of peptides requires coupling reagents that can be very sophisticated and expensive. In our case, we were interested in finding effective, economic and scalable conditions that do not lead to racemisation. Thus, we thought to use EDC as a coupling reagent and HOBt or DMAP as additives.

Even though EDC is more expensive than other carbodiimides, the corresponding urea is highly soluble in water, which is an advantage for the work-up steps. In our case, this was an important factor because we wanted to extend these conditions to the coupling of longer peptide fragments, in which the work-ups are more challenging.

In the next sections, we describe the assays that have been performed for the preparation of the dipeptide **5**. In all cases, the dipeptide was synthesized in solution and Fmoc/^{t}Bu protection scheme was choosen because it requires milder conditions than Boc/Bzl strategy (section 1.1.1.2, chapter 1). The product was analyzed by HPLC and its chromatographic purity was obtained by integration of the peak areas at 220 nm.

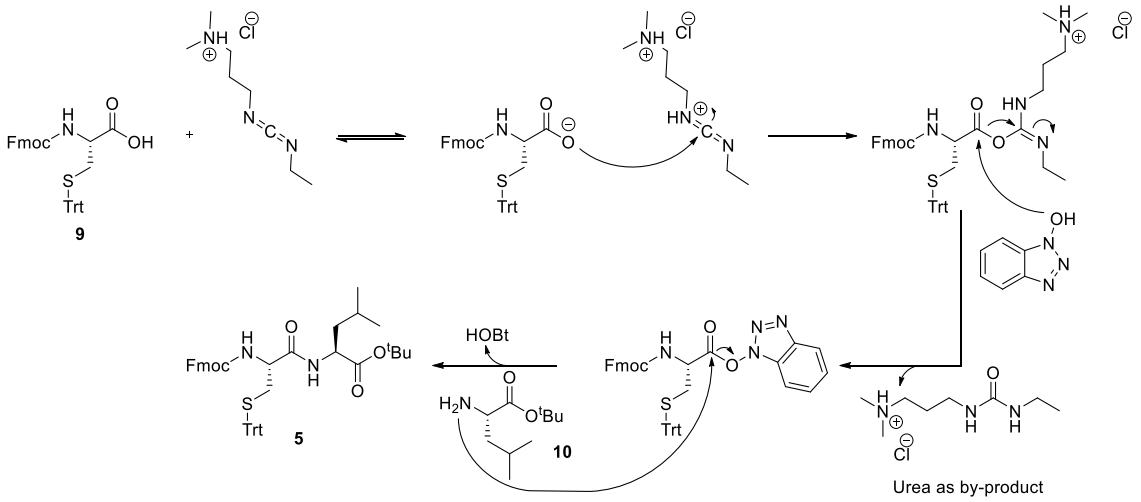
2.3.1.1. Using EDC·HCl, HOBt and DIPEA

The first synthesis of the dipeptide **5** was carried out using 1.1 eq of Fmoc-Cys(Trt)-OH (**9**), 1.0 eq of H_2N-Leu-O^{t}Bu·HCl (**10**), 1.1 eq EDC·HCl and 1.1 eq of HOBt at the scale of 0.25 mmol of **10**. We decided to add 2.1 eq of DIPEA because the leucine residue was in the hydrochloride form (scheme 2.13).



Scheme 2.13. Synthesis of **5** using EDC·HCl, HOBt and DIPEA.

Scheme 2.14 describes the mechanism of action of the coupling agent EDC·HCl in the presence of HOBt as additive.



Scheme 2.14. Mechanism of the formation of **5** using EDC·HCl and HOBt.

The HPLC chromatogram showed a 35 % of **5**, a 20 % of the amino acid derivative **9**, an 11 % of the N-terminal deprotected form of **5** (**11**, figure 2.8) and 25 % of DBF (figure 2.8), which was formed as a consequence of the partial removal of the Fmoc protecting group.

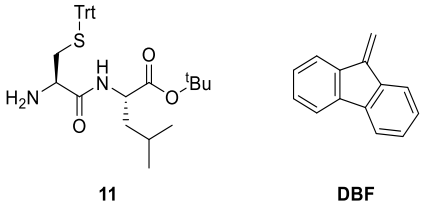
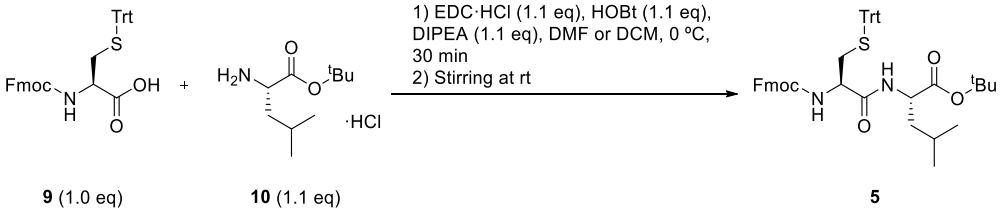


Figure 2.8. N-terminal deprotected form of **5** and DBF.

The premature release of the Fmoc group moved us to lower the amount of base. Moreover, due to the poor UV detection of **10**, we decided to use the **9** as the limiting reagent in order to quantify its consumption by HPLC. Thus, new assays of synthesis of **5** were carried out using 1.0 eq of **9**, 1.1 eq of **10**, 1.1 eq EDC·HCl, 1.1 eq of HOBt and 1.1 eq of DIPEA (scheme 2.15). Two solvents were tested (DMF and DCM) and chromatographic analyses were performed at different reaction times (table 2.7).



Scheme 2.15. Synthesis of **5** using EDC·HCl, HOBt and DIPEA.

The first reaction was performed using the conditions described above and the results are given after 17 h (entry 1, table 2.7). The HPLC chromatogram of the first experiment showed a 59 % of the dipeptide **5** and a 41 % of the amino acid derivative **9**. It was then decided to execute two additional experiments under the same reaction conditions but increasing the reaction times to 24 h and 48 h (entries 3 and 4, table 2.7) to see if conversion to **5** was improved with time. However, the HPLC analysis revealed a reduction of the conversion to **5** (30 % and 10 % respectively) together with an increment of **11** and other impurities such

as DBF, which indicated a low stability of the target peptide under the experimental conditions that were used.

Table 2.7. Reactions using the coupling system EDC·HCl/HOBt with DIPEA.

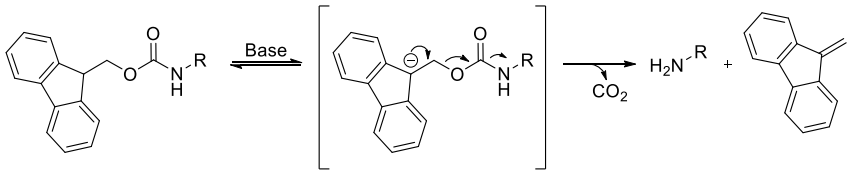
Entry[a]	Solvent	Time [h]	Chromatographic purities[b] [%]		
			5	9	Other
1	DMF	17	59	41	0
2	DMF	24	30	9	52
3	DMF	48	10	2	70
4	DCM	17	93	7	0
5	DCM	24	96	4	0
6	DCM	48	93	7	0

(a) All the reactions were carried out using 0.25 mmol (146.3 mg) of **9**.

(b) The percentages were obtained by integrating the peak areas at 220 nm.

At this point, we decided to change the solvent from DMF to DCM. Three reactions of 17 h, 24 h and 48 h were carried out in this solvent under the same conditions (entries 4, 5 and 6, table 2.7). The HPLC chromatograms showed a 93 %, 96 % and 93 % of **5** and a 7 %, 4 % and 7 % of the amino acid derivative **9**, respectively. Other significant impurities were not detected.

Scheme 2.16 shows the mechanism for the release of Fmoc mediated by a base. It is interesting to note that there was no evidence of Fmoc removal when the reaction was done in DCM, suggesting that the solvent plays an important role in the process. DMF is a polar aprotic solvent that solvates charged species efficiently, which could favour the formation of the intermediate species generated during the Fmoc removal.



Scheme 2.16. Mechanism for the Fmoc protecting group removal.

The Fmoc group is generally released with secondary amines such as piperidine, which present high nucleophilicity and behave like a scavenger of the resultant DBF (section 1.1.1.2.2). Nonetheless, for the reactions corresponding to entries 2, 3, and 4 of table 2.7, Fmoc was removed in the presence of DIPEA, a tertiary amine which is non-nucleophilic and thus, does not react with DBF.

Figure 2.9 shows the differences encountered in the chromatographic profiles when the reaction was carried out in DMF or in DCM.

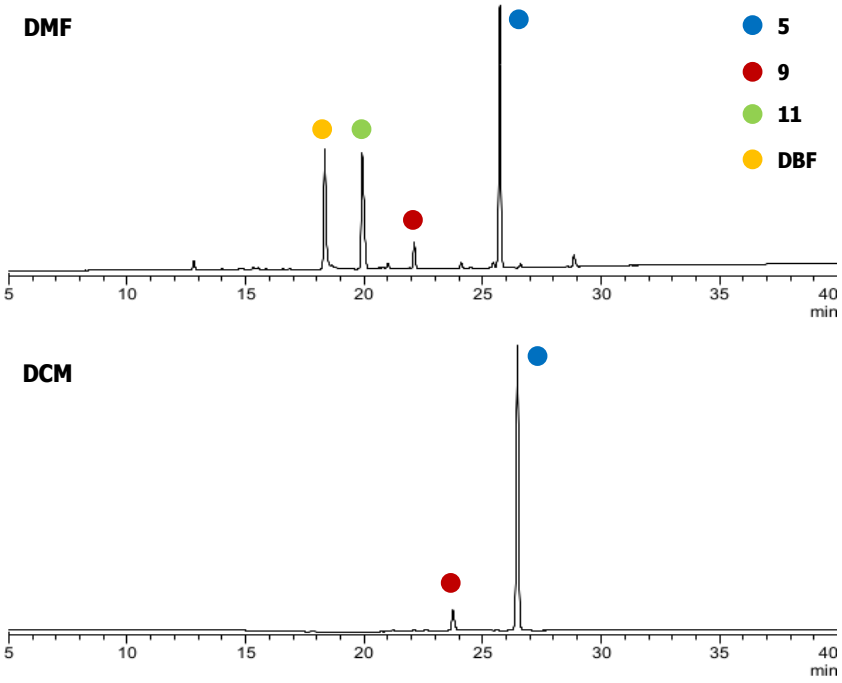
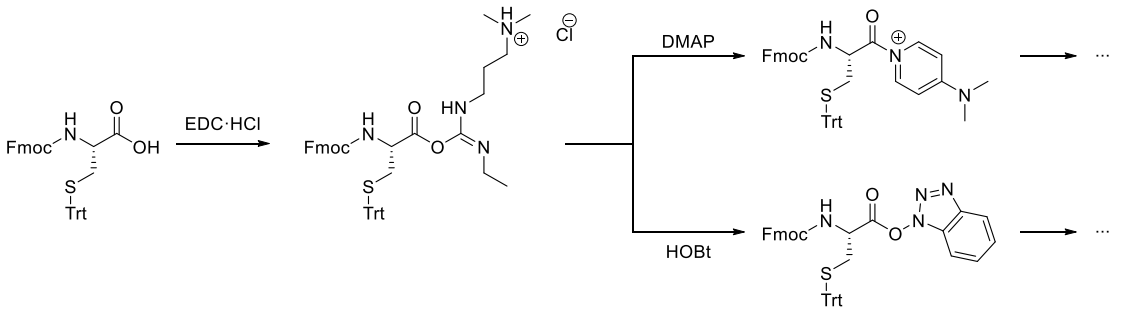


Figure 2.9. HPLC chromatograms of entries 2 and 5, table 2.7.

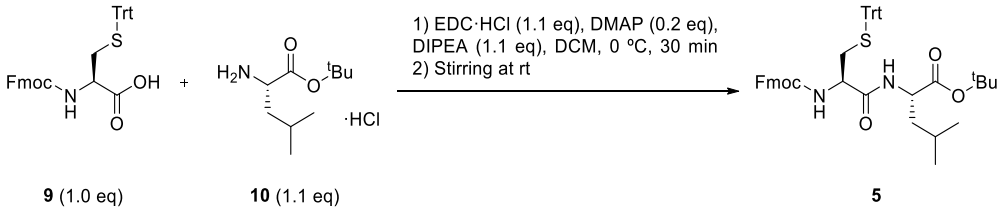
2.3.1.2. Using EDC·HCl, DMAP and DIPEA

4-Dimethylaminopyridine (DMAP) is a widely used reagent in the field of organic synthesis as a nucleophilic catalyst. It was found to be useful in the enhancement of peptide coupling reactions mediated by carbodiimides and symmetrical anhydrides.[111] Accordingly, it can be used as an additive to replace HOBt as they present similar mechanisms of action (scheme 2.17).



Scheme 2.17. Active esters generated from DMAP and HOBt.

The main drawback of DMAP as an additive is that it strongly catalyses the formation of the oxazolone intermediate that leads to racemisation in the presence of tertiary amines.[112] For this reason, DMAP was used in catalytic quantities to study its viability as an additive to improve the amide formation (scheme 2.18, table 2.8).



9 (1.0 eq) **10** (1.1 eq) **5**

Scheme 2.18. Synthesis of **5** using EDC·HCl, DMAP and DIPEA.

Table 2.8. Reactions using the coupling system EDC·HCl/DMAP with DIPEA.

Entry[a]	**Time** [h]	**Chromatographic purities**[b] [%]		
		5	**9**	**Other**
1	17	30	22	41
2	24	24	24	34
3	48	8	14	61
4[c]	17	52	-	44

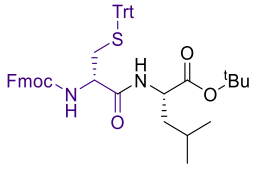
(a) All the reactions were carried out using 0.25 mmol (146.3 mg) of **9**.
(b) The percentages were obtained by integrating the peak areas at 220 nm.
(c) 0.4 eq of EDC·HCl were added after 3 h of reaction time.

Nonetheless, the HPLC chromatogram of the first experiment (table 2.8) showed no improvement in the conversion to the desired product **5** together with the detection of **11** and DBF. The main difference between the first and the latter reaction was that the amino acid derivative **9** was not detected in one case (entry 4, table 2.8) while a 22 % of this reagent was observed in the other case (entry 1, table 2.8). This result could be a consequence of the addition of 0.4 equivalents of EDC·HCl after 3 h of reaction in the case of entry 1, when the in-process control at that time showed the presence of **9**.

When the reaction time was increased to 24 h and 48 h, the HPLC analysis showed a decrease of the conversion to **5** (34 % and 8% respectively) and an increment of the amounts of **11** (23 % and 33 %, respectively) and DBF (11 % and 25 %, respectively). This behaviour was similar to the observed when the reaction was done in DMF and using HOBt as additive.

It is worth mentioning that the N-terminal deprotected form of **5** is detected even though the reactions were performed using DCM as solvent. This result could be explained by the use of DMAP instead of HOBt as additive. The low stability of the Fmoc group could be a consequence of the increased basicity of the reaction media when using DMAP (pK_a of 9.6), which favours the removal of the protecting group.

This increased basicity could be also responsible for the detection of a 2 % of the epimer of **5** (**23**, figure 2.10) in all the reactions performed under these conditions. Figure 2.11 shows an example of the trends observed in the chromatographic analysis of the reactions described in table 2.8.



23

Figure 2.10. Epimer of **5** (**23**).

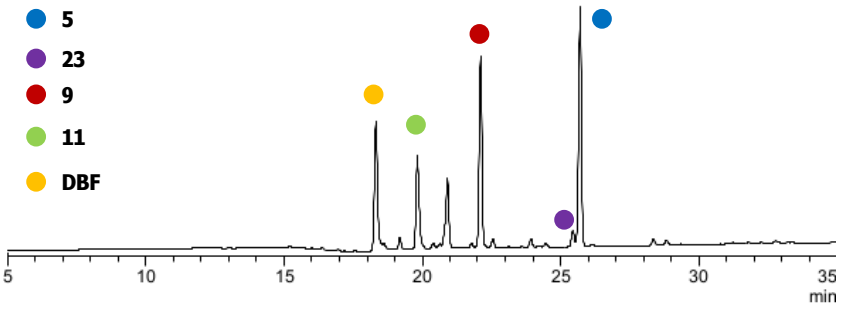
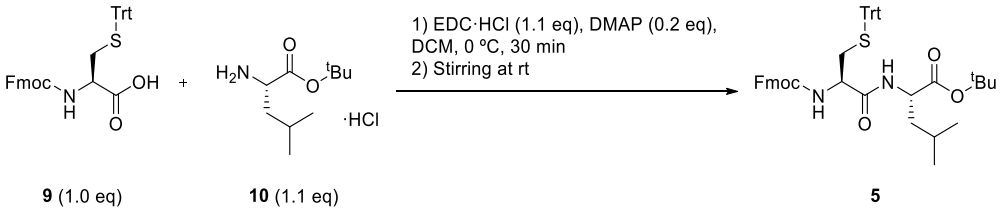


Figure 2.11. HPLC chromatogram of entry 1, table 2.8.

2.3.1.3. Using EDC·HCl and DMAP

The non-promising results obtained under the conditions described in the previous section could be attributed to the basic conditions of the reaction. For this reason, we carried out a different batch of reactions in which DIPEA was not used (scheme 2.19, table 2.9).



Scheme 2.19. Synthesis of **5** using EDC·HCl and DMAP.

Table 2.9. Reactions using the coupling system EDC·HCl/DMAP.

Entry[a]	**Time** [h]	**Chromatographic purities**[b] [%]		
		5	**9**	**Other**
1	17	78	16	6
2	24	76	16	8
3	48	76	17	6

(a) All the reactions were carried out using 0.25 mmol (146.3 mg) of **9**.
(b) The percentages were obtained by integrating the peak areas at 220 nm.

An improvement in the chromatographic purity of **5** was observed (table 2.9) when compared to those obtained when DIPEA was used (table 2.8). All reactions showed 76 % to 78 % of **5**, and 16% to 17 % of starting material **9** (table 2.9, figure 2.12 in the case of entry 1). **11** and the DBF were not detected in these reactions but an unknown impurity with an m/z of 631.3 was detected in a 6 % to 8 % of peak area.

Then, a second batch of reactions modifying the equivalents of EDC·HCl or DMAP was carried out in order to improve the yield of the target peptide. Table 2.10 summarizes the results obtained.

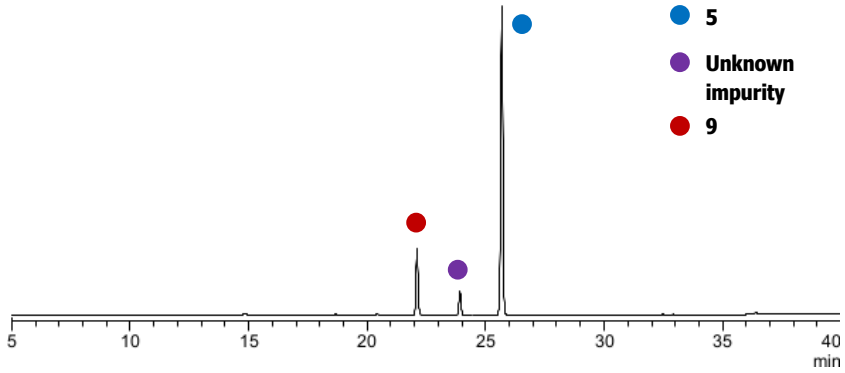


Figure 2.12. HPLC chromatogram of the crude of **5** obtained in the reaction of entry 1, table 2.9.

Table 2.10. Reactions with change in the EDC·HCl and DMAP equivalents.

Entry[a]	**EDC·HCl** [eq]	**DMAP** [eq]	**Chromatographic purities**[b] [%]		
			5	**9**	**Other**
1	1.1	0.4	71	15	14
2	1.1	1.0	84	0	15
3	1.5	0.2	92	0	8
4	1.1 + 0.4[c]	0.2	87	3	9

(a) All the reactions were carried out using 0.25 mmol (146.3 mg) of **9**.
(b) The percentages were obtained by integrating the peak areas at 220 nm.
(c) Added after 17 h.

Entries 1 and 2 (table 2.10, figure 2.13 (A) in the case of entry 2) correspond to those reactions in which the equivalents of DMAP were increased. When 0.4 equivalents of DMAP were used, the HPLC chromatogram showed a 71 % of **5** and a 15 % of starting material **9**. On the other hand, using a stoichiometric amount of DMAP (entry 2) led to an 84 % of **5** and starting material **9** was not detected. In both reactions was detected a rise of the unknown impurity (m/z of 631.3) to 14 % and 15 %, respectively. Moreover **11** and DBF were observed in a 1 % and 2 % respectively, in the reaction in which the equivalents of DMAP was stoichiometric (entry 2, table 2.10).

Entries 3 and 4 (table 2.10, figure 2.13 (B) in the case of entry 3) correspond to those reactions in which the equivalents of EDC·HCl were incremented. The HPLC chromatogram revealed a 87 % of dipeptide **5** when 1.5 eq were added from the beginning (entry 3), and the amount of desired product raised to a 92 % when the reaction was repeated under similar conditions but adding 0.4 equivalents of EDC·HCl after 17 h (entry 4). No starting material was observed in the former but a 3 % was detected in the latter. Moreover, the unknown impurity (m/z of 631.3) was also detected (6 % and 7 %, respectively).

Figure 2.13 compares the different chromatographic purities obtained when the reaction was carried out under different EDC·HCl and DMAP conditions.

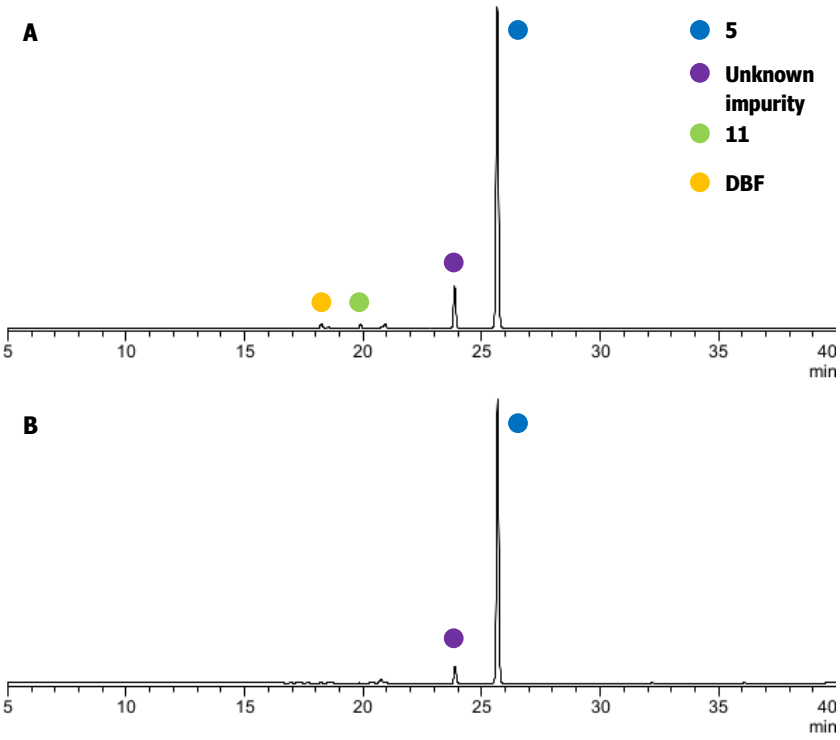
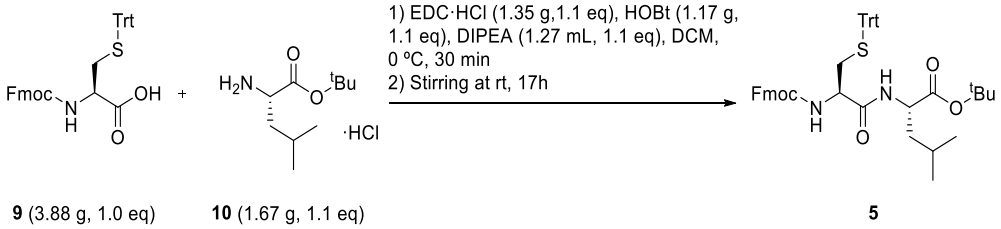


Figure 2.13. HPLC chromatogram of the crude of **5** obtained in the reactions of entries 2 (A) and 3 (B), table 2.10.

2.3.1.4. Synthesis of 5 at 5 g scale

Among all the tested conditions, the coupling system EDC·HCl/HOBt with DIPEA and DCM as solvent (section 2.3.1.1, entry 5, table 2.7) proved to be more successful for the synthesis of the desired dipeptide **5** and were the chosen conditions for scaling-up the reaction. Thus, as shown in scheme 2.20, reaction of 3.88 g of **9** with 1.67 g of **10** lead to 5.12 g of **5** (batch 5A) with a chromatographic purity of 98 % (figure 2.14).



9 (3.88 g, 1.0 eq) **10** (1.67 g, 1.1 eq) **5**

Scheme 2.20. Synthesis of **5** at 5 g scale.

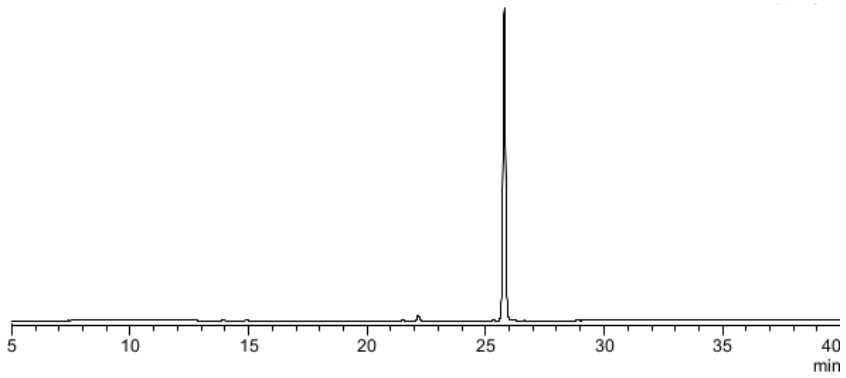
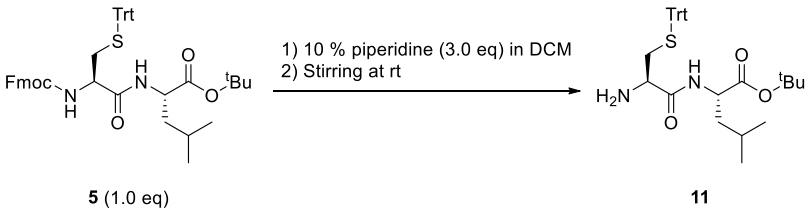


Figure 2.14. HPLC chromatogram of the scaled-up reaction of **5** (batch 5A).

Nonetheless, the result obtained in the reaction in which EDC·HCl and catalytic DMAP (entry 3, table 2.10) were used as coupling system might be an alternative since the desired dipeptide **5** was obtained with a chromatographic purity of 92 %.

2.3.2. Fmoc removal of the dipeptide 5

Once the dipeptide **5** was synthesised, the next step was the removal of the Fmoc group at the N^{α} position to obtain **11**. The reaction was carried out using a solution of 10 % piperidine in DCM (scheme 2.21).



Scheme 2.21. Removal of the N-terminal protecting group of **5**.

The use of secondary amines such as piperidine for removing the Fmoc group leads to the formation of an adduct between the by-product DBF and the secondary amine, that behaves like a scavenger of the by-product due to its high nucleophilicity (see scheme 1.7, section 1.1.1.2.2).

The reaction was monitored by HPLC and no starting material **5** was detected after 2.5 h. The HPLC analysis of the crude showed the presence of the DBF and the DBF adduct with the piperidine. The crude was purified by flash column chromatography (Hexanes/EtOAc 8:2 and DCM/MeOH 9:1) to afford 2.58 g (95 %) **11** (batch 11B) with a chromatographic purity of 99 % (figure 2.15).

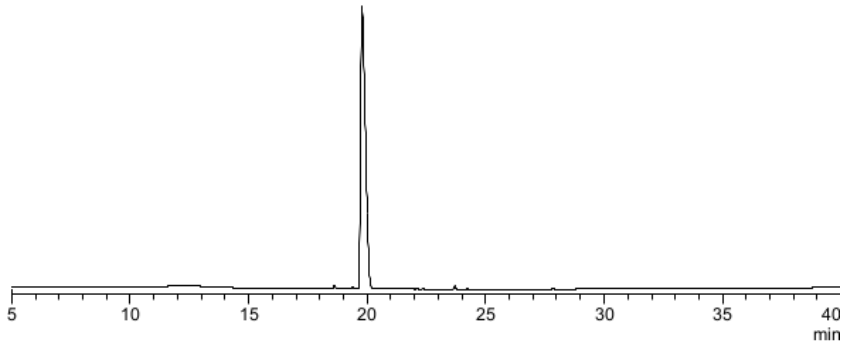
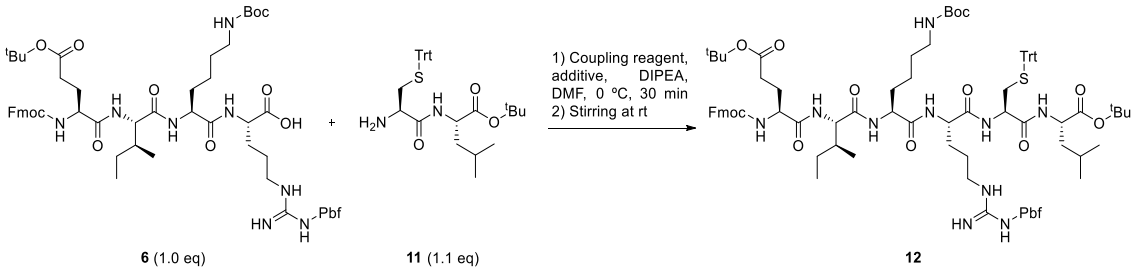


Figure 2.15. HPLC chromatogram of the purified **11** (batch 11B).

2.3.3. Synthesis of the hexapeptide 12

2.3.3.1. Using EDC·HCl as the coupling agent

One of the objectives of the present work was to find the optimal conditions for the synthesis of the dipeptide **5** in solution that could be extended to the coupling of longer peptide fragments. In this section, we discuss the preparation of the hexapeptide **12** using some of the conditions evaluated for the synthesis of the dipeptide **5** (scheme 2.22, table 2.11).



Scheme 2.22. General scheme for the synthesis of **12**.

Table 2.11. Experimental conditions used for the synthesis of **12**.

Entry	EDC·HCl [eq]	Additive [eq]	DIPEA [eq]	Time[c] [h]	Chromatographic purities[d] [%]			
					12	**6**	**24**	**Other**
1[a,b]	1.1	HOBt [1.1]	1.1	8	56	12	3	24
2[a,b]	1.1	DMAP [1.0]	-	6	44	5	27	21
3[a,b]	1.5	DMAP [0.2]	-	6	35	25	18	15

(a) The reactions were carried out using 0.26 mmol (300.9 mg) of **6**.
(b) Starting materials **6** and **11** arise from batch 18A and 11B respectively
(c) All the reaction times were determined monitoring the crude by HPLC.
(d) The percentages were obtained by integrating the HPLC peaks at 220 nm.

The first experiment was carried out using the most favorable conditions found for the synthesis of the dipeptide **5** (entry 1, table 2.11, figure 2.17). After 8 h, the reaction did not evolve further, leading to a crude containing only a 56 % of hexapeptide **12**. It is worth mentioning that a peak with the same mass of **12** was detected (3 %), which was assigned to the epimer **24**, shown in figure 2.16. Moreover, the obtained crude contained a 12 % of

starting material **6**, a 9 % of starting material **11** and a 15 % of other impurities. Some of these impurities correspond to the premature removal of the Fmoc group of the desired product **12**. Moreover, DBF was detected in a 6 % and the N-terminal deprotected form of **12** (**13**, figure 2.16) in an 8 %.

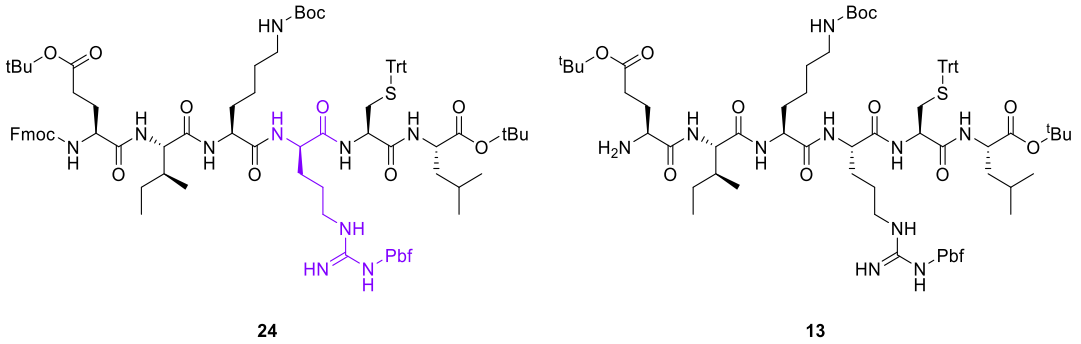


Figure 2.16. Epimer and N-terminal deprotected form of **12**.

HOBt was replaced for DMAP (entries 2 and 3, table 2.11, figure 2.17 in the case of entry 2) to improve the conversion of the reaction but the results were not outstanding. Thus, the conversion of **12** descended to 44 % (entry 2) and 35 % (entry 3) and the formation of the undesired epimer **24** increased significantly to 27 % and 18 %, respectively. Furthermore, a 5 % of starting material **6** and a 11 % of starting material **11** remained unreacted when the reaction was carried out in the presence of a stoichiometric quantity of DMAP (entry 2) and, when the amount of additive was lowered (entry 3), the percentages were 25 % and 15 % respectively.

For the reaction in which a stoichiometric amount of DMAP was used (entry 2), **13** was again detected in a 4 % and therefore, DBF was present in a 3 %. On the other hand, when a catalytic amount of DMAP was used (entry 3), **13** was not detected. As mentioned in section 2.3.1, this might be as a result of the increased basicity of the reaction when using DMAP, which favours the elimination of the Fmoc group when it is used in stoichiometric amounts.

The differences encountered in the formation of the epimer **24** in the presence of DMAP could be explained according to the amount of additive that was used in the reaction (stoichiometric, entry 2 or catalytic, entry 3). The highest amount of epimer was found when a stoichiometric amount of DMAP was used.

Figure 2.17 shows the differences detected in the chromatographic purity when the reaction was carried out using HOBt or DMAP.

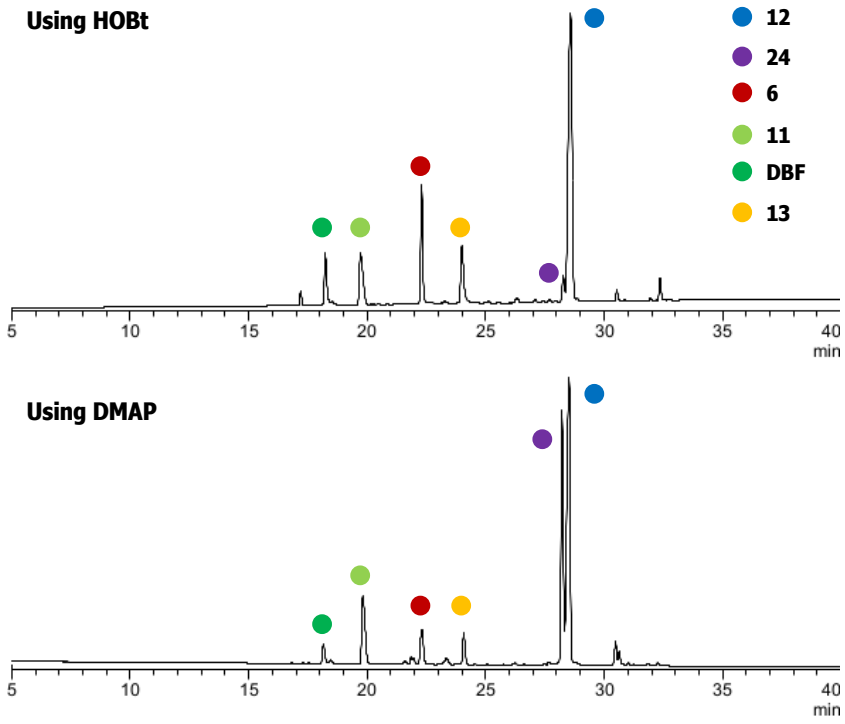
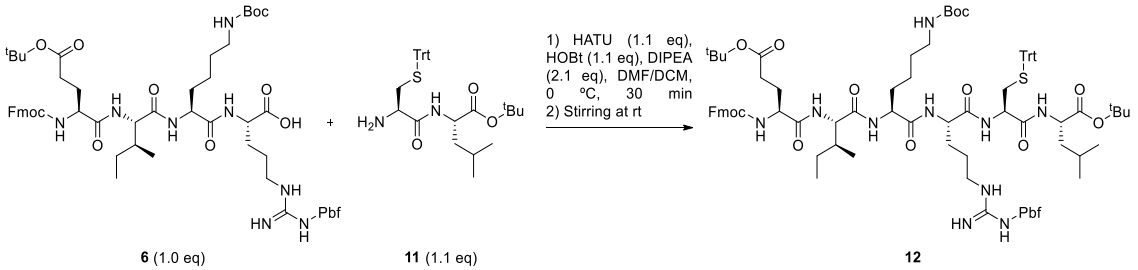


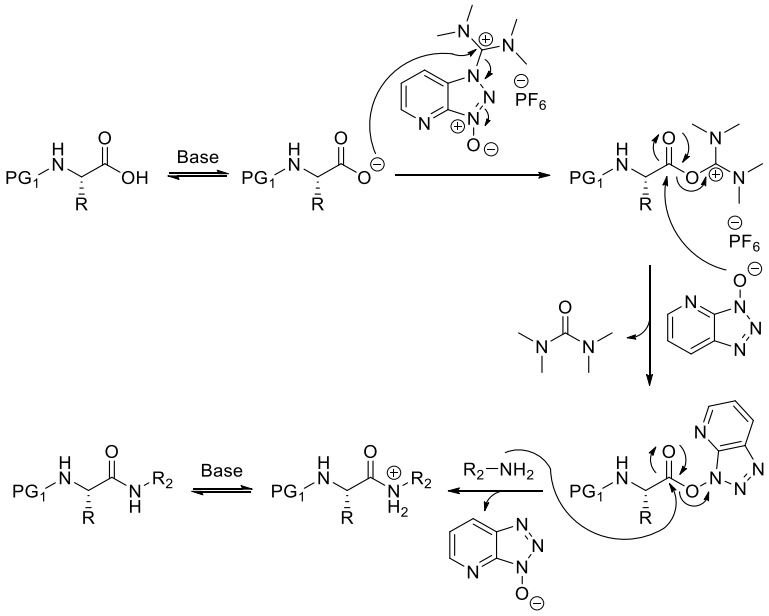
Figure 2.17. HPLC chromatograms of the synthesis of **12** using HOBt or DMAP (table 2.11).

2.3.3.2. Using HATU, HOBt and DIPEA

The previous results suggest that the conditions optimised for the synthesis of **5** does not suffice when applied to the coupling of large peptide fragments. Fortunately, substitution of EDC·HCl for HATU as the coupling agent improved the conversion to **12**. The reactions were performed using 1.1 eq of HATU, 1.1 HOBt and 2.1 eq of DIPEA (scheme 2.23, table 2.12). Scheme 2.24 describes the mechanism of action of HATU as coupling reagent. Activation of the carboxyl group takes place through reaction of the corresponding carboxylate with the coupling agent. Further reaction of the resulting intermediate with the hydroxybenzotriazolate anion gives the corresponding active ester that finally reacts with the N-terminal of the amino acid or the protected peptide to form the amide bond.



Scheme 2.23. Synthesis of **12** using HATU, HOBt and DIPEA.



Scheme 2.24. Mechanism of HATU as coupling reagent.

Table 2.12. Synthesis of **12** using HATU as the coupling agent.

	Solvent	**Time**[c] [h]	**Crude yield** [%]	**Chromatographic purities**[e] [%]			
				12	**6**	**24**	**Other**
Batch 12A[a]	DMF	6	>100[d]	88	-	-	9
Batch 12B[b]	DCM	o/n	>100[d]	93	1	-	6

(a) The reactions were carried out using 0.94 mmol (1.09 g) of **6**.
(b) The reactions were carried out using 0.51 mmol (0.60 g) of **6**.
(c) All the reaction times were determined monitoring the crude by HPLC.
(d) The crude yields were above 100 % (102 and 105 % respectively) most probably because the resulting solid was not completely dried when weighed.
(e) The percentages were obtained by integrating the HPLC peaks at 220 nm.

The first experiment was carried out using DMF as solvent (batch 12A, table 2.12). The HPLC chromatogram of the reaction crude showed an 88 % of hexapeptide **12** together with a 5 % of starting material **11** and 4 % of other impurities. It is worth mentioning that the epimer **24** was not detected.

When DMF was substituted by DCM (batch 12B, table 2.12, figure 2.18), the reaction was left stirring overnight due to the lower solubility of the reagents and to favour the completion of the reaction. Now, the HPLC chromatogram of the reaction crude showed a 93 % of target peptide (figure 2.18).

These results confirm that the optimal conditions found for the synthesis of the dipeptide **5** are not appropriate for the coupling of larger peptide fragments. The use of more efficient coupling reagents such as HATU lead to better results. Moreover, the presence of the epimer **24** could be explained by the fact that a protected peptide is more prone to empimerise during its activation due to the presence of an amide bond between the C-terminal residue and the next amino acid in the sequence, unlike a protected amino acid where the N^{α} is protected through a carbamate bond.

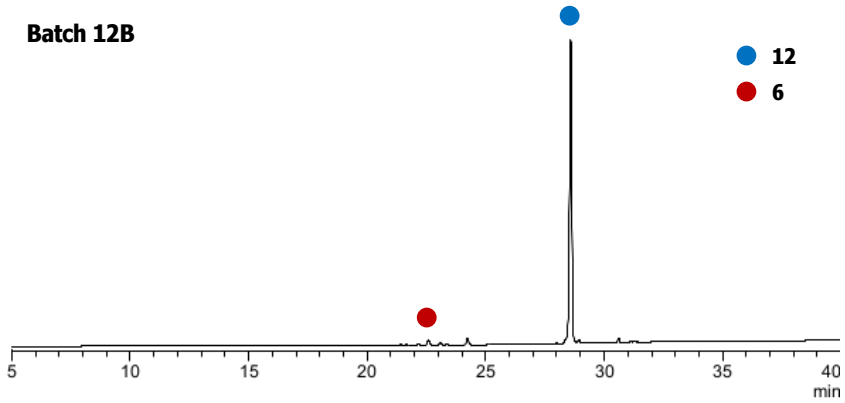
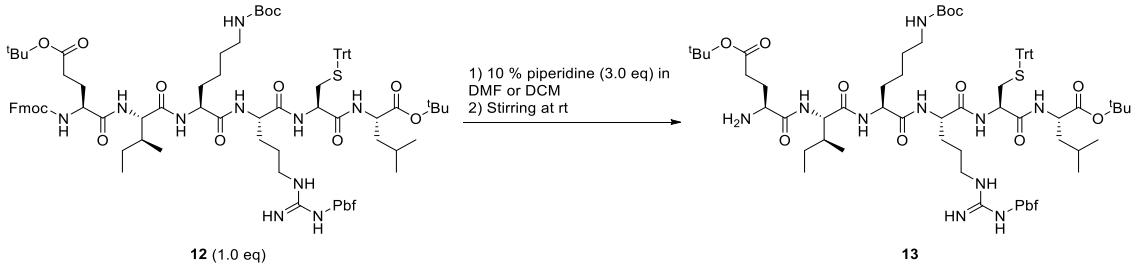


Figure 2.18. HPLC chromatogram of **12** using HATU as the coupling agent and DCM as solvent.

2.3.4. Fmoc removal of the protected hexapeptide 12

Following the convergent strategy proposed, the next step to achieve the synthesis of the target peptide **1** was the removal of the N-terminal protecting group of **12**. Two assays were performed with the batches obtained from the reactions described in table 2.12. The first reaction was carried out using a 10 % of piperidine in DMF while in the other one, DMF was substituted for DCM (scheme 2.25).



Scheme 2.25. Removal of the N-terminal protecting group of **12**.

The reactions were followed by HPLC until starting material **12** was not detected and the resulting crudes were purified by flash column chromatography (Hexanes/EtOAc 8:2 and DCM/MeOH 9:1) to remove the by-products DBF and its corresponding piperidine adduct. Table 2.13 summarises the results obtained after the purification of the reaction crudes.

Table 2.13. Results of the reactions performed for the synthesis of **13**.

	Batch of 12[c]	**Solvent**	**Time** [h]	**Crude yield** [%]	**Chromatographic purities**[d] [%]	
					13	**Other**
Batch 13A[a]	12A	DMF	1.5	78	88	12
Batch 13B[b]	12B	DCM	1.5	52	96	4

(a) The reaction was carried out using 0.95 mmol (1.60 g) of **12**.
(b) The reaction was carried out using 0.46 mmol (0.77 g) of **12**.
(c) See table 2.12.
(d) The percentages were obtained by integrating the peak areas at 220 nm.

The HPLC chromatogram of the purified product from batch 13A (1.09 g) showed a chromatographic purity of 88 % together with a 5 % of the remaining starting material **11**, that was present in the starting batch 12A. On the other hand, the HPLC chromatogram of the purified **13** from batch 13B (0.346 g) showed a higher chromatographic purity (96 %, figure 2.19).

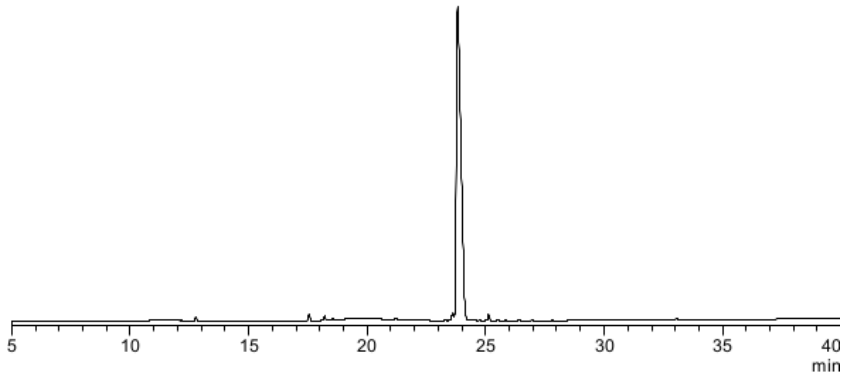


Figure 2.19. HPLC chromatogram of **13** obtained from batch 13B.

The explanation that might account for the differences encountered in the chromatographic purities of the crudes of **13** is that the batches of the precursor **12**, had different chromatographic purities. The highest purity of **13** was obtained when the batch 12B of **12**, which had the highest purity, was used. The different solvents used to perform the reaction seem that does not affect the purity nor the time of reaction.

2.3.5. Synthesis of the undecapeptide 14

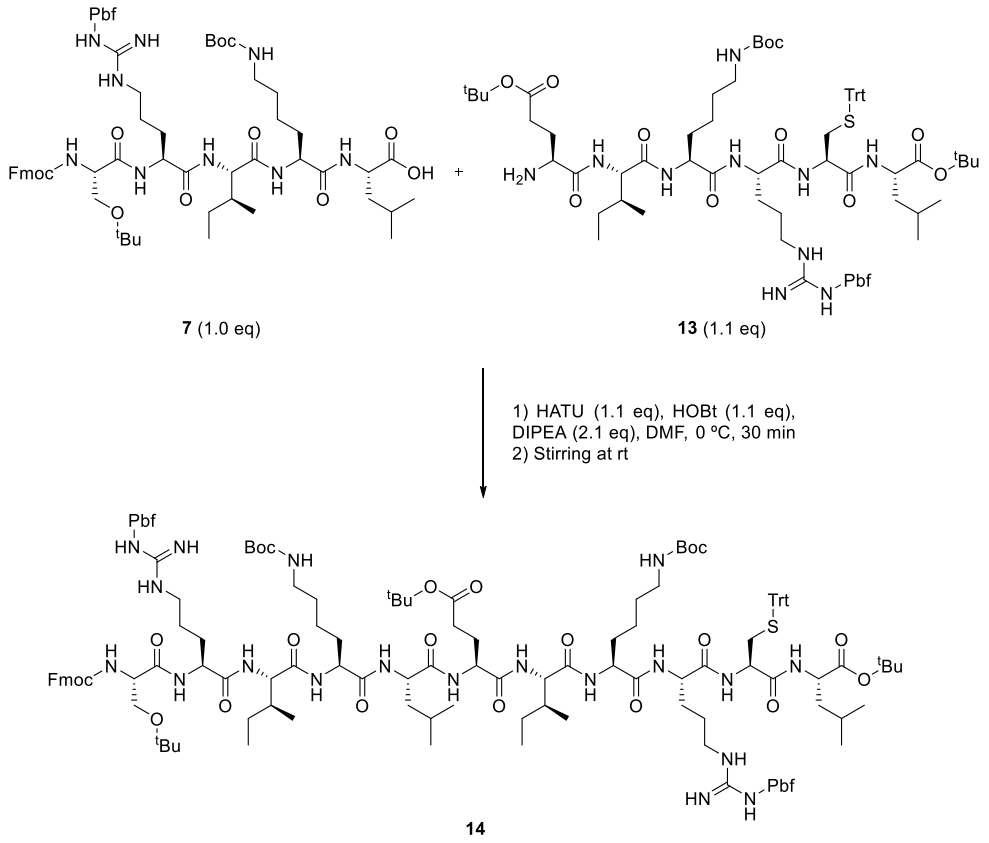
2.3.5.1. Using HATU, HOBt and DIPEA

The coupling between medium-sized protected fragments **13** and **7** that would lead to the target undecapeptide **14** was tested according to the results obtained in the synthesis of the hexapeptide **12** (section 2.3.3), where HATU proved to be more efficient than EDC·HCl. For this reason, two different assays using the former coupling reagent were performed using as starting material, the batches of **13** that were synthesised as described in section 2.3.4 (scheme 2.26). The results are summarised in table 2.14.

Table 2.14. Results of the reactions performed for the synthesis of **14**.

	Batch of 13[c]	**Time** [h]	**Crude yield** [%]	**Chromatographic purities**[d] [%]		
				14	**13**	**Other**
Batch 14A[a]	13A	4	96	83	5	6
Batch 14B[b]	13B	o/n	76	94	-	6

(a) The reaction was carried out using 0.31 mmol (0.389 g) of **7**.
(b) The reaction was carried out using 0.18 mmol (0.226 g) of **7**.
(c) See table 2.13.
(d) The percentages were obtained by integrating the peak areas at 220 nm.



Scheme 2.26. Preparation of the undecapeptide **14**.

The first reaction (batch 14A, table 2.14) was performed with the batch 13A (table 2.13, section 2.3.4). The reaction was monitored by HPLC and quenched by precipitation with water when it did not evolve further. The HPLC chromatogram of the crude showed that **14** was present with a chromatographic purity of 83 % (figure 2.20).

The second reaction (batch 14B, table 2.14) was performed with the batch 13B (table 2.13, section 2.3.4). The reaction was monitored by HPLC and an additional equivalent of DIPEA was added when the reaction did not evolve further. The reaction was quenched after stirring overnight and extractions were performed with EtOAc and acid/basic aqueous solutions. Nonetheless, formation of a precipitate was observed in the organic phase and it was filtrated off. The target peptide **14** was obtained in a 76 % yield and a chromatographic purity of 94 % (figure 2.20).

Two explanations could account for the differences encountered in the chromatographic purities of the two batches of **14**. First, the highest purity of **14** was obtained when the batch of **13** that was used to carry out the reaction had the highest purity. Second, the different work-up could also explain this result. The reaction that led to batch 14A was precipitated with water and no further purifications were carried out. On the other hand, the reaction of batch 14B was quenched with extractions with EtOAc. During this extractions, the desired peptide **14** precipitated. EtOAc might have solubilised impurities that water could not thus leading to a higher chromatographic purity.

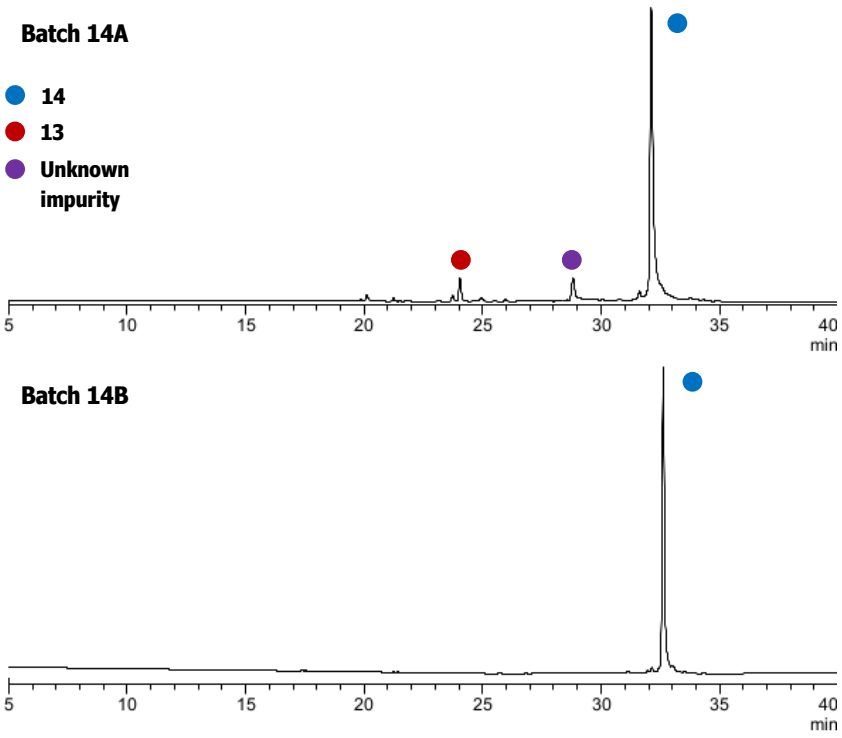


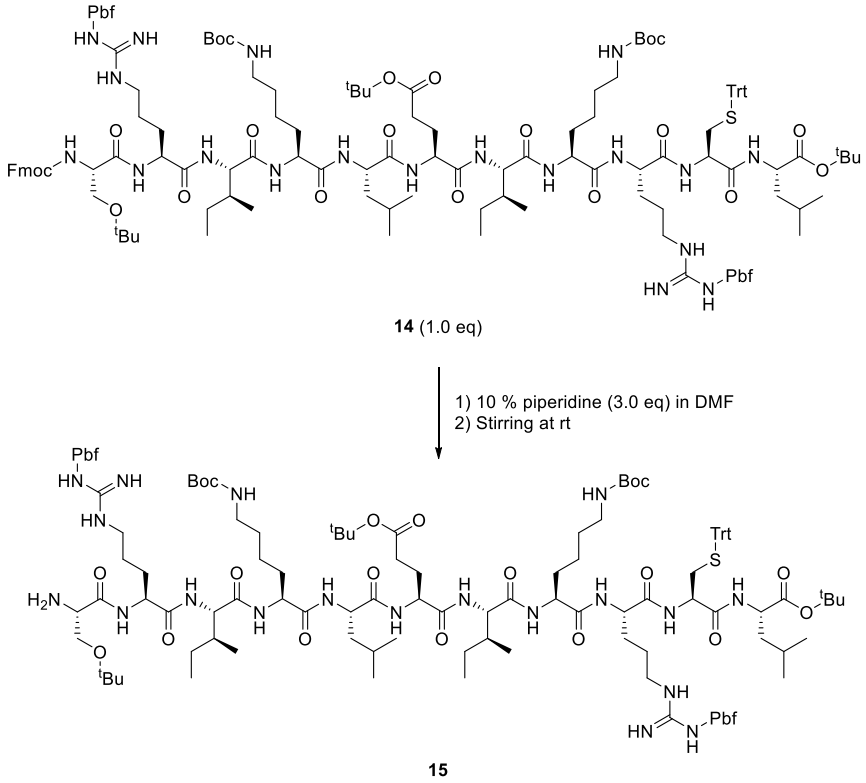
Figure 2.20. HPLC chromatograms of batch 14A and 14B.

2.3.6. Fmoc removal of the undecapeptide 14

The removal of the Fmoc protecting group of the intermediate **14** was the next step in the synthetic route for the preparation of the target peptide **1**. To do so, the batch 14B prepared as described in section 2.3.5 (table 2.14) was treated with a solution of 10 % piperidine in DMF to obtain the N-terminal deprotected **15** (scheme 2.27).

The reaction was monitored by HPLC until the starting material **14** was not detected. The crude peptide was quenched by precipitation with water. In this case, purification by flash column chromatography could not be carried out due to the insolubility of **15**. Therefore, the crude of **15** was triturated six times with Et_2O to remove DBF and its piperidine-adduct, obtaining 331.0 mg of a solid containing **15** (batch 15A, 103 % crude yield, figure 2.21) with a chromatographic purity of 91 %.

This is a promising result to purify an insoluble intermediate from the by-products that have been generated during the synthesis. As mentioned before, one of the main drawbacks of the solution-phase synthesis of peptides is that purification of the intermediates is difficult and usually takes a long timeframe. Usually, chromatographic techniques are required resulting in low recoveries of the target product. However, purification of the insoluble peptide **15** was achieved just with washings with Et_2O.



Scheme 2.27. Preparation of the N-terminal deprotected **15**.

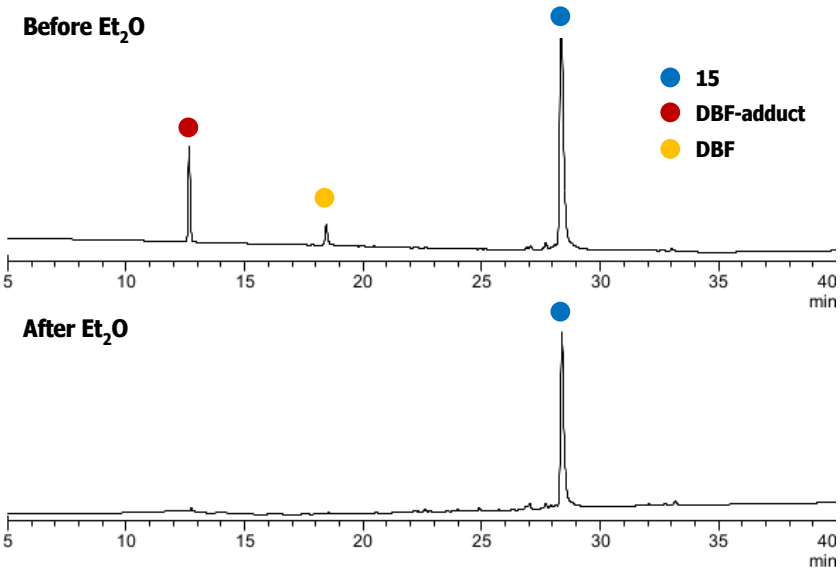
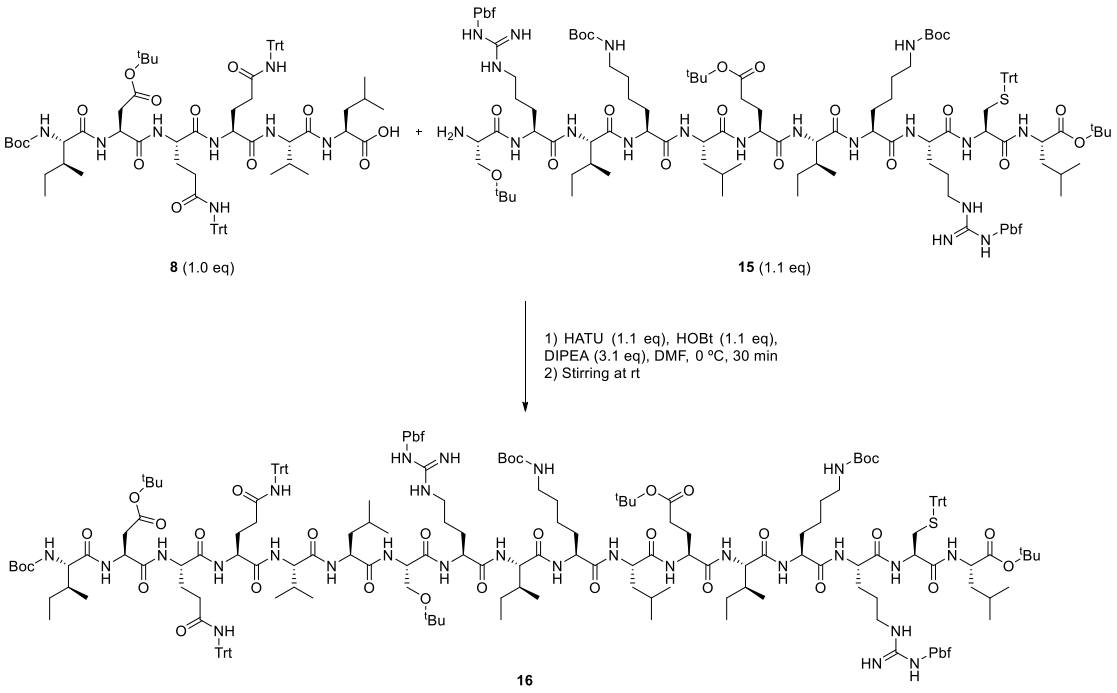


Figure 2.21. HPLC chromatograms of **15** (batch 15A) before and after purification.

2.3.7. Synthesis of the protected Fexapotide 16

This step corresponds to the last coupling reaction of the synthetic route and consists of making an amide bond between the C-terminal end of the hexapeptide **8** and the N-terminal deprotected undecapeptide **15**. The work scale was 0.282 g of **15** and 0.140 g of **8**. The

coupling reaction was performed using HATU and HOBt as the coupling reagent and additive, respectively (scheme 2.28).



Scheme 2.28. Preparation of **16**.

Generally, 2 equivalents of a base relative to the amount of HATU are used. In the particular case of the reaction performed to obtain **16**, an extra equivalent of DIPEA was added in order to achieve a faster reaction.

The reaction was monitored by HPLC until the starting material **8** was not detected and the crude was quenched by precipitation with water when the reaction did not evolve further. The HPLC analysis of the crude (batch 16A, 103 % crude yield, figure 2.23) contained the protected peptide **16** with a chromatographic purity of 62 % together with a 6 % of starting material **15** and 32 % of other impurities that could not be characterised. It is worth mentioning that a peak with the same mass than the target **16** was detected in an 8 %, which was assigned to the epimer of **16** (**25**, figure 2.22). The crude peptide was not purified and was used in the final deprotection step.

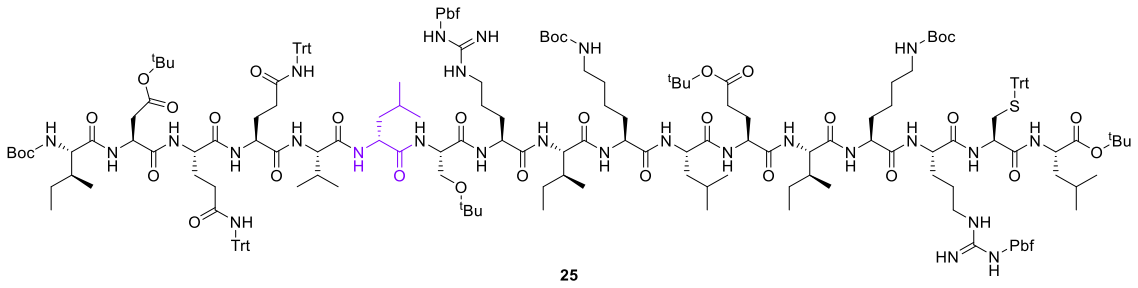


Figure 2.22. Epimer of **16** (**25**).

Further assays of this coupling reaction trying other coupling systems, lowering the temperature or using an excess of the more soluble **8** should be performed in order to minimise the formation of the epimer **25** and the presence of **15** in the reaction crude.

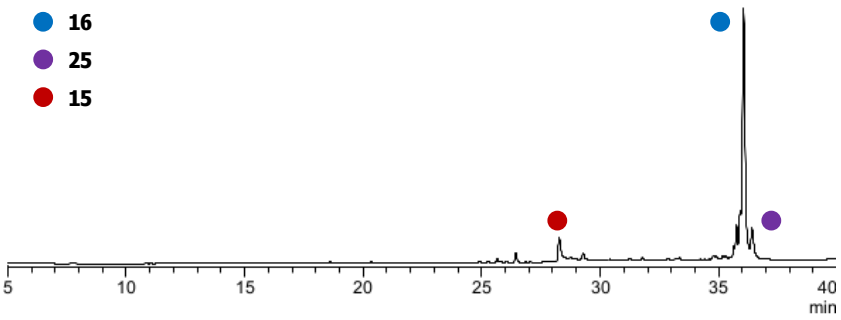
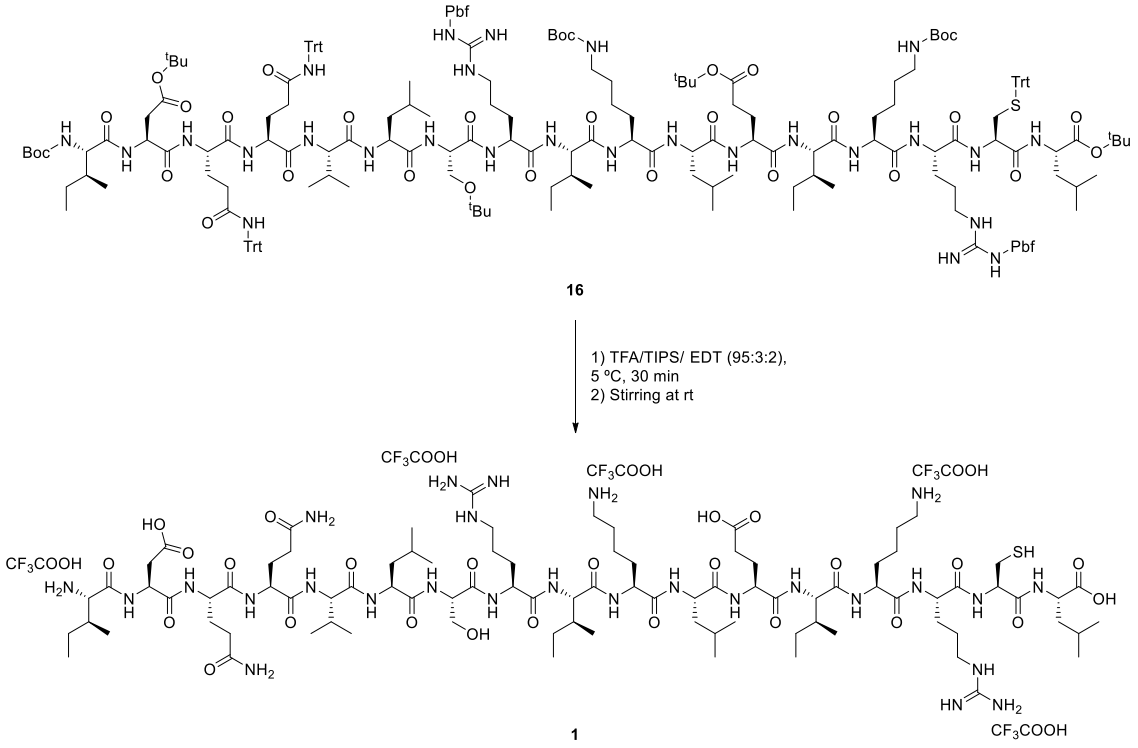


Figure 2.23. HPLC chromatogram of **16** (batch 16A).

2.3.8. Synthesis of Fexapotide

The final step of the synthetic route to achieve the target Fexapotide (**1**) was the removal of the side-chain, N-terminal and C-terminal protecting groups with an acidolytic treatment of **16** (scheme 2.29). The work-scale was 0.410 g of the crude of **16** obtained in the previous section.

The reaction was performed by adding the crude **16** over a cooled solution of TFA, TIPS and EDT (95:3:2). The reaction was left at 5 ºC for 30 min and at 20 ºC for 3.5 h. TIPS was used as a scavenger of the carbocations generated during the removal of the protecting groups, thus avoiding their reattachment to the peptide. EDT was used to avoid the formation of disulfide bonds between cysteines.



Scheme 2.29. Preparation of Fexapotide (**1**).

The target peptide was isolated by precipitation with MTBE and the HPLC chromatogram of the resulting solid (batch 1B, figure 2.26) contained the desired Fexapotide **1** with a chromatographic purity of 40 %, together with a 6 % of the diastereomeric form of **1**

resulting most probably from **25** (**26**, figure 2.24), a 4 % of the *tert*-butylated form of **1**, a 6 % of the side-chain deprotected form of **15** (**27**, figure 2.25) and a 44 % of other impurities that could not be characterised.

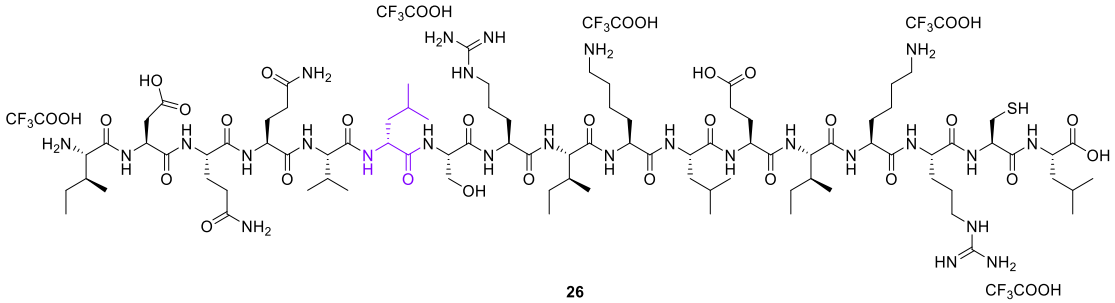


Figure 2.24. Epimer of **1** (**26**).

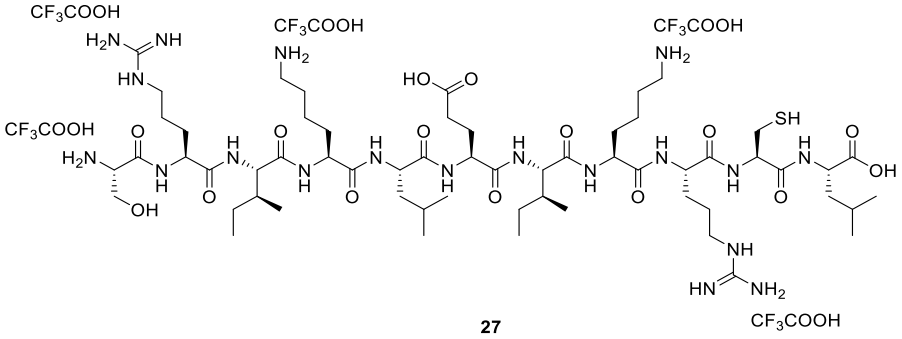


Figure 2.25. Side-chain deprotected form of **15** (**26**).

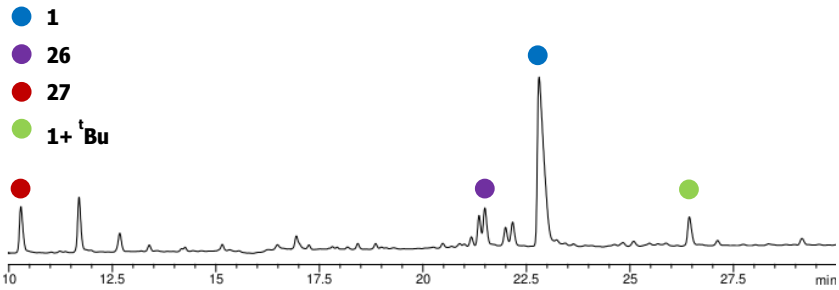


Figure 2.26. HPLC chromatogram of **1** (batch 1B).

2.4. Conclusions

The synthesis of the 17-residue Fexapotide following a convergent approach consisting on the solution synthesis of the protected C-terminal fragment Fmoc-Cys(Trt)-Leu-O^{t}Bu (**5**), the solid-phase synthesis of the protected peptides Fmoc-Glu(^{t}Bu)-Ile-Lys(Boc)-Arg(Pbf)-OH (**6**), Fmoc-Ser(^{t}Bu)-Arg(Pbf)-Ile-Lys(Boc)-Leu-OH (**7**) and the N-terminal Boc-Ile-Asp-(^{t}Bu)-Gln(Trt)-Gln(Trt)-Val-Leu-OH (**8**), and their assembly on solution has been studied.

2.4.1. Synthesis of the protected peptide fragments

The protected peptide fragments were synthesized on a 2-CTC resin manually (**6** at 2.22 mmol scale, **7** at 1.14 mmol scale) or mechanically (**6** at 6.13 and 10.14 mmol scales, **7** at 11.63 mmol scale, **8** at 11.09 and 7.18 mmol scales). The first Fmoc-amino acid was coupled to the resin using 3 or 6 eq of DIPEA in DCM with yields that ranged from 57 % (**6**, 1.5 h with mechanical stirring) to 70 % (**6**, 2 h with manual stirring). Further couplings were performed using DIC/HOBt (3 eq of each reagent) in DMF. No recouplings were needed for **6** and **7** (mechanical stirring), and recouplings were required for Lys and Arg for **7** following a manual procedure, and Gln (mechanical stirring) was recoupled in the case of **8**. The protected peptides were released from the resin by using a solution of 1 % TFA in DCM. After removing volatiles under reduced pressure, the peptide crudes were recovered as white solids with chromatographic purities between 91 % for **8** and 98 % for **6** and **7**.

Premature loss of peptide chains was detected in some of the syntheses, especially when higher work scales were used (around 10 g of 2-CTC resin). These results indicated that special attention had to be taken to the experimental conditions in order to avoid eventual acid traces during the synthesis. For future syntheses, it was decided to remove the N-terminal protecting group when the resin had to be left several hours before coupling a new amino acid to avoid the premature loss of chains.

The dipeptide **11** was obtained in solution in two steps from **9** and **10** (4.84 mmol scale). The full protected dipeptide **5** was obtained using EDC·HCl/HOBt with DIPEA (1.1 eq of each reagent) or EDC·HCl/DMAP (1.5 eq and 0.2 eq, respectively), with chromatographic purities of 98 % and 92 % respectively. The batch resulting from the synthesis using DIPEA was treated with 10 % of piperidine in DCM to remove the Fmoc group to afford **11** in a 95 % overall yield (99 % of chromatographic purity) after purification by flash column chromatography (Hexanes/EtOAc 8:2 and DCM/MeOH 9:1).

2.4.2. Solution-phase assembly of the protected peptide fragments

The fragment **12** was obtained in solution from the condensation of the dipeptide **11** with the tetrapeptide **6**. The best results were obtained when HATU/HOBt was used as the coupling system at 0 ºC with DCM or DMF as solvents (white solid with chromatographic purities of 93 % (DCM) and 88 % (DMF)). The use of EDC·HCl/HOBt and EDC·HCl/DMAP as coupling systems led to uncompleted reactions and low chromatographic purities of **12**. Moreover, the use of DMAP as additive led to high percentages of the epimer **24** (18-27 %).

Full removal of the Fmoc group from **12** in solution was achieved at rt after no starting material **12** was detected by HPLC using a solution of 10 % piperidine in DMF or DCM.

The resulting hexapeptide **13** was obtained with a chromatographic purity of 96 % after purification by flash column chromatography (Hexanes/EtOAc 8:2 and DCM/MeOH 9:1).

The pentapeptide **7** was coupled to the N-terminal deprotected hexapeptide **13** using HATU/HOBt as the coupling system at 0 ºC in DMF or DCM. The desired undecapeptide **14** was obtained with a 94 % of chromatographic purity after the work-up.

Full removal of the Fmoc group from **14** was achieved using a solution of 10 % piperidine in DMF at rt until no starting material **14** was detected by HPLC. The desired N-terminal undecapeptide **15** was obtained with a chromatographic purity of 91 % after a work-up that included washings with Et_2O. The use of a flash column chromatography was precluded due to solubility problems.

The heptadecapeptide **16** corresponding to the sequence of Fexapotide was obtained by coupling the hexapeptide **8** to the N-terminal deprotected undecapeptide **15**. The reaction was performed using the HATU/HOBt coupling system at 0 ºC for 30 min and proceeded until the disappearance of **8**. The crude peptide was obtained with a chromatographic purity of 62 % after precipitation with water, together with a 6 % of starting material **15** and 8 % of the epimer **25**.

Full deprotection of **16** was performed using a mixture of TFA, TIPS and EDT (95:3:2) for 4 h. After precipitation with MTBE, a white solid was obtained containing the desired Fexapotide **1** with a chromatographic purity of 40 %, together with a 6 % of the diastereomer **26**, a 4 % of the *tert*-butylated form of **1** and a 6 % of the side-chain deprotected undecapeptide **27**.

Chapter 3: Synthesis of Fexapotide through a three-fragment convergent approach

3.1. Synthetic strategy

In the previous chapters, the synthesis of the target peptide has been discussed. First, a linear solid-phase synthesis was proposed but it resulted to be inefficient and expensive due to the high number of recoupling steps that were needed. For this reason, it was decided to change the linear strategy to a convergent approach in which the synthesis of protected fragments that represent the sequence of the desired peptide were synthesised by the solid-phase strategy, and then were assembled in solution to yield the desired side-chain protected peptide. The final peptide was obtained by removing the side-chain protecting groups (scheme 2.1).

This convergent strategy was promising in terms of laboratory scale because it led to the target peptide with a higher chromatographic purity than the obtained using the linear approach. The fact of synthesising the protected fragments on solid-phase with high purities and the easy monitor of fragment coupling on solution could account for this result. Nonetheless, the results achieved were not good enough to scale-up the process, so further optimisation should be done.

As mentioned in chapter 2, it is worth noting that when a convergent approach is adopted, it is recommended that the peptide fragments synthesised by SPPS should not be longer than 5 amino acids.[11] However, following the guidelines of the company, it is easier and faster to accomplish all the regulatory requirements in terms of structure characterisation, manufacture process and impurities emerging from the synthetic process when the number of intermediates is as low as possible. In this sense, it is worth mentioning that the preparation of a protected peptide fragment by the solid-phase methodology is considered a single synthetic step in the industrial context. For this reason, a new approach lowering the number of peptide fragments was proposed. The peptide fragments **6** (tetrapeptide) and **7** (pentapeptide) were merged to form the nonapeptide **28** (figure 3.1).

Thus, instead of fully re-optimise the synthetic strategy described in chapter 2, we took advantage of the experience acquired to optimise the new synthetic path shown in scheme 3.1. This new route will allow us to explore not only the convergent strategy, but also to evaluate the extent and the ease use of SPPS for larger fragments from an industrial point of view. In this strategy, the desired target peptide **1** was divided into fragments **5**, **28** and **8**. Fragments **28** and **8** were synthesised in a solid support. These fragments were then condensed in solution-phase to afford the desired side-chain protected peptide **16**, which led to the target Fexapotide (**1**) when the side-chain protecting groups were removed. With this modification, the number of synthetic steps was considerably reduced from 11 steps in the first convergent approach to 8 steps in the proposed one. This is important because the work-up of reactions involving such large molecules are problematic and reduction of the number of steps can be envisaged as synthetic advantage, specially at industrial level.

In this chapter, the solid-phase synthesis of the protected peptide fragments and their condensation in solution to obtain the desired peptide **1** will be discussed. As mentioned before, all products and intermediates were analyzed by HPLC-UV or HPLC-MS and chromatographic purity was determined by integration of the peak areas at 220 nm.

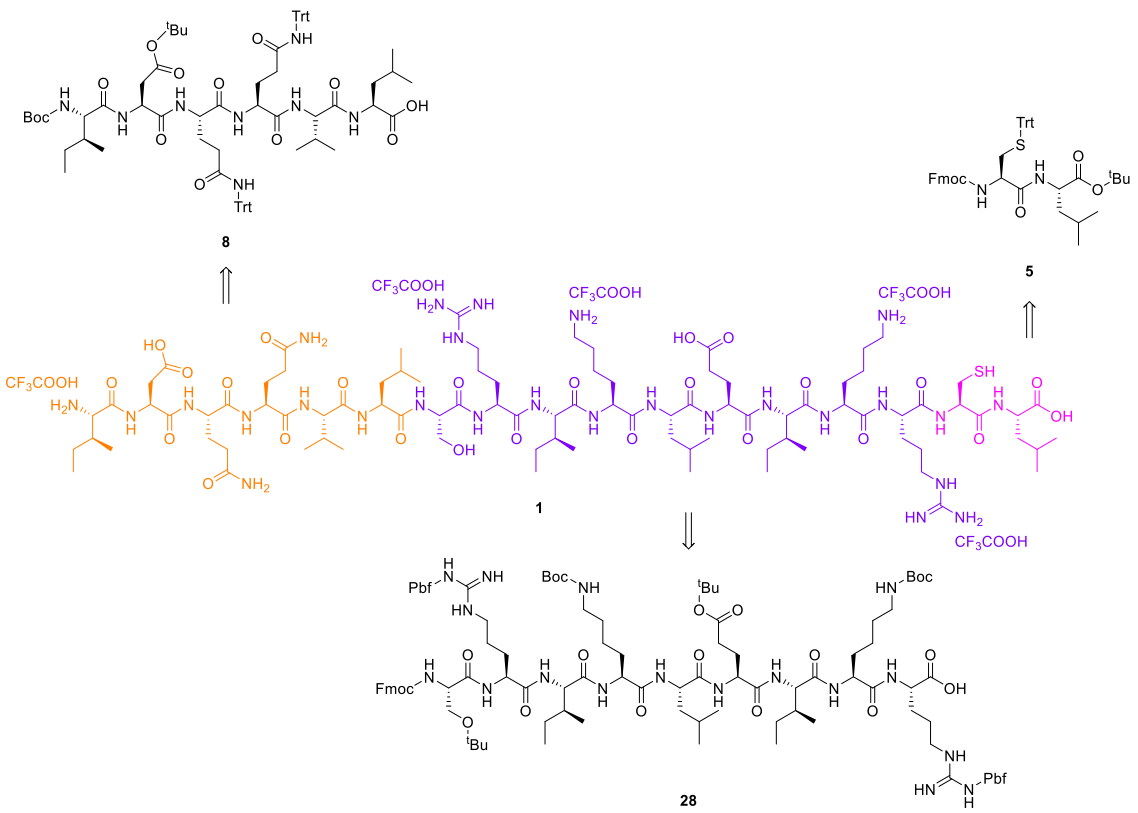
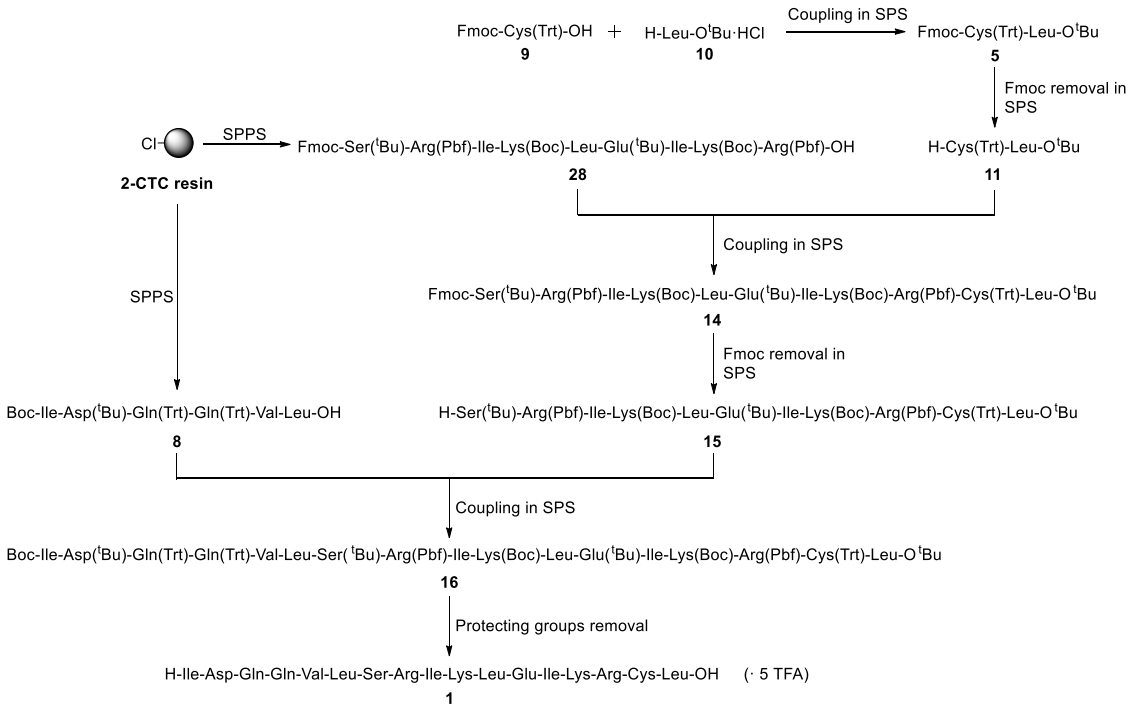


Figure 3.1. Fragmentation of **1** to follow a new convergent approach.



Scheme 3.1. New convergent synthesis proposed.

3.2. Solid-phase synthesis of the protected peptide fragments

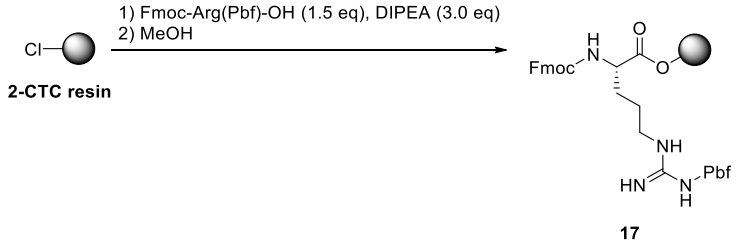
As mentioned in chapter 2, solid-phase peptide synthesis was used for the preparation of protected peptide fragments, being the 2-CTC resin the most suitable polymeric support for the designed strategy since it allows the release of the desired intermediates under very mild conditions such as 1-2 % of TFA or 25 % of hexafluoroisopropanol (HFIP).[98]

It must be pointed out that, in general, the SPPS syntheses described in this chapter are long and require several days to be completed. In consequence, the growing peptidyl-resin has to be left overnight inside the reactor several times along the process. As discussed in chapter 2, this can be troublesome because premature cleavage of the peptide can take place resulting in low final yields. For this reason, it was decided to remove the N-terminal protecting group when the resin had to be left several hours between couplings. The idea was that the free amino group may act as scavenger if there is presence of trace amount of acid in the environment that could induce premature cleavage.

3.2.1. Synthesis of the nonapeptide fragment 28

3.2.1.1. Incorporation of the first amino acid

The coupling of commercially available Fmoc-Arg(Pbf)-OH to the polymeric support was performed under conditions similar to those used in section 2.2.1.1 (scheme 3.2). The non-reacted active sites of the resin were capped with the addition of MeOH after 1.5 h of reaction.



Scheme 3.2. Incorporation of Fmoc-Arg(Pbf)-OH to the 2-CTC resin.

The amount of amino acid that was incorporated to the resin was determined by UV quantification at 301 nm of the piperidine adduct formed after the removal of the Fmoc protecting group. Table 3.1 summarise the substitution values obtained in the different syntheses of aminoacyl-resin **17** using different scales and reaction times.

The substitution values obtained for the incorporation of the first amino acid of **28** were similar to those obtained for the incorporation of the first amino acid of **6** (section 2.2.1.1, chapter 2) with the exception of batch 17D in which the substitution value was considerably lower. Accordance in the substitution values of both peptide fragments could be expected since the amino acid residue that was incorporated is Arg in both cases. In batch 17E, the amount of amino acid incorporated was not determined and thus, the initial substitution of the resin was assumed to continue the synthesis of this batch. Thus, we must assume that the excess of reactants used in batch 17E was larger than the ones used in the other batches.

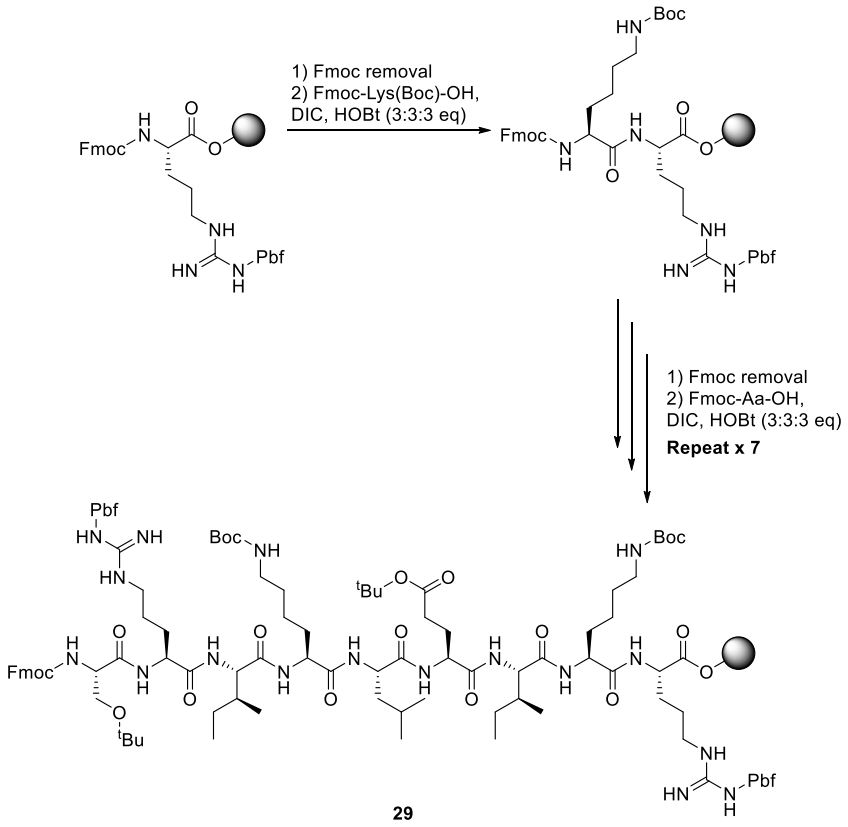
Table 3.1. Substitutions obtained for the coupling of the first amino acid on the 2-CTC resin in the different syntheses of **28**.

	2-CTC resin [g]	**Reaction time** [h]	**Substitution** [mmol/g]
Batch 17D	7.68	1.5	0.27
Batch 17E	5.23	1.5	-[a]
Batch 17F	4.98	1.5	0.55
Batch 17G	5.02	1.5	0.45

(a) The amount of amino acid was not determined. The initial substitution of the resin (1.40 mmol/g) was assumed.

3.2.1.2. Peptide chain elongation

Four independent syntheses of **28** were carried out using the four batches of aminoacyl-resin described in table 3.1. The coupling reactions were performed using the Fmoc-protected amino acid with the standard DIC/HOBt coupling system (3:3:3 eq) and were monitored with the ninhydrin test. The Fmoc protecting group was removed by using a 20 % solution of piperidine in DMF (scheme 3.3). As mentioned before, the N-terminal protecting group was removed when the resin had to be left several hours before coupling a new amino acid to avoid the premature loss of peptide chains that were detected during the synthesis of the protected peptide fragments of chapter 2. Table 3.2 summarises the number of coupling steps needed per amino acid in the different syntheses of **28**.



Scheme 3.3. Peptide chain elongation cycle to obtain the peptidyl-resin **29**.

Excluding the first synthesis arising from batch 17D, recouplings were needed for some of the amino acids. The peptidyl-resins coming from batch 17E and 17F needed two or three recouplings for some amino acids even though the coupling times were increased to 3 or 4 h. For this reason, in batch 17G, the number of equivalents of the reagents corresponding

to entries 5 to 7 were increased (4 equivalents). Under these conditions a single coupling was enough except for arginine and serine which had to be recoupled. The results obtained with the Arg derivative are not surprising because it has bulky protecting group. The differences encountered in the number of couplings needed per amino acid between batch 17D and the other batches might be a consequence of the different substitution values. Lower substitution values lead to lower peptide-chain interactions and thus, facilitate the completeness of coupling reactions. For this reason, in batch 17D, which presented the lowest substitution value, no recouplings were needed.

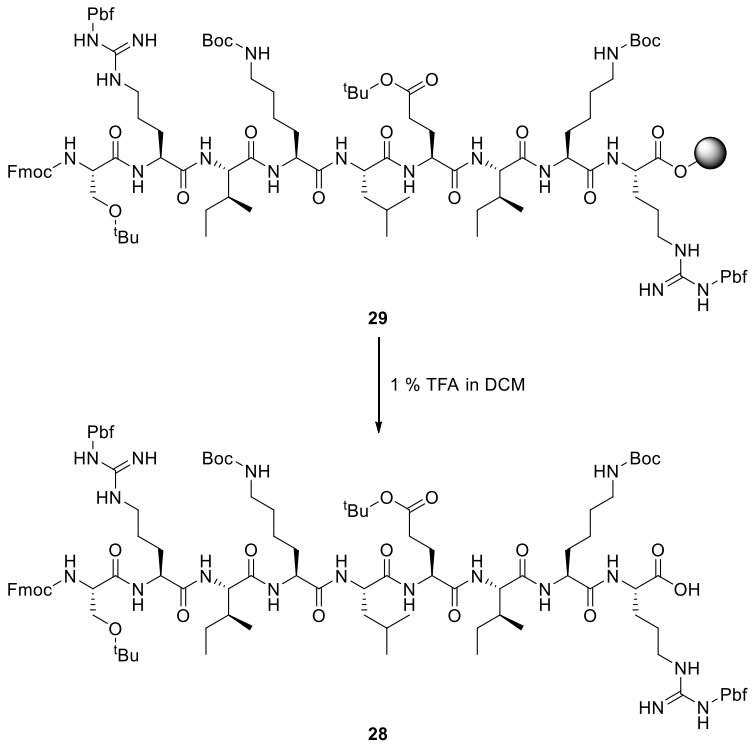
Table 3.2. Number of couplings needed per amino acid for the different syntheses of **28**.

		Number of couplings			
Entry	**Amino acid**	**Batch 17D**	**Batch 17E**	**Batch 17F**	**Batch 17G**
1	Fmoc-Lys(Boc)-OH	1	1	1	1
2	Fmoc-Ile-OH	1	2	2	1
3	Fmoc-Glu(^{t}Bu)-OH	1	1	2	1
4	Fmoc-Leu-OH	1	1	1	1
5	Fmoc-Lys(Boc)-OH	1	3	2	1
6	Fmoc-Ile-OH	1	2	1	1
7	Fmoc-Arg(Pbf)-OH	1	3	1	2
8	Fmoc-Ser(^{t}Bu)-OH	1	2	1	2

3.2.1.3. Cleavage of the peptide from the resin

The protected peptide was released from the peptidyl-resins from batches 17D to 17G by using a solution of 1 % TFA in DCM (scheme 3.4). Nonetheless, the work-up methodology was modified for the different batches. In the case of batch 17D and batch 17E, the filtrates were collected in a round-bottom flask containing Et_2O and the peptide was obtained after removing TFA under reduced pressure. For batch 17F, the filtrates were collected to a round-bottom flask containing Et_2O and the resulting precipitate was filtered and dried at reduced pressure. The post-cleavage work-up methodology of batch 17G is described in section 3.2.1.3.1.

The peptidyl-resin coming from batch 17D afforded 480.8 mg of crude peptide with a 51 % of chromatographic purity for **28** (figure 3.3). The main impurity (21 %) has a base peak of m/z of 1098.8 ($[M+2H]^{2+}$) which might correspond to the methylated target peptide. The impurities at 20.1 min and 20.7 min (2 % and 4 % respectively) have the same m/z of 1041.1 ($[M+2H]^{2+}$) and can be attributed to the peptides resulting from loss of the protecting group Boc from any of the two lysine residues of the peptide chain (**30** and **31**, figure 3.2).



Scheme 3.4. Removal of **28** from the peptidyl-resin **29**.

The peptidyl-resin coming from batch 17E yielded 5.04 g of crude peptide with a chromatographic purity of 88 % of the desired peptide **28** (figure 3.3).

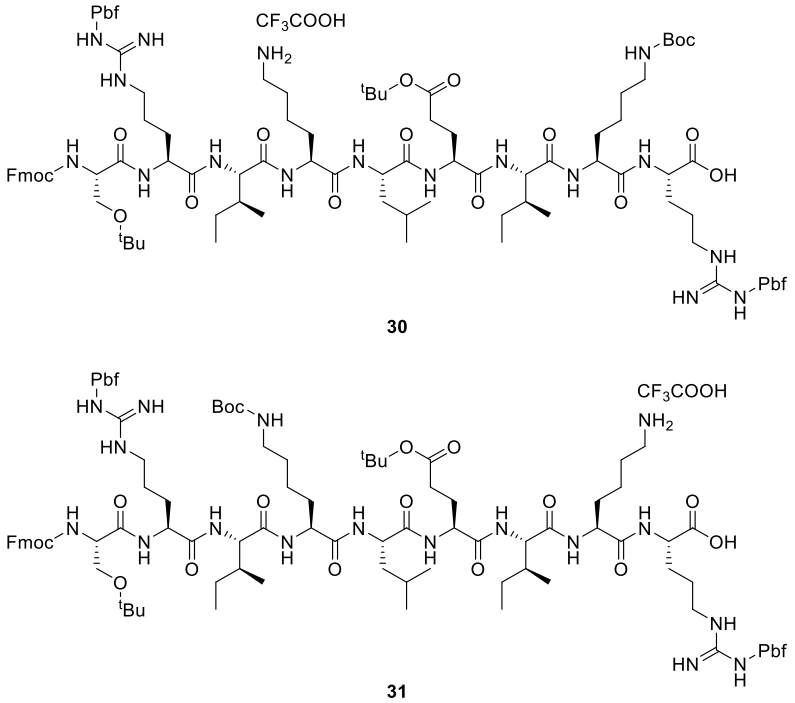


Figure 3.2. Loss of Boc from any of the two lysine residues of **28** (**30** and **31**).

The reason for the differences in the HPLC retention times when comparing the chromatograms showed in figure 3.3, is because the crudes were analysed under the same chromatographic conditions but with different instruments.

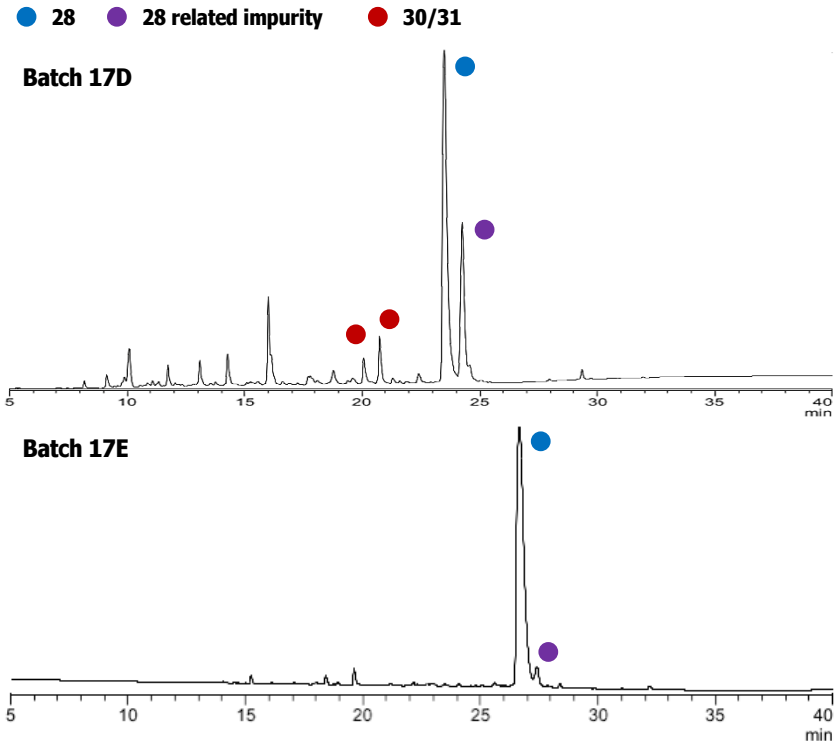


Figure 3.3. HPLC chromatograms of the peptide crude obtained from batches 17D and 17E.

The results suggest that the post cleavage work-up methodology carried out with the crudes resulting from batches 17D and 17E, which was removing volatiles under reduced pressure was not suitable, at least for batch 17D in which removal of some protecting groups was detected. Therefore, a new methodology was tested with the peptidyl-resin coming from batch 17F to avoid these impurities. After the treatment of this batch with 1 % TFA in DCM, the peptide was precipitated with Et_2O. Unfortunately, obstruction of the filter during the filtration step moved us to remove volatiles under vacuum resulting in 5.25 g of crude peptide with a 79 % of chromatographic purity (figure 3.4, A). The impurities with retention times of 8.37 min and 9.91 min (6 % and 9 % respectively), have the same m/z ($[M+2H]^{2+}$ of 1041.1) and can be attributed to **30** and **31**.

A small amount of crude peptide from batch 17F (157.6 mg) was purified by flash column chromatography with DCM/MeOH (9:1) containing 1 % of formic acid (figure 3.4, B). The target **28** was recovered in two fractions of 42.2 mg (80 % of chromatographic purity) and 69.0 mg (87 % of chromatographic purity). Nonetheless, purification of higher amounts of the crude (0.954 g), did not result in an improvement in the chromatographic purity. The retention times of the chromatograms shown in figure 3.4 are different to the retention times of the chromatograms shown in figure 3.3 because the crudes were analysed under different chromatographic conditions (see method D, section 5.3.3.3, experimental section).

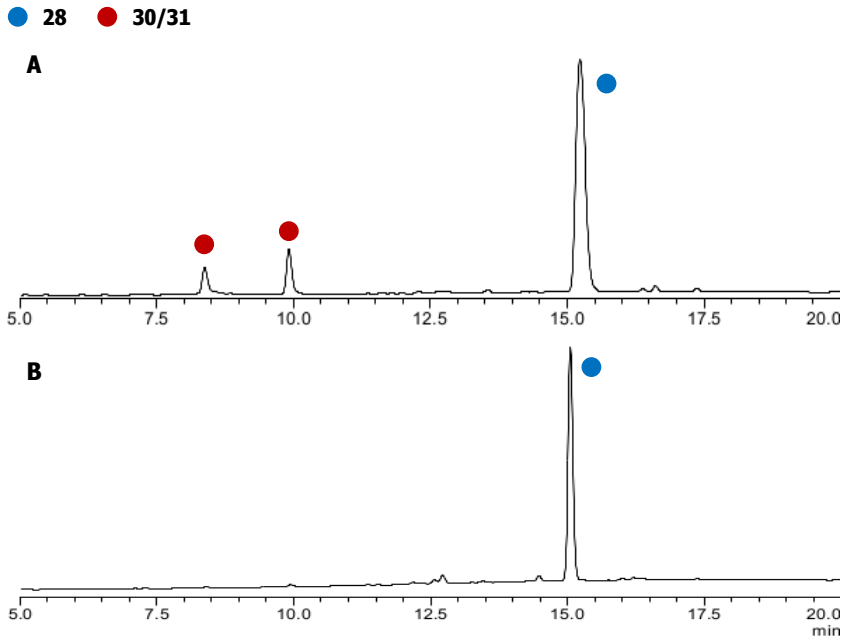


Figure 3.4. HPLC chromatogram of the peptide crude of batch 17F (A) and the peptide purified (B).

3.2.1.3.1. Study of a new peptidyl-resin cleavage methodology

A possible explanation for the premature loss of the Boc group could be that volatiles were removed under vacuum in the presence of TFA that was used in the cleavage step. Working with such quantities of peptide crudes required to increase the volume of solvents and thus, to extend the exposure of the peptide to TFA when concentrating under vacuum. To confirm that, alternative protocols for the work-up of the acidolytic crude were studied using the peptide crude from batch 17G. Thus, several solvents were tested in order to improve the granularity of the precipitate and, consequently, to avoid the filtration problems. Moreover, a base was added to the crude peptide solution to neutralise TFA and avoid the presence of the acid during the work-up. Table 3.3 summarises the conditions studied.

High chromatographic purities were obtained in all the assays. Nonetheless, the highest values were obtained under the conditions shown in entries 1, 2 and 3 (table 3.3). Moreover, in all cases filtration was achieved without problems. In general, by-products resulting from Boc removal were not detected, which confirmed that TFA neutralisation is required to keep peptide integrity, especially when working at larger work scales.

It is worth mentioning that when the work-up described in entry 3 was tested with a larger amount of peptidyl-resin 17G (2.95 g), the results did not change. In this case, 1.533 g (53 % crude yield) of **28** were obtained with a chromatographic purity of 94 % (figure 3.5).

Table 3.3. Work-up protocols studied with the peptide crude from batch 17G.

Entry	**Peptidyl-resin** [g]	**Base**[a]	**Solvent**	**Procedure**	**28** [g]	**Crude yield** [%]	**Purity**[b] [%]
1	1.13	Pyridine	Et_2O	The filtrates were collected to a round-bottom flask that contained Et_2O and pyridine. The suspension was filtered, washed with H_2O and Et_2O, and dried under reduced pressure.	0.52	47	94
2	1.23	TEA	Et_2O	The filtrates were collected to a round-bottom flask that contained Et_2O and TEA. The suspension was filtered, washed with H_2O and Et_2O, and dried under reduced pressure.	0.61	51	95
3	1.01	TEA	MTBE	The filtrates were collected to a round-bottom flask that contained MTBE and TEA. The suspension was filtered, washed with H_2O and Et_2O, and dried under reduced pressure.	0.53	53	94
4	1.04	TEA	Diisopropyl ether	The filtrates were collected to a round-bottom flask that contained diisopropyl ether and TEA. The suspension was filtered, washed with H_2O and Et_2O, and dried under reduced pressure.	0.68	67	91
5	1.18	TEA	DCM/Et_2O	The filtrates were collected to a round-bottom flask that contained DCM and TEA. The solution was concentrated under reduced pressure and Et_2O was added dropwise with an addition funnel. The suspension was filtered, washed with H_2O and Et_2O, and dried under reduced pressure.	0.60	52	92
6	1.04	TEA	THF/H_2O	The filtrates were collected to a round-bottom flask that contained THF and TEA. The solution was concentrated under reduced pressure and H_2O was added dropwise with an addition funnel. The suspension was filtered, washed with Et_2O and dried under reduced pressure.	0.34	33	90

(a) 1.2 eq relative to TFA.
(b) The percentages were obtained by integrating the peak areas at 220 nm.

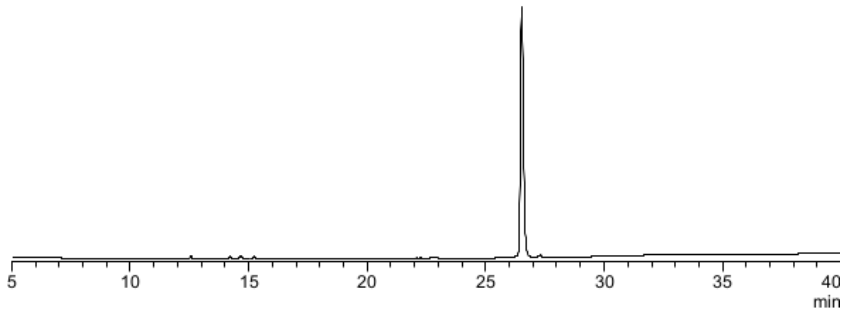


Figure 3.5. HPLC chromatogram of the peptide crude obtained from batch 17G.

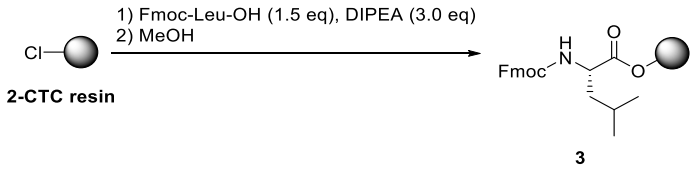
It is worth noting that the crude yields of the different assays that were carried out are low (33-67 %) and cannot be only explained with the losses that are usually experienced during a precipitation process. Probably, the low crude yields values are related with the premature loss of peptide chains during the synthesis of **28**. It therefore suggests that the removal of N-terminal protecting group when the resin had to be left several hours before coupling a new amino acid did not fully prevent the premature loss of peptide chains.

3.2.2. Synthesis of the hexapeptide fragment 8

This peptide fragment was synthesised again because we considered that the chromatographic purities of the batches of **8** coming from the peptidyl-resins 3C and 3D (see chapter 2) were not good enough.

3.2.2.1. Incorporation of the first amino acid

The coupling of commercially available Fmoc-Leu-OH to the polymeric support was performed following the methodology already mentioned in section 3.2.1.1 (scheme 3.5). Again, the non-reacted active sites of the resin were capped with the addition of MeOH after 1.5 h of reaction.



Scheme 3.5. Incorporation of Fmoc-Leu-OH to the 2-CTC resin.

The amount of amino acid that was incorporated to the resin was determined by UV quantification at 301 nm of the piperidine adduct resulting from the Fmoc removal. Table 3.4 summarises the substitution values obtained in the different syntheses of aminoacyl-resin **3** using different scales and reaction times.

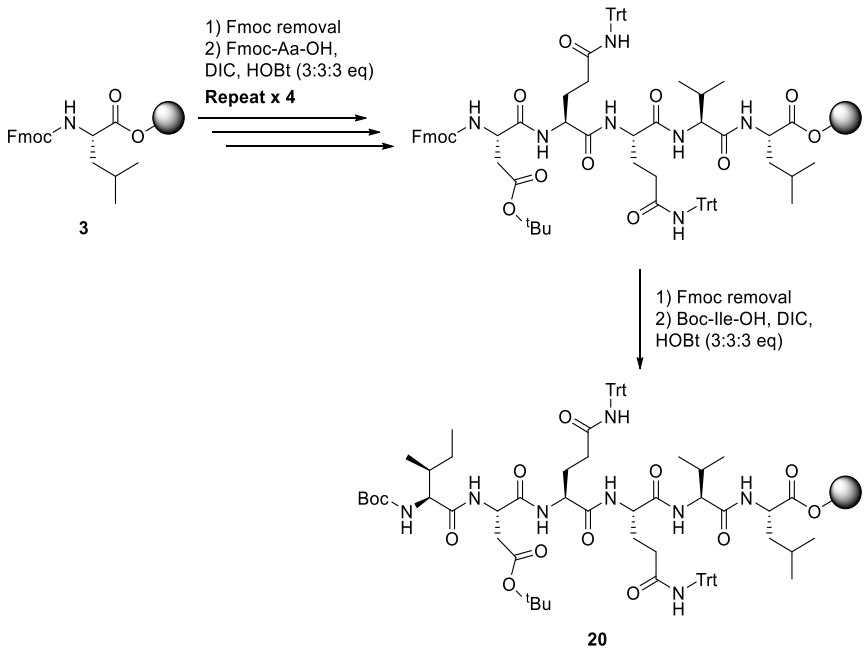
Table 3.4. Substitutions obtained for the coupling of the first amino acid on the 2-CTC resin in the different syntheses of **8**.

	2-CTC resin [g]	**Substitution** [mmol/g]
Batch 3E	10.2	0.72
Batch 3F	5.39	0.59

The substitution values obtained for the incorporation of the first amino acid were similar to those obtained in section 2.2.3.1.

3.2.2.2. Peptide chain elongation

Two independent syntheses of **8** were carried out using the batches of aminoacyl-resin described in table 3.4. The coupling reactions were performed under conditions similar to those used in the synthesis of the nonapeptide **28** (scheme 3.6). Coupling reactions were monitored with the ninhydrin test and recoupling was performed when the test was positive. As mentioned in section 3.2.1.2, the N-terminal protecting group was removed when the resin had to be left several hours before coupling a new amino acid. Table 3.5 summarises the number of couplings needed per amino acid in the different syntheses of **8**.



Scheme 3.6. Peptide chain elongation cycle to obtain the peptidyl-resin **20**.

Table 3.5. Number of couplings needed per amino acid for the different syntheses of **8**.

		Number of couplings	
Entry	**Amino acid**	**Batch 3E**	**Batch 3F**
1	Fmoc-Val-OH	1	1
2	Fmoc-Gln(Trt)-OH	2	1
3	Fmoc-Gln(Trt)-OH	3	1
4	Fmoc-Asp(^{t}Bu)-OH	2[a]	1
5	Boc-Ile-OH	4[a]	1

(a) The coupling reagent was changed to HATU.

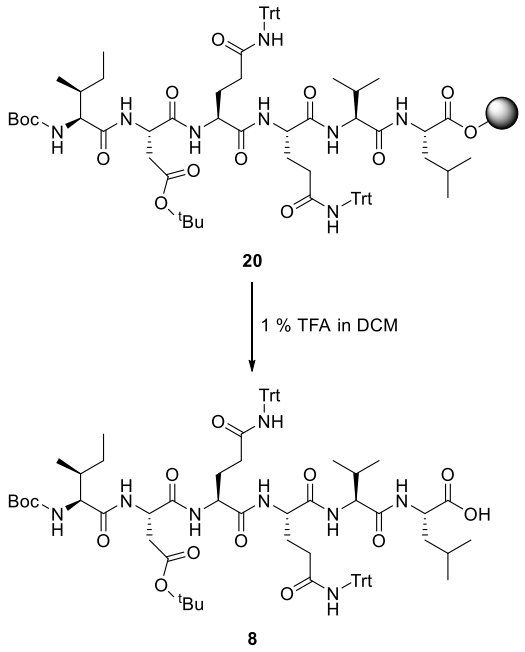
In batch 3E, recouplings were needed except for the second residue which was valine. The fact that Gln residues required two or three recouplings (entries 2 and 3), moved us to change the coupling reaction conditions for Asp and Ile residues (entries 4 and 5) for a more efficient coupling reagent such as HATU. Unfortunately, recouplings could not be avoided. On the other hand, recouplings were not required in batch 3F. This result suggests that the initial functionalisation of the resin may play an important role in the efficiency with which the synthesis proceeds.

The propensity of peptide chains to form ordered structures is the main cause of the highly sequence-specific variability in the synthetic efficiency encountered during peptide assembly. As the peptide grows on the resin, it can form secondary structures that cause peptide chain

aggregation, which results in lower reaction rates due to the poor accessibility of reagents to the reactive centres. In this sense, the predisposition of peptides to aggregate on the resin is higher when the loading values are also high. This could explain why recouplings were required in batch 3E where the resin loading was 0.72 mmol/g. This substitution value was higher to the one obtained in batch 3F (0.59 mmol/g), where recoupling steps were not required.

3.2.2.3. Cleavage of the peptide from the resin

The protected peptide was removed from the peptidyl-resin coming from batches 3E and 3F by using a solution of 1 % TFA in DCM (scheme 3.7). Table 3.6 summarises the work-up protocols used for each synthesis.



Scheme 3.7. Removal of **8** from the peptidyl-resin **20**.

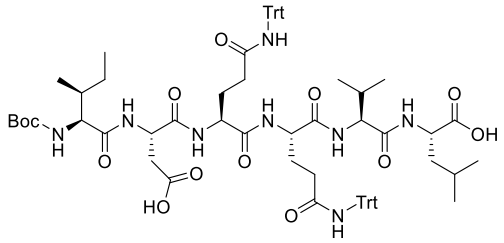
Table 3.6. Work-up protocols used for the different syntheses of **8**.

Peptidyl-resin	**Procedure**	**28** [g]	**Purity**[b] [%]
3E	The filtrates were collected in a round-bottom flask containing Et_2O and the peptide was obtained after removing volatiles under reduced pressure.	5.65	85
3F	The filtrates we collected to a round-bottom flask that contained DCM and TEA[a]. The solution was concentrated under reduced pressure and Et_2O was added dropwise with an addition funnel. The suspension was filtered, washed with H_2O and Et_2O, and dried under reduced pressure.	5.23	91

(a) 1.2 eq relative to TFA.
(b) The percentages were obtained by integrating the peak areas at 220 nm.

The peptidyl-resin coming from batch 3E afforded 5.65 g of crude peptide as a white solid with a chromatographic purity of 85 % (figure 3.7, A) after isolation following the protocol described in entry 1. The impurity with a retention time of 23.1 min (7 %) has an m/z of 1300.0 ($[M+H]^+$) and can be attributed to the loss of a *tert*-butyl protecting group (**32**, figure

3.6). As mentioned before, the loss of this protecting group could be attributed to the presence of TFA during removal of the volatiles under reduced pressure.



32

Figure 3.6. Loss of ^{t}Bu from the aspartic acid residue of **8** (**32**).

The post cleavage work-up protocol described in section 3.2.1.3.1 was used to isolate the peptide coming from batch 3F (entry 2). In this case, 5.23 g (98 %) of crude peptide were obtained as a white solid with a chromatographic purity of 91 % (figure 3.7, B). As expected, partially deprotected forms of the peptide were not detected when the remaining TFA was neutralised.

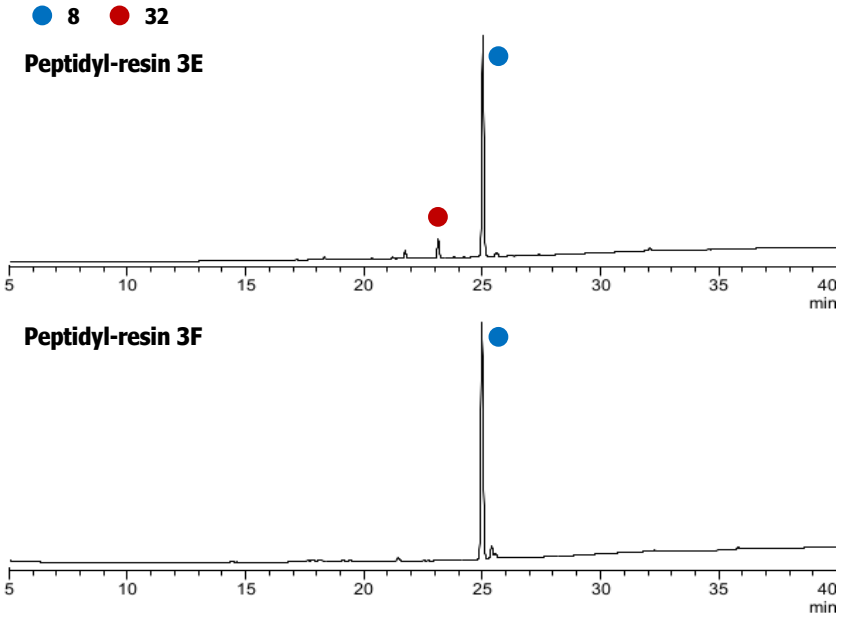


Figure 3.7. HPLC chromatograms of the peptide crudes obtained from batch 3E and 3F.

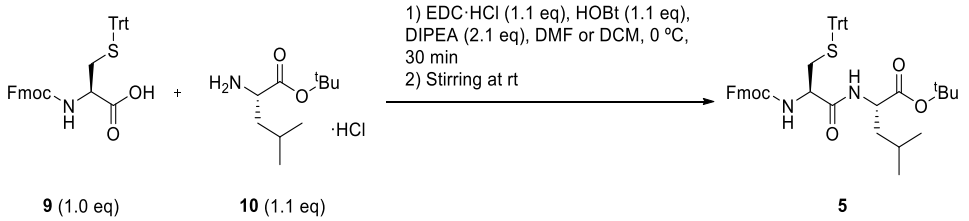
The functionalisation of the starting aminoacyl-resin 3E was 0.72 mmol/g and thus, the estimated amount of **8** was of 12.9 g. After performing the cleavage, the obtained amount of **8** was of 5.65 g (44 %). On the other hand, for the aminoacyl-resin 3F (functionalisation of 0.59 mmol/g), 5.3 g of **8** were expected. Surprisingly, after the cleavage of the peptidyl-resin 3F, 5.23 g (98 %) of the desired **8** were obtained, which represents an almost quantitative yield. Again, the result of batch 3E suggests that the removal of N-terminal protecting group when the resin had to be left several hours before coupling a new amino acid may not be enough to prevent the premature loss of peptide chains. The explanation of why peptide chains were prematurely lost is yet unknown, but further studies are necessary to identify the causes of these premature losses to assure the reproducibility of the synthesis.

3.3. Condensation of the protected peptide fragments in solution

3.3.1. Synthesis to obtain the dipeptide 11

In the previous chapter (section 2.3.1), the optimisation process for the synthesis of the protected dipeptide **5** was discussed and optimised. Nonetheless, in order to seek optimal conditions for amide bond formation between the N-terminal deprotected dipeptide **11** and the protected nonapeptide **28** to afford the protected undecapeptide **14**, more batches of **11** had to be prepared.

The best results were obtained when EDC·HCl was used as coupling reagent, HOBt as additive, DIPEA as base and DCM as solvent. The synthetic protocol consisted of adding a mixture of the coupling reagent and the base in DCM over a DCM solution of Fmoc-Cys(Trt)-OH, H_2N-Leu-O^{t}Bu·HCl and HOBt. However, we decided to modify the order of addition of reagents to avoid the solubility problems encountered when mixing the base and the coupling agent in DCM. Thus, neat DIPEA was now added dropwise with an automatic injector for 105 min over a solution containing Fmoc-Cys(Trt)-OH, H_2N-Leu-O^{t}Bu·HCl, EDC·HCl and HOBt. Moreover, the quantity of base was doubled (2.1 equivalents) and the reaction time was shortened from 17 h to 6 h (scheme 3.8). The reaction was monitored by HPLC and, after 6 h from the addition of the base, it was quenched by performing the work-up.



9 (1.0 eq) **10** (1.1 eq) **5**

Scheme 3.8. Synthesis of **5** using EDC·HCl, HOBt and DIPEA.

Table 3.7. Assays changing the order of addition of reagents.

	Solvent	**Time** [h]	**Crude Yield** [%]	**Chromatographic purities**[b] [%]		
				5	**1**	**Other**
Batch 5B[a]	DMF	6	99	96	0	4
Batch 5C[a]	DCM	6	93	87	5	8

(a) Both reactions were carried out using 6.60 mmol (3.87 g) of **9**.
(b) The percentages were obtained by integrating the peak areas at 220 nm.

The first reaction was performed using DMF as solvent while the second reaction was carried out using DCM (table 3.7). Figure 3.8 shows the chromatographic profiles of both reactions. As it can be seen, the HPLC chromatogram showed a 96 % of the dipeptide **5** and starting material **1** was not detected when DMF was used (batch 5B). The other peaks that were observed corresponded to **11** and DBF (2.2 % and 1.4 %, respectively). In the case of the reaction carried out using DCM as solvent (batch 5C), the HPLC chromatogram showed an 87 % of the dipeptide **5**, a 5 % of **1** and 8 % of by-products (1.4 % of **11** and 1.9 % of DBF, among other unidentified impurities).

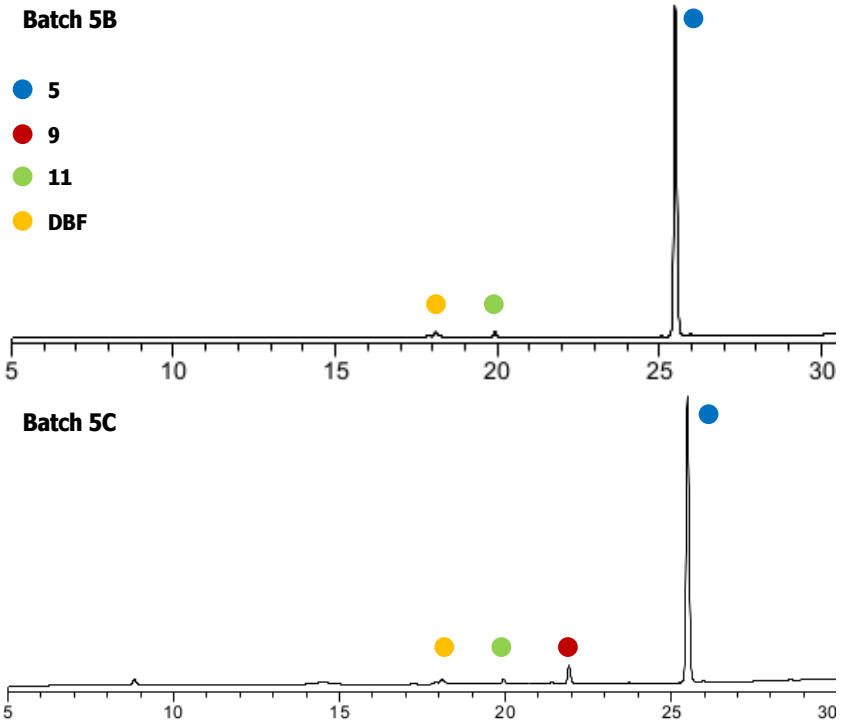


Figure 3.8. HPLC chromatograms of batches 5B and 5C.

Table 3.8 compares the results obtained for the synthesis of the dipeptide **5** described in section 2.3.1.1 using 1.1 equivalents of DIPEA (entries 1 and 2) with those achieved using 2.1 equivalents of base and using the new addition order of reagents (entries 3 and 4).

Table 3.8. Assays performed using different solvents and equivalents of DIPEA.

Entry	Solvent	DIPEA [eq]	Time [h]	Chromatographic purities[c] [%]		
				5	1	Other
1[a]	DMF	1.1	17	40	4	47
2[a]	DCM	1.1	17	91	7	0
3[b]	DMF	2.1	6	96	0	4
4[b]	DCM	2.1	6	87	5	8

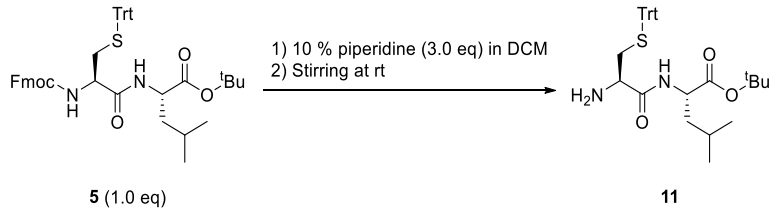
(a) Both reactions were carried out using 0.25 mmol (146.3 mg) of **9**.
(b) Both reactions were carried out using 6.60 mmol (3.87 g) of **9**.
(c) The percentages were obtained by integrating the peak areas at 220 nm.

This table shows that the chromatographic purity slightly decreased when DCM was used as solvent (entries 2 and 4). However, a remarkable improvement in the yield of **5** and a much lower percentage of impurities were observed when using DMF as solvent under the new conditions (entries 1 and 3). More specifically, the percentages of **11** and DBF decreased from 25 % to 2.2 % and from 22 % to 1.4 %, respectively.

So, according to the results obtained, the fact of adding the DIPEA alone to the mixture of reagents and/or increasing the number of equivalents of the base did not improve the chromatographic profile when the reaction was performed in DCM. However, DMF afforded much better results. The need of a long reaction time (17h, entry 1) could account for these results, which could be explained in terms of time exposure of reagents to the solvent. Those results suggest that long reaction times may compromise the stability of the Fmoc protection or favour other side reactions when DMF containing a base is used. In the

case of reactions performed in DCM (entry 4), premature Fmoc removal could be explained by the use of a higher quantity of base.

Th next step of the synthesis is the Fmoc removal of the dipeptide to obtain the desired dipeptide **11**. This reaction was performed by treating the protected dipeptide **5** with a solution of 10 % piperidine (3.0 eq) in DCM for 2.5 h (scheme 3.9). The target peptide **11** (batch 11C) was obtained in an 84 % yield (chromatographic purity of 99 %, figure 3.9) after the work-up and flash column chromatography (Hexanes/EtOAc 8:2 and DCM/MeOH 9:1).



Scheme 3.9. Removal of the N-terminal protecting group of **5**.

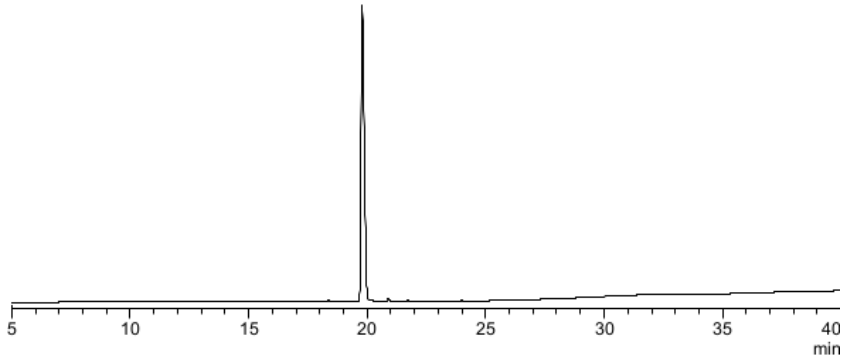


Figure 3.9. HPLC chromatogram of the purified **11** (batch 11C).

3.3.2. Optimisation of the synthesis of the undecapeptide 14

In the first convergent approach, the undecapeptide intermediate **14** was obtained from the condensation in solution of the protected pentapeptide **7** and the N-terminal deprotected hexapeptide **13** which, in turn, was obtained from the assembly in solution of the protected hexapeptide **6** and the N-terminal deprotected **11**.

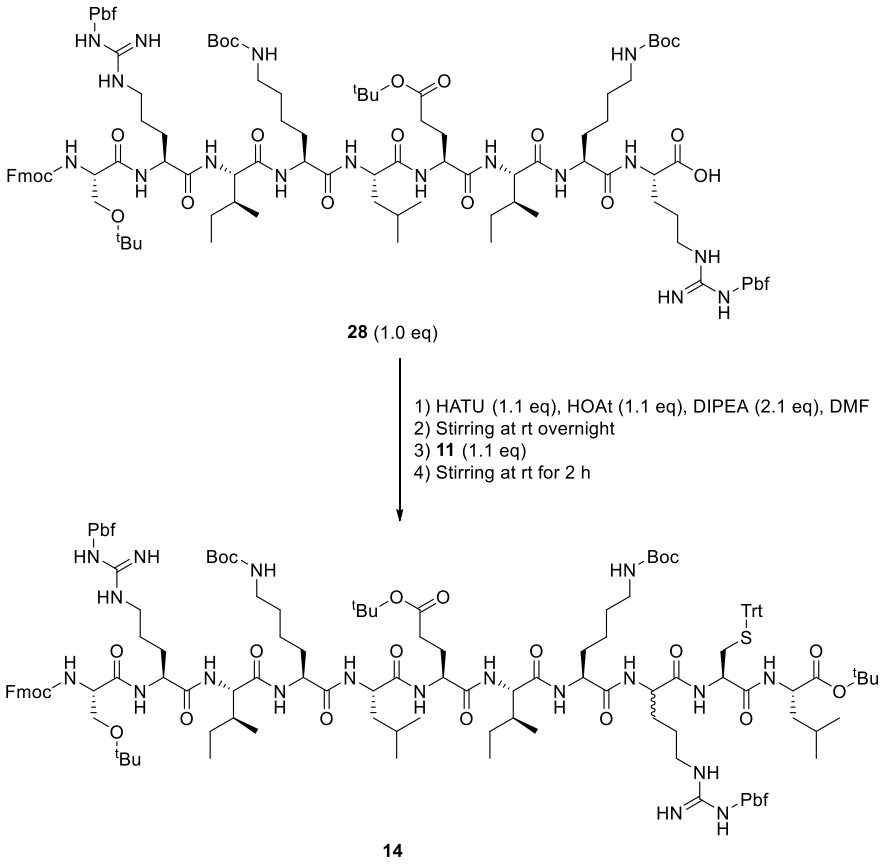
It is worth mentioning that during the coupling reactions, epimerisation was detected when EDC·HCl and HATU were used as coupling reagents. As mentioned in chapter 2, the risk of epimerisation is higher when coupling two peptide fragments, since the C-terminal residue that is being activated has at the N^{α}-position an amide instead of a carbamate. Amino protecting groups bound as carbamates, are less prone to induce the formation of the oxazolone intermediate when compared to amides due to the electronic withdrawing properties of the carbamate group that destabilises the anion that would be formed by proton abstraction.[53]

We must point out that although some epimerisation may be acceptable in laboratory studies, it become an important draw back in large industrial synthesis aimed to the production of pharmaceutical drugs with very demanding purity standards. In this context, the coupling reaction between the protected nonapeptide **28** and the N-terminal deprotected dipeptide **11**

was studied carefully aiming at optimizing the reaction conditions to obtain the minimum level of epimerization. With this purpose, in the present study the temperature was decreased from 0 ºC (the one used for the reactions discussed in chapter 2) to -20 ºC. Moreover, other conditions such as the use of different coupling reagents were examined to achieve the best possible results.

3.3.2.1. Test of epimerisation during fragment condensation

When coupling longer peptide fragments, it is sometimes hard to detect by HPLC if epimerisation has taken place because of the close retention times that epimers may have. To make sure that the HPLC method and the column used were able to differentiate the desired product from its epimer, a preliminary experiment was performed in which the protected nonapeptide **28** was preactivated overnight at room temperature in the presence of HATU and DIPEA to induce epimerisation (scheme 3.10). Afterwards, the N-terminal deprotected dipeptide **11** was added and left for 2 h. Finally, **14** was precipitated in water to remove the excess of reagents and salts; and the resulting crude was triturated with Et_2O.



Scheme 3.10. Synthesis of the undecapeptide **14** under epimerisation conditions.

As shown in figure 3.11, the HPLC-MS analysis of the crude obtained revealed two peaks corresponding to the desired undecapeptide **14** (retention time of 27.6 min in figure 3.11, A) and the corresponding epimer (**33**, figure 3.10, retention time of 28.9 min in figure 3.11, A). The epimer of **28** (**34**, figure 3.10) could also be detected. The N-terminal deprotected dipeptide **11** was not observed probably because the trituration with Et_2O effectively removed the excess of it from the crude. These results confirmed that the analytical conditions that were used were suitable to determine if epimerisation took place under the experimental conditions.

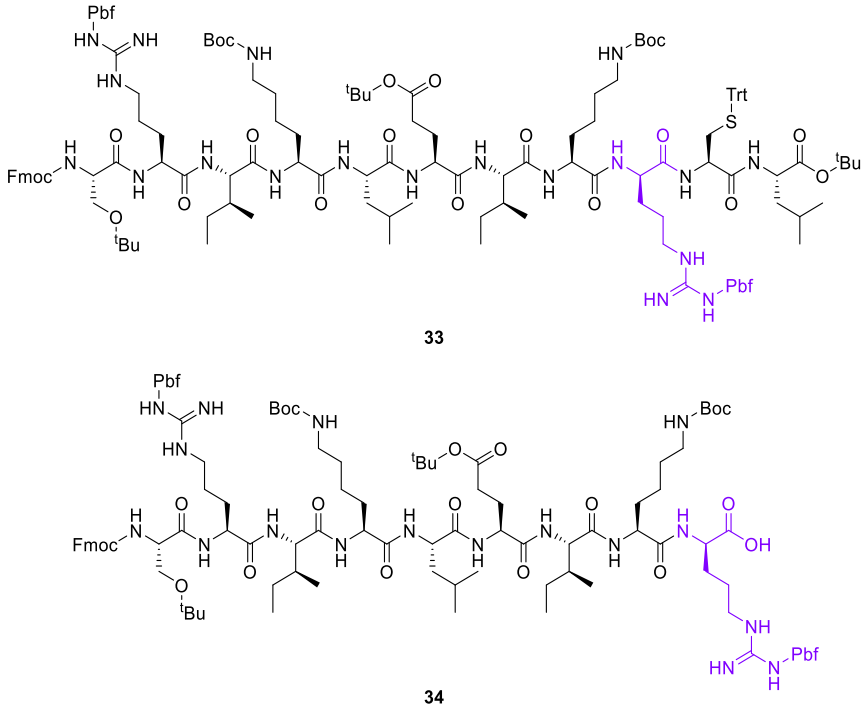


Figure 3.10. Epimers of **14** (**33**) and **28** (**34**).

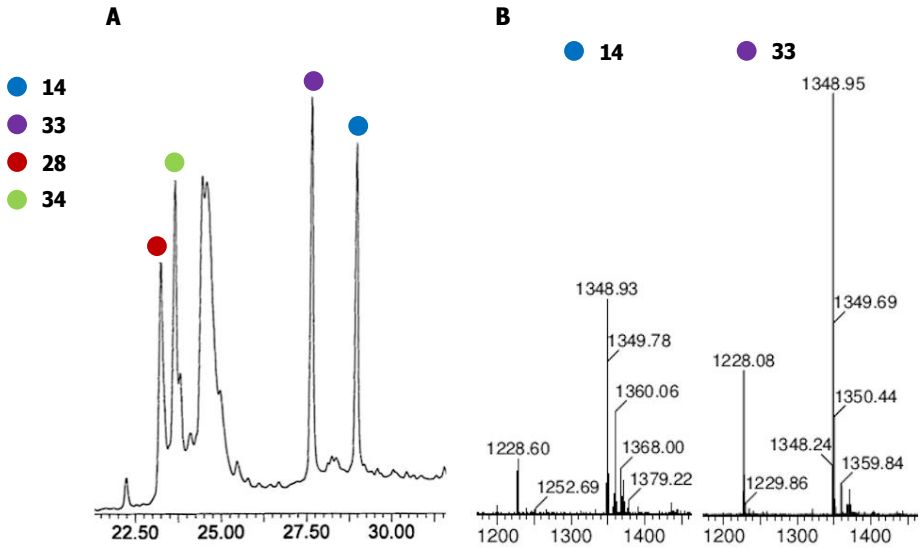
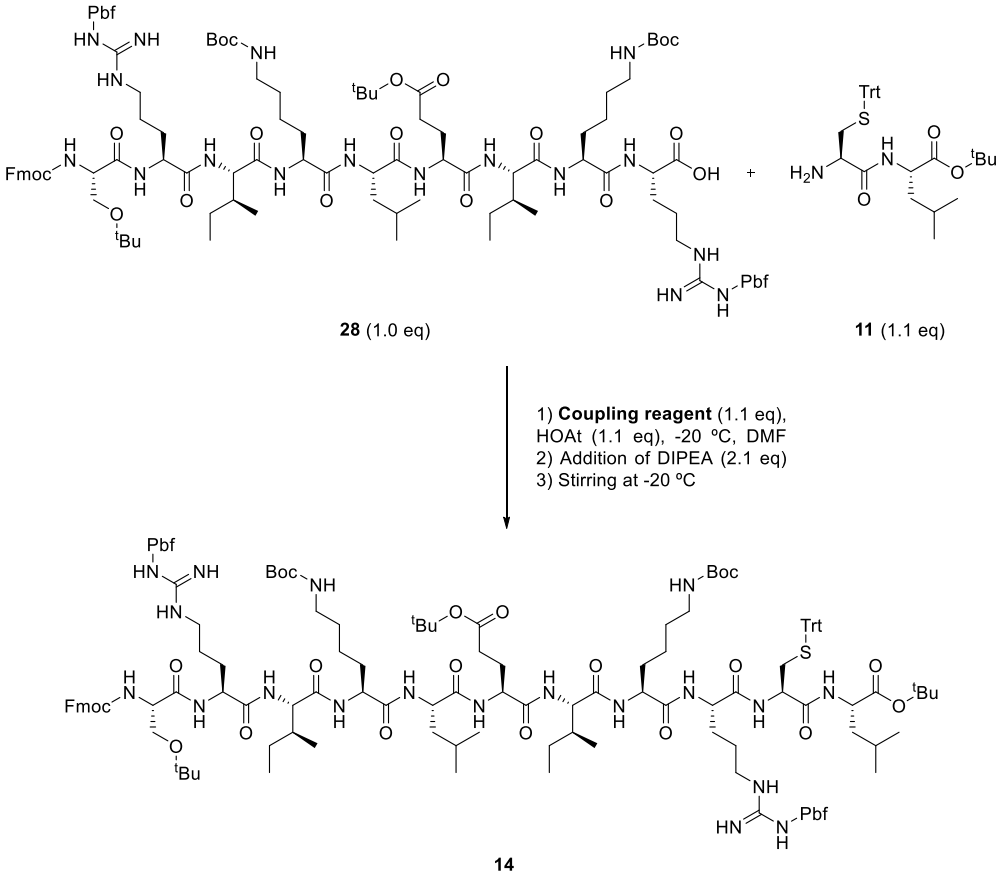


Figure 3.11. HPLC (A) and MS spectra (B) of the resulting reaction crude when forcing epimerisation.

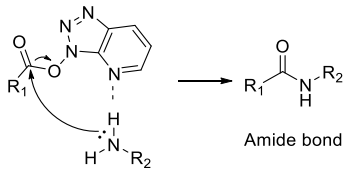
3.3.2.2. Assays using different coupling reagents

A battery of reactions (entries 1-4, table 3.9) were performed with the aim of finding the best coupling reagent considering the availability and price of each of them. The general procedure involved the dropwise addition of DIPEA in DMF during 30 min over a DMF solution with the protected nonapeptide **28**, the N-terminal deprotected dipeptide **11**, the coupling reagent and the additive (if necessary) at -20 ºC (scheme 3.11). Again, the reaction mixture was precipitated in water to remove the excess of reagents and salts, and triturated with Et_2O to remove the excess of the N-terminal deprotected dipeptide **11**. The reactions were monitored by HPLC-UV until starting material **28** was not detected.



Scheme 3.11. Synthesis of **14** using different coupling reagents.

The additive employed in these syntheses was HOAt, a more efficient analogue of HOBt because it has a nitrogen atom located at the position 7 of the benzotriazole which causes two main effects.[113] First, the electron-withdrawing effect makes the HOAt ester a better leaving group, thus increasing the reactivity of the amine towards the ester. Second, the position of this nitrogen leads to a neighbouring effect that favours the nucleophilic attack of the amine to the activated carboxylic acid to form the amide bond and consequently, epimerisation is reduced (scheme 3.12).



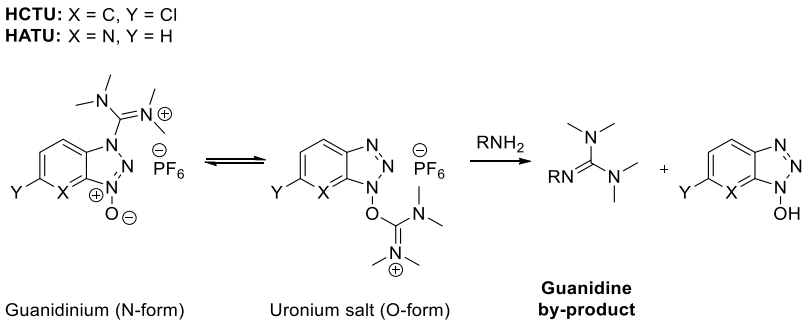
Scheme 3.12. Neighbouring effect of HOAt.

Table 3.9. Comparison of the reactions performed using different coupling reagents.

Entry[a]	Coupling reagent	Additive	Time [h]	Crude yield [%]	Chromatographic purities[b] [%]		
					14	**33**	**Other**
1	EDC·HCl	HOAt	3	89	94	1.8	3
2	PyOAP	-	1	87	95	0.5	3
3	HCTU	HOAt	2	87	95	0.7	3
4	HATU	HOAt	1	88	95	0.3	3

(a) All reactions were carried out using 0.17 mmol (370.0 mg) of **28**.
(b) The percentages were obtained by integrating the peak areas at 220 nm.

The results obtained in the reaction corresponding to entry 1 showed that the carbodiimide proved to be inadequate in terms of epimerisation as the HPLC chromatogram showed the highest percentage of **33** (1.8 %). The other coupling agents (PyAOP, HCTU and HATU) are known to be more efficient than carbodiimides.[113] The presence of HOAt in reactions containing HATU or HCTU prevents the formation of guanidine by-products (scheme 3.13).[114,80] The results revealed that the use of PyAOP, HCTU/HOAt or HATU/HOAt were better coupling conditions than EDC·HCl/HOAt because the epimer was formed in lower percentages (0.3 % to 0.7 %) and the reactions were faster (entries 2, 3 and 4, respectively). Figure 3.12 compares the HPLC chromatographic purity of the crude obtained when EDC·HCl or HATU were used as coupling reagents.



Scheme 3.13. Formation of the guanidine by-product when using HCTU or HATU.

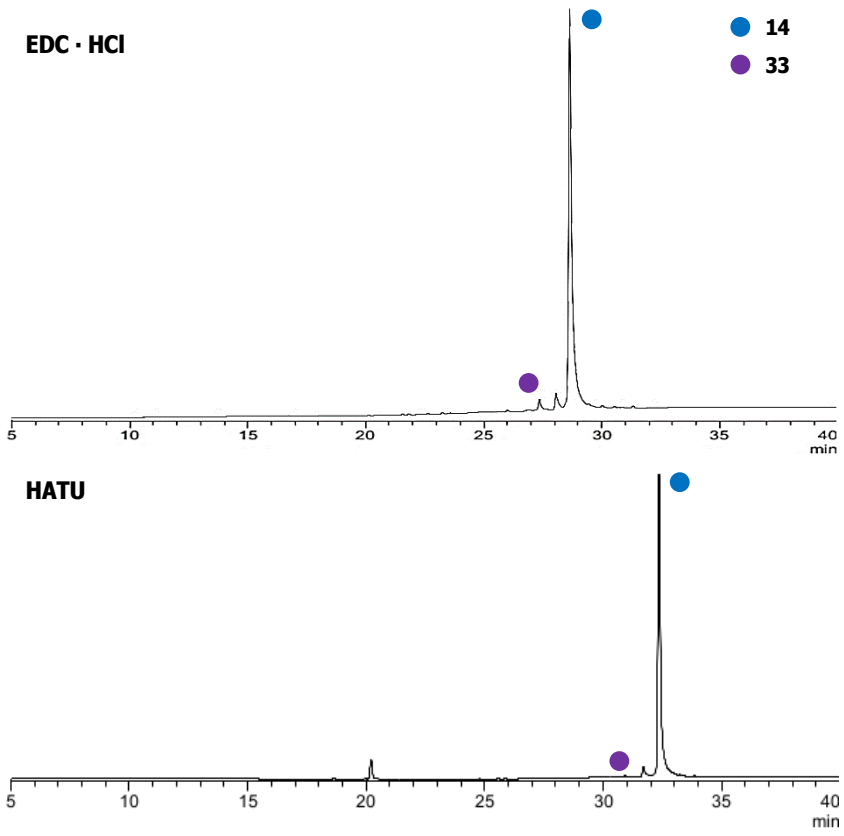


Figure 3.12. HPLC chromatograms of the reactions corresponding to entries 1 and 4, table 3.9.

The reason why there are some differences in the retention time of the HPLC chromatograms showed in figure 3.12 is due to the fact that the crudes were analysed with a different HPLC instrument but under the same chromatographic conditions. Moreover, the

signal with a retention time of 20.2 min in the HATU chromatogram does not belong to the sample as it was also observed in the preceding blanks that were injected before the reaction sample.

Considering the results obtained with HATU as coupling reagent and HOAt as additive, a new battery of reactions was designed in order to optimise experimental conditions such as the order of addition of reagents, temperature, reagent concentrations or reaction scale. Table 3.10 summarises these assays.

Table 3.10. Reaction conditions using HATU/HOAt.

	Temperature [ºC]	**DMF**[c] [mL]	**Order of addition**[d]	**Time**[e] [h]	**Crude yield** [%]	**Chromatographic purities**[f] [%]		
						14	**33**	**Other**
Batch 14D[a]	-20	4+1	Protocol 1	1	88	92	1.9	6
Batch 14E[a]	-20	5	Protocol 2	1	74	91	0.9	8
Batch 14F[a]	-20	4+1	Protocol 3	1	82	92	1.2	7
Batch 14G[a]	-40	4+1	Protocol 3	1	84	93	1.1	6
Batch 14H[a]	-20	8+2	Protocol 3	1	84	93	0.6	6
Batch 14I[a]	-20	8+2	Protocol 4	3	83	86	0.5	13
Batch 14J[b]	-20	18+6	Protocol 3	1	87	93	1.0	6

(a) The reactions were carried out using 0.17 mmol (370.0 mg) of **28**.
(b) The reaction was carried out using 0.40 mmol (884.0 mg) of **28**.
(c) The DMF volume is referred as "volume in the round-bottom flask + volume in the syringe".
(d) Protocol 1: Round-bottom flask (RBF) containing **28**, HOAt and HATU. Syringe containing **11** and DIPEA.
Protocol 2: RBF containing **28**, **11**, HOAt and HATU. Syringe containing neat DIPEA.
Protocol 3: RBF containing **28**, **11**, HOAt and HATU. Syringe containing DIPEA diluted in DMF.
Protocol 4: RBF containing **28**, **11** and HOAt. Syringe containing HATU and DIPEA.
(e) Time after the addition of the base that was added for 30 min.
(f) The percentages were obtained by integrating the peak areas at 220 nm.

In general, all the assays afforded the desired product with a high chromatographic purity (between 91 % and 93 %) and crude yield (between 74 % and 88 %). The starting material **28** was consumed in all the reactions, except for the reaction in which HATU was added simultaneously with DIPEA (batch 14I). This might be a consequence of a reaction that probably took place when HATU was mixed with DIPEA in DMF because the solution became orange after mixing. The reaction in which the N-terminal deprotected dipeptide **11** was added together with the base (batch 14D) lead to a higher percentage of epimer (1.9 %). The fact that **11** was mixed with the base before starting the reaction and that cysteine is one of the amino acids most sensitive to racemisation could account for this result.

The volume of DMF to carry out the reaction is important in terms of epimerisation since the protected nonapeptide **28** could start its preactivation before the addition of the base as the N-terminal deprotected dipeptide **11** can deprotonate the C-terminal carboxyl group of **28**, thus leading to the formation of active intermediates in the presence of the coupling reagents. This was proven when two assays using different volumes of DMF were carried out (batches 14F and 14H). The HPLC chromatogram of the crude obtained from the reaction performed using the lowest volume of DMF showed a 1.2 % of the epimer **33** while only a 0.6 % of the epimer was detected when the volume of DMF was doubled. The reaction described in batch 14E of table 3.10 confirms that the volume of solvent to carry out the reaction is important to minimise epimerisation since only 0.9 % of **33** was detected even though the base was added neat. Moreover, the percentage of epimer was lower when compared to the percentage obtained in the reaction of batch 14F, which had the same final volume of DMF (5 mL). These differences could be accounted to the different initial

volumes of solvent (5 mL in the former compared to 4 mL in the latter). Finally, the reaction proved to be very fast because the starting **28** was also consumed at -40 ºC (batch 14G) and a similar percentage of **14** was achieved when compared to the reaction carried out at -20 ºC (batch 14F). Curiously, the formation of **33** was not reduced.

With these results, the reaction was slightly scaled-up (batch 14J) following a protocol similar to that used in the reaction described in entry 5. The HPLC chromatogram of the reaction crude showed a 93 % of the desired protected undecapeptide **14**, a 1 % of the epimer **33** and a 6 % of other unknown impurities (figure 3.13).

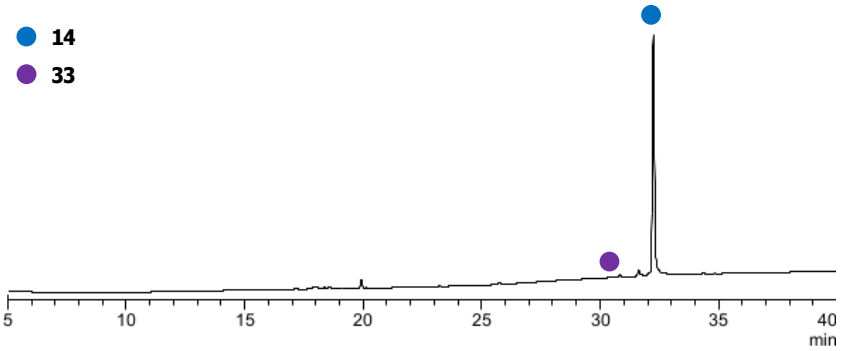


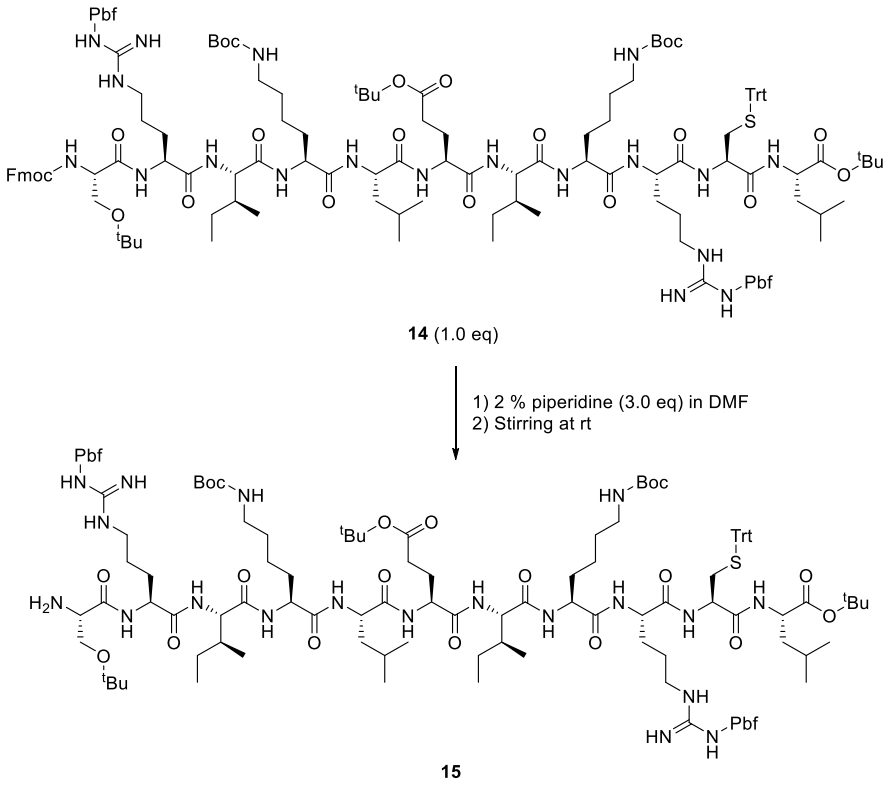
Figure 3.13. HPLC chromatogram of the crude corresponding to the reaction of batch 14J (table 3.10).

With the first convergent approach, described in chapter 2, the intermediate **14** was obtained with chromatographic purities of 83 % and 94 %, the latter one with lower yields. Thus, with this new approach, the chromatographic profiles and yields of the reactions to obtain the undecapeptide **14** have improved enough to be considered a good starting point for the industrial scaling-up of this step.

3.3.3. Fmoc removal of the protected undecapeptide 14

The next step of the synthetic route was the Fmoc removal of the protected undecapeptide **14** to obtain the N-terminal deprotected undecapeptide **15**. In the synthetic approach of chapter 2, the Fmoc group of **14** was removed by using a solution of 10 % piperidine in DMF (section 2.3.6). In this new synthetic approach, piperidine was diluted to a 2 % (scheme 3.14).

Table 3.11 summarises the results achieved on the four assays that were performed. The reaction was monitored by HPLC and after 1 h, the starting material **14** was no longer detected. The desired product was precipitated with water, filtrated and triturated six times with Et_2O (batches 15B and 15C). However, some filtration problems were encountered in the two first assays, associated with the obstruction of the filter. Consequently, it was decided to replace the filtration of the solid for centrifugation (batches 15D and 15E). A final trituration of the resulting crude with Et_2O lead to **15** with good chromatographic purities. We must point out that this result confirms the effectiveness of such trituration to remove the by-products generated during the Fmoc removal, as previously observed in chapter 2 (section 2.3.6).



Scheme 3.14. Preparation of **15**.

Table 3.11. Results of the reactions carried out for the synthesis of **15**.

	14 [g]	**Work-up**	**Crude yield** [%]	**Chromatographic purities**[a] [%]			
				15	**DBF**	**DBF-adduct**	**Other**
Batch 15B	1.33	Filtration	80	86	1.7	0.4	11
Batch 15C	0.93	Filtration	69	88	2.5	0.7	8
Batch 15D	0.37	Centrifugation	97	92	-	-	8
Batch 15E	0.32	Centrifugation	89	94	-	-	6

(a) The percentages were obtained by integrating the peak areas at 220 nm.

The HPLC analyses of the reactions that are shown in batch 15B and 15C showed an 86 % and an 88 % of N-terminal deprotected undecapeptide **14** together with a 2.1 % and 3.2 % Fmoc by-products, respectively. On the other hand, these by-products were not detected in the crudes resulting from centrifugation (batches 15D and 15E), and thus the chromatographic purity of the target peptide was slightly higher (92 % and 94 %, respectively).

It is worth mentioning that the Fmoc group was fully removed from the intermediate **14** even though the solution of piperidine in DMF was diluted to 2 %. Moreover, the purity and yield of the target peptide **15** was improved by changing the filtration for centrifugation as shown in figure 3.14.

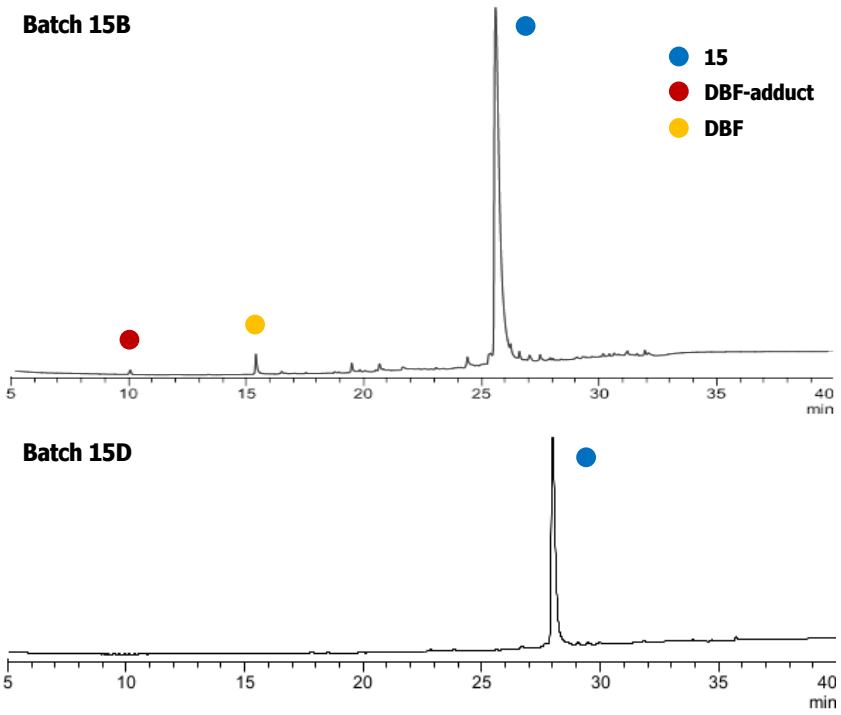
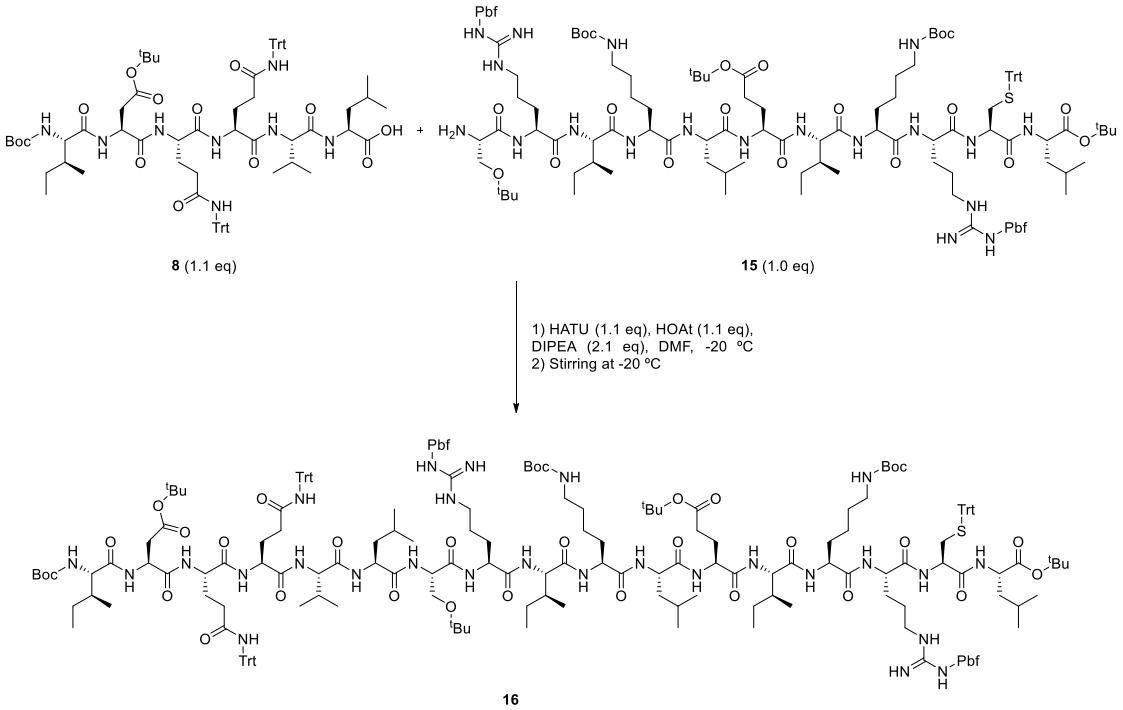


Figure 3.14. HPLC profiles of the crudes of **15** resulting from filtration and centrifugation (the differences in the retention times are due to the fact that both analysis were performed using the same chromatographic conditions but in different HPLC instruments).

3.3.4. Optimisation of the synthesis of the protected peptide 16

The next step of the synthetic route consisted of the coupling the protected hexapeptide **8** with the N-terminal deprotected undecapeptide **15** to obtain the protected Fexapotide **16** (scheme 3.15).

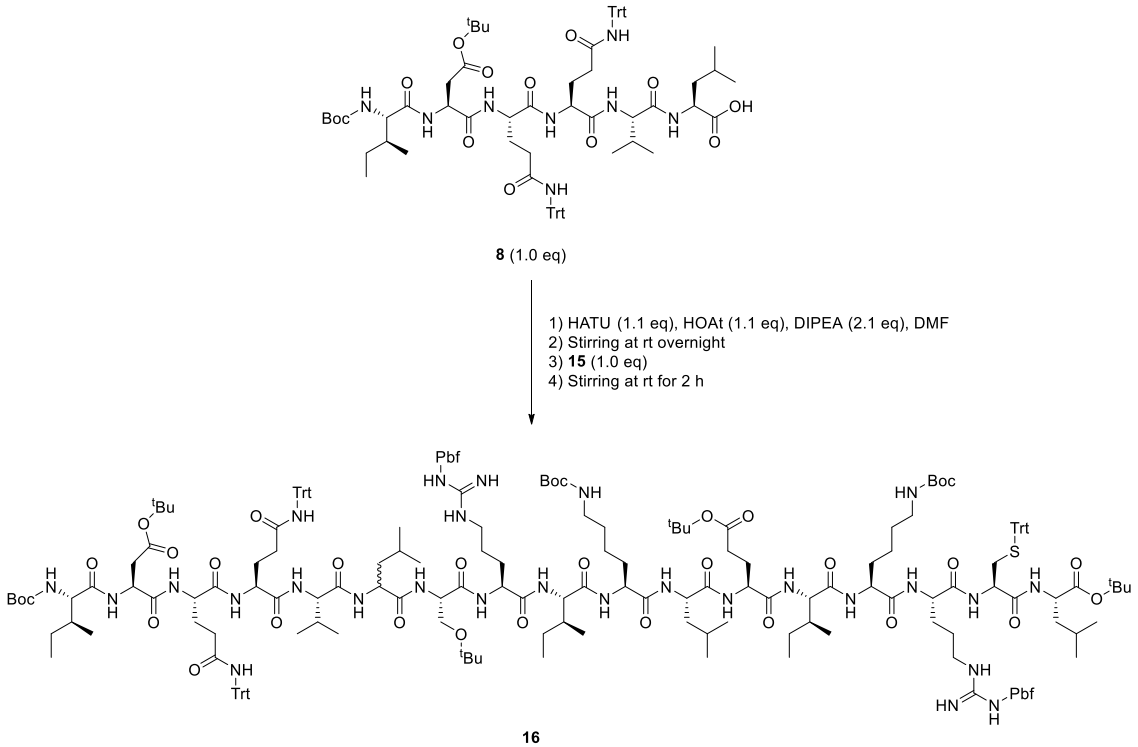


Scheme 3.15. Final chain assembly to obtain the protected Fexapotide **16**.

The target Fexapotide **1** and the precursor of **1**, which is the protected Fexapotide **16**, were considered HP-APIs. Working with APIs that have high potency and cytotoxicity present several challenges for pharmaceutical companies such as handling, containment and cost. Therefore, solid state handling and reactions in which one of these substances was present were carried out in an isolator following strict security protocols (section 5.2.2, experimental section).

3.3.4.1. Test of epimerisation during fragment condensation

As mentioned in section 3.3.2.1, to make sure that the HPLC method and the column that were going to be used were able to differentiate the desired product from its epimer, a preliminary experiment was performed in which the protected hexapeptide **8** was preactivated overnight at rt in the presence of HATU and DIPEA to induce epimerisation. Afterwards, the N-terminal deprotected undecapeptide **15** was added and stirred for 2 h. Finally, the crude was precipitated in water to remove the excess of reagents and salts, and triturated with Et_2O (scheme 3.16).



Scheme 3.16. Forcing the epimerisation of the protected peptide **16**.

The resulting crude was analysed by HPLC-MS revealing two peaks that were attributed to the desired **16** and its corresponding epimer (**25**) in a proportion of 64:36 (figure 3.15). No other relevant impurities were observed. Therefore, the analytical conditions were considered suitable to determine if epimerisation takes place during fragment coupling under standard experimental conditions.

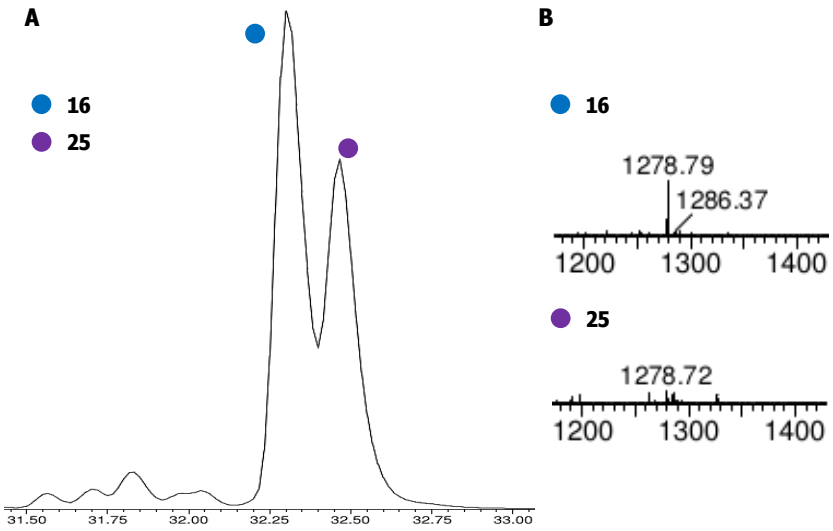


Figure 3.15. (A) HPLC of the resulting crude from the reaction carried out under conditions shown in scheme 3.16. (B) MS spectra ($[M+2H+Na]^{3+}$) of the two main peaks of the crude.

3.3.4.2. Assay of a new work-up methodology for removing the excess of 8

The coupling between the protected hexapeptide **8** and the N-terminal deprotected undecapeptide **15** is performed by using the former in excess. Consequently, **15** should not be detected if the reaction proceeds quantitatively.

In the previous coupling step, the excess of one of the starting materials was removed by washing the crude that was obtained with Et_2O (chapter 3, section 3.3.2.2). Nonetheless, removing the excess of **8** with washings resulted to be problematic due to the higher insolubility of **8** when compared to the dipeptide **11**, which moved us to set up a new work-up methodology. In general, a precipitation with water was usually carried out once the reaction reached full conversion. Now, an excess of base was added to the crude with the aim of forming the salt of the C-terminal peptide fragment and thus, avoid its precipitation in water.

Therefore, the protected hexapeptide **8** was dissolved in DMF (1 mL), DIPEA (5.0 eq) was added and the resulting mixture was left stirring for 1 h when water (9 mL) was added. The formation of a precipitate was not observed, and the reaction mixture was placed in an ice bath where additional water (9 mL) was added. Precipitation was again not observed. Figure 3.16 shows the analyses by HPLC and HPLC-MS of the aqueous solution.

The HPLC chromatogram showed the presence of **8** (54.2 %) and two more products at retention times of 22.8 min (26 %) and 23.1 min (14 %), with a m/z of 1299.7 and 1299.8, respectively. Moreover, the mass of these products was 56 g/mol lower than the mass of the protected hexapeptide **8**, the retention times of these products were very similar and they eluted around 2 min faster than **8**. Altogether, indicated the possibility of aspartamide formation.

This important side reaction can take place under acidic or basic conditions, and consists of an intramolecular cyclisation through the aspartic acid side-chain that leads to a five membered succinimide ring with the concomitant loss of the side-chain protecting group ^{t}Bu (**35**, scheme 3.17).[115,116,117] The succinimide intermediate **35** opens in the presence of water, to form the α (**36**) and β (**37**) peptides, both with the same mass. The aspartamide formation

is highly sequence-dependent and can be minimised using bulky protecting groups for the β-carboxylic acid.[118]

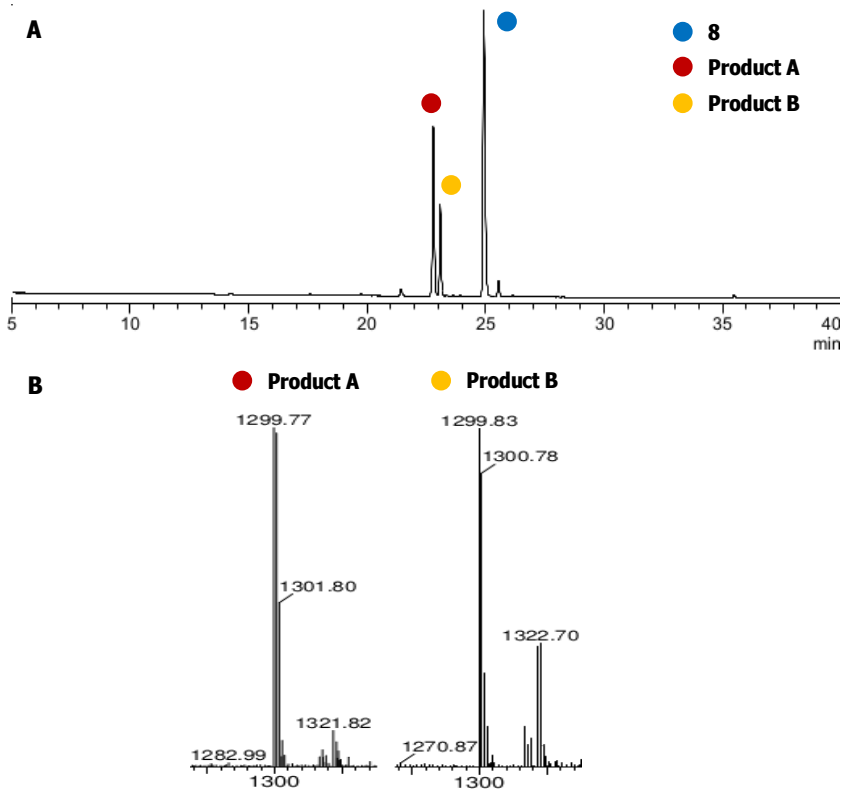
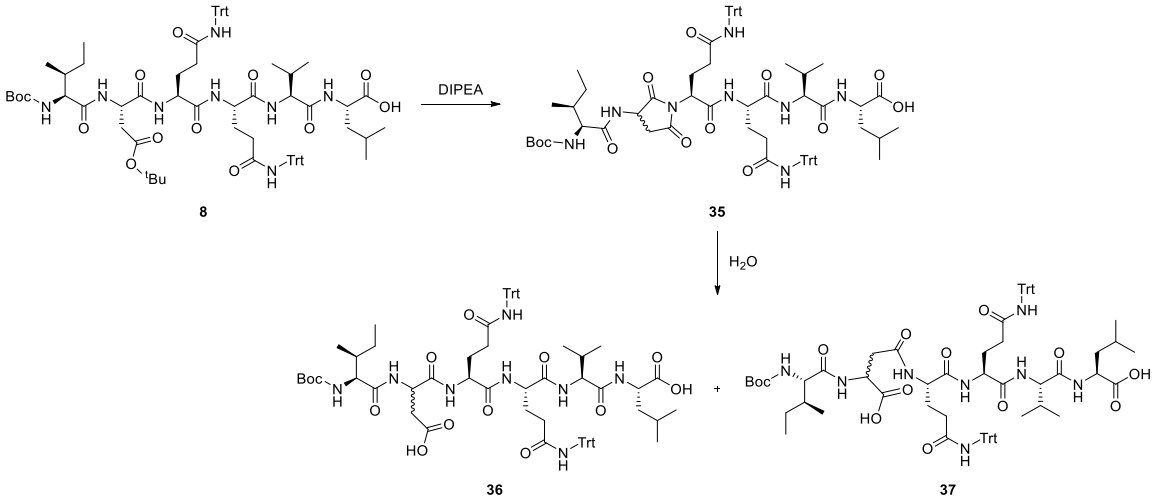


Figure 3.16. HPLC of the aqueous solution (A) and MS spectra of the main peaks (B).

The expected mass (m/z) for the α- and β-peptides (**36** and **37**) resulting from the protected hexapeptide **8** is 1298.7, which was coincident with the mass found for the by-products detected in the chromatographic analysis of the basic aqueous solution (products A and B, figure 3.16). The fact that precipitation in water was not observed, led us think that this work-up methodology could be useful to remove the excess of **8**. However, the absence of potential aspartamide formation in the protected Fexapotide **16** had to be proved.



Scheme 3.17. Formation of aspartamide in the presence of a base.

3.3.4.3. Reaction assay including DIPEA in the work-up methodology

Following the protocol shown in the scheme 3.15, the protected hexapeptide **8** (1.1 eq), the N-terminal deprotected undecapeptide **15** (0.07 mmol, 1.0 eq) and HOAt (1.1 eq) were weighed and dissolved in DMF. Once the solution was clear, it was placed in a MeOH/ice bath (-20 ºC) and HATU (1.1 eq) was added. Then, DIPEA (2.1 eq) was weighted over DMF and added to the cold solution dropwise with an automatic injector for 30 min. The reaction was monitored by HPLC-UV and after 4.5 h, less than 1 % of starting material **15** was detected. Finally, the reaction mixture was placed in an ice bath. The crude containing the desired product was precipitated with water, centrifuged and lyophilised.

Analysis of the white solid obtained showed the desired **16** with a chromatographic purity of 74 %, together with a 2 % of **8**. At this point, the new work-up methodology was tested to remove the excess of the starting material.

The solid was dissolved in DMF and DIPEA was added (4.5 eq relative to **8**). The resulting mixture was left stirring for 30 min when the product was precipitated by the addition of water. The suspension was centrifuged, the supernatant was separated and the solid was lyophilised to dryness. Finally, the aqueous solution and the obtained solid were analysed by HPLC (figure 3.17).

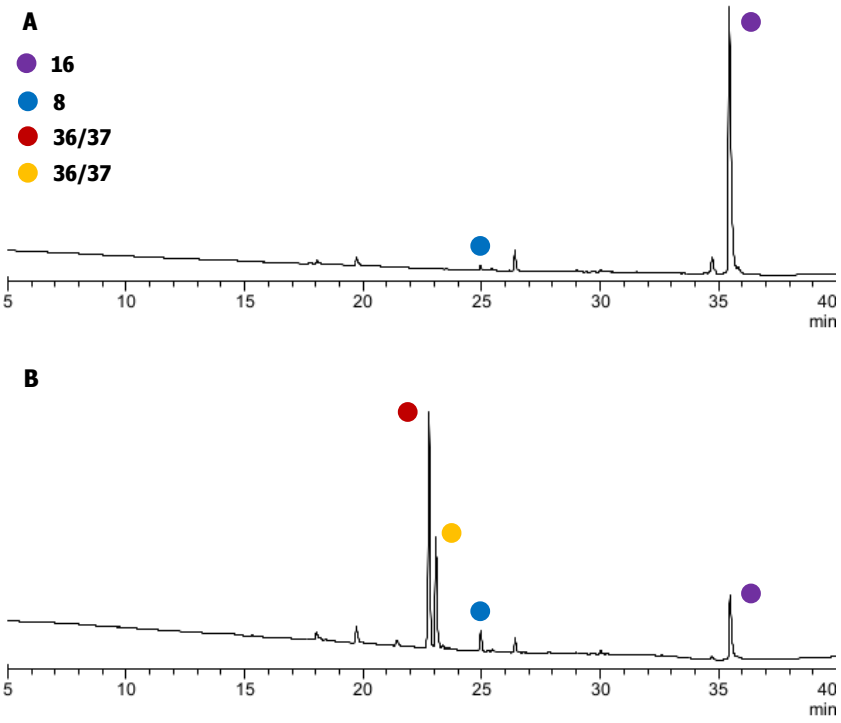


Figure 3.17. HPLC chromatograms of the solid (A, batch 16B) and the aqueous solution (B).

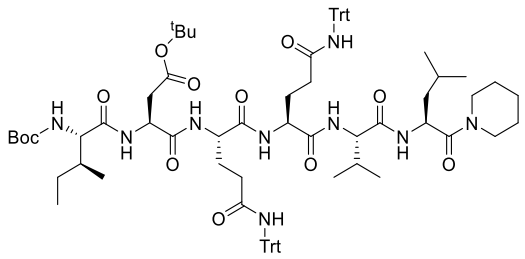
The HPLC chromatogram of the crude presented the protected Fexapotide **16** (batch 16B) with a chromatographic purity of 82 %, a 0.4 % of starting material **8** and a 4 % of an unknown impurity (26.4 min) with a m/z of $[M+H]^+$ 1422.8 (figure 3.17, A). On the other hand, the HPLC chromatogram of the aqueous phase showed the presence of **36** and **37** in a 47 % and 22 %, respectively. The hexapeptide **8**, the impurity with a m/z of $[M+H]^+$ 1422.8 and the protected peptide **16** were detected in a 4 %, 2 % and 18 % respectively.

These results suggested that the new work-up methodology was valid for removing the excess of **8** since a decrease from a 2 % to a 0.4 % could be observed. The HPLC analysis

of the aqueous phase confirmed that aspartamide was formed on the **8** sequence but not with the **16** sequence because the peaks with the mass of the corresponding aspartamide by-products of **16** were not detected. The target protected peptide detected in the aqueous solution was a consequence of the suspension of a small part of the solid that remained after centrifugation.

An accurate LC-MS/MS analysis of the crude was performed to determine the structure of the impurity with a m/z of $[M+H]^+$ 1422.8. The LC-MS/MS software suggested that the molecular formula of this product was $C_{83}H_{107}N_9O_{12}$. Comparing to the molecular formula of the protected hexapeptide **8** ($C_{78}H_{98}N_8O_{13}$), it could be said that both structures were related because of the small difference in the molecular formula.

This result suggested that probably a substitution at the C-terminal of **8** provoked by an amine had occurred during the activation of the carboxylic acid by the coupling reagents. Unexpectedly, the molecular formula was coincident with the incorporation of piperidine and therefore, the by-product with an m/z of 1422.8 was the piperidide form of **8** (**38**, figure 3.18).



38

Figure 3.18. Proposed structure of the impurity with a m/z of $[M+H]^+$ 1422.8.

The piperidine, that led to the formation of the piperidide impurity, might have come from the intermediate **15** because this peptide was obtained by treatment of the protected undecapeptide **14** with piperidine to remove the Fmoc protecting group. Therefore, the presence of the **38** suggested that some piperidine remained in the solid of **15** and reacted when exposed to the protected hexapeptide **8** when was being activated by the coupling reagents. Another batch of **15** had to be used to follow the study.

3.3.4.4. Fragment condensation using another batch of 15

The reaction was performed under the same conditions but the batch of N-terminal deprotected undecapeptide **15** was changed to find out if the piperidide **38** was again detected. The reaction was carried out twice to confirm the results. Table 3.12 summaries the results that were obtained.

Table 3.12. Results using another batch of **15**.

	Batch of 15	**Time** [h]	**Crude yield** [%]	**Chromatographic purities**[b] [%]			
				16	**8**	**38**	**Other**
Batch 16C[a]	15B	4.5	70	56	-	17	27
Batch 16D[a]	15B	2	86	54	-	14	32

(a) The reaction was carried out using 0.07 mmol (169.7 mg) of **15**.
(b) The percentages were obtained by integrating the peak areas at 220 nm.

The HPLC chromatograms of the reactions showed again the presence of the piperidide **38** and at higher percentages. The crude obtained in the reaction of batch 16C contained the piperidide **38** in a 17 % and an unknown impurity in a 9 %, together with minor impurities. This unknown impurity had a similar retention time and mass (m/z of $[M+2H]^{2+}$ 1243.7) to the N-terminal deprotected undecapeptide **15** (m/z of $[M+2H]^{2+}$ 1237.2).

On the other hand, the crude obtained for the reaction of batch 16D contained the piperidide **38** in a 14 %, together with a 4 % of **15** and a 12 % of the impurity with a m/z of $[M+2H]^{2+}$ 1243.7.

These results suggested that the amount of piperidine retained in this batch of **15** (batch 15B, table 3.11, section 3.3.3) was higher when compared to the one used for the reaction described in section 3.3.4.3 according to the amount of piperidide that was formed. Moreover, the consumption of the protected hexapeptide **8** to form the piperidide led to an incomplete conversion and consequently, starting material **15** was not consumed and could be detected in the HPLC (figure 3.19).

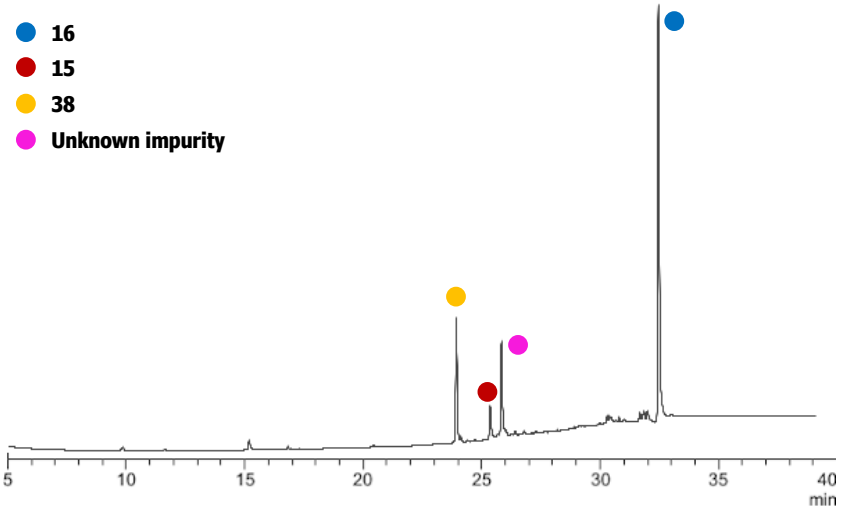


Figure 3.19. HPLC chromatogram of the obtained crude of batch 16D.

These results moved us to perform the reaction again with a different batch of N-terminal deprotected undecapeptide **15**. Table 3.13 summarises the results obtained for these new batches.

Table 3.13. Results using different batches of **15**.

	Batch of 15	**Time** [h]	**Crude Yield** [%]	**Chromatographic purities**[f] [%]			
				16	**8**	**38**	**Other**
Batch 16E[a]	15E	2	89	78	-	6	16
Batch 16F[b]	15D	1	-	75	2	6	17
Batch 16G[c]	15D[d]	3	>100[e]	73	3	7	17

(a) The reaction was carried out using 0.09 mmol (230.0 mg) of **15**.
(b) The reaction was carried out using 0.007 mmol (17.8 mg) of **15**, the temperature was not controlled, and the product was not isolated.
(c) The reaction was carried out using 0.07 mmol (169.3 mg) of **15**.
(d) **15** was mixed with water.
(e) The crude yield was above 100 % (108 %) most probably because the resulting solid was not completely dried when weighed.
(f) The percentages were obtained by integrating the peak areas at 220 nm.

The HPLC chromatograms of the reactions showed that the piperidide **38** was again detected but at lower percentages (6 % and 7 %).

A slurry with water was performed to the batch of N-terminal deprotected undecapeptide **15** (15D) to remove the piperidine but the HPLC profile of the reaction crude that was obtained using the peptide that was washed with water (batch 16G) still contained the by-product in a 7 %.

These results suggested that piperidine was retained in the batches of **15** used to perform the reactions and that washings of **15** with water did not decrease the percentage of the by-product. It is worth mentioning that the percentage of the piperidide impurity formed is lower in the reactions described in table 3.13 when compared to the ones performed in table 3.12. Thus, the latter reactions reached full conversion and consequently, starting material **15** was not detected. Figure 3.20 shows the HPLC chromatogram of the crude obtained for batch 16G.

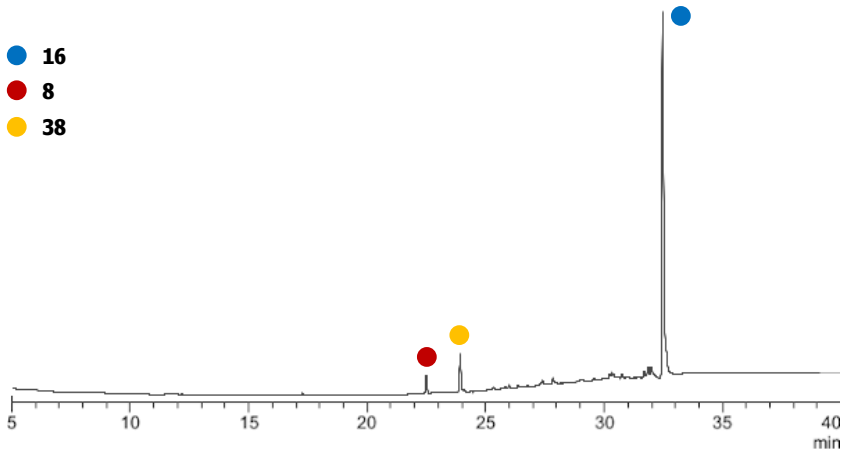
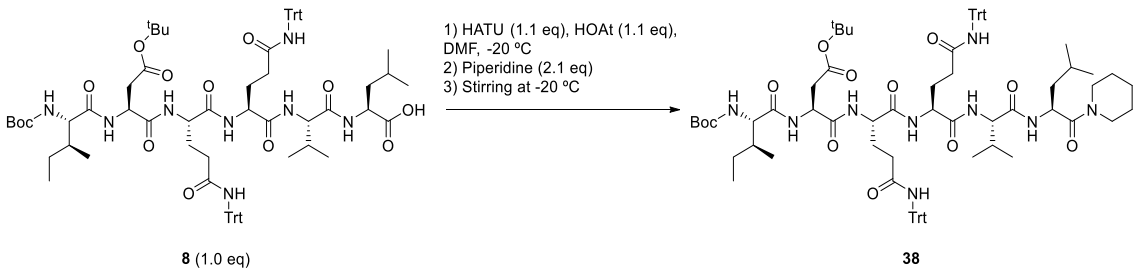


Figure 3.20. HPLC chromatogram of the crude of batch 16G.

3.3.4.5. Synthesis and solubility studies of 38

The piperidide **38** was synthesised to confirm the results described in section 3.3.4.4, and to perform solubility studies. A knowledge of the solubility properties of **38** would be useful to remove the by-product from a crude containing the protected Fexapotide **16**.

The synthesis of the piperidide **38** was carried out as follows: the protected hexapeptide **8** (0.59 mmol, 1.0 eq), HATU (1.1 eq) and HOAt (1.1 eq) were dissolved together in DMF at -20 ºC. Diluted piperidine (2.1 eq) in DMF was then added with an automatic injector (scheme 3.18). The reaction was monitored by HPLC and the reaction crude was precipitated with water when no starting material **8** was detected.



Scheme 3.18. Synthesis of the piperidide **38**.

The HPLC analysis of the crude showed the piperidide **38** with a chromatographic purity of 92 % (figure 3.21).

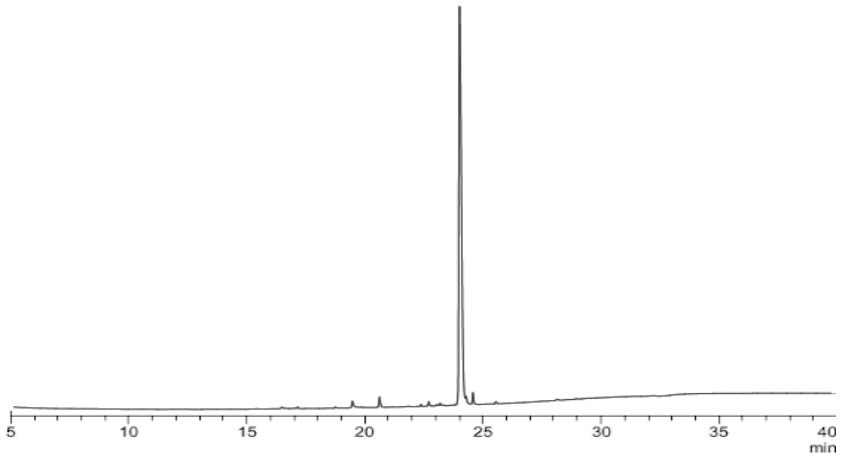


Figure 3.21. HPLC analysis of the piperidide **38**.

To perform the solubility studies, the piperidide **38** (around 40 mg) was weighed in 15 different test tubes. To each tube, 120 μL of a solvent was added and the temperature was set to 40 ºC (table 3.14). The solubility was checked every 10 min (up to 1.5 h) and additional volumes (60 μL or 120 μL) of the solvent were added in some test tubes if poor solubility was detected. The temperature was lowered to 25 ºC after 1.5 h, the mixtures were left overnight and solubility was again checked. Finally, the temperature was set to 5 ºC and the solubility was checked again after 30 min and 2 h. Table 3.14 summarises the results obtained.

Table 3.14. Solubility results of the piperidide **38**.

Entry	**Solvent**	**r.t**	**Time (min)**							
			–	10	30	60	90	o. n.	30	120
			40 ºC					**25 ºC**	**5 ºC**	
1	MeOH	×	×	×	×[b]	×[b]	×	×	×	×
2	EtOH	✓	✓	✓	✓	✓	✓	×	×	×
3	iPrOH	✓	✓	✓	✓	✓	✓	×	×	×
4	ACN	×	×	×	×[b]	×[b]	×	×	×	×
5	Acetone	×	×	×	×[b]	×[b]	×	×	×	×
6	THF	✓	✓	✓	✓	✓	✓	✓	(-)	(-)
7	Toluene	×	Gel	Gel	Gel[b]	Gel[b]	Gel	×	×	×
8	DMF	✓	✓	✓	✓	✓	✓	✓	×	×
9	MeOH/H_2O[a]	×	×	×	×	×	×	×	×	×
10	EtOH/H_2O[a]	✓	✓	×	×	×[c]	×	×	×	×
11	iPrOH/H_2O[a]	✓	✓	✓	✓	✓	✓	×	×	×
12	ACN/H_2O[a]	×	×	×	×	×	×	×	×	×
13	Acetone/H_2O[a]	✓	✓	✓	×	×[b]	×	×	×	×
14	THF/H_2O[a]	×	×	×	×	×[b]	✓	(-)	(-)	(-)
15	DMF/H_2O[a]	✓	✓	✓	✓	✓	✓	✓	×	×

(a) 9:1 (v/v).
(b) Addition of 60 μL.
(c) Addition of 120 μL.
✓: Yes; ×: No; (-): Cloudy.

It was found that the piperidide **38** was soluble in EtOH, iPrOH, THF and DMF when the temperature was set at 40 ºC. Nonetheless, DMF was discarded because the target **16** was also soluble in DMF. In accordance with these results, the solvents EtOH, iPrOH and THF were tested with a batch of **16** that contained the piperidide **38**.

3.3.4.5.1. Solubility test for removing the 38 present in a batch of 16

About 38 mg of a sample of **16** that contained a 7 % of the piperidide **38** (from batch 16G, table 3.13) were weighed in three test tubes and 120 μL of iPrOH, EtOH and THF were added, respectively. The temperature was set at 40 ºC and the mixture was left stirring for 4 h. Then, the suspensions that were obtained in iPrOH and EtOH were filtered and the resulting solids were dried under vacuum. The THF solution was left overnight at 25 ºC but formation of a precipitate was not observed.

The HPLC profiles of the solids obtained from the assays with iPrOH and EtOH showed, respectively, a 2 % and 0.6 % of the piperidide **38** and the chromatographic purity of the target peptide raised from a 73 % to 84 %, in the case of the latter solvent. The experiment was repeated to confirm the result that was obtained with EtOH, resulting in this case, a decrease of the piperidide impurity from a 7 % to a 0.8 %.

3.3.4.6. Assays of purification with EtOH

These encouraging results achieved with EtOH moved to explore the use of this solvent under the conditions described in section 3.3.4.5 with other batches of **16** that contained piperidide. Table 3.15 summarizes the results and figure 3.22 shows the chromatograms before and after the treatment with EtOH. The reason why there are differences in the retention times given by the analysis of the solid resulting from the batch 16B (A, table 3.15), compared to the rest of analyses shown in figure 3.22, is the fact that this crude was analysed with a different HPLC instrument although under the same chromatographic conditions.

Table 3.15. Results of the purification of **16** with EtOH.

Entry	Batch of 16	Time [h]	Chromatographic purities[a] [%]			
			16	8	38	Other
1	16B	4	90	-	-	10
2	16D	4	69	-	-	31
3	16E + 16G	4	74	-	-	26

(a) The percentages were obtained by integrating the peak areas at 220 nm.

These results confirm that the treatment of the protected Fexapotide **16** with EtOH at 40 ºC for 4 h is effective to remove the piperidide **38** and the excess of starting material **8** since they were no longer detected. Nonetheless, the main drawback is that the yield of protected Fexapotide **16** recovered after the treatment is generally low.

The crude obtained in batch 16B contained the protected Fexapotide **16** with a chromatographic purity of 82 %, a 0.4 % of starting material **8** and a 4 % of the piperidide **38**. When this crude was treated with EtOH at 40 ºC (entry 1, table 3.15), the HPLC analysis of the resulting solid showed that the chromatographic purity of **16** increased up to a 90 % (figure 3.22).

When the treatment with EtOH was performed to batch 16D that was a crude that contained the protected Fexapotide **16** with a chromatographic purity of 54 %, together with a 4 % of starting material **15**, a 14 % of the piperidide **38** and a 12 % of an unknown impurity, the

HPLC analysis of the resulting solid after the treatment with EtOH showed that the chromatographic purity of the intermediate **16** increased up to a 69 %, and that the unknown impurity decreased to a 2 % (figure 3.22).

Finally, the crudes batch 16E and batch 16G, contained the protected Fexapotide **16** with a chromatographic purity of 73-78 % and a 6-7 % of the piperidide **38**. However, the chromatographic purity of **16** did not improve in this case (74 %) after the treatment with EtOH at 40 ºC but the piperidide **38** was no longer detected (figure 3.22).

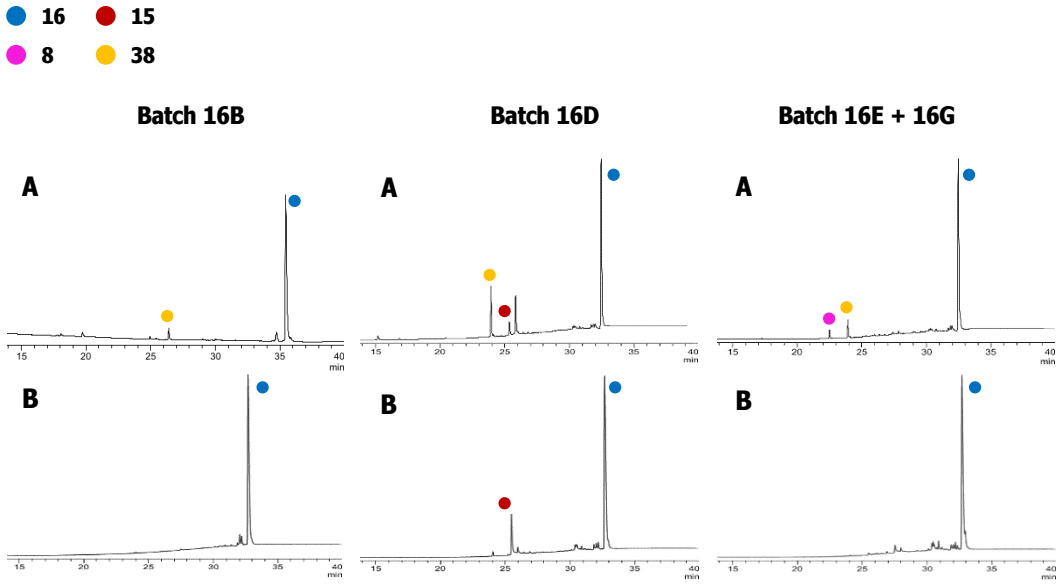


Figure 3.22. Comparison of the HPLC chromatograms of the **16** crudes before (A) and after (B) the treatment with EtOH (table 3.15).

3.3.4.7. Reaction changing the batch of 15

In order to remove the piperidine remaining from the solid of N-terminal deprotected undecapeptide **15**, another coupling assay with **8** was performed under similar conditions to those described in section 3.3.4.4, but now washing the intermediate **15** with an acidic solution to remove piperidine by forming the more water-soluble salt. Table 3.16 summarises the results obtained.

Table 3.16. Results of the reactions performed after washing **15** with an acidic solution.

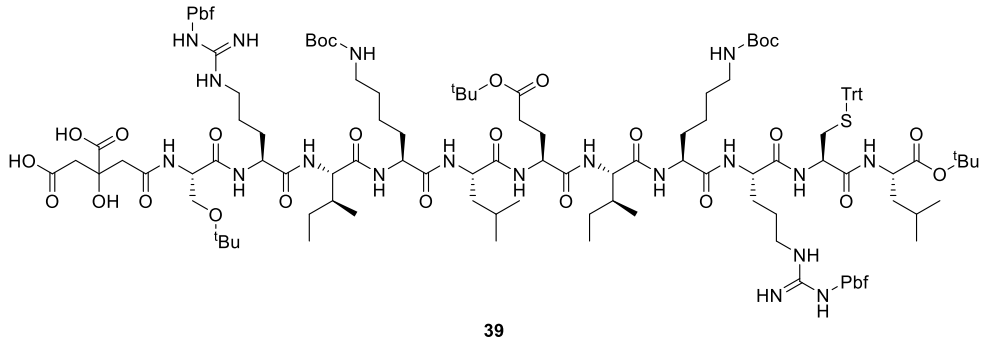
	Batch of 15	**Time** [h]	**Chromatographic purities**[f] [%]			
			16	**8**	**38**	**Other**
Batch 16H[a]	15B[d]	4	50	26	2	22
Batch 16I[b]	15B[e]	1	23	16	-	61
Batch 16J[c]	15B[e]	3	67	10	-	23

(a) The reaction was carried out using 0.02 mmol (47.9 mg) of **15**.
(b) The reaction was carried out using 0.006 mmol (16.0 mg) of **15**, the temperature was not controlled, and the product was not isolated.
(c) The reaction was carried out using 0.03 mmol (78.9 mg) of **15**.
(d) **15** was mixed with a 2.5 % citric acid aqueous solution.
(e) **15** was mixed with a 2.0 N NaH_2PO_4 aqueous solution (pH 3).
(f) The percentages were obtained by integrating the peak areas at 220 nm.

The HPLC profiles of the reactions showed that the piperidide **38** was only detected (2 %) in one of the reactions when the slurry was performed using an aqueous citric acid solution

to the N-terminal deprotected undecapeptide **15** (batch 16H, figure 3.25). It is worth mentioning that the same batch of **15** gave a 14-17% of piperidide when used directly without a prior washing (batches 16C and 16D, table 3.12, section 3.3.4.4). However, the percentage of protected Fexapotide **16** slightly decreased (from 54-56 % to 50 %). Moreover, a 26 % of starting material **8** was observed, while the protected hexapeptide was not detected when **15** was used without prior washings of the solid.

Some other impurities could be detected in the reaction crude and identified. For example, an impurity with a m/z of $[M+2H]^{2+}$ 1324.2 (7 %) was assigned to a peptide resulting from the coupling of citric acid to the N-terminal deprotected **15** (**39**, figure 3.23). This result suggested that some citric acid remained in the solid after washing the intermediate **15** and was assembled to the undecapeptide in the presence of the coupling reagents.

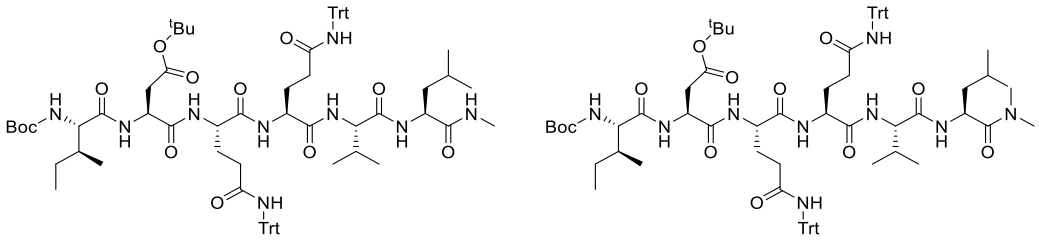


39

Figure 3.23. Citric acid related impurity (**39**).

Again, the consumption of the N-terminal deprotected undecapeptide **15** to form this impurity could explain the uncomplete conversion to form the target peptide **16**.

Two of the other impurities detected, had a m/z of $[M+H]^+$ 1368.7 and 1382.7 (0.6 % and 0.4 %, respectively), which might be attributed to N-methylated (**40**) and N,N-dimethylated (**41**) derivatives of the protected hexapeptide **8** (figure 3.24). The reason why these by-products were formed is still unknown. The amines that DMF might contain may explain this result but it has not been demonstrated yet.



40 **41**

Figure 3.24. Possible impurities **40** and **41**.

Other two experiments were carried out using an aqueous NaH_2PO_4 solution to wash the N-terminal deprotected undecapeptide **15**.

The reaction of batch 16I (table 3.16) was performed in a qualitative way with the aim of finding out if the piperidine could be removed under these conditions. Therefore, the scale of the reaction was low, the temperature was not controlled and the target protected peptide

16 was not isolated. The HPLC chromatogram of the in-process control showed that the piperidide **38** was not detected under these conditions.

The same batch of **15** (batch 15B) was used to perform another reaction under controlled experimental conditions and raising the scale. The HPLC analysis of the resulting solid (batch 16J, figure 3.25, A) contained the protected Fexapotide **16** in a 67 %, a 10 % of starting material **8**, 1.4 % of the potential methylated peptides (**40** and **41**) and 4 % of an unknown product, among other minor impurities. As in the preceding assay, the piperidide was not detected.

The protocol described in section 3.3.4.5 consisting of dissolving the protected Fexapotide **16** in EtOH at 40 ºC was used with the obtained crude of 16J (table 3.16) to remove the starting material **8** and other impurities. The HPLC profile of the solid (figure 3.25, B) showed the protected Fexapotide **16** with a chromatographic purity of 84 %. Even though the chromatographic purity was improved, only a 60 % of the solid containing the protected Fexapotide **16** was recovered after the treatment with EtOH, which indicated that part of the desired product was solubilised by EtOH.

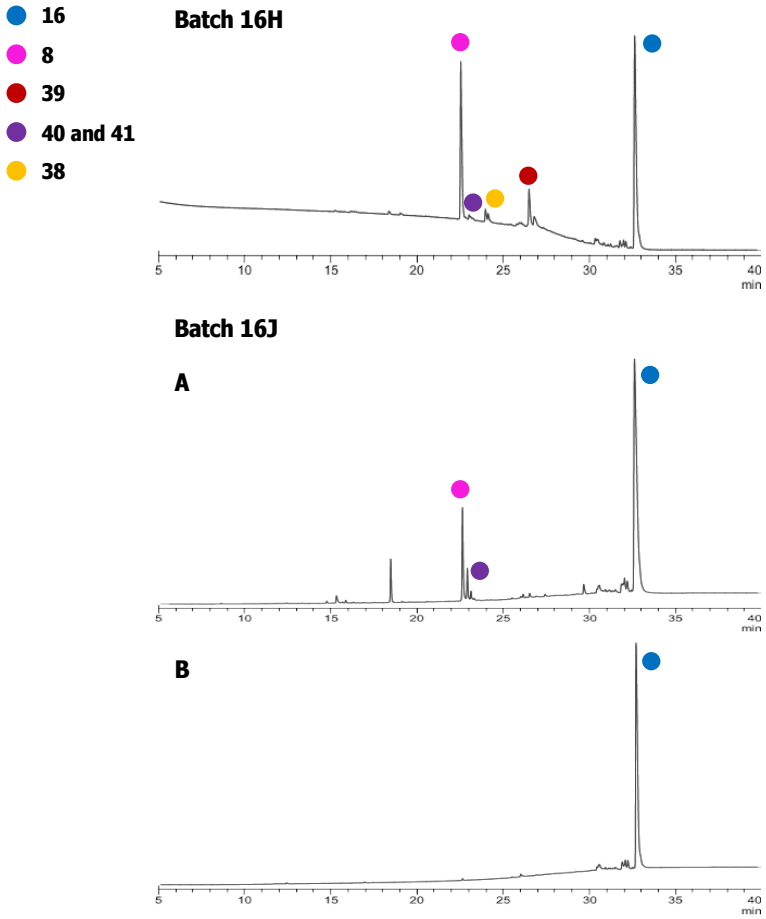
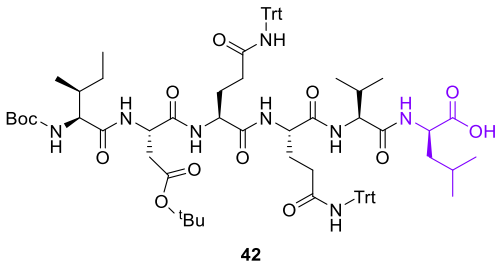


Figure 3.25. HPLC chromatograms of the solids corresponding to batch 16H and batch 16J.

The results presented in table 3.16 suggested that washing the N-terminal deprotected undecapeptide **15** with an acidic aqueous solution decreased the formation of the piperidide impurity and thus was effective to remove the piperidine retained in **15**. The use of an aqueous solution of NaH_2PO_4 led to better results as the chromatographic profiles were cleaner than those obtained when citric acid was used.

3.3.4.8. Preliminary assays using different potential source of amines

A batch of qualitative reactions were carried out, to find out the source that led to the formation of certain impurities related to the protected hexapeptide **8**, such as those described in section 3.3.4.7. Therefore, the scale of the reaction was lowered, the temperature of reaction was not controlled and the target peptide **16** was not isolated. Since the reaction temperature nor the addition of reagents was not controlled, the epimer of **16** (**25**) and the epimer of **8** (**42**, figure 3.26) were detected. Table 3.17 summarises the results obtained.



42

Figure 3.26. Epimer of **8** (**42**).

Table 3.17. Results of the reactions performed to obtain **16** using different potential sources of amines.

Entry	Modified component	Batch of 15	Time [h]	Chromatographic purities[g] [%]			
				16	**8**	**8 related impurities**	**Other**
1[a]	8[b]	15C[f]	1	42	20	4	34
2[a]	DMF[c]	15C[f]	1	19	16	8	57
3[a]	DIPEA[d]	15C[f]	1	33	10	5	51
4[a]	THF[e]	15C[f]	1	50	11	-	39

(a) The reaction was carried out using 0.005 mmol (14.0 mg) of **15**.
(b) **8** was mixed with a 2.0 N NaH_2PO_4 aqueous solution (pH 3).
(c) DMF GC-quality.
(d) DIPEA from a different bottle.
(e) Reaction performed using THF as solvent.
(f) **15** was mixed with a 2.0 N NaH_2PO_4 aqueous solution (pH 3).
(g) The percentages were obtained by integrating the peak areas at 220 nm.

As mentioned in section 3.3.4.7, some impurities might be formed because of the presence of methylamine and dimethylamine in the reaction mixture. The protected hexapeptide **8** (batch 8F, section 3.2.2.3) was considered as the first potential source of amines. A slurry of the peptide with an aqueous NaH_2PO_4 solution was carried out to try to remove the amines remaining in the solid as consequence of treating the peptide-solution resulting from the cleavage of the peptide from the resin with triethylamine to neutralise the TFA. The HPLC profile of the in-process control of the reaction (entry 1, table 3.17) showed that the the impurities **40** and **41** that might be attributed to the to N-methylated and N,N-dimethylated derivatives of **8** were detected in a 2 % each.

Another source of amines could be DMF, which is known that decomposes to give dimethylamine. To that moment, reactions were performed using an industrial quality grade DMF. Therefore, it was decided to move to a DMF of GC-quality grade. The HPLC of the in-process control of the reaction (entry 2, table 3.17) showed the presence of the impurities **40** and **41** in a 5 % and 3 %, respectively.

DIPEA was also considered a source of amines and, therefore, another batch of base coming from a different bottle was used to execute the assay. The HPLC analysis of the in-process control of the reaction (entry 3, table 3.17) showed the presence of the impurities in a 3 % and 2 %, respectively.

Finally, it was decided to change the solvent of the reaction (DMF) and use THF. Under these conditions, the reaction mixture did not become clear and the HPLC sample had to be diluted with NMP. The HPLC analysis of the in-process control of the reaction (entry 4, table 3.17, figure 3.27) showed that the impurities **40** and **41** were not detected.

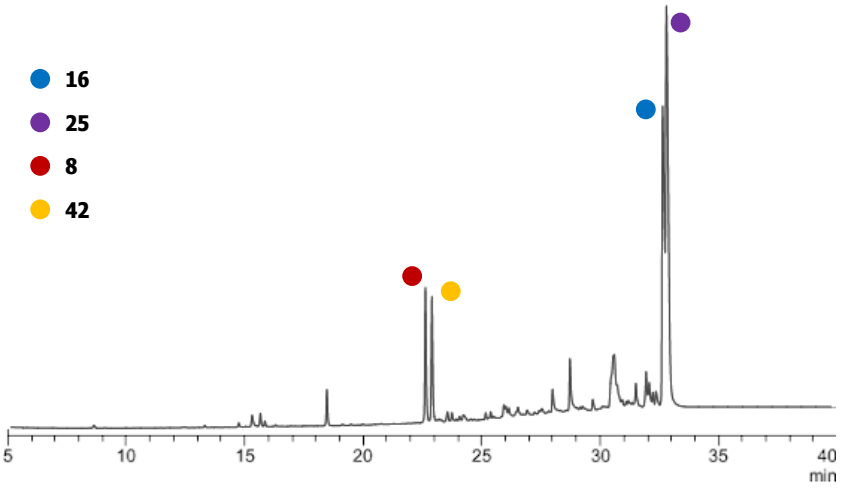


Figure 3.27. HPLC chromatogram of the in-process control of entry 4, table 3.17.

The fact that the impurities were not detected only when THF was used suggested that the source that caused the formation of these impurities was most probably DMF. Other solvents should be tested to perform this reaction in order to improve the results in terms of chromatographic quality. In this sense, NMP could be an excellent candidate to be used as the solvent for the reaction. These results indicate that the quality of the solvent is critical in this kind of reactions to achieve the desired peptide with the required quality.

3.3.4.9. Reaction with change in the solvent

Considering the results described in section 3.3.4.8, the reaction was performed in NMP under similar conditions. Table 3.18 summarises the results obtained.

Table 3.18. Results of the reactions performed for the synthesis of **16** using NMP

Entry	Batch of 16	Batch of 15	Time [h]	Crude yield [%]	Chromatographic purities[d] [%]			
					16	8	8 related impurities	Other
1[a]	-	15C[c]	1	-	50	17	-	33
2[b]	16K	15C[c]	4	83	66	9	-	25

(a) The reaction was carried out using 0.005 mmol (14.0 mg) of **15**.
(b) The reaction was carried out using 0.11 mmol (261.8 mg) of **15**.
(c) **15** was mixed with a 2.0 N NaH_2PO_4 aqueous solution (pH 3).
(d) The percentages were obtained by integrating the peak areas at 220 nm.

The reaction was first performed at a lower scale (entry 1), because the goal was to analyse the chromatographic profile of impurities in the reaction crude when NMP was used as solvent. For this reason, the temperature was not controlled and the target peptide **16** was

not isolated. The HPLC chromatogram of the in-process control of the reaction contained the protected Fexapotide **16** in a 50 % (diastereomeric mixture 89:11), a 16 % of starting material **8** (diastereomeric mixture 54:46) and a 2 % of starting material **15**. It is worth mentioning that the impurities **40** and **41** were not detected.

With these results, it was decided to carry out the same reaction controlling experimental conditions such as temperature and the addition of the base (entry 2, table 3.18). The HPLC analysis of the crude of batch 16K (figure 3.28, A) contained a 66 % of the protected Fexapotide **16** and 9 % of starting material **8**, among other minor unknown impurities.

In order to remove the starting material **8** and other impurities, the solid resulting from the reaction crude was mixed with EtOH at 40 ºC. As shown in chromatogram B of figure 3.28, the starting material **8** and some impurities were clearly removed, raising the chromatographic purity of the target peptide from 66 % to 88 %. Moreover, the solid recovered from this treatment (about 85 %) was higher than those obtained in other purification assays.

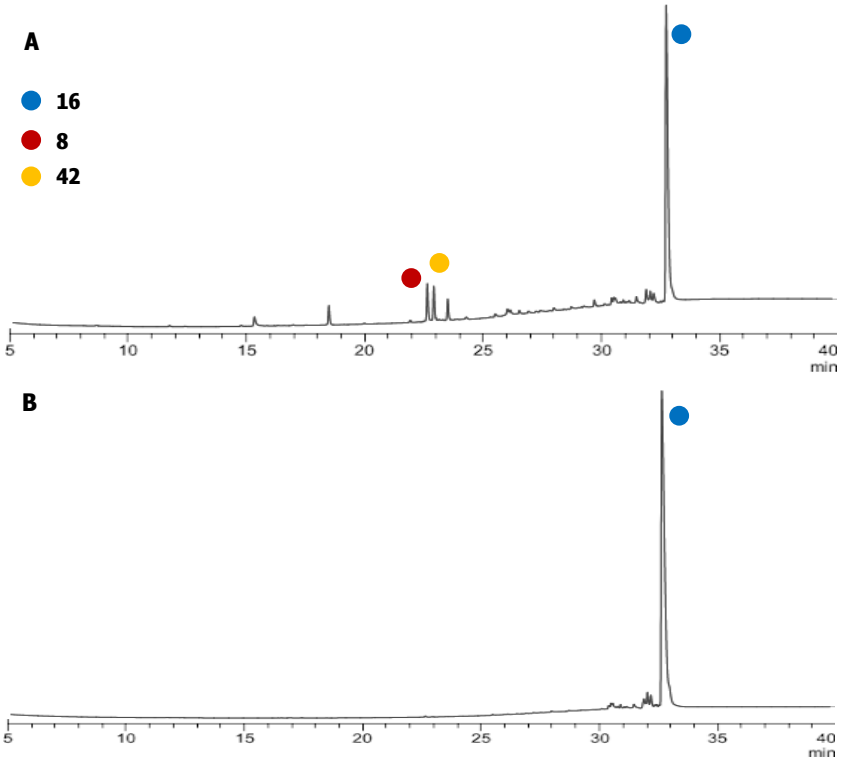
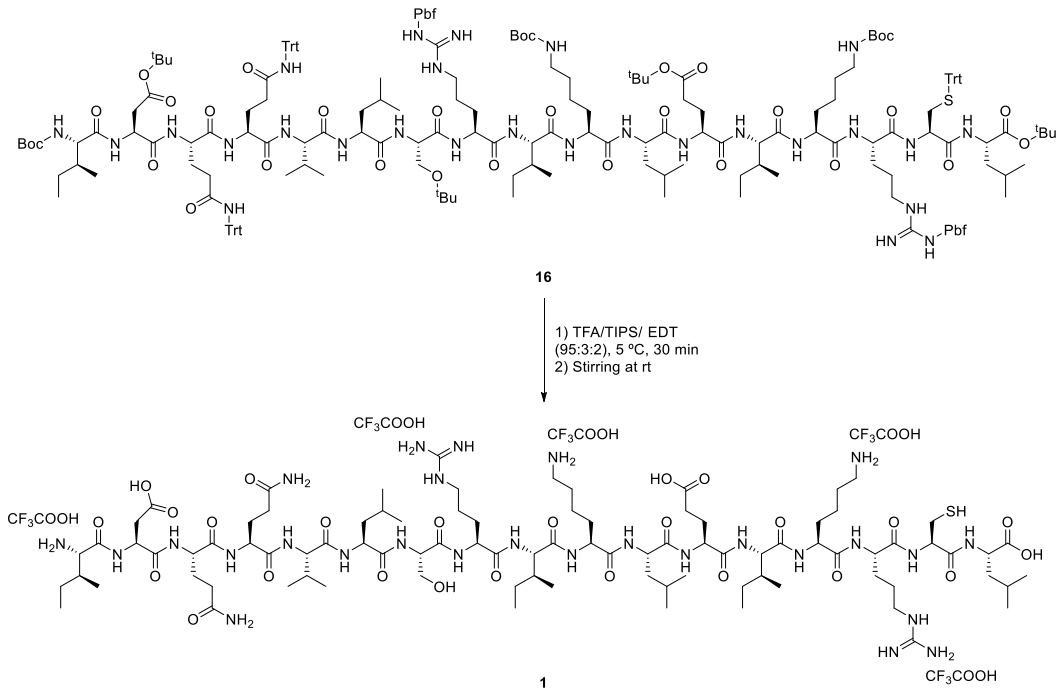


Figure 3.28. HPLC chromatogram of the obtained crude of batch 16K using NMP as solvent before (A) and after the EtOH treatment (B).

3.3.5. Synthesis of Fexapotide

The final step of the synthetic route to achieve the target Fexapotide (**1**) was the removal of the side-chain, N-terminal and C-terminal protecting groups with an acidolytic treatment of the protected Fexapotide **16** (scheme 3.19).



Scheme 3.19. Full deprotection of Fexapotide **1**.

The crude containing **16** was added over a cooled solution of TFA, TIPS and EDT (95:3:2) and the mixture was left stirring at 5 ºC for 30 min and at 20 ºC for 3.5 hours. The resulting target peptide crude was precipitated with MTBE. TIPS was added as a scavenger of the carbocations generated during the removal of the protecting groups to avoid undesired reactions of the peptide provoked by these species. EDT was used to avoid disulfide formation in cysteine containing peptides.

Three reactions were carried out using different crudes of protected peptide **16**. Unlike the reaction of entry 1 where only one crude was used, three crudes and two crudes with similar chromatographic purities for **16** were combined to perform the reactions of entries 2 and 3, respectively. Table 3.19 summarises the results obtained.

Table 3.19. Results of the reactions performed for the synthesis of **1**.

	Batch of 16	**Acidolytic cocktail** [mL]	**Crude yield** [%]	**Chromatographic purities** [%]	
				1	**Other**
Batch 1C[a]	16C	4	>100[d]	50[e]	50[e]
Batch 1D[b]	Purified 16B + 16J + 16K	6	84	71[f]	29[f]
Batch 1E[c]	Purified 16D + 16E + 16G	3	67	57[f]	43[f]

(a) The reaction was carried out using 0.05 mmol (177.3 mg) of **16**.
(b) The reaction was carried out using 0.06 mmol (238.6 mg) of **16**.
(c) The reaction was carried out using 0.02 mmol (75.1 mg) of **16**.
(d) The crude yield was above 100 % (114 %) most probably because the resulting solid was not dried.
(e) The percentages were obtained by integrating the peak areas at 220 nm.
(f) The percentages were obtained by integrating the peak areas at 210 nm.

The first reaction was performed using **16** from batch 16C. The HPLC profile of the reaction contained the desired Fexapotide **1** (50 % of chromatographic purity) and the full deprotected peptide resulting from the piperidide **38** (**43**, figure 3.29) as the main products among other minor impurities. In order to get more insight into the real impurity amount in the mixture, the ^{1}H-NMR spectrum of the reaction crude was recorded (figure 3.30, B) and

the zone between 8.5 ppm and 9.0 ppm where the protons of one amide of **1** and one amide of the impurity was deconvoluted. A 12 % of the impurity was found after integration of the signals corresponding to the amide protons, a result that was more closely aligned to the one described in section 3.3.4.4 for the synthesis of batch 16C, the precursor of **1**, in which the impurity was present in a 17 %. These results suggested that the HPLC response factor had a major impact when the protecting groups were removed, some of them with relevant properties as chromophores. So, in this case, the chromatographic purities are somehow misleading.

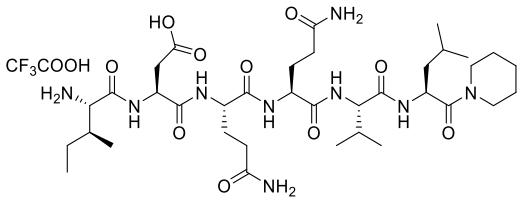


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Figure 3.29. Side-chain deprotected form of **38** (**43**).

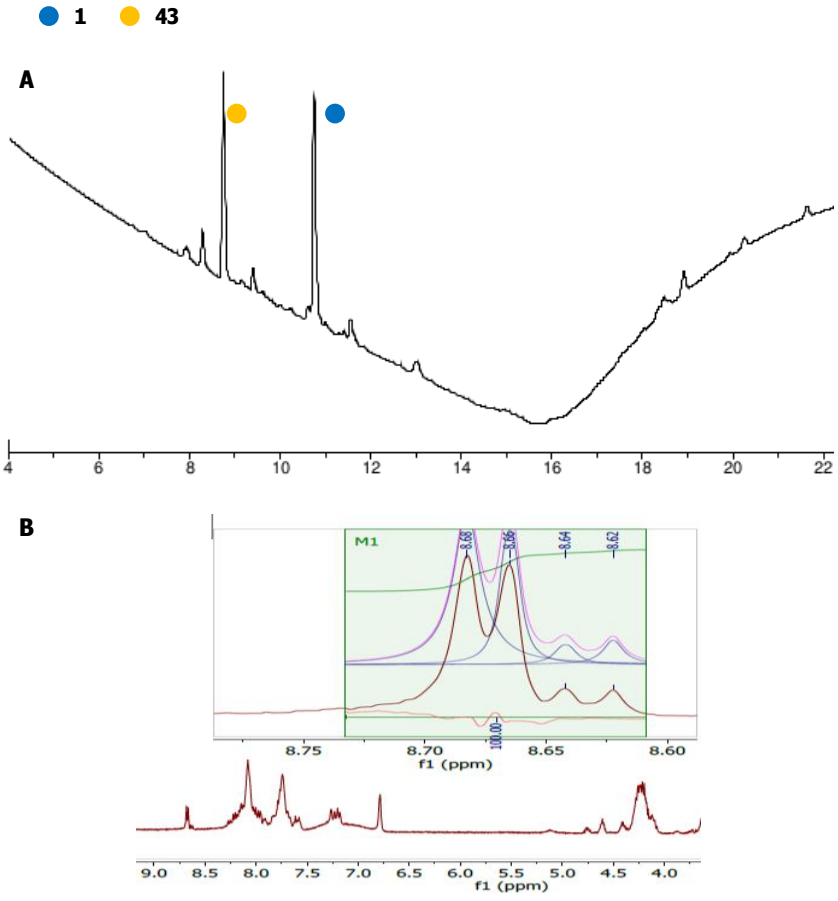


Figure 3.30. HPLC chromatogram (A) and a NMR deconvolution study (B) of the **1** crude obtained under conditions of entry 1, table 3.19.

Considering the chromatographic results obtained in the first assay, it was decided to modify the chromatographic conditions such as the gradient, HPLC column and wavelength detection, which was shifted from 220 nm to 210 nm.

The HPLC analysis of the solid obtained after the work-up of the reaction to obtain batch 1D (figure 3.31), contained the desired Fexapotide **1** with a chromatographic purity of 71 % and the ^{t}butylated form of **1** in a 7 %, among other unknown impurities. In the case of batch 1E, the HPLC profile (figure 3.31) showed **1** with a chromatographic purity of 57 %, the full deprotected peptide corresponding to **15** (**27**) in a 7 % and the *tert*-butylated form of **1** in a 6 %, among other unknown impurities.

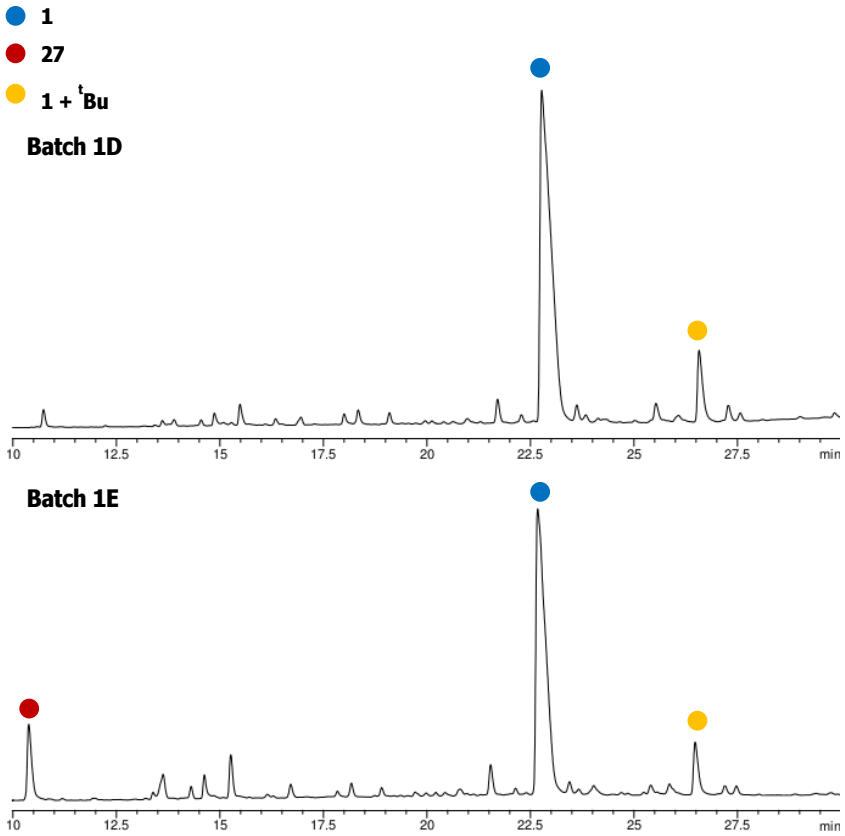


Figure 3.31. HPLC chromatograms of the crudes corresponding to batch 1D (A) and batch 1E (B).

The *tert*-butylated form of **1**, which was also detected in the synthesis of **1** described in chapter 2, might have been formed due to the reaction of the target peptide with carbocations resulting from the elimination of protecting groups. For example, *tert*-butylation of cysteine has been observed after removing the Boc group or after a global deprotection using the Fmoc/^{t}Bu strategy.[119]

However, according to the literature, the formation of the *tert*-butylated form of **1** could be induced by the use of MTBE in the peptide precipitation step. MTBE is preferred to other ethers such as diethyl ether with regards to safety concerns, especially in peptide industrial production. MTBE could behave as an alkylating agent and might be able to cause *tert*-butylation via the isobutene intermediate in the presence of strong acids. Moreover, it has been described that the use of MTBE in the work-up might lead to the undesired tert-butylation, particularly when the peptide contains susceptible residues such as Trp, Tyr, Cys or Met. [120,121,122] Substitution of MTBE for another ether as antisolvent for peptide precipitation might supress this side reaction and therefore, should be considered.

Apart from the *tert*-butylation problem above mentioned, the purity of the precursor **16** must be considered when conclusions about the purity of **1** have to be driven. For example, the crude obtained in batch 1D (table 3.19) had a better chromatographic purity than that of the

crude afforded by the reaction of batch 1E because the precursor **16** had a higher chromatographic purity. The crude used for the reaction to obtain batch 1E contained the protected peptide **15**, which appeared in the chromatographic analysis of the corresponding reaction crude.

3.3.6. Purification of Fexapotide

As mentioned before, the target peptide **1** was considered a HP-API and thus, solid-state handling and reactions in which this substance was present were carried out in an isolator. The use of preparative HPLC together with the lyophilisation technique have proved to be very useful for the isolation and purification of peptides. However, the use of these techniques resulted to be impossible because there was not the required instrumentation inside the isolator. Therefore, the purification of Fexapotide **1** was tested using other techniques.

3.3.6.1. Purification by precipitation

Precipitation of peptides and proteins can be carried out taking advantage of its isoelectric point (pI). The pI is the pH of a solution at which the net charge of a protein or peptide becomes zero.

When the pH of the solution is above the pI, the surface of the protein is predominantly negatively charged and will present intermolecular repulsive forces that favour solubility. Besides, when the pH of the solution is below the pI, the surface of the protein is predominantly positively charged and repulsion is again experienced. However, when the pH of the solution equals the pI, the negative and positive charges are balanced and the repulsive electrostatic forces are reduced while the attractive forces predominate causing the aggregation and precipitation.[123]

The pI has traditionally been determined by isoelectric focusing. This method uses a pH gradient to establish the pH at which the proteins have no net charge. However, it can also be theoretically predicted. In the case of Fexapotide, the Chemicalize (ChemAxon technology) online platform was used to conduct this calculation, which gave a pI of 9.5.

3.3.6.1.1. Assay performing a pH gradient to 9.5

The methodology that was carried out consisted on the dissolution of the target **1** with an acidic aqueous solution of KH_2PO_4 at pH 2.0 and then, the pH was adjusted to 9.5 with a basic aqueous solution of 1.5 % NH_3 in H_2O. The resulting mixture was left stirring until formation of a precipitate was observed and filtrated. The solid was transferred into a reactor vessel that contained TFA (to form the TFA salt of **1**), stirred for 30 min and precipitated with MTBE.

Table 3.20. Results of the purification of **1** by precipitation at pH 9.5.

Entry	Batch of 1	Chromatographic purities[b] [%]		
		1	**1+ ^{t}Bu**	**Other**
1[a]	1E	22	8	70

(a) The assay was carried out using 0.01 mmol (35.9 mg) of **1**.
(b) The percentages were obtained by integrating the peak areas at 210 nm.

The HPLC chromatogram of the resulting solid (figure 3.32) showed the desired Fexapotide **1** with a chromatographic purity of 22 %, the *tert*-butylated form of **1** in an 8 % and the

dimeric form of **1** was detected in a 34 %, among other minor impurities. The formation of the dimer could be explained in terms of disulfide formation, a process that is favoured under basic conditions.

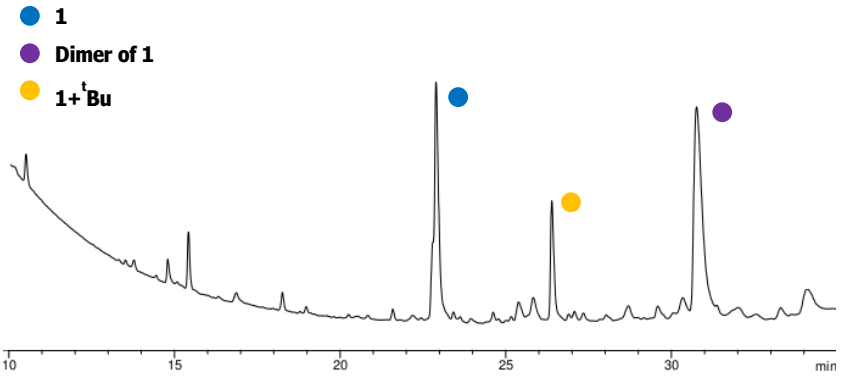


Figure 3.32. HPLC chromatogram of **1** after precipitation at pH 9.5.

In order to recover the reduced peptide, the solid was dissolved in a 1.7 mg/mL TCEP·HCl aqueous solution and stirred for 4 h at room temperature. The HPLC analysis of the in-process sample (figure 3.33) revealed that the dimer was reduced to **1**, and showed a chromatographic purity of 61 %.

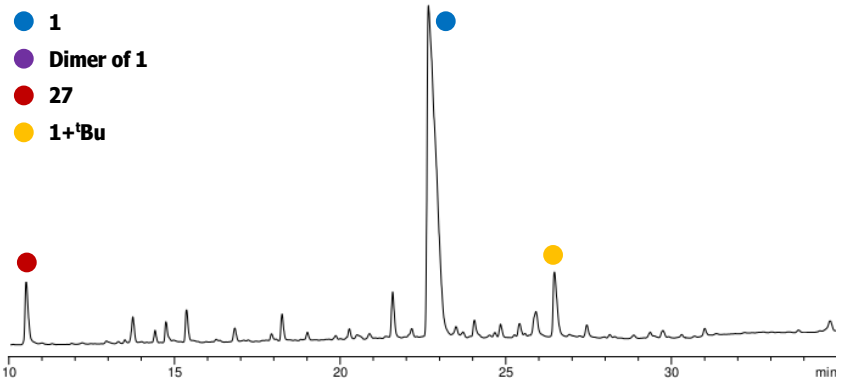


Figure 3.33. HPLC chromatogram of the in-process sample of the disulfide bond reduction.

The crude resulting from batch 1E (section 3.3.5) contained Fexapotide **1** with a chromatographic purity of 57 %, the deprotected form of **15** in a 7 % and the *tert*-butylated form of **1** in a 6 %. Precipitation by adjusting the pH at 9.5 improved the chromatographic purity of **1** to a 61 %.

Nonetheless, an additional reduction step had to be performed. That moved us to consider the use of TCEP·HCl as additive and reduce a little bit the pH for precipitation to avoid disulfide formation.

3.3.6.1.2. Assay performing a pH gradient to 8.7

The pI was predicted with another online platform (isoelectric point calculator, IPC), which is based on the following approaches:

- In the case of proteins isoelectric point mostly depends on seven charged amino acids: glutamic acid (δ-carboxyl group), aspartic acid (β-carboxyl group), cysteine

(thiol group), tyrosine (phenol group), histidine (imidazole side chains), lysine (ε-amino group) and arginine (guanidino group).

- Additionally, the charge of protein terminal groups (amino and carboxyl) should be taken into account. Each of them has its unique acid dissociation constant referred to as pK.

- The net charge of the protein is in tight relation with the solution (buffer) pH. Keeping in the main this, the Henderson-Hasselbach equation is used to calculate protein charge at a given pH.

The most critical issue for pI determination is the use of appropriate pKa values. In this sense, The IPC platform considers the pKa values of several databases. The predicted pI for Fexapotide was 8.7.

The assay was carried out under similar conditions to those described in section 3.3.6.1.1. The target **1** was dissolved in a 4.0 mg/mL TCEP·HCl in H_2O (pH 2.3) and the pH was adjusted to 8.7 with a basic aqueous solution of 1.5 % NH_3 in H_2O. The resulting mixture was left stirring until formation of a precipitate was observed and filtrated. The solid was transferred into a reactor vessel that contained TFA, stirred for 30 min and precipitated with MTBE. The procedure was repeated twice to confirm the results (table 3.21).

Table 3.21. Results of the purification of **1** by precipitation at pH 8.7.

Entry	Batch of 1	Chromatographic purities[c] [%]		
		1	**1+ ^{t}Bu**	**Other**
1[a]	1D	80	8	12
2[b]	1D	80	8	12

(a) The assay was carried out using 0.01 mmol (30.2 mg) of **1**.
(b) The assay was carried out using 0.03 mmol (67.7 mg) of **1**.
(c) The percentages were obtained by integrating the peak areas at 210 nm.

The HPLC chromatograms of the corresponding solids showed the desired Fexapotide **1** with a chromatographic purity of 80 % and the *tert*-butylated form of **1** in an 8 %. The dimeric form of **1** was not detected in this case due to the presence of TCEP·HCl in the mixture.

The crude resulting from batch 1D (section 3.3.5) contained the Fexapotide **1** with a chromatographic purity of 71 % and the *tert*-butylated form of **1** in a 7 %. Now, the precipitation by adjusting the pH at 8.7 increased up the chromatographic purity to an 80 %.

Figure 3.34 compares the HPLC chromatograms of batch 1D before and after performing a pH gradient to 8.7.

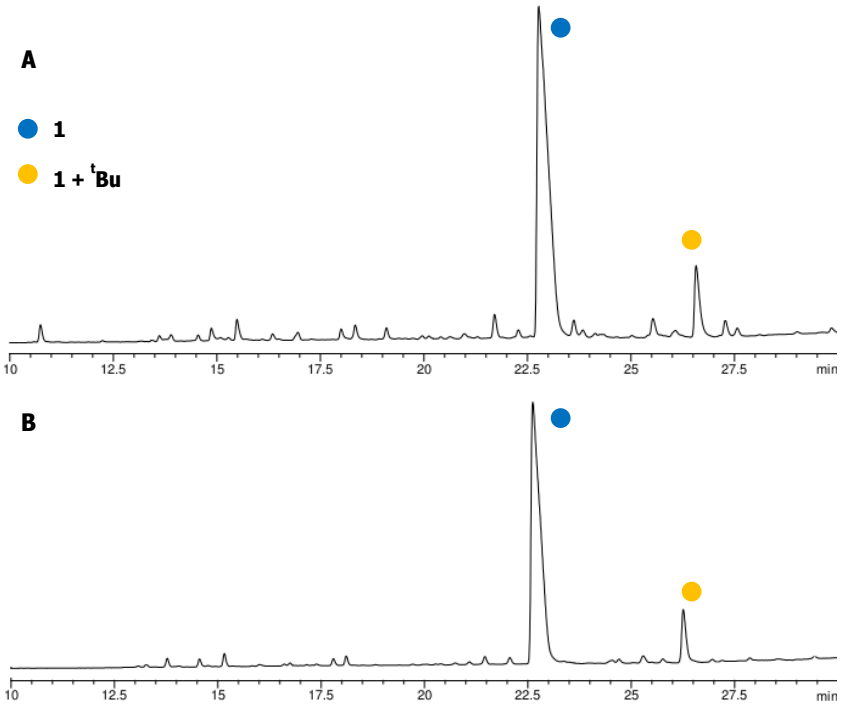


Figure 3.34. Comparison of the crude of 1D before (A) and after (B) the gradient to pH 8.7.

Due to the low recovery of crude after precipitation, the aqueous phases were analysed and it was found that **1** still remained in the solution. Therefore, the pH was readjusted to 8.7 and left stirring for 2 h but formation of a new precipitate was not observed. The pH was then adjusted to pH 9.5 and left stirring for 24 h. Now, a precipitate was observed, filtered and analysed by HPLC. The chromatographic profiles showed the desired Fexapotide **1** with chromatographic purities of 52 % and 54 % (figure 3.35).

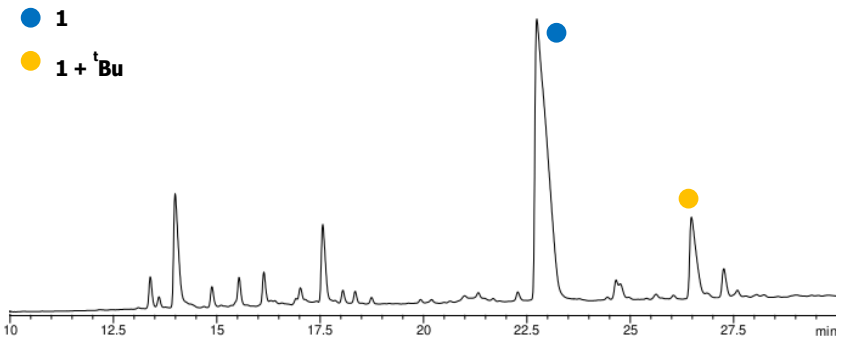


Figure 3.35. HPLC chromatogram of batch 1D when the pH was readjusted to 9.5.

These results confirmed that lower chromatographic purities are obtained when the pH was adjusted to 9.5 when compared to the results obtained at pH 8.7 and that the chromatographic purity was increased from 71 % to an 80 % when the gradient to pH 8.7 was performed to precipitate the desired **1**.

3.4. Conclusions

The synthesis of the 17-residue Fexapotide following a convergent approach consisting on the solution synthesis of the protected C-terminal fragment Fmoc-Cys(Trt)-Leu-O^{t}Bu (**5**), the solid-phase synthesis of the protected peptides Fmoc-Ser(^{t}Bu)-Arg(Pbf)-Ile-Lys(Boc)-Leu-Glu(^{t}Bu)-Ile-Lys(Boc)-Arg(Pbf)-OH (**28**), and the N-terminal Boc-Ile-Asp-(^{t}Bu)-Gln(Trt)-Gln(Trt)-Val-Leu-OH (**8**), and their assembly on solution has been studied.

3.4.1. Synthesis of the protected peptide fragments

The protected peptide fragments were synthesized on a 2-CTC resin (**28** at 2.51, 3.80 and 4.11 mmol scales, **8** at 3.91 and 9.53 mmol scales). The first Fmoc-amino acid was coupled to the resin using 3 eq of DIPEA in DCM with yields that ranged from 23 % (**28**, 1.5 h with mechanical stirring) to 67% (**8**, 1.5 h with mechanical stirring). Further amino acid couplings were performed using DIC/HOBt (3 eq of each reagent) in DMF. Recouplings were needed for Ile, Glu, Lys, Arg and Ser in the case of **28** (mechanical stirring), and for Gln, Asp and Ile in the case of **8** (mechanical stirring).

The protected peptides were released from the resin by using a solution of 1 % TFA in DCM. Precipitation of peptide crudes in Et_2O or MTBE containing TEA afforded white solids with chromatographic purities of 95 % for **28** and 91 % for **8**. Even though, the N-terminal protecting group was removed when the resin had to be left several hours before coupling a new amino acid, it did not prevent the premature loss of peptide chains.

The dipeptide **5** was synthesised following a similar approach that the one mentioned in section 2.4 but adding neat DIPEA to the reaction mixture instead of mixing the base with the coupling agent to avoid solubility problems. Under these conditions, **5** was obtained with a chromatographic purity of 87 % (6.16 mmol scale) when DCM was used as solvent, and 96 % (6.59 mmol scale) when the solvent was DMF.

The Fmoc group was removed from **5** using a 2 % of piperidine in DCM. The N-terminal deprotected peptide **11** was obtained in 84 % yield (0.92 mmol, 99 % of chromatographic purity) after purification by flash column chromatography (Hexanes/EtOAc 8:2 and DCM/MeOH 9:1).

3.4.2. Solution-phase assembly of the protected peptide fragments

The fragment **14** was obtained by the assembly of the dipeptide **11** with the nonapeptide **28**. Several coupling conditions using DMF as solvent at -20 °C were tried to minimize epimerization: EDC·HCl/HOAt, PyOAP, HCTU/HOAt and HATU/HOAt. Reaction times (full conversion of the limiting starting material) ranged from 1 h for PyOAP, HCTU/HOAt and HATU/HOAt, to 3 h for EDC·HCl/HOAt. Moreover, phosphonium and uranium salts afforded better results against epimerisation (0,5%) than the carbodiimide (1,8%). In all cases, the peptide crudes were isolated as white solids with chromatographic purities of around 95% and yields ranging from 87 % to 89 %.

The coupling system that afforded the best results (HATU/HOAt) was studied under different experimental conditions such as the order of addition of reagents, temperature, reagent concentrations or reaction scale. In general, all the assays afforded the desired product with high chromatographic purities (between 91 % and 93 %) being the best results

those that when mixing **28**, **11**, HOAt and HATU dissolved in DMF and adding dropwise with a syringe a solution of DIPEA in DMF at -20 ºC. Under these conditions, the undecapeptide **14** was obtained in 87 % yield as a white solid with a 93 % of chromatographic purity, together with a 1 % of the epimer **33**.

The N-terminal deprotected undecapeptide **15** was obtained by treatment of **14** with 2 % of piperidine in DMF. The desired peptide was isolated in 69-97 % yield with a chromatographic purity between 86-94 % after precipitation and purification with Et_2O.

The hexapeptide **8** was coupled to the N-terminal deprotected fragment **15** to afford the heptadecapeptide **16** corresponding to the full-length sequence of Fexapotide. The use of the conditions mentioned above for the obtention of the undecapeptide **14** afforded peptide crudes of **16** with the absence of the limiting starting material after 2 – 4.5 h and with chromatographic purities of 50 %-78 %. In this particular case, the formation of the piperidide **38** resulting from the reaction of **8** with piperidine that remained from the intermediate **15**, was detected. The amount of this by-product ranged from 6 % to 17 % depending on the batch of **15** that was used. Different experiments were performed to remove the amount of piperidine from **15**. Thus, the piperidide **38** was lowered to a 2 % after prior washings of **15** with a 2.5 % aqueous solution of citric acid or NaH_2PO_4 at pH 3. Moreover, the by-product was synthesised on solution (92 % of chromatographic purity) and its solubility in different solvents was explored, giving positive results with EtOH, iPrOH and THF. The treatment of a batch of **16** containing 7 % of the piperidide **38** (73 % of chromatographic purity) with EtOH at 40 °C for 4 h minimised the presence of the by-product to a 0.6 % and raised the chromatographic purity to 84 %.

Careful analysis of hexapeptide **8** revealed the presence of potential peptide impurities coming from DMF. Therefore, the heptadecapeptide **16** was prepared as mentioned before but using NMP as solvent. These impurities were not detected in the reaction crude but a 9 % of **8** and 1 % of **15** remained after 4 h with a 66 % of chromatographic purity for the desired peptide. Washings of the crude with EtOH at 40 °C removed **8** and raised the chromatographic purity of **16** to 88 %.

Full deprotection of **16** (starting chromatographic purities between 50 %-71 %) was performed using a mixture of TFA, TIPS and EDT (95:3:2) for 4 h. After precipitation with MTBE, the Fexapotide crudes were recovered with chromatographic purities between 50 % and 71 % (crude yields 67-84 %), together with 6-7 % of a *tert*-butylated form of **1**. Further purification by precipitation at the pI was assayed considering the theoretically predicted pI values of 9.5 and 8.7. The chromatographic purity raised from 57 % to 61 % when the peptide was precipitated at pH 9.5, and from 71 % to 80 % when it was precipitated at pH 8.7.

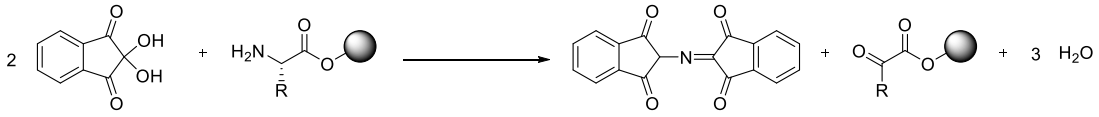
Chapter 4: Exploring novel methodologies to follow the coupling process and Fmoc removal on solid-phase

There are very few methodologies for the in-process control of the reactions involved in SPPS. Some of the existing methodologies for the reaction monitoring are qualitative, are usually performed once the reaction has ended and thus, *in situ* modifications cannot be performed.

Determination of the total amount of amino acid that has been incorporated into the resin is usually determined by the Fmoc UV-Vis quantification or by weighing the resin before and after the addition of the amino acid. When the syntheses are carried out in a polypropylene syringe, these determinations can be easily carried out. However, it becomes cumbersome when the scale is increased, and the syntheses take place in a reactor vessel. As mentioned before, the main goal of the project is to design a synthetic methodology to prepare a therapeutic peptide suitable for pilot plant scale-up. To execute the Fmoc quantification by UV-Vis an aliquot of the aminoacyl-resin must be taken from the reactor vessel, dried under vacuum, and weighed. On the other hand, to quantify by weight, the whole resin must be removed from the reactor vessel once the first amino acid has been added and dried it under vacuum. Working in a pilot plant implies the use of large volume reactor vessels. Remove the resin and/or an aliquot of resin from a bigger reactor vessel is a tedious job and time-consuming. For this reason, a methodology based on the quantitative determination by HPLC of the remaining amino acid in the reaction mixture at different times was developed to estimate the amount of amino acid incorporated.

Fmoc removal during the peptide chain elongation on solid support is usually not quantified nor qualitative tested. From the industrial point of view, every reaction must be carefully monitored by HPLC or NMR to control the formation of by-products and to ensure the completion of the reaction. Thus, in the second part of the present chapter, an HPLC methodology to determine the completion of the Fmoc removal reaction was also designed. This methodology is based on the quantification of the by-products generated during the Fmoc removal reaction that are present in the supernatant of the solid-phase reaction mixture.

Finally, the elongation of the peptide chain during peptide synthesis is usually qualitatively monitored with the ninhydrin or Kaiser test. This test is based on the reaction of ninhydrin with amines (scheme 4.1). It is a very sensitive test for primary amines, visualized by an intense blue colour.



Scheme 4.1. Ninhydrin test reaction with primary amines.

Usually, the colour is developed mainly in the resin beads and partly in the supernatant. The intensity of the colour depends on the nature of the amino terminus to be detected. As the resin sample must be heated, possible hidden NH_2 -groups may become more accessible and thus detectable. However, prolonged heating as well as overheating should be avoided as it may cause cleavage of Lys(Boc) or Fmoc removal by the presence of pyridine, present in the test reagent solutions.

The fact that the ninhydrin test is qualitative, it becomes not suitable from the industrial point of view. Moreover, to perform the test, an aliquot of the peptidyl-resin must be taken from the reactor vessel, with the inconveniences that this entails. Accordingly, in the third

part of the chapter, a novel in-process reaction monitoring methodology based on the HPLC analysis of the supernatant from the reaction mixture was developed.

4.1. Preliminary studies for the development of a new reaction monitoring methodology for SPPS

4.1.1. Incorporation of the first amino acid

Because of the volatility of DCM, the experiments were performed in DMF to accurately weight the supernatant aliquot for quantification purposes. The experiment was carried out at a scale of 5.30 g of resin using 3.98 g of amino acid.

The proposed methodology is outlined in figure 4.1. The first step consisted on the determination of the ratio between the amino acid concentration (mg/mL) and the peak area of the HPLC chromatogram. To do so, the amino acid dissolved in DMF was transferred to the reactor vessel containing the resin. Then, an aliquot of the supernatant was weighed in a volumetric flask, diluted with ACN and analysed by HPLC. The amino acid concentration (mg/mL) in the resulting solution was calculated through the equation 1.

(1) $$\text{g of aliquot}\frac{\text{g of Aa in the reaction}}{\text{g of Aa in the reaction + g of DMF}} = \text{g of Aa in the volumetric flask}$$

(2) $$\text{g of aliquot}\frac{\text{g of Aa in the reaction}}{\text{g of Aa in the reaction + g of DMF + g of DIPEA}} = \text{g of Aa in the volumetric flask}$$

We assume that no reaction processes take place at this point and consequently, this experiment was considered as a control and allowed us to determine the ratio above mentioned. Then, DIPEA was added to the reactor vessel to start the reaction. To determine by HPLC the amount of remaining amino acid, aliquots of the supernatant at different reaction times were taken, weighed in a volumetric flask, diluted with ACN and analysed by HPLC. At this point, the unreacted amount of amino acid present in the diluted solution can be estimated using the ratio previously determined in the control experiment and the value provided by equation 2.

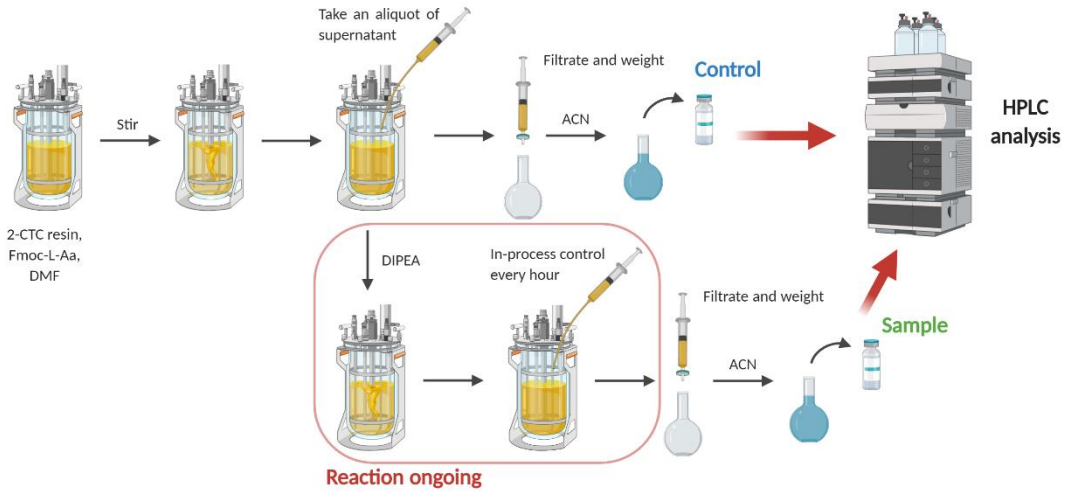


Figure 4.1. Scheme of the procedure.

Figure 4.2 summarises the results obtained in this assay, where a decreasing tendency of the amount of amino acid in the reaction mixture can be observed once DIPEA was added. The amount of amino acid decreased from 3.98 g (entry 1, table 5.38, experimental section) to

2.37 g after 3 h of reaction, which indicates that 1.61 g of amino acid were incorporated to the resin (loading of 0.66 mmol/g).

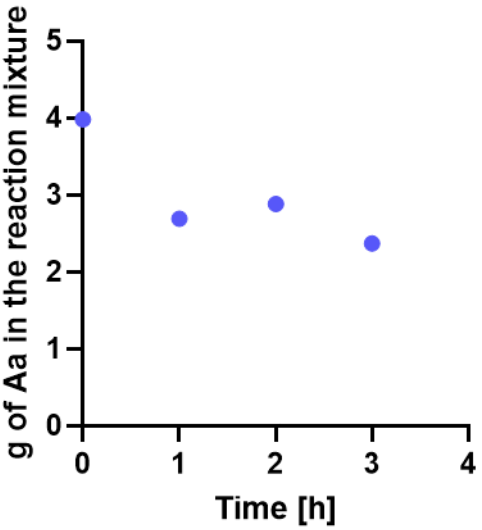


Figure 4.2. Amino acid consumption during the reaction.

Another experiment was performed under similar conditions where two samples were taken at each time in order to get an insight into the reproducibility of the method (figure 4.3). Moreover, the amount of amino acid incorporated to the resin was compared in this case with the result obtained with the quantification by weight. The experiment was carried out at a scale of 5.31 g of resin using 3.97 g of amino acid.

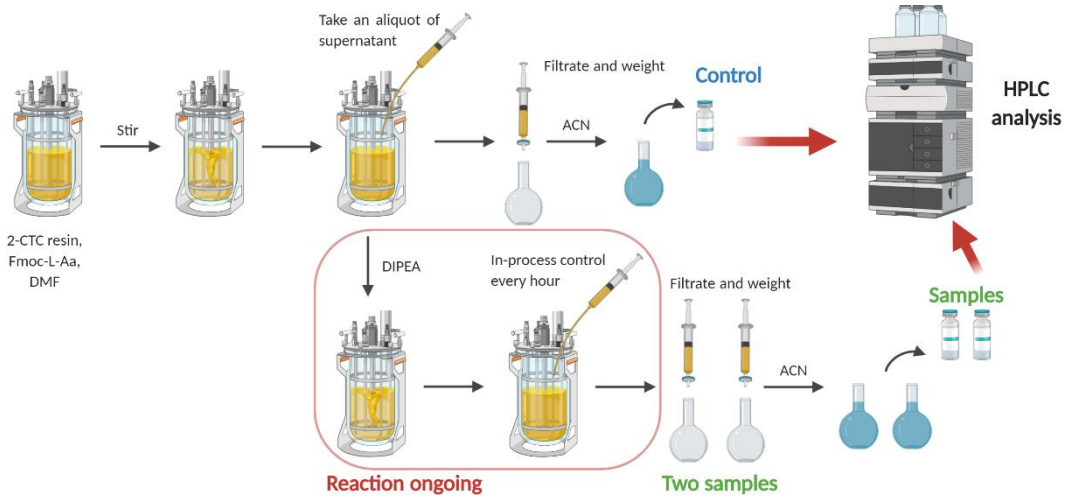


Figure 4.3. Scheme of the procedure when two samples were removed at each in-process control.

As shown in figure 4.4, no significant differences between the two aliquots taken at the same reaction time could be observed. After 3 h of reaction, the loading determined by HPLC analysis was found to be 0.67 mmol/g (1.66 g of amino acid incorporated) and 0.64 mmol/g (1.54 g of amino acid incorporated) for the two samples that were taken at this reaction time.

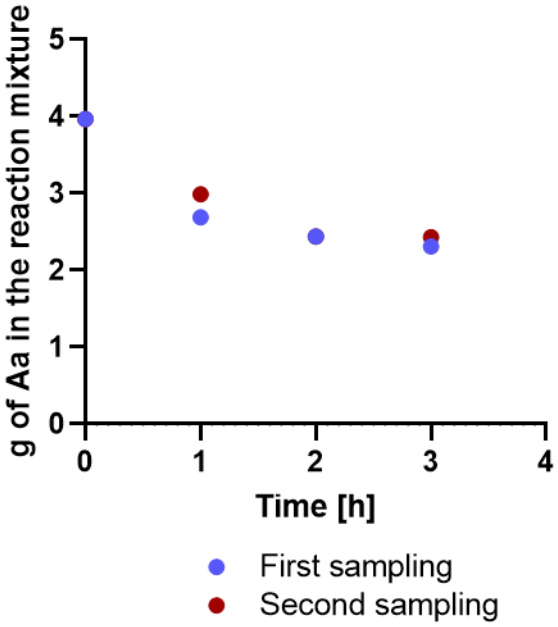


Figure 4.4. Amino acid consumption during the reaction in which two aliquots of supernatant were taken.

Quantification by weight afforded a loading of 0.57 mmol/g. It is worth noting again that this determination may be relatively imprecise because the loading is determined by weighting a resin that is located inside a quite heavy reactor vessel, so the associated error is not negligible. However, the possibility of losing amino acid bound to the resin during washings cannot be discarded according to the precedents in our laboratory. This premature release of amino acid or peptide chains is now under study.

Another experiment was performed following the procedure described in figure 4.1 in order to compare the results obtained by the HPLC methodology with those obtained following the existing methodologies of Fmoc quantification and quantification by weight. The experiment was carried out at a scale of 4.97 g of resin using 3.69 g of amino acid.

Figure 4.5 summarises the results of this assay where, again, a decreasing tendency of the amount of amino acid in the reaction mixture was observed once DIPEA was added, that decreased from 3.69 g (table 5.40, experimental section) to 1.92 g after 4 h of reaction, which indicated that 1.77 g of amino acid were incorporated, yielding a loading of 0.74 mmol/g.

Quantification by UV-Vis and by weight afforded a loading of 0.67 mmol/g and 0.50 mmol/g respectively. The quantification provided by both the HPLC and the UV-Vis Fmoc methodologies are similar. Nonetheless, the result obtained using the quantification by weight, again, differs significatively.

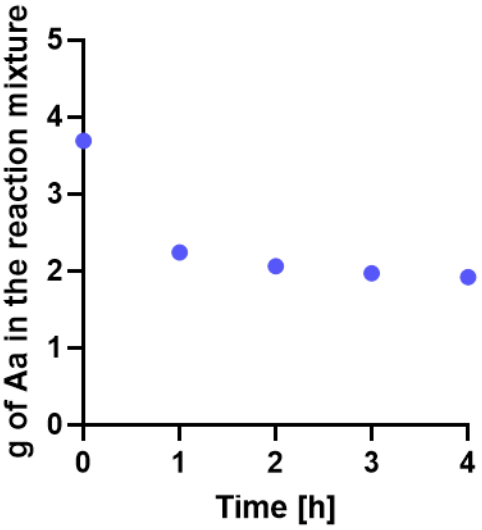


Figure 4.5. Amino acid consumption during the reaction.

Interestingly, the results obtained when the reaction was scaled up from 5 g to 10 g of 2-CTC resin were more coherent. As shown in figure 4.6, the amount of amino acid decreased from 7.69 g (table 5.41, experimental section) to 5.10 g after 4 h of reaction, which indicates that 2.59 g of amino acid were incorporated to the resin, leading to a loading of 0.54 mmol/g.

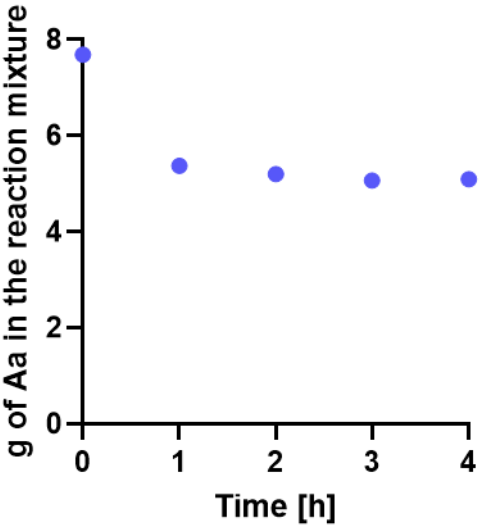


Figure 4.6. Amino acid consumption of the reaction in which the scale was increased.

Quantification by UV-Vis and by weight afforded a loading of 0.64 mmol/g and 0.58 mmol/g respectively. These results suggest that the weighing error was less notable when the scale of work was higher.

Finally, an assay was carried out using 10 g of 2-CTC resin and 7.50 g of amino acid, following the procedure described in figure 4.1 to determine the total amount of amino acid incorporated to the resin. In this case, the ratio between the amino acid concentration (mg/mL) and the peak area of the HPLC analysis was also determined from a standard

solution containing the amino acid (figure 4.7). The goal was to confirm that the control or standard solutions would lead to the same results.

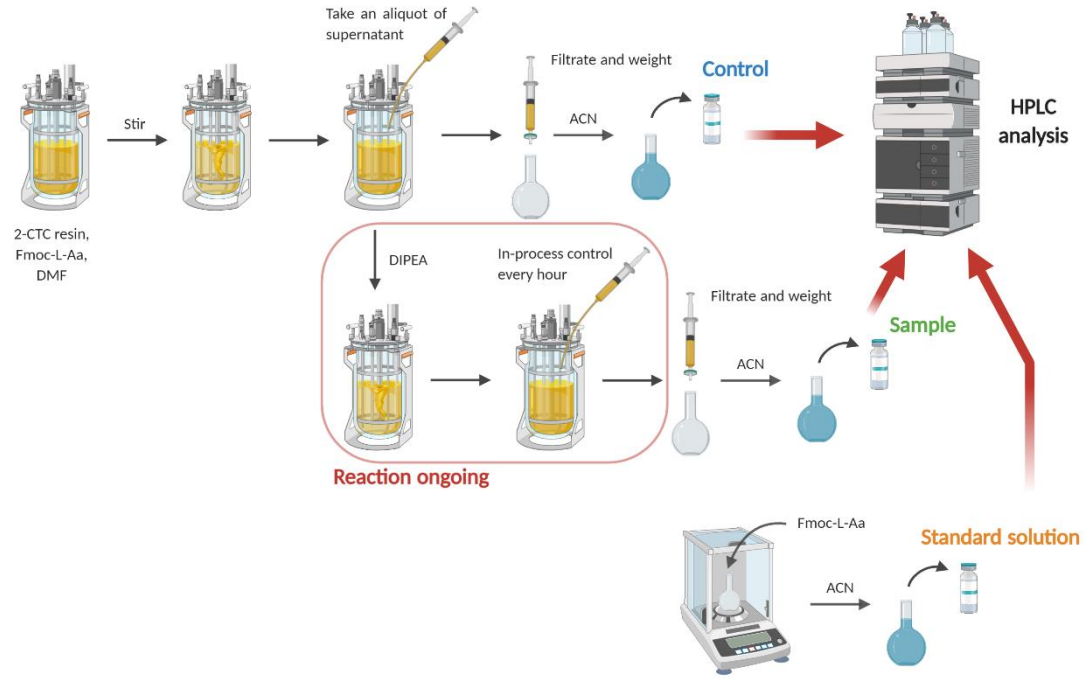


Figure 4.7. Scheme of the procedure when a standard solution was added to the methodology.

Figure 4.8 summarises the results obtained in this assay. Significant differences in the calculated amount of amino acid remaining in the supernatant were encountered when the calculations were done using the control solution or the standard solution as reference. Thus, after 3 h of reaction time, 5.00 g of amino acid were still in the supernatant according to the quantification carried out with the control solution (table 5.42, experimental section) while 3.48 g of amino acid were present according to the results obtained when the standard solution was used for quantification. The calculated loadings were 0.56 and 0.80 mmol/g, respectively.

Surprisingly, quantification by the UV-Vis Fmoc method afforded a loading of 0.63 mmol/g which suggests that the use of a standard solution does not improve the method and is counterproductive. This result may be accounted by the fact that the control solution is prepared under the same conditions than the in-process control samples and, in consequence, systematic errors are minimised.

In summary, those experiments reveal that the *precise* determination of the amount of amino acid that has been incorporated into the resin is very difficult and that may yield to significant differences when it has been compared to other methodologies. The HPLC method (based in the control solution as reference) and the typical UV-Vis Fmoc quantification led to similar results, being the former more convenient from an experimental point of view. The direct weighting methodology using big heavy reaction vessels is not practical and presumably leads to significant errors.

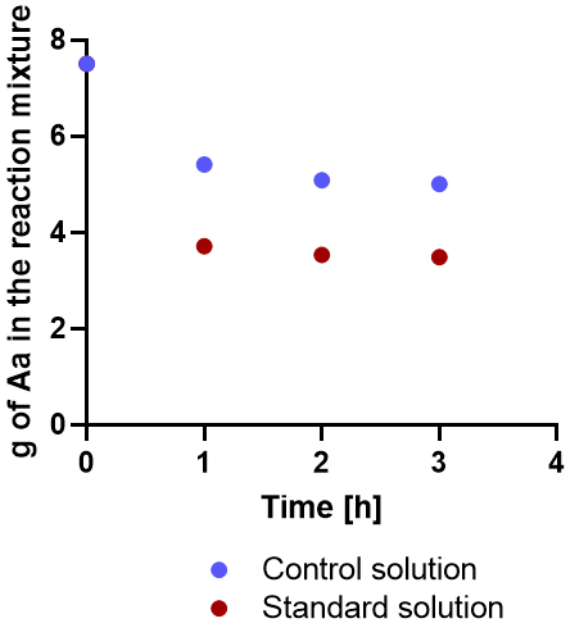
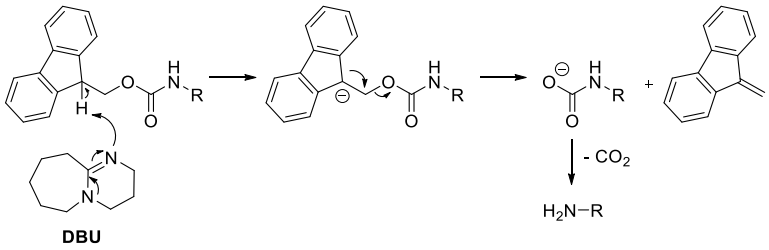


Figure 4.8. Amino acid consumption of the reaction when a standard solution was incorporated.

4.1.2 Fmoc removal

4.1.2.1. Using 1,8-diazabicycloundec-7-ene (DBU)

As stated before, another point of interest for the industrial in-process control was the removal of the Fmoc protecting group. When a non-nucleophilic base such as DBU is used for the removal of the N-terminal protecting group Fmoc, the only by-product specie that is generated is the dibenzofulvene (DBF, scheme 4.2).



Scheme 4.2. Fmoc removal using DBU.

Initially, the proposed HPLC methodology for the Fmoc removal monitoring was based on the quantification of the total amount of DBF in the supernatant and therefore, an external standard of DBF was required. To prepare such standard, DBF was isolated after the treatment with DBU of a Fmoc protected aminoacyl resin.

The experimental procedure consisted of adding a solution of 5 % DBU in DMF to the peptidyl-resin and weighing an aliquot of supernatant in a volumetric flask at different reaction times. The samples were diluted in ACN and the resulting solutions were analysed by HPLC (figure 4.9).

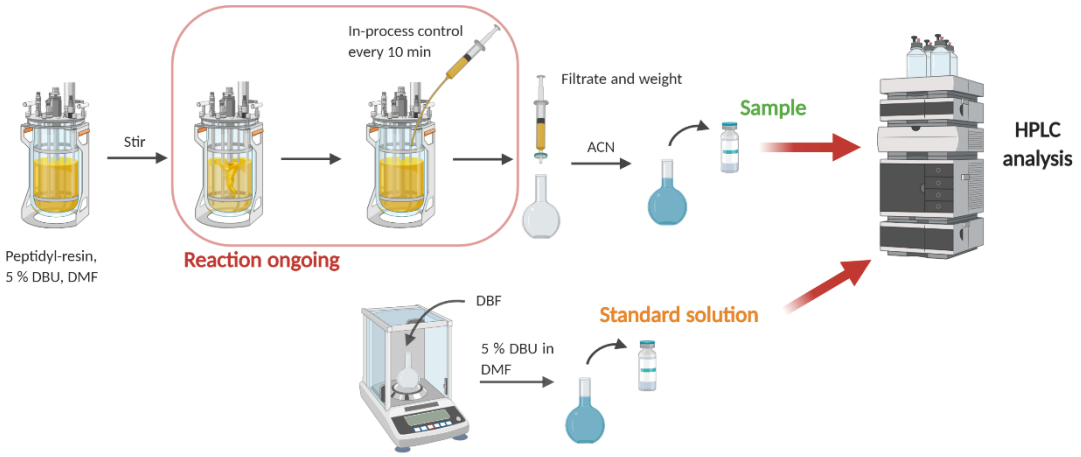


Figure 4.9. Procedure for the Fmoc removal monitoring using a standard solution of DBF.

The purpose was to calculate the DBF concentration in the supernatant comparing its peak areas with those of the standard DBF solution. Unfortunately, the poor stability of the DBF standard led to the presence of degradation impurities that increased with time (see figure 4.10). This problem, together with the low solubility of DBF in ACN, moved us to discard this methodology.

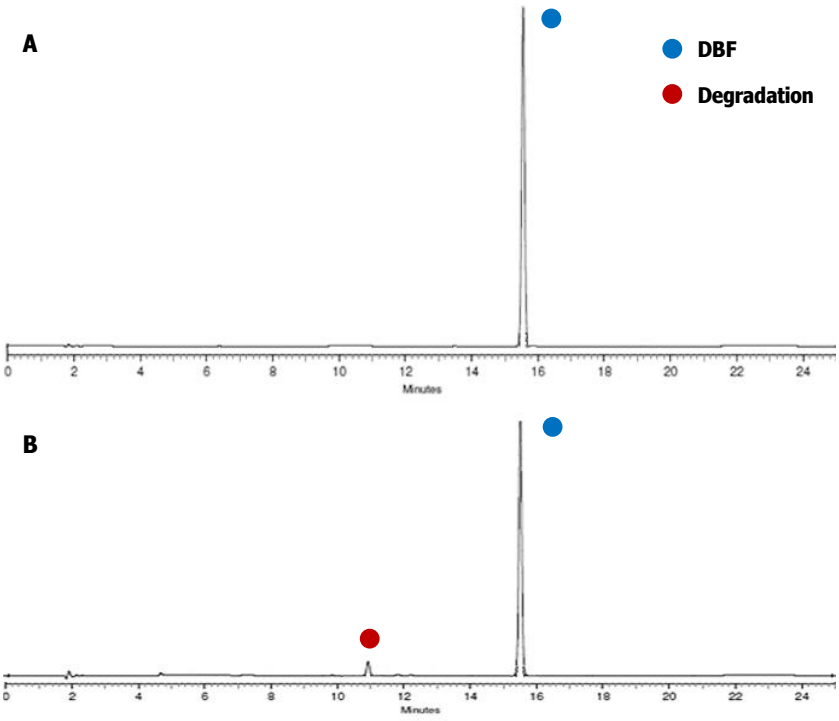


Figure 4.10. HPLC chromatograms of the DBF solutions in ACN. Chromatogram A belongs to the analysis of the sample that was performed just after the isolation of DBF while chromatogram B corresponds to the analysis of the standard solution of DBF after several hours.

4.1.2.1.1. HPLC monitoring of two consecutive DBU treatments

As a result of not being able to quantify the amount of DBF formed with the use of an external DBF standard solution, a new methodology was proposed. This methodology consisted of treating the peptidyl-resin with a solution of 5 % DBU in DMF and weighing

an aliquot of the supernatant in a volumetric flask after 10 min and 20 min. Then, the reaction mixture was filtered by suction and the procedure was repeated. The in-process control solutions were analysed by HPLC (figure 4.11).

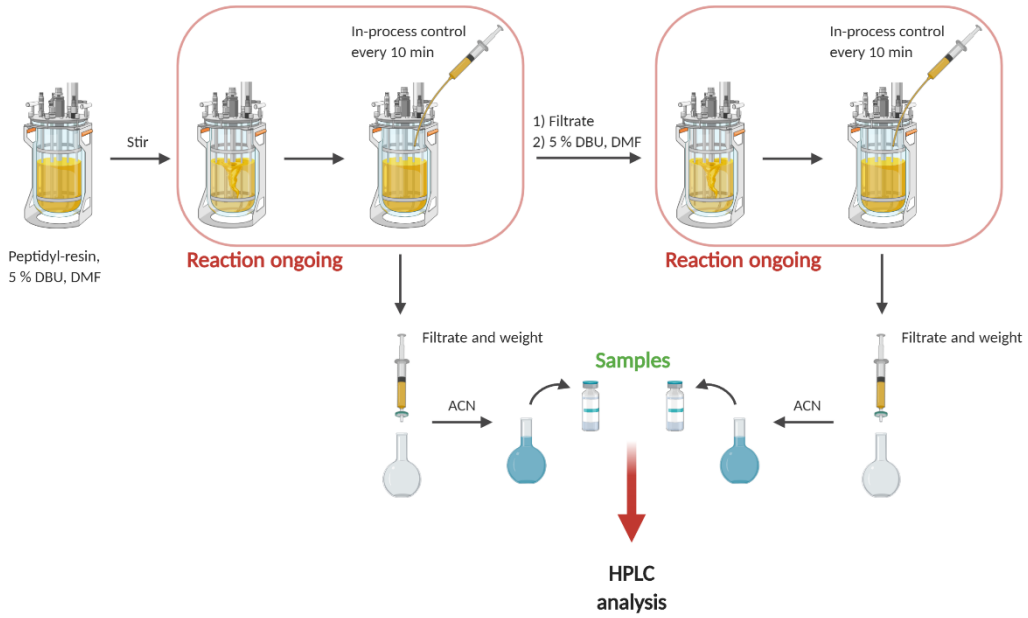


Figure 4.11. Scheme of the procedure for the Fmoc removal monitoring of two consecutive DBU treatments.

With this methodology, the amount of DBF formed could not be determined but completion of the reaction may be stablished if DBF is not detected after the second DBU treatment. Figure 4.12 summarises the results obtained in the first assay.

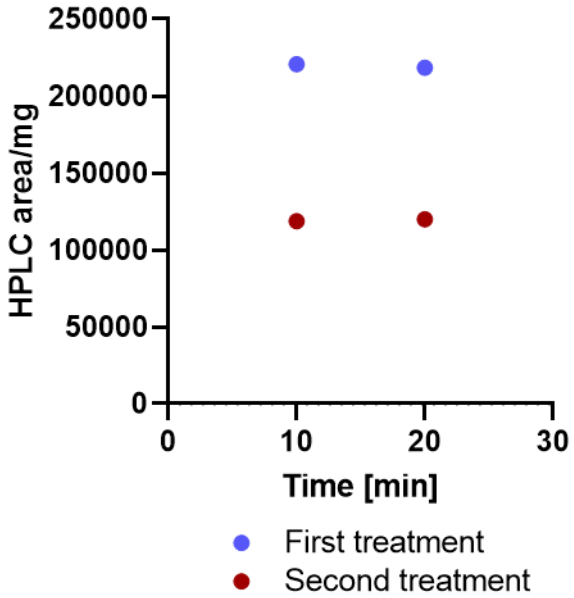


Figure 4.12. Comparison of the DBF formation: two consecutive treatments with DBU.

It was observed that DBF was still detected after the second treatment of the resin. This result suggested that the Fmoc group was not completely removed in the first treatment

and/or that DBF was not completely removed from the resin when the reaction mixture was filtrated off by suction.

Thus, a second experiment involving a washing steep in-between the base treatmets was carried out (figure 4.13). In this case, the HPLC analysis showed a residual formation of DBF after the first treatment (figure 4.14).

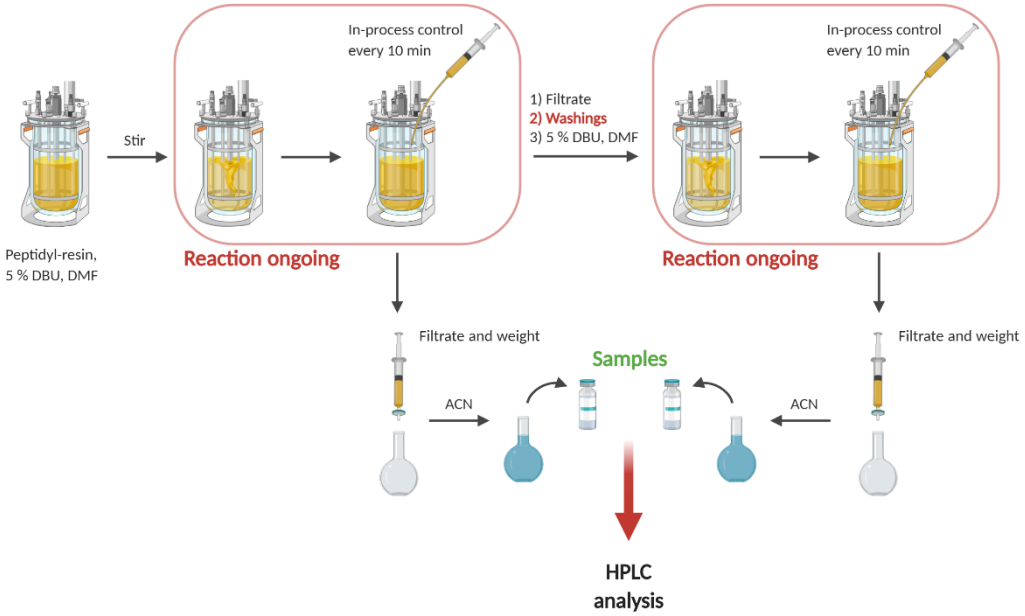


Figure 4.13. Scheme of the procedure for the Fmoc removal monitoring of two DBU treatments with washings in between.

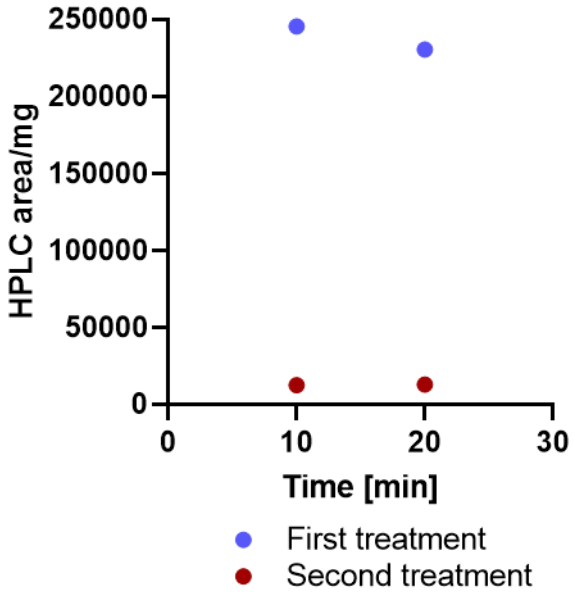


Figure 4.14. Comparison of the DBF formation: two treatments with DBU with washings in between.

Finally, one last experiment was carried out using three consecutive treatments with DBU to determine and ensure that the Fmoc group was completely removed after the second treatment (figure 4.15).

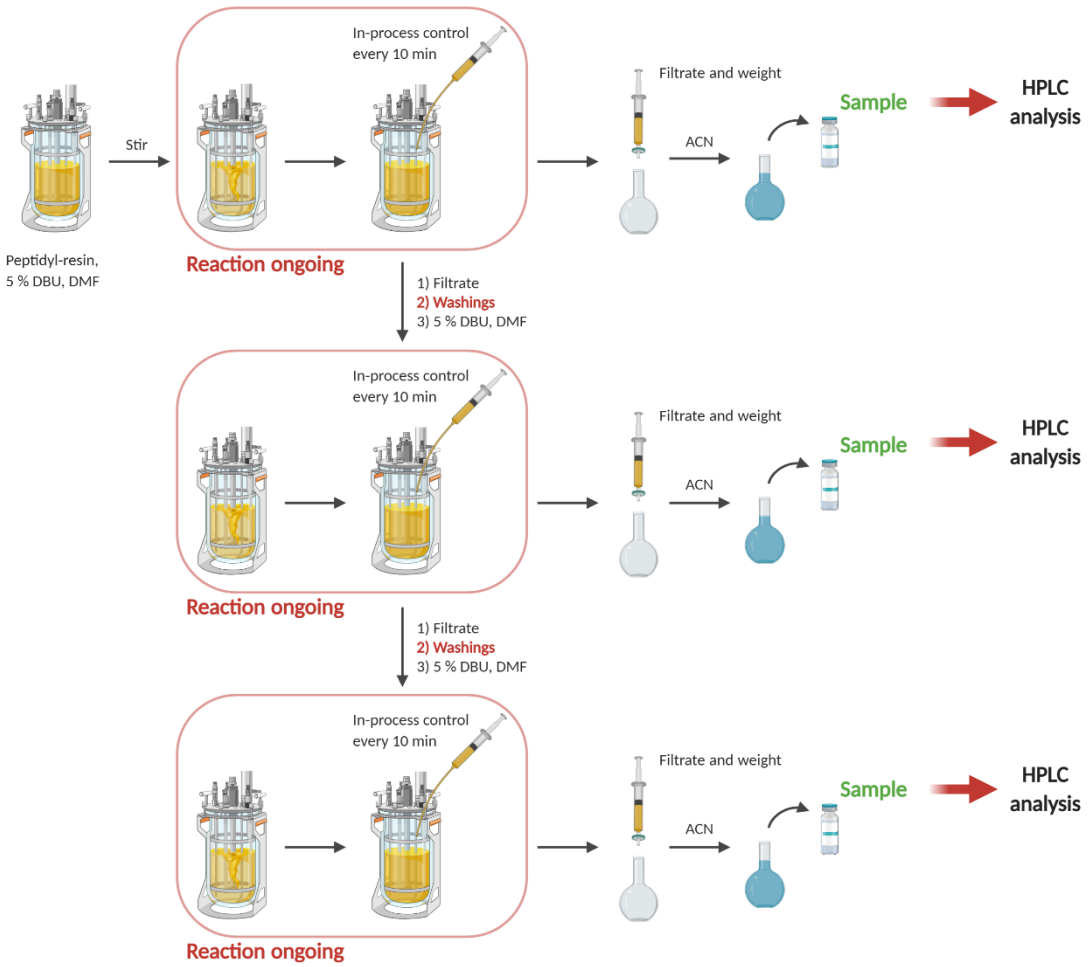


Figure 4.15. Scheme of the procedure for the Fmoc removal monitoring of three DBU treatments with washings in between.

As shown in figure 4.16, the HPLC analysis did not detect the presence of DBF in the supernatant of the third treatment. In summary, those experiments show that a first DBU treatment removes the majority of Fmoc groups, although a second treatment is necessary to ensure a quantitative deprotection. Moreover, this technique provides a convenient way to perform the necessary in-process control at industrial level.

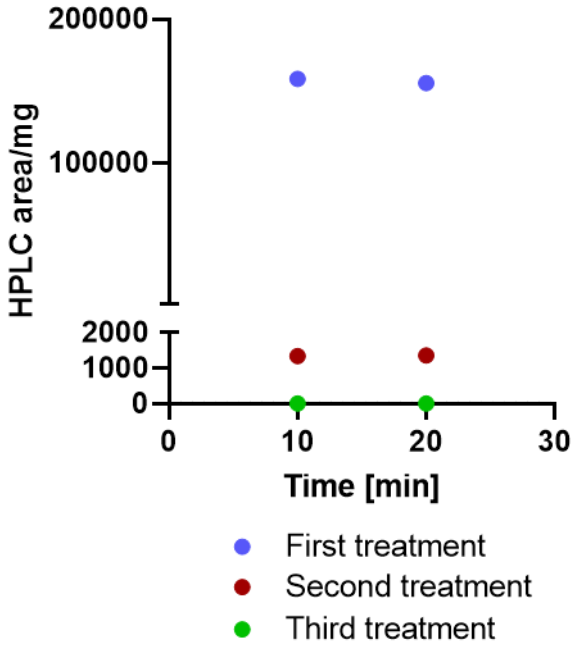


Figure 4.16. Comparison of the DBF formation: three treatments with DBU with washings in between.

4.1.2.2. Using piperidine

A methodology similar to the described in the precedent section was assayed but using piperidine instead of DBU as base. The peptidyl-resin was treated with a solution of 20 % piperidine in DMF and aliquots of supernatant were analysed by HPLC as described in the previous section (figure 4.17).

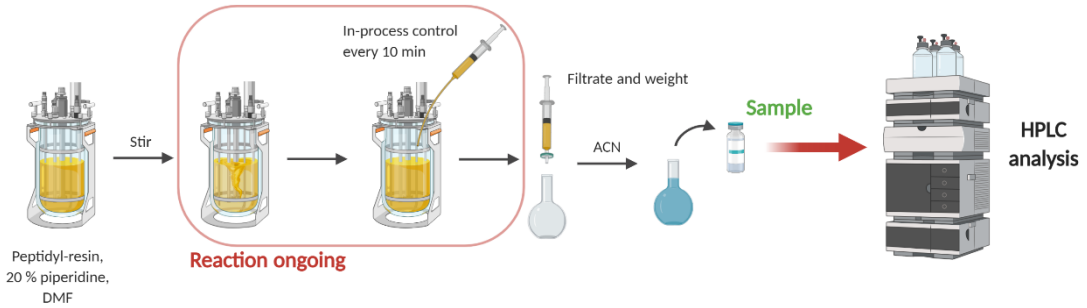
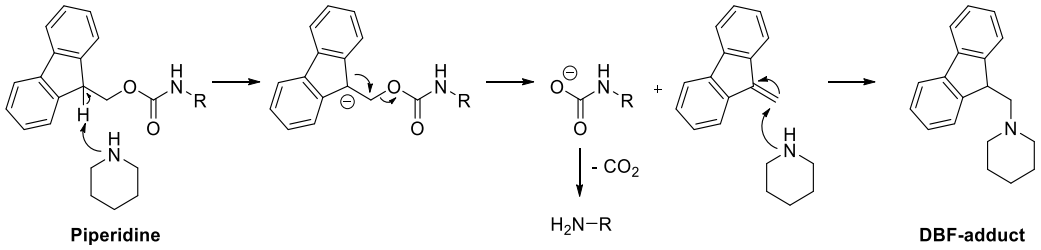


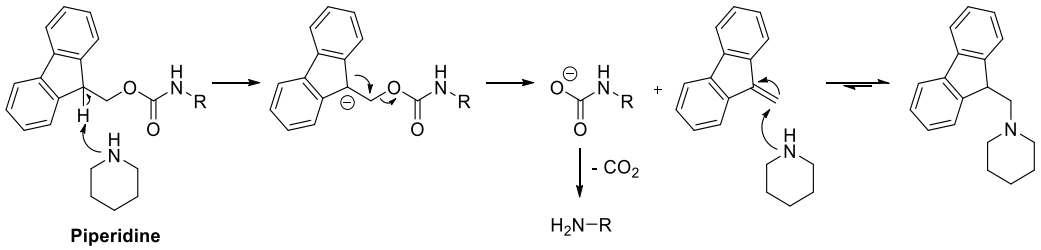
Figure 4.17. Scheme of the procedure for the Fmoc removal monitoring using a piperidine solution.

In this case, the piperidine adduct resulting from the Fmoc removal was analysed, bearing in mind that the total consumption of the DBF to form this by-product was expected due to the large excess of piperidine that was used (scheme 4.3).



Scheme 4.3. Fmoc removal using piperidine.

The first analysis showed that after 2 h of reaction, there was still a 4 % of DBF in the supernatant (figure 4.18) suggesting the possibility of an equilibrium between the DBF and the piperidine adduct (scheme 4.4).



Scheme 4.4. Formation of the DBF-piperidine adduct during Fmoc removal.

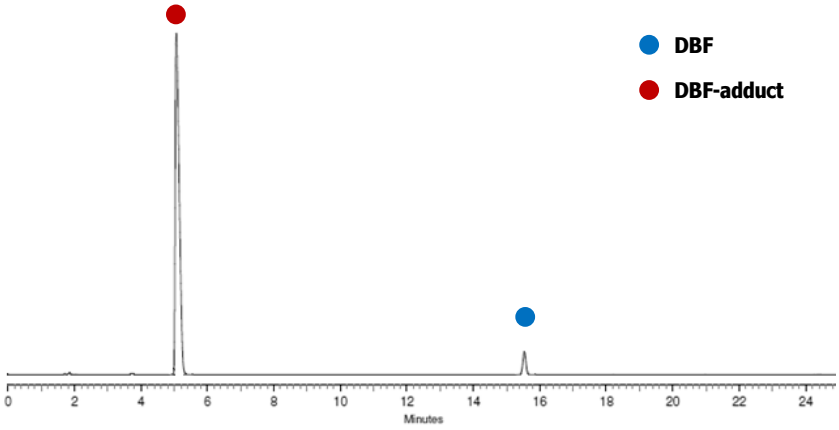


Figure 4.18. HPLC chromatogram of the in-process control analysis at 2 h of reaction time.

Following a protocol like the one described in section 4.1.2.1, an experiment involving three consecutive treatments with piperidine was monitored by HPLC. In this case, only one aliquot was taken after 5 min of the first treatment and after 2 min of the second and third treatments (figure 4.19). Figure 4.20 summarises the results obtained in this assay.

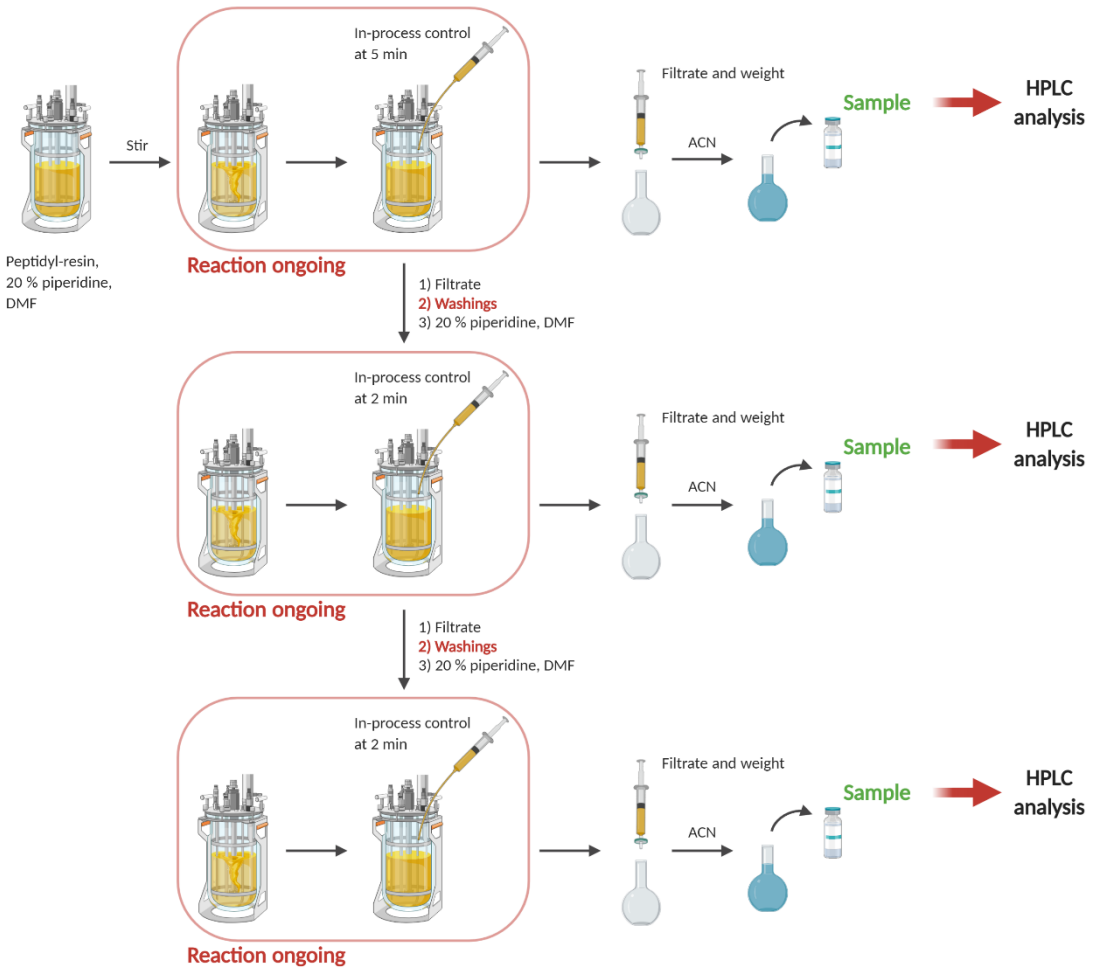


Figure 4.19. Scheme of the procedure for the Fmoc removal monitoring using three piperidine treatments with washings in between.

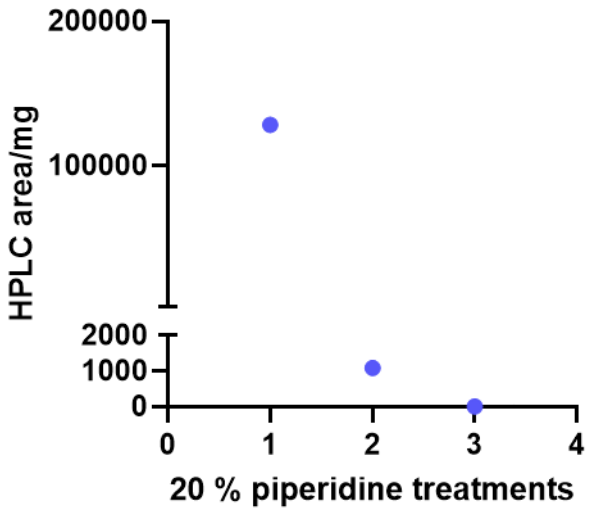


Figure 4.20. DBF formation after three treatments with piperidine with washings in between.

As seen in the experiments in which DBU was used to remove the Fmoc, the DBF-adduct was detected after the second treatment of the peptidyl-resin with piperidine but the HPLC

area/mg ratio obtained in the second treatment is considerably lower than the ratio obtained after the first treatment. This suggested that the Fmoc group was not completely removed after the first treatment. On the other hand, the DBF-adduct was again not detected after the third treatment of the peptidyl-resin which indicated that the Fmoc group was completely removed after the second treatment. These results confirm that the third treatment of the peptidyl-resin with a base is not necessary since the Fmoc group has been completely removed during the second treatment and that the treatments performed with DBU can be shortened to decrease the time of the overall process.

4.1.3. Elongation of the peptide chain

Finally, a methodology for the in-process control monitorization of the SPPS chain-elongation reactions was also evaluated. The proposed methodology consists on dissolving the amino acid to be incorporated (Fmoc-Val-OH, in this particular case) and HOBt in DMF. The resulting solution is transferred into the reactor vessel and stirred mechanically. At this point, an aliquot of the supernatant is weighed in a volumetric flask and diluted with ACN. Then, DIC is added to the mixture to start the reaction and left stirring for 1.5 h when an aliquot of supernatant is weighed in a volumetric flask and diluted with ACN. Finally, both sample solutions are analysed by HPLC (figure 4.21).

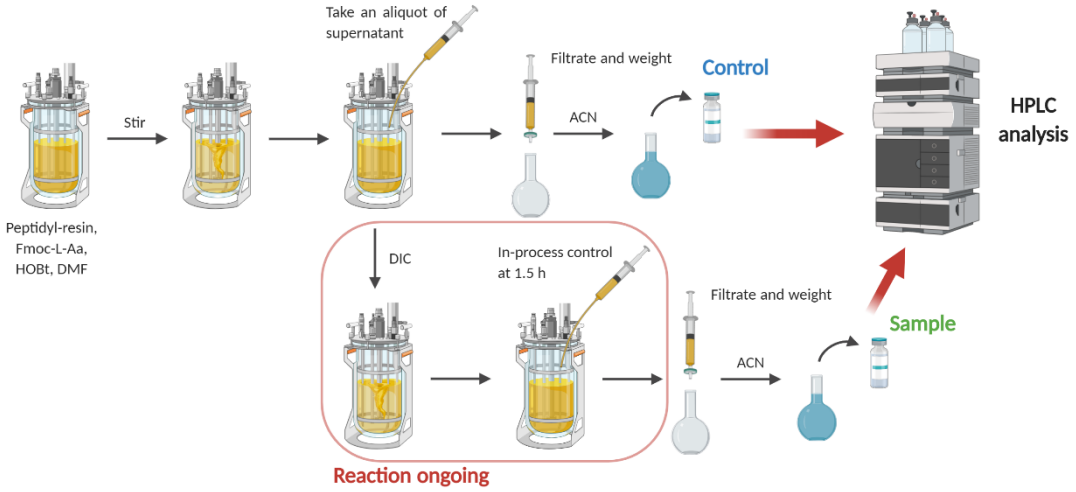


Figure 4.21. Methodology proposed for the coupling reactions monitoring on solid-phase.

The aliquot taken before the addition of the carbodiimide was considered as the control solution and used to establish the ratio between the amino acid concentration (mg/mL) and the peak area of the HPLC chromatogram. With this result, the idea was to calculate the remaining amount of amino acid in the supernatant after 1.5 h of reaction. Unfortunately, the total amount of non-reacted amino acid in the supernatant could not be quantified due to the presence of several unidentified peaks in the chromatogram (figure 4.22) that might correspond to the intermediates generated during the coupling reaction.

It is worth noting that we expected that the eluent used in the HPLC analysis should have hydrolysed those reactive intermediates, but it was not the case. In consequence, it was not possible to calculate the extend of the reaction by this method because the response factor of the intermediates was unknown.

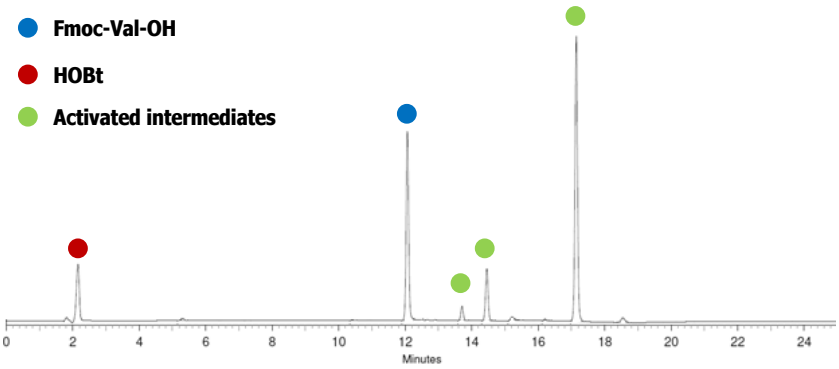


Figure 4.22. HPLC chromatogram of the in-process control at 1.5 h.

To solve this problem, a 0.1 % of NH_3 was added to the ACN used to dilute the aliquots expecting that the activated amino acid species would react faster and quantitatively with NH_3 to form the amino acid carboxamide. Figure 4.23 shows the HPLC profile of the in-process control of a sample that was diluted with this diluent.

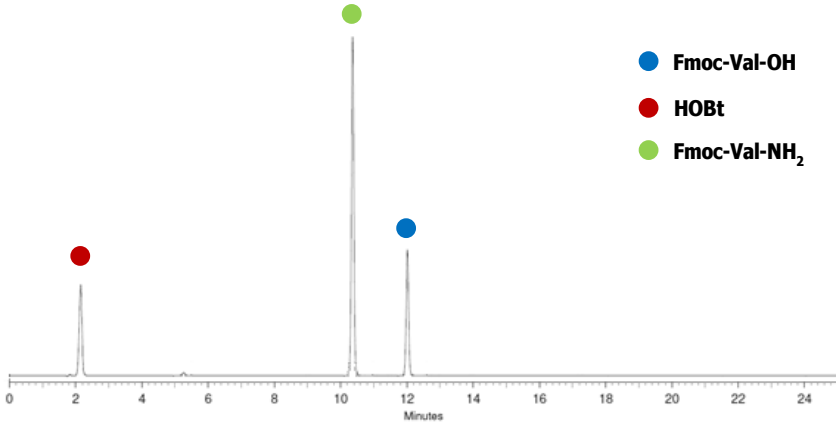


Figure 4.23. HPLC chromatogram of the in-process control using 0.1 % NH_3 in ACN as sample diluent.

By using this diluent, the total amount of non-reacted amino acid in the supernatant can be estimated assuming that the carboxamide has an HPLC response factor very similar to the response factor of the amino acid. Additional experiments will have to be carried out at later development stages to determine more precisely the response factors of both species in order to get more accurate results.

This result moved us to assay a new protocol similar to the described in figure 4.21 but the samples were now diluted with the ACN that contains 0.1 % NH_3. In this case, the reaction was left stirring for 1 h before analysing the supernatant (figure 4.24). In this case, a standard solution containing the amino acid was also prepared with the aim to compare the results obtained when the calculations were done using as reference this solution or the control solution. The results are summarised in tables 4.1 and 4.2 and show that both approaches led to similar results (1.68 and 1.65 eq of amino acid in the supernatant of the reaction mixture).

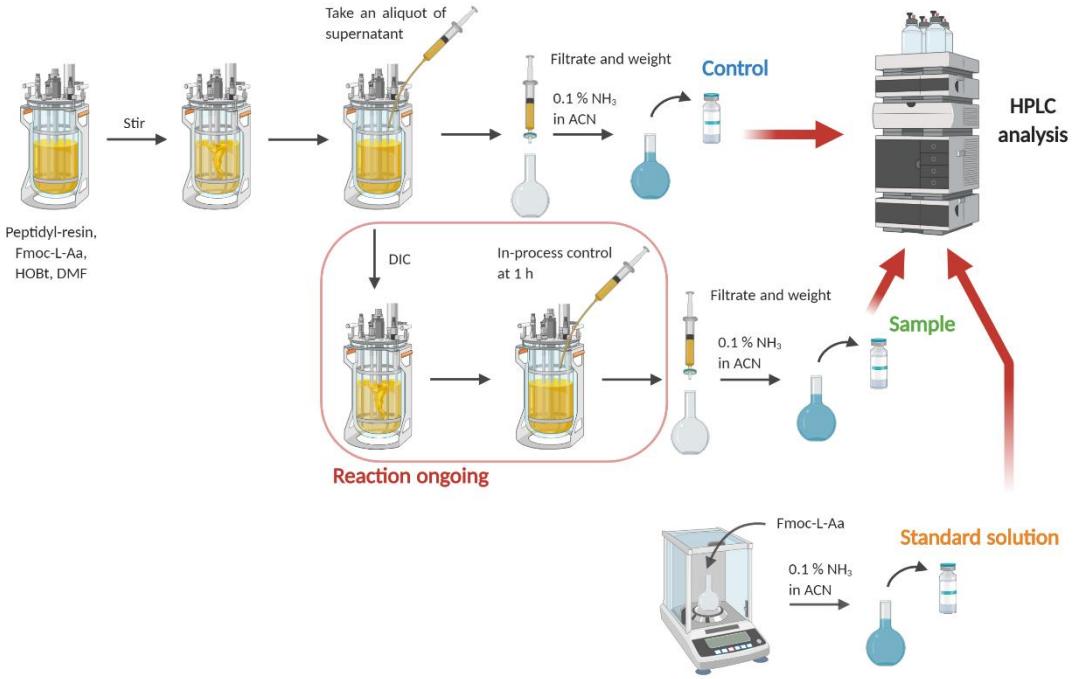


Figure 4.24. Scheme of the methodology proposed for the coupling reactions monitoring using 0.1 % NH_3 in ACN as sample diluent.

Table 4.1. Results obtained of the HPLC quantification of Aa in the supernatant using the control solution as reference.

Entry		**Time** [h]	
		0 (control)	1 (in-process)
1	g of aliquot	0.220	0.223
2	HPLC area	21254455	10389563
3	mg/mL in the volumetric flask	0.459[a]	0.224[b]
4	g of Aa in the volumetric flask	0.046	0.022
5	g of Aa in the reaction mixture	10.97	5.95
6	eq of Aa in the reaction mixture	3.10	1.68

(a) Calculated with experimental data.
(b) Calculated by HPLC.

Table 4.2. Results obtained of the HPLC quantification of Aa in the supernatant using the standard solution as reference.

Entry		**Time** [h]	
		External standard	1 (in-process)
1	g of aliquot	0.06316	0.223
2	HPLC area	14827967	10389563
3	mg/mL in the volumetric flask	0.316[a]	0.221[b]
4	g of Aa in the volumetric flask	-	0.022
5	g of Aa in the reaction mixture	-	5.85
6	eq of Aa in the reaction mixture	-	1.65

(c) Calculated with experimental data.
(d) Calculated by HPLC.

Unfortunately, those results revealed an important drawback for this methodology. In general, amino acids are used in a large excess. In this case, we used 3 eq. of Fmoc-Val-OH,

so we expected at least 2 eq. left in the supernatant. However, the calculated amount was about 1.6 eq. Additionally, this over-consume of reactive suggested that coupling was quantitative, but the ninhydrin test was positive. We interpret those results assuming that part of the amino acid was non-covalently bound to the resin and, therefore, we must consider that the method is not suitable to quantify the amount of amino acid that has been truly incorporated to the peptide chain.

4.1.3.1. Simulation of a coupling reaction

In order to evaluate if the amino acid was non-covalently bound to the polymeric support we designed an experiment in which the coupling reaction environment was simulated. To do so, Fmoc-Aa-OH (6.25, 3.0 eq) and HOBt (2.65 g, 2.8 eq) were weighed and dissolved in DMF. The solution was transferred to the reactor that contained the 2-CTC resin, the reactive points of which were previously blocked with MeOH, and the resulting suspension was stirred for 3 h. Under these conditions, the amino acid cannot covalently bind into the polymeric support.

Then, aliquots of the supernatant were weighed at different reaction times in a volumetric flask and diluted with ACN. 1 mL of these solutions were then transferred to a 10 mL volumetric flasks with a volumetric pipette and diluted again with ACN. The resulting solutions were analysed by HPLC. In parallel, a standard solution of Fmoc-Aa was prepared for HPLC quantification purposes (figure 4.25).

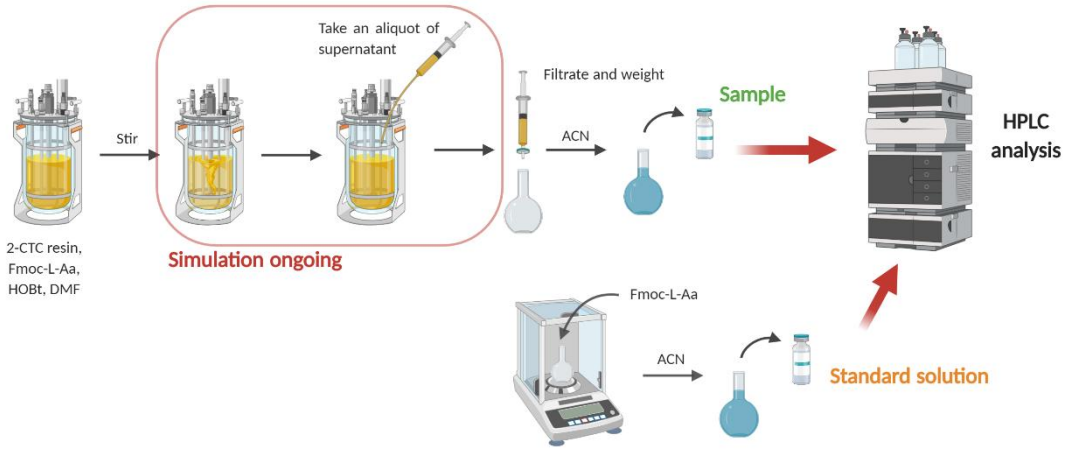


Figure 4.25. Scheme of the methodology proposed for the simulation of a coupling reaction.

Table 4.3 summarises the results obtained in this assay. The amino acid concentration and the amount of amino acid in the supernatant can be calculated by HPLC by comparing the HPLC area of the sample with the HPLC area of the standard solution. In this experiment, the concentration of amino acid in the standard solution was 0.3183 mg/mL and the experimental HPLC area was 17241106.

It was observed that the amount of the amino acid in the supernatant was about a 35 % lower than the expected. These results confirmed that the amino acid most probably was partially non-covalently bound to the polymeric support, thus giving lower values for the amino acid concentration in the supernatant. As a conclusion, it can be said that a deep wash of the resin under different conditions should be performed prior to quantification in order to remove as much as possible unreacted amino acid. In this sense, experiments that have

been recently carried out in our laboratory, have revealed that washing steps are crucial in order to avoid undesired results such as over coupling of amino acids to the peptide chain.

Table 4.3. Results obtained for the simulation of a coupling reaction.

Entry	**Theoretical Aa** [g]	**Aa in the supernatant**[a] [g]	**Aa adsorbed** [%]
1	6.25	4.11	34
2	6.25	4.07	35
3	6.25	4.04	35
4	6.25	4.04	35

(a) Calculated by HPLC.

4.2. Conclusions

Different methodologies for the in-process control of the solid-phase incorporation of the first amino acid, the Fmoc removal and the elongation of the peptide chain were explored. The results are summarised in the present section.

Incorporation of the first amino acid

A methodology based on the quantitative determination by HPLC of the remaining amino acid in the reaction mixture was studied in order to determine the total amount of amino acid that was incorporated into the resin. The results obtained were compared to those achieved with the existing Fmoc and by weight quantifications. When using a lower scale, after 4 h of reaction, the loading determined by HPLC analysis (0.74 mmol/g) was found to be lower than the values obtained UV-Vis (0.67 mmol/g) and by weight (0.50 mmol/g). The unexpected low value encountered when quantifying by weight was probably due to the error associated to the weighing procedure because this error was considerably reduced when higher amounts of resin were used (around 10 g of resin). Thus, under these conditions the loading determined by HPLC analysis after 4 h of reaction was found to be 0.54 mmol/g while the quantification by UV-Vis and by weight afforded loadings of 0.64 mmol/g and 0.58 mmol/g respectively. These results should be considered very preliminary due to the possibility that some amino acid could be non-covalently bound to the resin according to the results obtained in the study of the elongation of the peptide chain. Therefore, more work has to be done in order to confirm the suitability of this methodology.

Fmoc removal

The quantitative determination by HPLC of the by-products generated during the removal of the Fmoc group under basic conditions was studied to determine the completion of the reaction. When DBU was the base, the quantification of the by-product DBF on the supernatant was monitored using an external solution of DBF as standard. However, this protocol was discarded due to the low stability and solubility of DBF in DMF. In the case of piperidine, it was analysed the piperidine adduct resulting from the reaction of DBF with the base, expecting a total consumption of this by-product. However, both by-products were always detected in the reaction mixture, probably as a result of the establishment of an equilibrium process. Finally, a methodology based on that the completion of the reaction could be determined if the DBF or the DBF-adduct were not detected in the HPLC analysis after a second treatment of the peptidyl-resin with DBU or piperidine, proved to be the best methodology.

Elongation of the peptide chain

A methodology based on the quantitative determination by HPLC of the remaining amino acid in the supernatant was studied to establish the completion of the coupling reactions. The total amount of non-reacted amino acid could not be quantified due to the presence of several unidentified peaks in the chromatogram that might correspond to the activated species resulting from the reaction of the amino acid with the coupling reagent. A 0.1 % of NH_3 was added as nucleophile to the ACN used to dilute the reaction aliquots in order to decompose such species. Under these conditions the total amount of non-reacted amino acid in the supernatant could be estimated through the chromatographic peaks of the remaining amino acid and its amide. Unfortunately, we have found that this methodology is not suitable for the in-process control of the reaction, due to a partial non-covalent bonding of the amino acid to the resin that cannot be quantified. Fortunately, in the framework of another PhD thesis, promising results are being achieved by NMR analysis of samples obtained from the acidolytic cleavage of resin aliquots.

Concluding remarks

The work of this thesis constitutes the starting point of a project in collaboration with Esteve Química devoted to develop a robust and economic synthetic methodology based on the solid-phase strategy to produce the seventeen-residue therapeutic peptide Fexapotide at a plant-pilot scale according to the regulatory requirements of the pharmaceutical company. This preliminary study has been performed at laboratory scale.

Firstly, the linear approach was assayed but this strategy presented difficulties when trying to couple some amino acid residues and it was assumed that the scale up of the process following this methodology would be challenging and expensive. Therefore, our efforts were focused on the development of a convergent approach.

Following the requirements to address a convergent approach to minimise synthetic problems, that is, the use of fragments no longer than 5 amino acids and the absence of amino acids prone to racemise at the C-terminal end of the fragment, the first convergent approach that was proposed consisted on four fragments: the dipeptide **5**, the tetrapeptide **6**, the pentapeptide **7** and the hexapeptide **8**. The dipeptide was synthesised in solution-phase due to the need of protection at the C-terminal, and the rest were synthesised on solid-phase. Further condensation of the fragments in solution afforded the full protected Fexapotide, which led to the target peptide after removing the protecting groups.

This convergent synthetic strategy led to the target peptide with a much higher chromatographic purity than the obtained using the linear synthetic strategy. These promising results moved to try another convergent approach following the guidelines of the company based on regulatory requirements. Thus, it is easier and faster to accomplish these regulatory requirements in terms of structure characterisation, manufacture process and impurities emerging from the synthetic process when the number of intermediates is as low as possible. For this reason, the nonapeptide **28** was considered instead of the fragments **6** and **7**. This alternative convergent approach afforded better results in terms of chromatographic purity when compared to the other proposed strategies and was considered suitable for the company in terms of manufacture process and cost.

A preliminary study was also addressed towards the design of a reaction monitoring protocol to follow the synthetic process on solid phase, because the methodologies developed so far for the reaction monitoring are usually performed once the reaction has ended and thus, *in situ* modifications cannot be performed. Moreover, from the pharmaceutical company's point of view, the existing methodologies are not quantitative enough nor accessible to perform an in-process control of the synthetic steps. For this reason, several methodologies were assayed in the framework of the thesis to monitor different solid-phase synthetic steps such as the incorporation of the first amino acid on the solid support, the Fmoc protecting group removal and the elongation of the peptide chain. The results obtained should be considered very preliminary and more work must be done to confirm the suitability of these methodologies.

Another PhD thesis is currently studying the scale-up of the process using the three-fragment convergent approach and the use of NMR to follow the synthetic steps, with very promising results in both cases.

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Experimental Section

5.1. Materials and methods

5.1.1. Solvents and reagents

Table 5.1. Reagents and solvents used in the present thesis.

Product	Brand
2-CTC resin[a]	Irish-Biotech
TFA	Fisher Bioreagents
DIC	TCI
Piperidine	Carlo Erba Reagents
DMF	
MeOH	
iPrOH	
ACN	
NMP	VWR
DCM	
Acetone	
THF	Scharlau
Et_2O	
HFIP	TCI
HOBt·H_2O	Esteve Química
HATU	
EDC·HCl	
Amino acids	
HCTU	
DMAP	Acros Organics
PyAOP	Fluorochem
HOAt	
DIPEA	Sigma Aldrich

(a) The functionalisation of the resins for the peptide syntheses were 0.97 mmol/g, 1.10 mmol/g, 1.40 mmol/g and 1.60 mmol/g.

5.2. Instrumentation

5.2.1. General basic instrumentation

Table 5.2. Instruments used in the present thesis.

Instrument	Brand	Model
HPLC-MS	Waters	2695/2697 separation module
	Agilent	1200 Series
HPLC	Shimadzu	LC-20AD
	Agilent	1100/1260 Series
UV-Vis spectroscopy	Varian	Cary 100
Analytical balance	Mettler	Toledo AB254
Water purification system	Millipore	Milli-Q Plus Water Purification System

Table 5.2 (continued)

Magnetic Stirrer	Scilogex	MS-H-Pro+
NMR	Varian Bruker	400 Hz
Rotatory evaporator	BUCHI	R-200
Ultra-sonic bath	P-Selecta	Ultrasons UB-1488
Orbital Stirrer	Ika	KS250 basic
MALDI-TOF	Applied Biosystems	4700 Proteomics Analyzer 2011

5.2.2. Isolator used for the solid-state handing and reactions involving HP-APIs

The target Fexapotide **1** and the precursor of **1**, which is the protected Fexapotide **16**, were considered high-potency active pharmaceutical ingredients (HP-APIs). Working with APIs that have high potency and cytotoxicity present several challenges for pharmaceutical companies such as handling, containment and cost. Therefore, solid state handling and reactions in which one of these substances was present were carried out in an ProSys® isolator such as the one shown in the figure below which works with an internal pressure of -100 Pa.

1: Main room, 2: Airlock room, 3: Continuous line, 4: RTP, 5: HMI, 6: LOP, 7: Fan

5.3. Chromatography

5.3.1. Thin Layer Chromatography (TLC)

TLC was performed on silica gel F254 plates supplied by Merk. The sample was applied on the silica plate (stationary phase) and was eluted with an appropriate solvent mixture (mobile phase). Separation of compounds was accomplished due to different polarity. An UV-Vis lamp was used to detect the spots that absorbed UV light at 254 nm, and TLC staining solutions were used to detect compounds that did not absorb UV light.

TLC staining solutions:

- Basic $KMnO_4$: 40 g of K_2CO_3 and 6 g f $KMnO_4$ were dissolved in 600 mL of water. Then, 5 mL of 10 % NaOH were added. This staining solution oxidases compounds containing diols, alkenes, reactive methylenes, phenols, thiols or phosphines.

- Ninhydrin: 20 g of ninhydrin were dissolved in 600 mL of EtOH. This staining solution produces blue spots in the presence of primary amines when heated. Secondary amines are sometimes detected but the stain is not intense.

5.3.2. Column chromatography on silica gel

Column chromatography was performed using flash chromatography with silica gel (pore size of 60Å, 230 – 400 mesh and 40 - 63 μm of particle size).

5.3.3. High performance liquid chromatography (HPLC)

5.3.3.1. Analytical HPLC

Analytical HPLC was performed using different instruments:

- Shimadzu instrument 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. The column used was an Aeris™ WIDEPORE (C4 of 150×4.6 mm) with 3.6 μm of particle size and a pore size of 200Å.

- Hitachi Lachrom Elite instrument composed by a L-2130 pump, an automatic injector and an autosampler L-2200, a variable wavelength detector L-2400 and a column over L-2300. The column used was an ACE (C4 of 150×4.6 mm) with 3.0 μm of particle size and a pore size of 100 Å.

- Agilent instrument composed by an Agilent 1100 series quaternary pump, an automatic injector Agilent 1100 series, a dual variable wavelength detector Agilent 1100 series and a micro vacuum degasser device Agilent 1100 series. The column used was an Aeris™ WIDEPORE (C4 of 150×4.6 mm) with 3.6 μm of particle size and a pore size of 200 Å.

- Agilent instrument composed by an Agilent 1260 series quaternary pump, an automatic injector Agilent 1260 series, a dual variable wavelength detector Agilent 1260 series and a micro vacuum degasser device Agilent 1260 series. The column used was an Aeris™ WIDEPORE (C4 of 150×4.6 mm) with 3.6 μm of particle size and a pore size of 200 Å.

5.3.3.2. HPLC-MS

HPLC-MS was performed using different instruments:

- Waters instrument composed by a Waters 2695 separation module, an automatic injector, a Waters 2996 photodiode array detector and a Waters ESI-MS Micromass ZQ 4000 spectrometer. The column used was an Aeris™ WIDEPORE (C4 of 150×4.6 mm) with 3.6 μm of particle size and a pore size of 200 Å.

- Waters instrument composed by a Waters 2795 separation module, an automatic injector, a Waters 2996 photodiode array detector and a Waters ESI-MS Micromass ZQ 2000 spectrometer. The column used was an Aeris™ WIDEPORE (C4 of 150×4.6 mm) with 3.6 μm of particle size and a pore size of 200 Å.

- Agilent instrument composed by an Agilent 1200 series binary pump, an automatic injector Agilent 1200 series, a diode array detector Agilent 1200 series, a vacuum degasser device Agilent 1200 series and a 6320 Ion Trap LC/MS spectrometer. The columns used were an Aeris™ WIDEPORE (C4 of 150×4.6 mm) with 3.6 μm of particle size and a pore size of 200 Å, an Aeris™ PEPTIDE XB-C18 (150×3.0 mm) with 2.6 μm of particle size and pore size of 100 Å and a Kinetex® EVO (C18 of 150×3.0 mm) with 2.6 microns of particle size and pore size of 100 Å.

High resolution mass LC-MS/MS analyses were performed on an Agilent instrument composed by an Agilent 1200 series binary pump, an automatic injector Agilent 1200 series, a diode array detector Agilent 1200 series, a vacuum degasser device Agilent 1200 series and a Bruker Ultra-High Resolution QTOF (UHR-TOF MS). The columns used were an Aeris™ WIDEPORE (C4 of 150×4.6 mm) with 3.6 μm of particle size and a pore size of 200 Å, an Aeris™ PEPTIDE XB-C18 (150×3.0 mm) with 2.6 μm of particle size and pore size of 100 Å and a Kinetex® EVO (C18 of 150×3.0 mm) with 2.6 μm of particle size and pore size of 100 Å.

5.3.3.3. HPLC methods

Tables 5.3-5.8 summaries the different chromatographic conditions used in the present project.

Table 5.3. Chromatographic conditions: Method A.

Eluent A: 0.036% TFA in ACN
Eluent B: 0.045% TFA in H_2O

Time (min)	%A	% B
0	20	80
2	20	80
30	100	0
35	100	0
36	20	80
45	20	80

Column	Phenomenex® C18 250 mm × 4.0 mm, 3.2 μm, 320 Å
Column T	25 °C
Sample T	20 °C
Injection V	10 μL
Flow	1 mL/min

Table 5.4. Chromatographic conditions: Method B.

Eluent A: 0.036% TFA in ACN
Eluent B: 0.045% TFA in H_2O

Time (min)	%A	% B
0	5	95
2	5	95
30	100	0
40	100	0
41	5	95
50	5	95

Column	Aeris Widepore C4 150 mm × 4.6 mm, 3.6 µm, 200 Å
Column T	25 °C
Sample T	20 °C
Injection V	10 µL
Flow	1 mL/min

Table 5.5. Chromatographic conditions: Method C.

Eluent A: ACN
Eluent B: 10 mM CH_3COONH_4 pH = 2.8

Time (min)	%A	% B
0	30	70
15	70	30
20	30	70
25	30	70

Column	ACE C4 150 mm × 4.6 mm, 3.0 µm, 100 Å
Column T	40 °C
Sample T	20 °C
Injection V	5 µL
Flow	1 mL/min

Table 5.6. Chromatographic conditions: Method D.

Eluent A: 0.036% TFA in ACN
Eluent B: 0.045% TFA in H_2O

Time (min)	%A	% B
0	60	40
15	100	0
18	100	0
20	60	40
30	60	40

Column	Aeris Widepore C4 150 mm × 4.6 mm, 3.6 µm, 200 Å
Column T	25 °C
Sample T	20 °C
Injection V	10 µL
Flow	1 mL/min

Table 5.7. Chromatographic conditions: Method E.

Eluent A: 0.036% TFA in ACN
Eluent B: 0.045% TFA in H_2O

Time (min)	%A	% B
0	0	100
5	32	68
11	32	68
22	74	26
27	74	26
28	0	100
32	0	100

Column	XBRIDGE C18 50 mm × 3.0 mm, 3.5 µm, 130 Å
Column T	40 °C
Sample T	20 °C
Injection V	10 µL
Flow	1 mL/min

Table 5.8. Chromatographic conditions: Method F.

Eluent A: 0.036% TFA in ACN
Eluent B: 0.045% TFA in H_2O

Time (min)	%A	% B
0	20	80
1	20	80
15	30	70
40	45	55
41	45	55
41.5	20	80
45	20	80

Column	Kinetex® EVO C18 150 mm × 3.0 mm, 2.6 µm, 100 Å
Column T	25 °C
Sample T	20 °C
Injection V	10 µL
Flow	0.4 mL/min

5.3.4. MALDI-TOF

Mass spectra were recorded on a MALDI-TOF Applied Biosystem 4700 with a N_2 laser of 337 nm using ACH matrix (10 mg/mL of ACH in ACN-H_2O-TFA (1:1:0.1 v/v)).

5.3.5. Nuclear Magnetic Resonance (NMR)

^{1}H-NMR spectra were recorded on a Varian MERCURY 400 spectrometer. 2D spectra (COSY, TOCSY, HSQC and HMBC) and ^{13}C-NMR spectra were performed on a Bruker 400 spectrometer provided with a cryoprobe. Chemical shifts (δ) are quoted in ppm and referenced according to the corresponding deuterated solvent.

5.4. Analytical Methods

5.4.1. Ninhydrin test

Ninhydrin or Kaiser Test is a colorimetric test used to qualitatively 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 to give a dark blue coloured compound known as Ruhemann's purple.

To perform the assay, three solutions were prepared:

- Reagent A: 5 g of ninhydrin in 100 mL of EtOH.
- Reagent B: 80 g of phenol in 20 mL of EtOH.
- Reagent C: 2 mL of KCN 0.001 M in 98 mL of pyridine distilled over ninhydrin.

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

5.5. Solid-Phase Peptide Synthesis (SPPS)

5.5.1. General considerations

Solid-phase synthesis was carried out manually in polypropylene syringes fitted with polyethylene filter discs or in a 150 mL glass reactor vessel fitted with a porous glass filter disc. Stirring was performed with a Teflon bar or in an orbital shaker when a syringe was used and with a mechanical stirrer coupled to a Hastelloy rod with pitched blade paddles on its end in the case of the reactor vessel. All the peptides were synthesised using the Fmoc/^{t}Bu protection strategy. Solvents and all soluble reagents were removed by vacuum suction.

5.5.2. Resin conditioning and incorporation of the first amino acid

The 2-CTC resin was used to carry out the solid-phase peptide synthesis in the present thesis.

Resin conditioning consisted of washes with DCM (1 × 30 min, 1 × 1 min), DMF (2 × 1 min) and DCM (2 × 1 min).

Incorporation of the first amino acid onto the 2-CTC resin was achieved as follows: a solution of the Fmoc-protected amino acid (1.5 eq) in DCM, with the minimal quantity of DIPEA to dissolve the amino acid was added to the resin. Then, DIPEA (3.0 eq) was added and the reaction mixture was stirred for 1.5 h. Next, 0.8 mL of MeOH/g of resin were added and the reaction was stirred for 30 min. After this time, the reaction mixture was removed by vacuum suction and the resin was washed according to the protocol reported in each chapter. After the second wash of the resin with MeOH or iPrOH, a small aliquot of resin was taken to perform the Fmoc quantification.

5.5.3. Fmoc quantification and loading determination

The loading of the first amino acid that was attached to the resin was determined using UV-Vis spectrophotometry by quantifying the dibenzofulvene adduct formed after the Fmoc removal of the amino acid. Thus, an small aliquot that was taken from the resin and was

dried in the vacuum desiccator overnight. Afterwards, around 25 mg of aminoacyl-resin were weighed in two different 100 mL volumetric flasks and then diluted to the mark with 20 % piperidine in DMF. The solutions were left in the ultra-sonic bath for 30 min and the solutions were diluted in another two 100 mL volumetric flasks and diluted to the mark with 20 % piperidine in DMF. The UV absorbance (A) at 301 nm of the resulting solutions was then measured and the concentration of dibenzofulvene adduct (c) was calculated by the Lambert-Beer's law (ε = 7800 M^{-1} cm^{-1} at 301 nm with a l=1 cm). The loading of the aliquot was determined by dividing the total number of mmol of amino acid that was incorporated into the resin (calculated from the amount of dibenzofulvene adduct) by the number of g of aminoacyl resin that was weighed:

$$A = c \cdot \varepsilon \cdot l$$

$$\text{Loading determination} = \frac{\text{mmol of amino acid}}{\text{g of aminoacyl resin}}$$

5.5.4. Determination of the total amount of the first amino acid incorporated to the resin

In order to know the total amount of the first amino acid that was incorporated into the resin without the need of drying the whole quantity of resin obtained after coupling the amino acid, the following equation was developed:

$$\text{Mmols of amino acid} = R_{Ti}F_f \times \frac{[1 + F_i(M_{OMe}-M_{HCl})/1000]}{[1 - F_f(M_{Aa}-M_{OMe})/1000]}$$

From a weighed dry aliquot of the aminoacyl resin (R_{Af}) with a loading (F_f) determined by the Fmoc quantification, the number of mmols of amino acid that has been incorporated to this aliquot can be calculated but the total number of mmols coupled to the resin remains unknown. To find that, let's consider the aliquot of 2-CTC (R_{Ai}) corresponding to R_{Af} before the incorporation of the amino acid. Before the addition of the first amino acid, this aliquot of 2-CTC has an initial loading (F_i). Thus, the mass of the aliquot after the incorporation of the first amino acid (R_{Af}) could be calculated by the following equation in which Δm is the increase on the mass due to the incorporation of the first amino acid:

(1) $$R_{Ai} + \Delta m = R_{Af}$$

The increase on the mass Δm is affected by two components. The first component is related to the quantity of amino acid incorporated (Δm_{Aa}), the other is related to the number of reactive points that have been blocked (Δm_{OMe}). Therefore, equation 1 is modified to the following one:

(2) $$R_{Ai} + \Delta m_{Aa} + \Delta m_{OMe} = R_{Af}$$

The quantity of amino acid incorporated to the aliquot of resin (Δm_{Aa}) can be calculated by multiplying the number of mmols on the final aminoacyl resin aliquot ($R_{Af}F_f$) by the difference between the molar masses of the deprotonated amino acid and HCl ($M_{Aa} - M_{HCl}$). That is to say, the deprotonated amino acid is attached to the resin and HCl is generated when this occurs. If mass is expressed in grams and the loading in mmols per gram, it must be divided by 1000 if the molar mass is expressed in grams per mol:

(3) $$\Delta m_{Aa} = (R_{Af}F_f)(M_{Aa} - M_{HCl})/1000$$

This can also be applied to the number of reactive points that have been blocked using MeOH (Δm_{OMe}) that is, the number of mmols of the initial reactive points minus the number of mmols of the coupled amino acid ($R_{Ai}F_i$ - $R_{Af}F_f$). In the capping reaction, deprotonated MeOH is incorporated into the resin while HCl is generated:

(4) $$\Delta m_{OMe} = (R_{Ai}F_i - R_{Af}F_F)\times(M_{OMe} - M_{HCl})/1000$$

If equations 3 and 4 are introduced to equation 1 we obtain the following equation:

(5) $$R_{Ai} + (R_{Af}F_f)(M_{Aa} - M_{HCl})/1000 + (R_{Ai}F_i - R_{Af}F_f)(M_{OMe} - M_{HCl})/1000 = R_{Af}$$

From this equation, it can be found that the mass of the initial aliquot of 2-CTC (R_{Ai}) is:

(6) $$R_{Ai} = R_{Af} \times \frac{[1 - F_f(M_{Aa} - M_{OMe})/1000]}{[1 + F_i(M_{OMe} - M_{HCl})/1000]}$$

The total number of mmols of amino acid that has been attached to the initial amount of resin (R_{Ti}) can be calculated using equation 7:

(7) $$\text{Mmols of amino acid} = \frac{R_{Af}F_f}{R_{Ai}} \times R_{Ti}$$

Substitution of equation 6 into 7 leads to:

(8) $$\text{Mmols of amino acid} = R_{Ti}F_f \times \frac{[1+F_i(M_{OMe} - M_{HCl})/1000]}{[1-F_f(M_{Aa} - M_{OMe})/1000]}$$

This equation allows the calculation of the total number of mmols of amino acid that has been attached to the 2-CTC resin without the need of working with the total amount of aminoacyl resin, which would require high volumes of solvents, especially when performing medium to large scale syntheses.

5.5.5. Elongation of the peptide chain on the resin

The aminoacyl resin was washed consecutively with DMF (2 × 1 min), DCM (2 × 1 min) and DMF (2 × 1 min). Then, two treatments of 10 mL/g of resin with a solution of 20 % piperidine in DMF were performed and the aminoacyl resin was washed according to the protocol reported in each chapter. The Fmoc-protected amino acid and HOBt were weighted and dissolved in a vial, and then added to the syringe or to the reactor vessel. The reaction mixture was stirred for 1 h manually or mechanically at 200 rpm when the reactor vessel was used. Then, a ninhydrin test was performed and, if a positive test was obtained, the reaction mixture was left for another hour. If the colorimetric test was still positive, the resin was washed according to the protocol reported in each chapter and re-coupling with the same amino acid was performed under similar conditions. Once the colorimetric test was negative, the Fmoc group was removed and the next amino acid was coupled following the protocol described for each amino acid.

5.5.6. Fmoc removal

The peptidyl-resin was washed consecutively with DMF (2 × 1 min), DCM (2 × 1 min) and DMF (2 × 1 min). Then, two treatments of 10 mL/g of resin with a solution of 20 % piperidine in DMF were performed. Afterwards, the peptidyl-resin was washed according to the protocol reported in each chapter.

5.5.7. Cleavage of the peptide from the resin

5.5.7.1. Methodology 1

The peptidyl-resin was washed with DMF (2 × 1 min), DCM (2 × 1 min), MeOH (2 × 1 min) and DCM (2 × 1 min). A solution of TFA/TIPS/H_2O (90:5:5, v/v) was added to the peptidyl-resin and was stirred manually for 1.5 h when the resulting solution was filtered to a conical centrifuge tube containing Et_2O and centrifuged. The supernatant was removed and the solid was dried under vacuum.

5.5.7.2. Methodology 2

The peptidyl-resin was washed with DCM (3 × 1 min) and a solution of 1 % TFA in DCM (10 mL/g of resin) was added. The reaction was left for 5 min when the resulting solution was filtered to conical centrifuge tube containing Et_2O and centrifuged. The supernatant was removed and the procedure was repeated two more times. The solid was dried under vacuum.

5.5.7.3. Methodology 3

The peptidyl-resin was washed with DCM (3 × 1 min) and a solution of 1 % TFA in DCM (10 mL/g of resin) was added. The reaction was left for 5 min and the resulting solution was filtered to a round bottom flask containing Et_2O. The procedure was repeated two more times. The resin was washed twice with DCM and the filtrates were collected in the same round bottom flask. The peptide was obtained after removing volatiles under reduced pressure.

5.5.7.4. Methodology 4

The peptidyl-resin was washed with DCM (3 × 1 min) and a solution of 1 % TFA in DCM (10 mL/g of resin) was added. The reaction was left for 5 min and the resulting solution was filtered to a round bottom flask containing 2.8 mL of Et_2O/mL of DCM in which the filtrates were collected. The procedure was repeated two more times. The resin was washed twice with DCM and the filtrates were collected in the round bottom flask. The precipitated peptide was filtered with a filter crucible (num. 3) and dried at reduced pressure

.

Chapter 1

5.6. Solid-phase peptide synthesis of 1 following a linear strategy

5.6.1. Amino acid coupling protocol

Tables 5.9, 5.10 and 5.11 summarise the protocols that were used for the on-resin amino acid coupling. The reaction mixtures were stirred manually. Coupling conversions were checked by the ninhydrin colorimetric test and re-couplings were carried out if required.

Table 5.9. Amino acid coupling protocol 1.

Step	Treatment	Conditions
1	Washes	DMF (2 × 1 min), DCM (2 × 1 min) and DMF (2 × 1 min)
2	Coupling	Fmoc-protected amino acid, additive, DIC (3:3:3) in DMF (1 h)
3	Washes	DMF (2 × 1 min), DCM (2 × 1 min), DMF (2 × 1 min) and DCM (2 × 1 min)
4	Colorimetric test	Ninhydrin test

Table 5.10. Amino acid coupling protocol 2.

Step	Treatment	Conditions
1	Washes	DMF/NMP (2 × 1 min), DCM (2 × 1 min) and DMF/NMP (2 × 1 min)
2	Coupling	Fmoc-protected amino acid, HATU, DIPEA (3:3:6) in DMF/NMP (1 h)
3	Washes	DMF/NMP (2 × 1 min), DCM (2 × 1 min), DMF/NMP (2 × 1 min) and DCM (2 × 1 min)
4	Colorimetric test	Ninhydrin test

Table 5.11. Amino acid coupling protocol 3.

Step	Treatment	Conditions
1	Washes	DMF/NMP (2 × 1 min), DCM (2 × 1 min) and DMF/NMP (2 × 1 min)
2	Coupling	Fmoc-protected amino acid, HATU, HOAt, DIPEA (3:3:3:6) in DMF/NMP (1 h)
3	Washes	DMF/NMP (2 × 1 min), DCM (2 × 1 min), DMF/ NMP (2 × 1 min) and DCM (2 × 1 min)
4	Colorimetric test	Ninhydrin test

5.6.2. Fmoc removal protocol

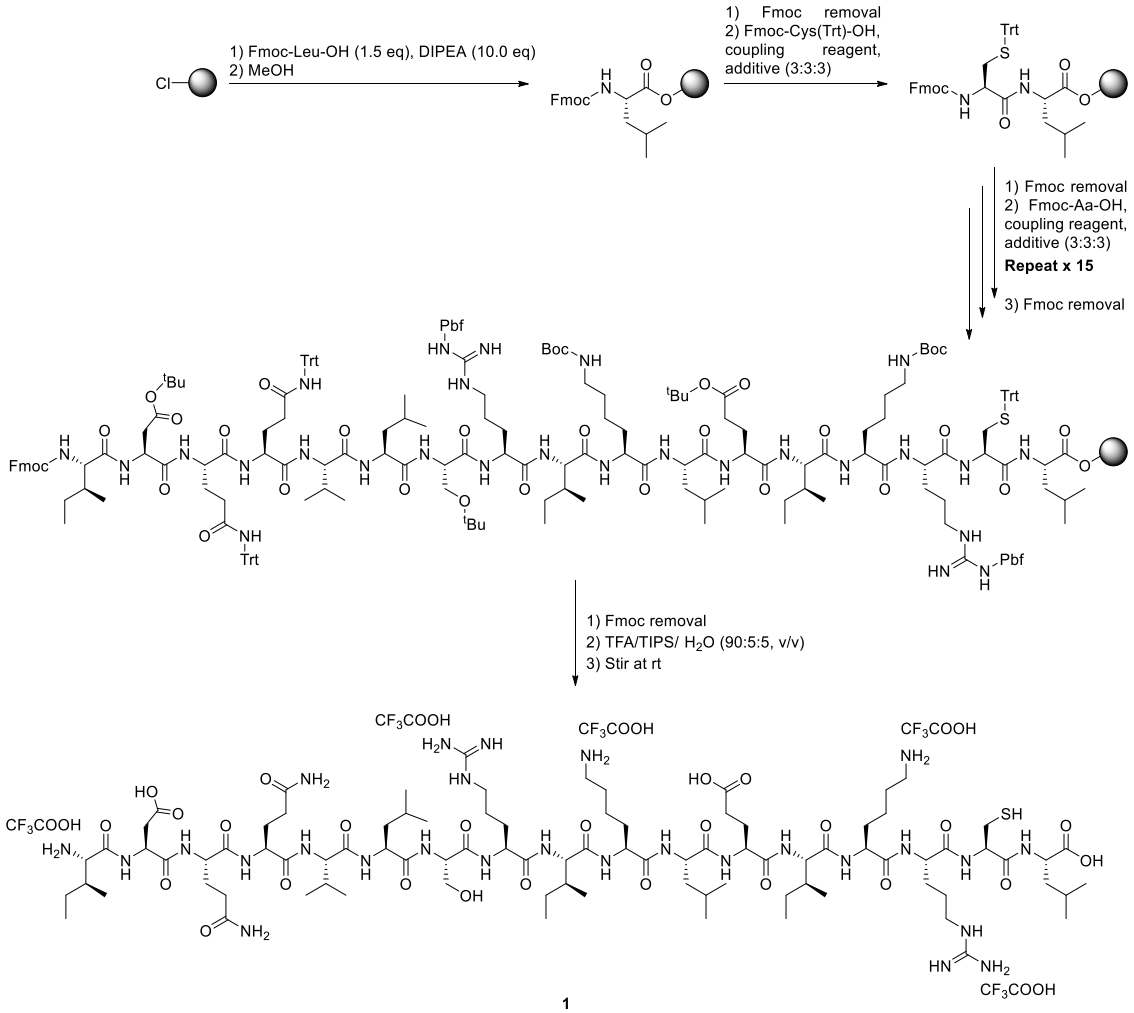
Table 5.12 summarises the protocol that was used for the N-terminal deprotection. All Fmoc removal treatments were performed at rt and the reaction mixture was shaken manually.

Table 5.12. Fmoc removal protocol 1.

Step	Treatment	Conditions
1	Washes	DMF (2 × 1 min), DCM (2 × 1 min) and DMF (2 × 1 min)
2	Fmoc removal	20 % piperidine in DMF (1 × 5 min and 1 × 10 min)
3	Washes	DMF (2 × 1 min), DCM (2 × 1 min) and DMF (2 × 1 min)

5.6.3. Synthesis of 1

5.6.3.1. Batch 1A



2-CTC resin (0.10 g, 1.1 mmol/g) was added into a polypropylene syringe fitted with a polyethylene filter disc. The resin was conditioned and the first coupling was performed using 58.3 mg (1.90 eq) of Fmoc-Leu-OH and 142.0 mg (10.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 1.5 h when 0.8 mL of MeOH/g of resin were added and stirred for 30 min. The final loading was found to be 0.29 mmol/g (0.032 mmol, section 5.5.3). For the peptide elongation, the standard protocols described in tables 5.9-5.11 (section 5.6.1) and table 5.12 (section 5.6.2) were followed. The amounts of reagents for this synthesis are given in table 5.13. For entries 1 to 3 the protocol described in table 5.9 was performed with Oxyma as additive. As a result of the elevated number of re-couplings (8 in total), the couplings described in entries 3[b] to 5 were carried out following the protocol of table 5.10.

The protocol described in table 5.11 was used for the couplings of entries 6 to 8 and 11 to 16, and DMF was substituted by NMP. Finally, entries 9[b] to 10 were performed using the protocol of table 5.9 with change in the number of eq of the reagents that were increased from 3.0 to 6.0. After the cleavage of the peptidyl-resin (methodology 1, section 5.5.7), 71.0 mg (0.027 mmol, 84 %) of crude peptide were obtained as a white solid with a 6 % of chromatographic purity (10.9 min, figure 5.1). **MS (ESI):** m/z calc. for $[M+H]^+$ of $C_{90}H_{163}N_{27}O_{25}S$ 2055.2; found 2056.0.

Table 5.13. Amounts of reagents used for the linear synthesis of **1**.

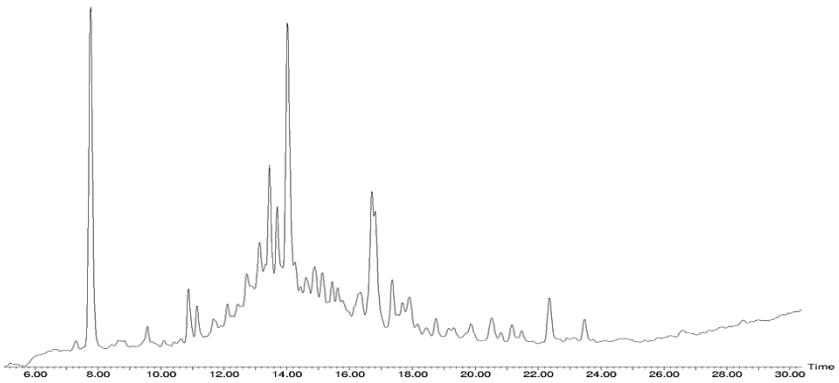
Entry	**Amino acid** [mg, eq]		**Oxyma** [mg, eq]	**DIC** [µL, eq]	**Reaction time** [h]
1	Fmoc-Cys(Trt)-OH	108, 5.8 94, 5.0	17, 3.7 16, 3.5	15, 3.0	1
2	Fmoc-Arg(Pbf)-OH	78, 3.6 79, 3.7 68, 3.2	18, 3.8 35, 7.5 28, 6.0	15, 3.0	1
3	Fmoc-Lys(Boc)-OH	70, 4.5 50, 3.2 49, 3.2	26, 5.5 26, 5.5 37, 7.9	15, 3.0	1
Entry	**Amino acid** [mg, eq]		**HATU** [mg, eq]	**DIPEA** [µL, eq]	**Reaction time** [h]
3[b]	Fmoc-Lys(Boc)-OH	59, 3.8 52, 3.4	50, 4.0 52, 4.2	34, 6.0	1
4	Fmoc-Ile-OH	84, 6.8 82, 6.7 107, 8.8 90, 7.4 95, 7.8	95, 7.2 110, 8.4 90, 6.9 84, 6.4 84, 6.4	72, 12.0	1
5	Fmoc-Glu(^{t}Bu)-OH	101, 6.6 95, 6.2	82, 6.2 80, 6.1	72, 12.0	1
Entry	**Amino acid** [mg, eq]		**HATU/HOAt** [mg, eq]	**DIPEA** [µL, eq]	**Reaction time** [h]
6	Fmoc-Leu-OH	84, 6.9 86, 7.1	81, 6.2 / 25, 5.3 78, 5.9 / 23,4.9	72, 12.0	1
7	Fmoc-Lys(Boc)-OH	100, 6.2 100, 6.2	79, 6.0 / 29, 6.2 82, 6.3 / 29, 6.2	72, 12.0	1
8	Fmoc-Ile-OH	78, 6.4 85, 6.9 80, 6.6	87, 6.6 / 29, 6.2 85, 6.5 / 28, 6.0 88, 6.7 / 29, 6.2	72, 12.0	1
Entry	**Amino acid** [mg, eq]		**HATU** [mg, eq]	**DIPEA** [µL, eq]	**Reaction time** [h]
9	Fmoc-Arg(Pbf)-OH	134, 6.0 134, 6.0	74, 5.6 77, 5.9	72, 12.0	1
Entry	**Amino acid** [mg, eq]		**HOAt** [mg, eq]	**DIC** [µL, eq]	**Reaction time** [h]
9[b]	Fmoc-Arg(Pbf)-OH	138, 6.2	32, 6.8	32, 6.0	1
10	Fmoc-Ser(^{t}Bu)-OH	80, 6.0 81, 6.1	28, 6.0 30, 6.4	32, 6.0	1
Entry	**Amino acid** [mg, eq]		**HATU/HOAt** [mg, eq]	**DIPEA** [µL, eq]	**Reaction time** [h]
11	Fmoc-Leu-OH	82, 6.7	74, 5.6 / 25, 5.3	72, 12.0	1
12	Fmoc-Val-OH	104, 8.8	111, 8.5 / 42, 8.9	108, 18.0	1
13	Fmoc-Gln(Trt)-OH	127, 6.0	74, 5.6 / 25, 5.3	72, 12.0	1
14	Fmoc-Gln(Trt)-OH	127, 6.0	75, 5.7 / 28, 6.0	72, 12.0	1
15	Fmoc-Asp(^{t}Bu)-OH	87, 6.1	77, 5.9 / 24, 5.1	72, 12.0	1

Table 5.13 (continued)

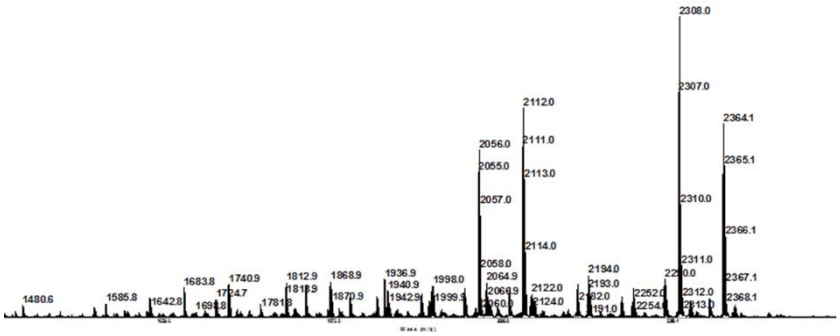
16	Fmoc-Ile-OH	78, 6.4	76, 5.8	28, 6.0	72, 12.0	1

Figure 5.1. Analysis of the acidolytic crude of 1[a]

HPLC analysis[b]



MALDI-TOF



(a) Chromatographic conditions: Method A (Section 5.3.3.3).
(b) Waters 2695, UV detection at 220 nm.

Chapter 2

5.7. Solid-phase peptide synthesis

5.7.1. Amino acid coupling protocol

Table 5.14 summarises the protocol that was used for the solid-phase synthesis of the protected fragments. The reaction mixtures were stirred manually (syringe) or mechanically (reactor vessel) at 200 rpm. Coupling conversions were checked by the ninhydrin colorimetric test and re-couplings were carried out if required.

Table 5.14. Amino acid coupling protocol.

Step	Treatment	Conditions
1	Washes	DMF/NMP (2 × 1 min), DCM (2 × 1 min) and DMF/NMP (2 × 1 min)
2	Coupling	Fmoc-protected amino acid, HOBt, DIC (3:3:3) in DMF/NMP (1 h)
3	Colorimetric test	Ninhydrin test
4	Washes	DMF/NMP (2 × 1 min), DCM (2 × 1 min) and DMF/NMP (2 × 1 min)
5	Colorimetric test	Ninhydrin test

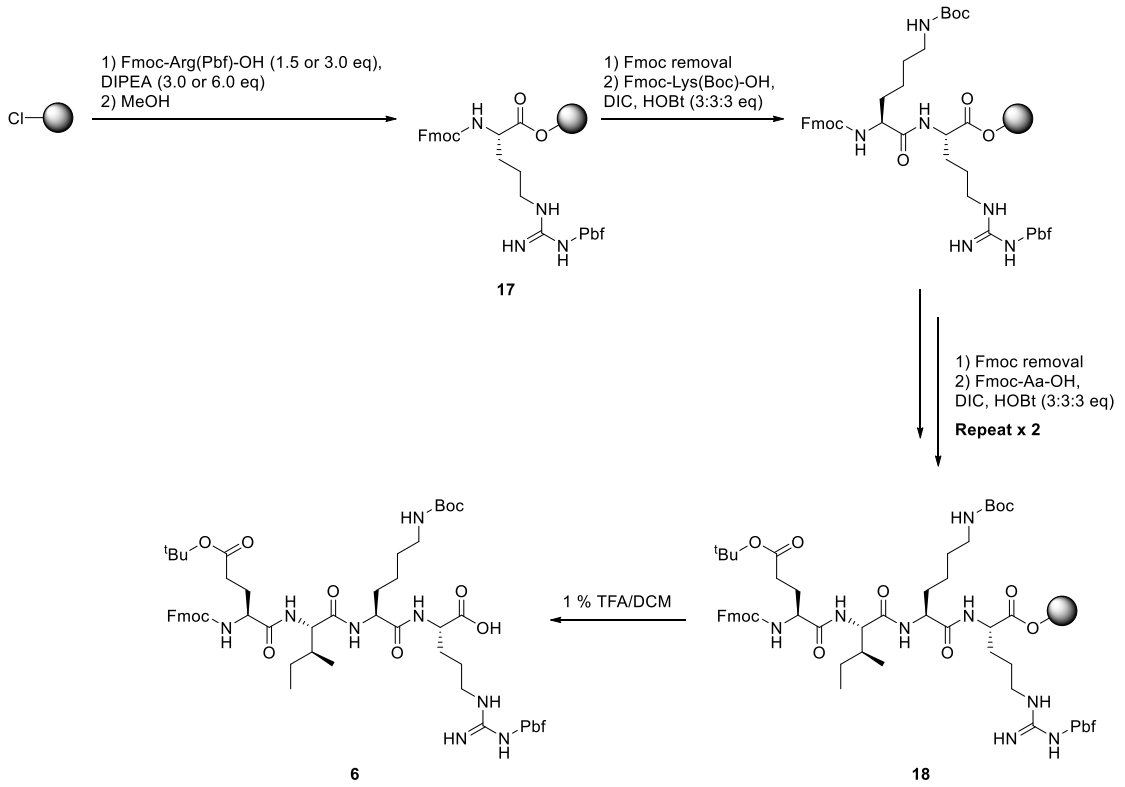
5.7.2. Fmoc removal protocol

Table 5.15 summarises the protocol that was used to remove the Fmoc N-terminal protecting group. All treatments were performed at 25 °C and the reaction mixture was stirred manually (syringe) or mechanically (reactor vessel) at 200 rpm.

Table 5.15. Fmoc removal protocol.

Step	Treatment	Conditions
1	Washes	DMF (2 × 1 min), DCM (2 × 1 min) and DMF (2 × 1 min)
2	Fmoc removal	20 % piperidine in DMF (1 × 5 min and 1 × 10 min)
3	Washes	DMF (2 × 1 min), DCM (2 × 1 min) and DMF (2 × 1 min)

5.7.3. Synthesis of 6



5.7.3.1. From peptidyl-resin 17A

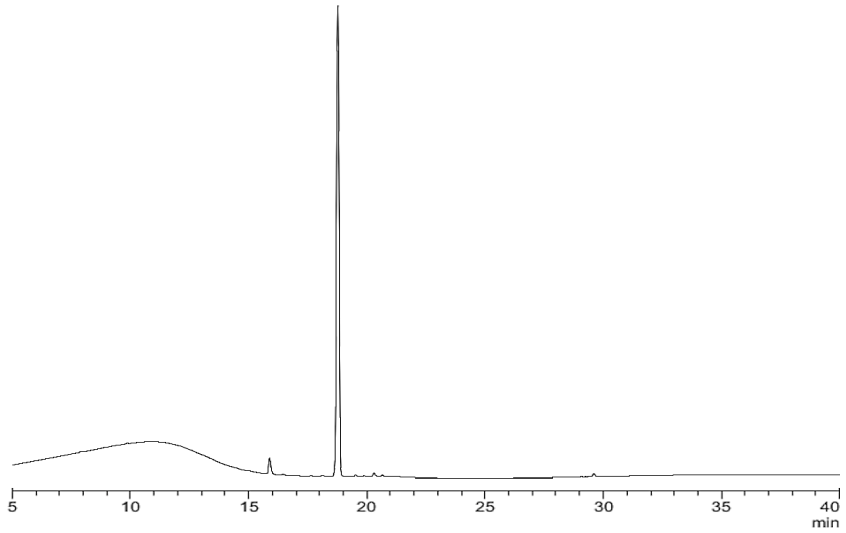
2-CTC resin (1.99 g, 1.60 mmol/g) was added into a polypropylene syringe fitted with a polyethylene filter disc. The resin was conditioned and the first coupling was performed with 3.10 g (1.5 eq) of Fmoc-Arg(Pbf)-OH and 1.23 g (3.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 2 h when 0.8 mL of MeOH/g of resin were added and the resulting reaction mixture was stirred for 30 min. The final loading was found to be 0.64 mmol/g (2.22 mmol, section 5.5.3). The standard protocols described in table 5.14 (section 5.7.1) and table 5.15 (section 5.7.2) were followed for the peptide elongation. The amounts of reagents for this synthesis are given in table 5.16. After the cleavage of the peptide from the resin (methodology 2, section 5.5.7), 2.52 g (2.14 mmol, 96 %) of crude peptide were obtained as a white solid with a 96 % of chromatographic purity (18.9 min, figure 5.2). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{60}H_{86}N_8O_{14}S^+$ 1175.6; found 1175.5.

Table 5.16. Amounts of reagents used for the synthesis of **6** (batch 17A).

Entry	**Amino acid** [g, eq]		**HOBt·H$_2$O** [g, eq]	**DIC** [mL, eq]	**Reaction time** [h]
1	Fmoc-Lys(Boc)-OH	3.22, 3.1	1.10, 3.2	1.03, 3.0	1
2	Fmoc-Ile-OH	2.35, 3.0	1.06, 3.1	1.03, 3.0	1
3	Fmoc-Glu(^{t}Bu)-OH	2.95, 3.0	1.05, 3.1	1.03, 3.0	1

Figure 5.2. HPLC chromatogram of 6[a]

Crude analysis[b]**:** Batch 17A



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Waters 2695, UV detection at 220 nm.

5.7.3.2. From peptidyl-resin 17B

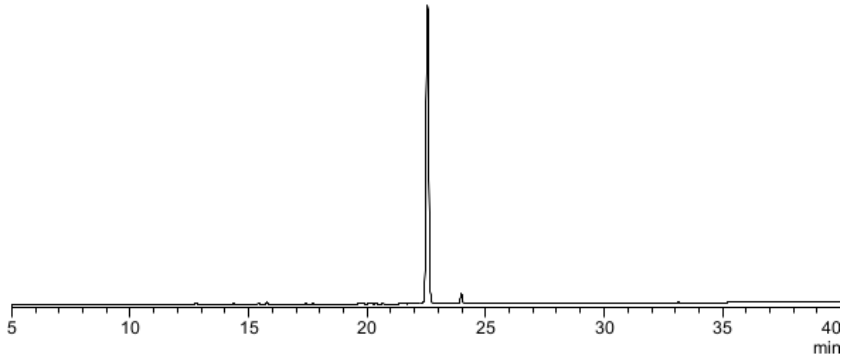
2-CTC resin (10.58 g, 0.97 mmol/g) was added into a 150 mL glass reactor vessel fitted with a porous glass filter disc. The resin was conditioned and the first coupling was performed using 20.13 g (3.0 eq) of Fmoc-Arg(Pbf)-OH and 8.16 g (6.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 1.5 h when 0.8 mL of MeOH/g of resin were added and the resulting reaction mixture was stirred for 1 h. The final loading was found to be 0.42 mmol/g (6.13 mmol, sections 5.5.3 and 5.5.4). For the peptide elongation, the standard protocols described in table 5.14 (section 5.7.1) and table 5.15 (section 5.7.2) were followed. The amounts of reagents for this synthesis are given in table 5.17. A final mass increment of 1.40 g (1.19 mmol, 19 %) was determined drying the resin under vacuum. The expected mass increment of the resin was of 7.21 g (6.13 mmol). After the acidolytic treatment of 3.92 g the peptidyl-resin (methodology 3, section 5.5.7), 0.96 g (0.82 mmol) of crude peptide were obtained as a white solid with a 98 % of chromatographic purity (22.5 min, figure 5.3). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{60}H_{86}N_8O_{14}S$ 1175.6; found 1175.5.

Table 5.17. Amounts of reagents used for the synthesis of **6** (batch 17B).

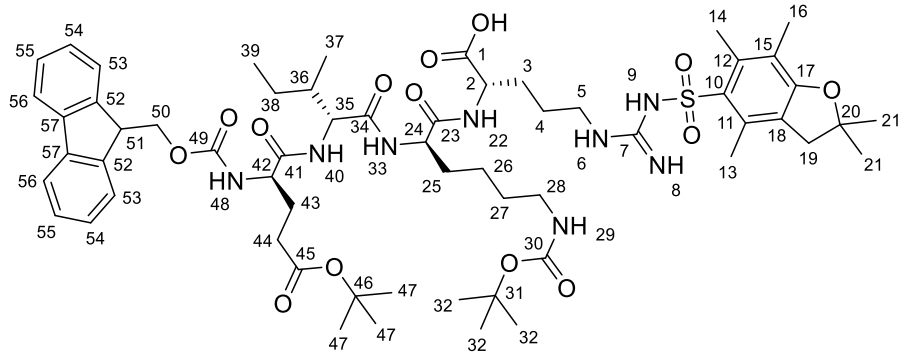
Entry	**Amino acid** [g, eq]		**HOBt·H$_2$O** [g, eq]	**DIC** [mL, eq]	**Reaction time** [h]
1	Fmoc-Lys(Boc)-OH	8.79, 3.1	2.82, 3.0	2.85, 3.0	2
2	Fmoc-Ile-OH	6.54, 3.0	3.06, 3.3	2.85, 3.0	2
3	Fmoc-Glu(^{t}Bu)-OH	8.51, 3.1	2.98, 3.2	2.85, 3.0	2

Figure 5.3. HPLC chromatogram of 6[a]

Crude analysis[b]**:** Batch 17B



NMR characterisation[c]



^{1}H NMR ($(CD_3)_2SO$, 400 MHz): δ 7.99 (m, 2H, H_{22}, H_{33}), 7.88 (d, *J* = 7.51 Hz, 2H, H_{56}), 7.70 (m, 3H, H_{40}, H_{53}), 7.54 (d, *J* = 8.39 Hz, 1H, H_{48}), 7.41 (td, *J* = 7.53, 1.11 Hz, 2H, H_{55}), 7.31 (td, *J* = 7.45, 1.19 Hz, 2H, H_{54}), 6.71 (t, *J* = 5.72 Hz, 1H, H_{29}), 4.25 (m, 2H, H_{50}), 4.23 (m, 1H, H_{24}), 4.20 (m, 2H, H_{35}, H_{51}), 4.12 (m, 1H, H_2), 4.06 (m, 1H, H_{42}), 3.02 (q, *J* = 6.33 Hz, 2H, H_5), 2.95 (s, 2H, H_{19}), 2.86 (q, *J* = 6.72 Hz, 2H, H_{28}), 2.47 (s, 3H, H_{14}), 2.42 (s, 3H, H_{13}), 2.21 (m, 2H, H_{44}), 1.99 (s, 3H, H_{16}), 1.90 – 1.46 (m, 7H, H_3 or H_4, H_{25} or H_{26}, H_{36}, H_{43}), 1.45 – 0.96 (m, 32H, H_3 or H_4, H_{21}, H_{25} or H_{26}, H_{27}, H_{32}, H_{47}, H_{38}), 0.79 (m, 3H, H_{37}), 0.76 (m, 3H, H_{39}). **^{13}C NMR** ($(CD_3)_2SO$, 100 MHz): δ 173.3 (C_1), 171.7 (C_{45}), 171.4 (C_{23}), 171.1 (C_{41}), 170.7 (C_{34}), 157.4 (C_{17}), 156.1 (C_7), 155.9 (C_{49}), 155.5 (C_{30}), 143.7 (C_{52}), 140.7 (C_{57}), 137.2 (C_{15}), 134.2 (C_{10}), 131.4 (C_{11}), 127.6 (C_{55}), 127.1 (C_{54}), 125.3 (C_{53}), 124.3 (C_{18}), 120.1 (C_{56}), 116.2 (C_{12}), 86.3 (C_{20}), 79.6 (C_{31} or C_{46}), 77.3 (C_{31} or C_{46}), 65.7 (C_{50}), 56.6 (C_{35}), 53.8 (C_{42}), 52.3 (C_{24}), 51.6 (C_2), 46.6 (C_{51}), 42.5 (C_{19}), 39.8 (C_5, C_{28}), 36.7 (C_{36}), 31.7 (C_{25} or C_{26}), 31.5 (C_{44}), 29.3 (C_{27}), 28.5 (C_3 or C_4), 28.3 (C_{21}, C_{32} or C_{47}), 27.7 (C_{32} or C_{42}), 27.4 (C_{43}), 25.4 (C_3 or C_4), 24.2 (C_{38}), 22.6 (C_{25} or C_{26}), 18.9 (C_{13}), 17.6 (C_{14}), 15.2 (C_{37}), 12.2 (C_{16}), 11.0 (C_{39})

(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.
(c) Bruker 400 spectrometer.

5.7.3.3. From peptidyl-resin 17C

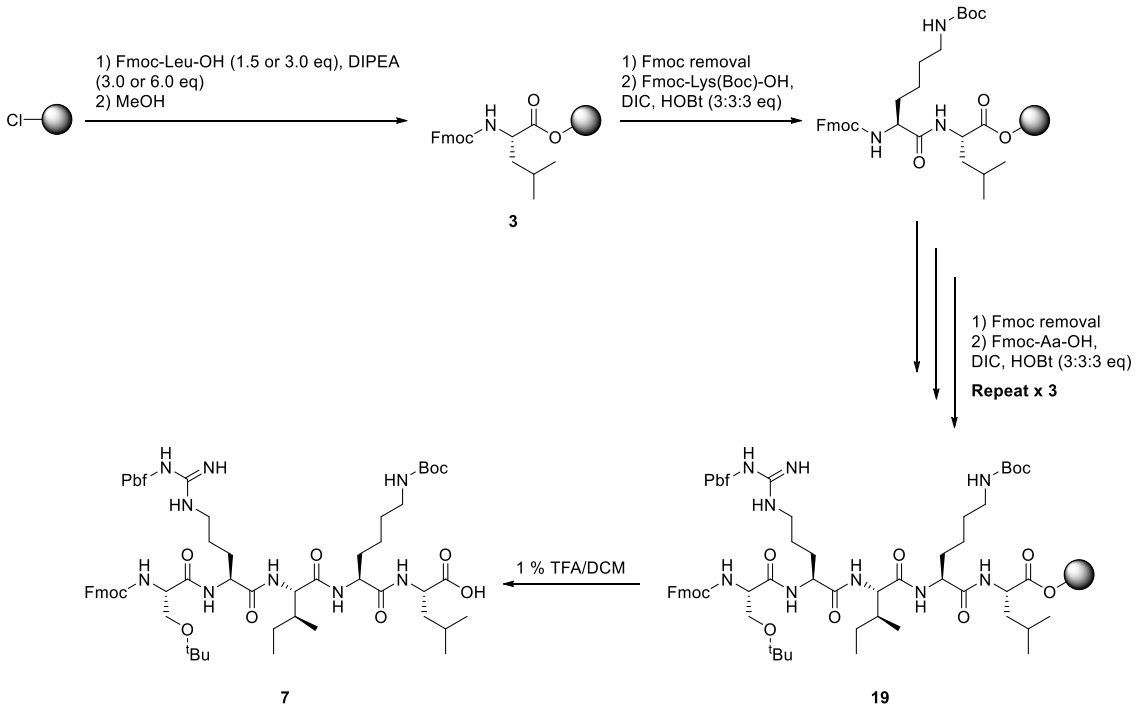
2-CTC resin (11.1 g, 1.60 mmol/g) was added into a 150 mL glass reactor vessel fitted with a porous glass filter disc. The resin was conditioned and the first coupling was performed using 34.3 g (3.0 eq) of Fmoc-Arg(Pbf)-OH and 13.7 g (6.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 1.5 h when 0.8 mL of MeOH/g of resin were added and the resulting reaction mixture was stirred for 1 h. The final loading was found to be 0.57 mmol/g (10.1 mmol, sections 5.5.3 and 5.5.4). For the peptide elongation, the standard protocols described in table 5.14 (section 5.7.1) and table 5.15 (section 5.7.2) were followed. The amounts of reagents for this synthesis are given in table 5.18. The resin was dried under

vacuum and weighed after the addition of the last amino acid. There was no mass increment of the resin.

Table 5.18. Amounts of reagents used for the synthesis of **6** (batch 17C).

Entry	Amino acid [g, eq]		HOBt·H_2O [g, eq]	DIC [mL, eq]	Reaction time [h]
1	Fmoc-Lys(Boc)-OH	16.9, 3.6	5.73, 3.7	4.71, 3.0	2
2	Fmoc-Ile-OH	9.44, 2.6	5.34, 3.4	4.71, 3.0	2
3	Fmoc-Glu(^{t}Bu)-OH	14.6, 3.3	6.43, 4.1	4.71, 3.0	2

5.7.4. Synthesis of 7



5.7.4.1. From peptidyl-resin 3A

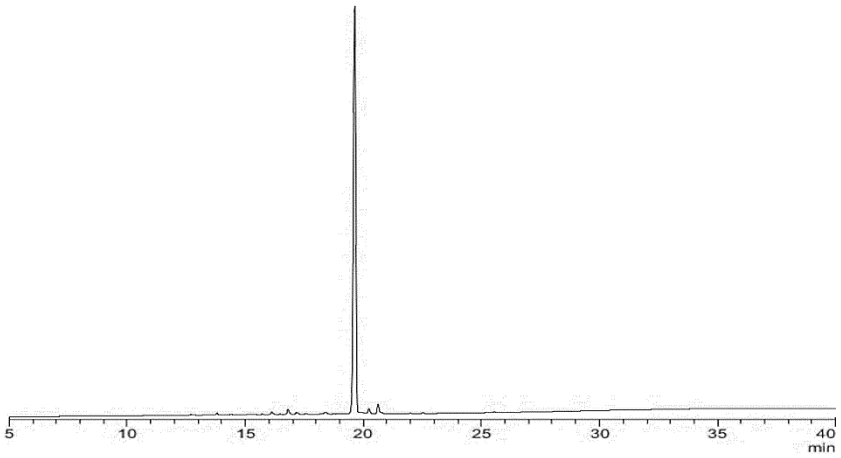
2-CTC resin (1.05 g, 1.60 mmol/g) was added into a polypropylene syringe fitted with a polyethylene filter disc. The resin was conditioned and the first coupling was performed with 0.89 g (1.5 eq) of Fmoc-Leu-OH and 0.65 g (3.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 2 h when 0.8 mL of MeOH/g of resin were added and the resulting reaction mixture was stirred for 30 min. The final loading was found to be 0.79 mmol/g (1.14 mmol, section 5.5.3). The standard protocols described in table 5.14 (section 5.7.1) and table 5.15 (section 5.7.2) were followed for the peptide elongation. The amounts of reagents for this synthesis are given in table 5.19. A final mass increment of 1.43 g (1.14 mmol, 100 %) was determined after drying the resin under vacuum. The expected mass increment on the resin was of 1.43 g (1.14 mmol). After the cleavage of the peptide from the resin (methodology 2, section 5.5.7), 1.45 g (1.16 mmol, quantitative) of crude peptide were obtained as a white solid with a 94 % of chromatographic purity (19.5 min, figure 5.4). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{64}H_{95}N_9O_{14}S$ 1246.7; found 1246.7.

Table 5.19. Amounts of reagents used for the synthesis of **7** (batch 3A).

Entry	Amino acid [g, eq]		HOBt·H_2O [g, eq]	DIC [mL, eq]	Reaction time [h]
1	Fmoc-Lys(Boc)-OH	1.62, 3.0 1.63, 3.0	0.53, 3.0 0.53, 3.0	0.53, 3.0	1 1
2	Fmoc-Ile-OH	1.24, 3.0	0.53, 3.0	0.53, 3.0	1
3	Fmoc-Arg(Pbf)-OH	2.27, 3.1 2.19, 2.9	0.54, 3.1 0.51, 2.9	0.53, 3.0	1 1
4	Fmoc-Ser(^{t}Bu)-OH	1.36, 3.1	0.52, 2.9	0.53, 3.0	1

Figure 5.4. HPLC chromatogram of 7[a]

Crude analysis[b]**:** Batch 3A



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Waters 2695, UV detection at 220 nm.

5.7.4.2. From peptidyl-resin 3B

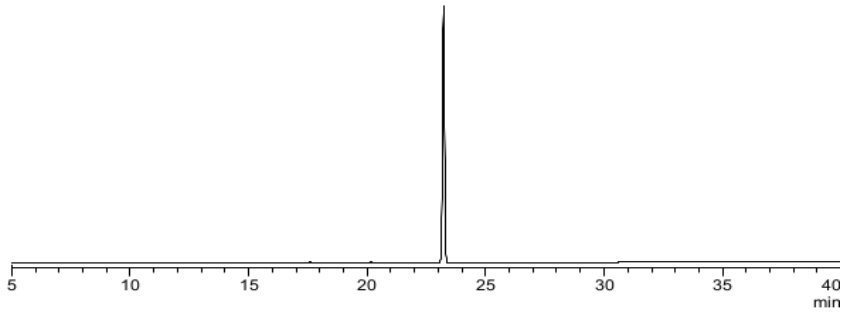
2-CTC resin (10.3 g, 0.97 mmol/g) was added into a 150 mL glass reactor vessel fitted with a porous glass filter disc. The resin was conditioned and the first coupling was performed using 10.7 g (3.0 eq) of Fmoc-Leu-OH and 7.42 g (6.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 1.5 h when 0.8 mL of MeOH/g of resin were added and the resulting reaction mixture was stirred for 1 h. The final loading was found to be 0.83 mmol/g (11.6 mmol, sections 5.5.3 and 5.5.4). For the peptide elongation, the standard protocols described in table 5.14 (section 5.7.1) and table 5.15 (section 5.7.2) were followed. The amounts of reagents for this synthesis are given in table 5.20. A final mass increment of 6.60 g (5.30 mmol, 45 %) was determined drying the resin under vacuum. The expected mass increment on the resin was of 14.5 g (11.6 mmol). After the cleavage of the peptide from the resin (methodology 3, section 5.5.7), 6.70 g (5.37 mmol, 46 %) of crude peptide were obtained as a white solid with a 98 % of chromatographic purity (23.2 min, figure 5.5). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{64}H_{95}N_9O_{14}S$ 1246.7; found 1246.5.

Table 5.20. Amounts of reagents used for the synthesis of **7** (batch 3B).

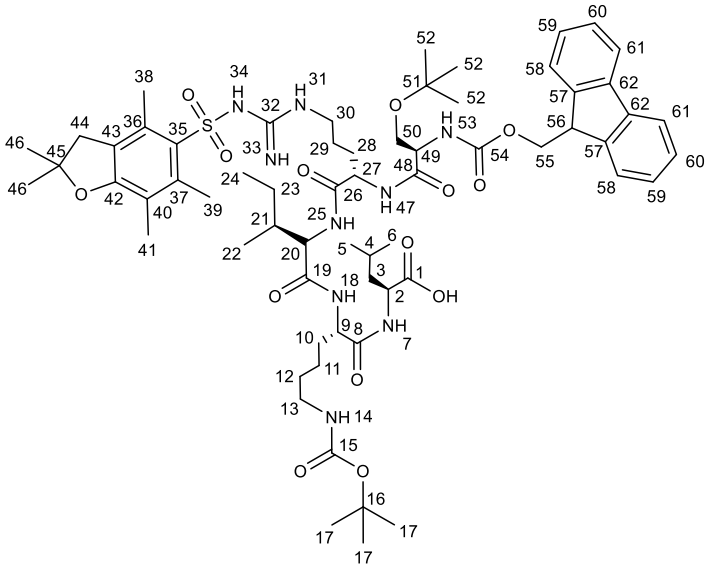
Entry	**Amino acid** [g, eq]		**HOBt·H_2O** [g, eq]	**DIC** [mL, eq]	**Reaction time** [h]
1	Fmoc-Lys(Boc)-OH	15.5, 2.8	5.38, 3.0	5.40, 3.0	1
2	Fmoc-Ile-OH	12.3, 3.0	5.55, 3.1	5.40, 3.0	1
3	Fmoc-Arg(Pbf)-OH	22.0, 2.9	4.21, 2.4	5.40, 3.0	2
4	Fmoc-Ser(^{t}Bu)-OH	13.1, 2.9	4.44, 2.5	5.40, 3.0	1

Figure 5.5. HPLC chromatogram of 7[a]

Crude analysis[b]**:** Batch 3B



NMR characterisation[c]



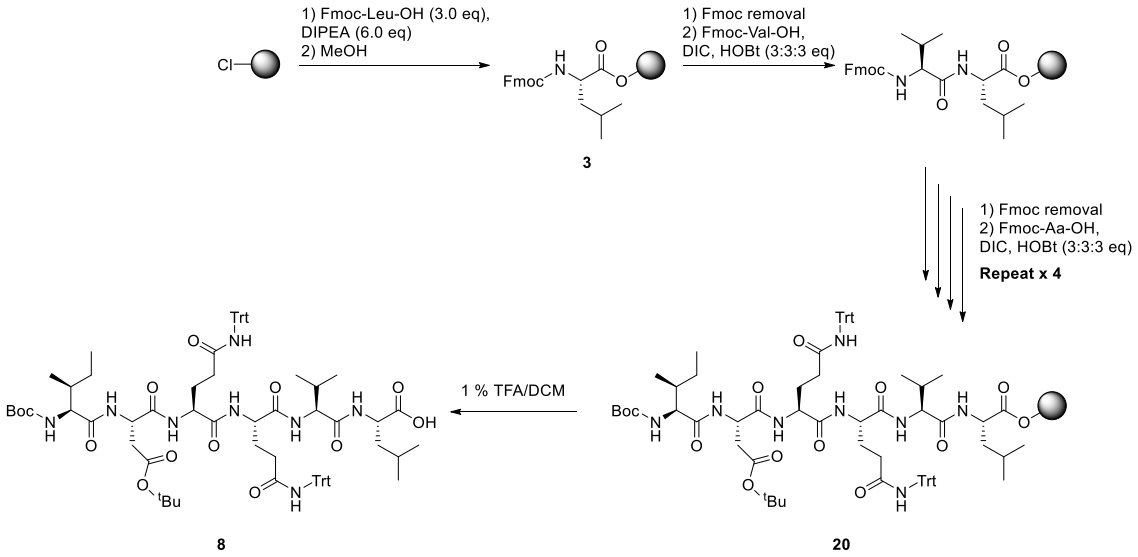
^{1}H NMR ($(CD_3)_2SO$, 400 MHz): δ 8.05 (d, *J* = 7.97 Hz, 1H, H_7), 7.96 (d, *J* = 8.01 Hz, 1H, H_{47}), 7.92 – 7.83 (m, 3H, H_{18}, H_{61}), 7.79 (d, *J* = 8.69 Hz, 1H, H_{25}), 7.69 (dd, *J* = 7.38, 3.18 Hz, 1H, H_{58}), 7.40 (t, *J* = 7.39 Hz, 2H, H_{60}), 7.36 – 7.29 (m, 3H, H_{53}, H_{59}), 6.68 (t, *J* = 5.59 Hz, 1H, H_{14}), 4.32 (m, 1H, H_{27}), 4.26 (m, 2H, H_{55}), 4.22 (m, 1H, H_9), 4.21 (m, 1H, H_{56}), 4.20 (m, 1H, H_2), 4.16 (m, 1H, H_{20}), 4.10 (m, 1H, H_{49}), 3.44 (m, 2H, H_{50}), 3.00 (d, *J* = 6.34 Hz, 2H, H_{30}), 2.91 (s, 2H, H_{44}), 2.84 (q, *J* = 6.69 Hz, 2H, H_{13}), 2.45 (s, 3H, H_{39}), 2.39 (s, 3H, H_{38}), 1.97 (s, 3H, H_{41}), 1.69-1.39 (m, 8H, H_3, H_4, H_{10} or H_{11}, H_{21}, H_{28} or H_{29}), 1.40 – 0.99 (m, 32H, H_{10} or H_{11}, H_{12}, H_{17}, H_{23}, H_{28} or H_{29}, H_{46}, H_{52}), 0.84 (d, *J* = 6.57 Hz, 3H, H_5 or H_6), 0.78 (m, 3H, H_5 or H_6), 0.77 (m, 3H, H_{22}), 0.76 (m, 3H, H_{24}). **^{13}C NMR** ($(CD_3)_2SO$, 100 MHz): δ 173.9 (C_1), 171.5 (C_8), 171.0 (C_{26}), 170.5 (C_{19}), 169.8 (C_{48}), 157.4 (C_{42}), 156.2 (C_{32}, C_{54}), 155.6 (C_{15}), 143.7 (C_{57}), 140.6 (C_{62}), 137.3 (C_{40}), 134.0 (C_{35}), 131.5 (C_{36}), 127.7 (C_{60}), 127.1 (C_{59}),125.2 (C_{58}), 124.4 (C_{43}), 120.1 (C_{61}), 116.3 (C_{37}), 86.3 (C_{45}), 77.4 (C_{16}), 72.9 (C_{51}), 65.8 (C_{55}), 61.8 (C_{50}), 56.7 (C_{20}), 55.4 (C_{49}), 52.2 (C_9), 52.0 (C_{27}), 50.0 (C_2), 46.6 (C_{56}), 42.4 (C_{44}), 40.0 (C_{30}), 39.8 (C_3), 39.7 (C_{13}), 36.7 (C_{21}), 31.6 (C_{10} or C_{11}), 29.5 (C_{28} or C_{29}), 29.2 (C_{12}), 28.2 (C_{17}, C_{46}),

Figure 5.5 (continued)

27.1 (C_{52}), 25.2 (C_{28} or C_{29}), 24.1 (C_4, C_{23}), 22.8 (C_5 or C_6), 22.4 (C_{10} or C_{11}), 21.10 (C_5 or C_6), 18.9 (C_{38}), 17.6 (C_{39}), 15.1 (C_{22}), 12.2 (C_{41}), 11.0 (C_{24})

(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.
(c) Bruker 400 spectrometer.

5.7.5. Synthesis of 8



5.7.5.1. From peptidyl-resin 3C

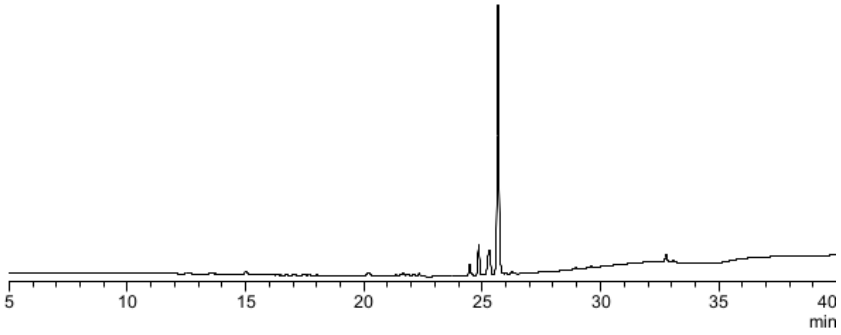
2-CTC resin (10.1 g, 0.97 mmol/g) was added into a 150 mL glass reactor vessel fitted with a porous glass filter disc. The resin was conditioned and the first coupling was performed with 10.8 g (3.1 eq) of Fmoc-Leu-OH and 7.59 g (6.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 1.5 h when 0.8 mL of MeOH/g of resin were added and the resulting reaction mixture was stirred for 1 h. The final loading was found to be 0.79 mmol/g (11.1 mmol, sections 5.5.3 and 5.5.4). For the peptide elongation, the standard protocols described in table 5.14 (section 5.7.1) and table 5.15 (section 5.7.2) were followed. The amounts of reagents for this synthesis are given in table 5.21. A final mass increment of 2.14 g (1.58 mmol) was determined after drying the resin under vacuum. The expected mass increment on the resin was of 15.0 g (11.1 mmol). After the acidolytic treatment of 4.23 g the peptidyl-resin (methodology 3, section 5.5.7), 0.74 g (0.55 mmol) of crude peptide were obtained as a white solid with a 73% of chromatographic purity (25.7 min, figure 5.6). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{78}H_{98}N_8O_{13}^+$ 1355.7; found 1356.7.

Table 5.21. Amounts of reagents used for the synthesis of **8** (Batch 3C).

Entry	Amino acid [g, eq]		HOBt·H_2O [g, eq]	DIC [mL, eq]	Reaction time [h]
1	Fmoc-Val-OH	11.2, 3.0	5.15, 3.0	5.00, 3.0	1
2	Fmoc-Gln(Trt)-OH	20.0, 2.9	5.13, 3.0	5.00, 3.0	1
3	Fmoc-Gln(Trt)-OH	21.4, 3.2	4.89, 2.8	5.00, 3.0	2
4	Fmoc-Asp(^{t}Bu)-OH	14.0, 3.1	4.95, 2.8	5.00, 3.0	1
5	Boc-Ile-OH	7.89, 3.1	5.32 , 3.1	5.00, 3.0	2

Figure 5.6. HPLC chromatogram of 8[a]

Crude analysis[b]**:** Batch 3C



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.7.5.2. From peptidyl-resin 3D

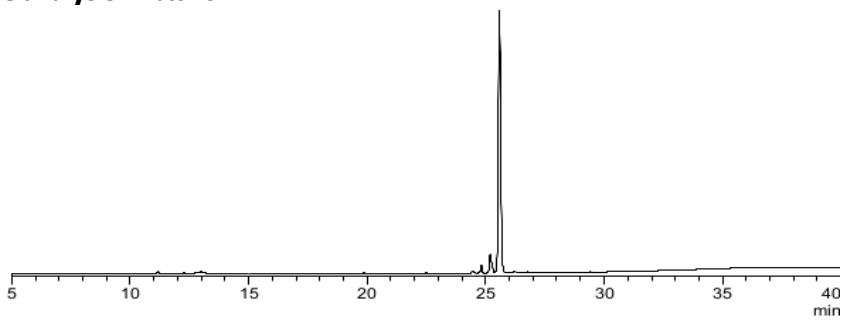
2-CTC resin (10.2 g, 1.10 mmol/g) was added into a 150 mL glass reactor vessel fitted with a porous glass filter disc. The resin was conditioned and the first coupling was performed with 11.9 g (3.1 eq) of Fmoc-Leu-OH and 8.66 g (6.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 1.5 h when 0.8 mL of MeOH/g of resin were added and the resulting reaction mixture was stirred for 1 h. The final loading was found to be 0.56 mmol/g (7.18 mmol, sections 5.5.3 and 5.5.4). The standard protocols described in table 5.14 (section 5.7.1) and table 5.15 (section 5.7.2) were followed for the peptide elongation. The amounts of reagents for this synthesis are given in table 5.22. A final mass increment of 9.68 g (7.14 mmol, 99 %) was determined after drying the resin under vacuum. The expected mass increment on the resin was of 9.74 g (7.18 mmol). After the acidolytic treatment of 0.13 g the peptidyl-resin (methodology 3, section 5.5.7), 64.5 mg of crude peptide were obtained as a white solid with a 91 % of chromatographic purity (25.6 min, figure 5.7). **MS (ESI)**: m/z calc. for $^+$ $[M+H]^+$ of $C_{78}H_{98}N_8O_{13}^+$ 1355.7; found 1356.8.

Table 5.22. Amounts of reagents used for the synthesis of **8** (batch 3D).

Entry	Amino acid [g, eq]		HOBt·H_2O [g, eq]	DIC [mL, eq]	Reaction time [h]
1	Fmoc-Val-OH	7.37, 3.0	3.77, 3.4	3.33, 3.0	2
2	Fmoc-Gln(Trt)-OH	14.4, 3.3	3.77, 3.4	3.33, 3.0	2
3	Fmoc-Gln(Trt)-OH	13.9, 3.2 7.68, 1.7	3.44, 3.1 1.60, 1.5	3.33, 3.0 1.66, 1.5	2 2
4	Fmoc-Asp(^{t}Bu)-OH	8.98, 3.0	3.53, 3.2	3.33, 3.0	2
5	Boc-Ile-OH	5.31, 3.2	3.47, 3.1	3.33, 3.0	2

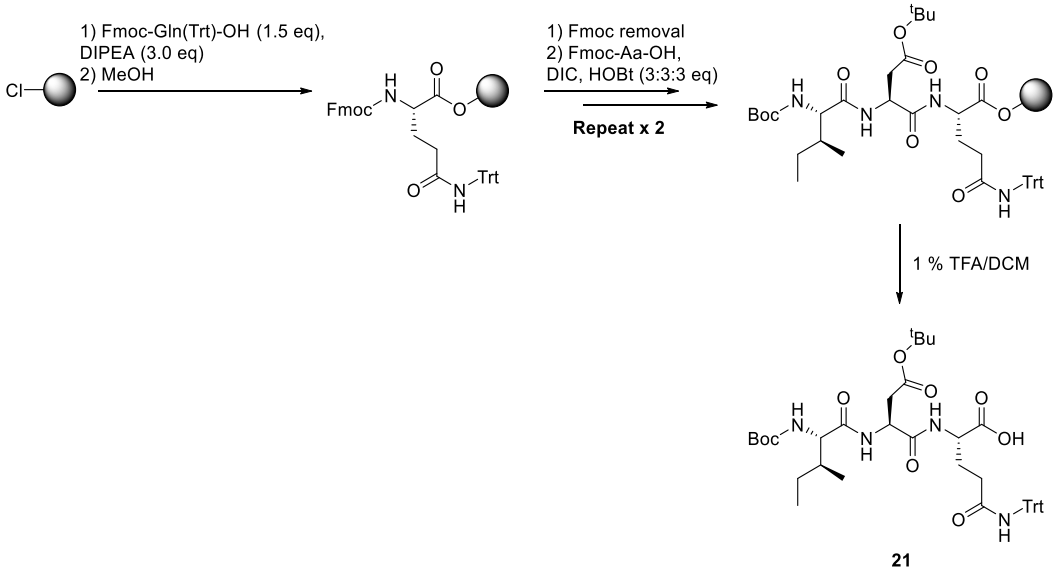
Figure 5.7. HPLC chromatogram of 8[a]

Crude analysis[b]**:** Batch 3D



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.7.5.3. Synthesis of the tripeptide 21



2-CTC resin (0.211 g, 1.10 mmol/g) was added into a polypropylene syringe fitted with a polyethylene filter disc. The resin was conditioned and the first coupling was performed with 216.0 mg (1.5 eq) of Fmoc-Gln(Trt)-OH and 0.115 mL (3.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 2 h when 0.8 mL of MeOH/g of resin were added and the resulting reaction mixture was stirred for 30 min. The final loading was found to be 0.34 mmol/g (0.07 mmol, section 5.5.3). For the peptide elongation, the standard protocols

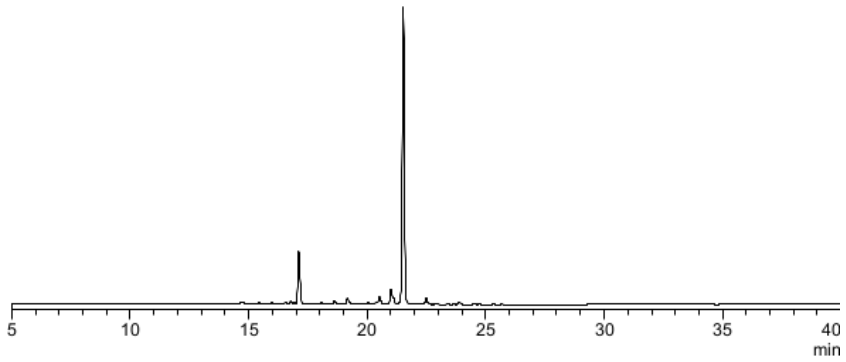
described in table 5.14 (section 5.7.1) and table 5.15 (section 5.7.2) were followed. The amounts of reagents for this synthesis are given in table 5.23. After the acidolytic treatment of the peptidyl-resin (methodology 2, section 5.5.7), 0.045 g (0.058 mmol) of crude peptide were obtained as a white solid with a 75% of chromatographic purity (21.5 min, figure 5.8). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{43}H_{56}N_4O_9^+$ 772.9; found 773.5.

Table 5.23. Amounts of reagents used for the synthesis of **21**.

Entry	**Amino acid** [mg, eq]		**HOBt·H$_2$O** [mg, eq]	**DIC** [mL, eq]	**Reaction time** [h]
1	Fmoc-Asp(^{t}Bu)-OH	90.6, 3.1	36.5, 3.3	0.033, 3.0	1
2	Boc-Ile-OH	59.5, 3.6	35.6, 3.2	0.033, 3.0	1
		53.4, 3.2	33.0, 3.0	0.033, 3.0	1

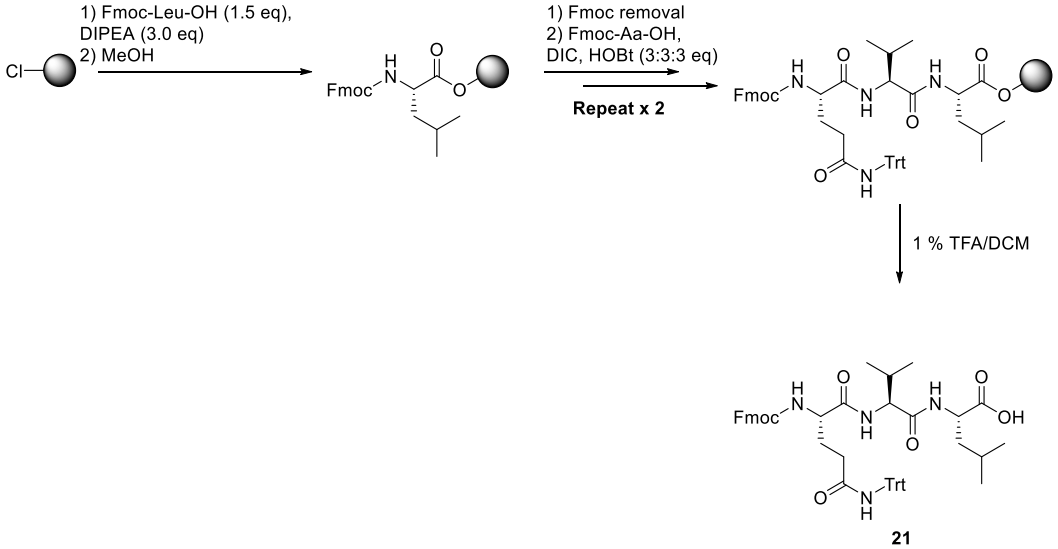
Figure 5.8. HPLC chromatogram of 21[a]

Crude analysis[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.7.5.4. Synthesis of the tripeptide 22



2-CTC resin (0.207 g, 1.10 mmol/g) was added into a polypropylene syringe fitted with a polyethylene filter disc. The resin was conditioned and the first coupling was performed with

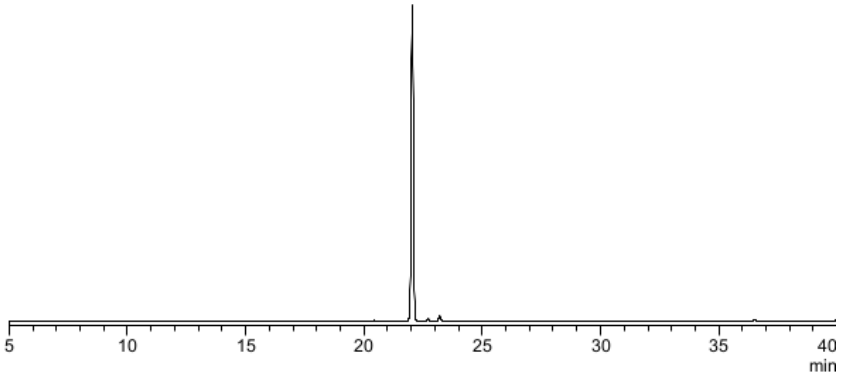
118.1 mg (1.5 eq) of Fmoc-Leu-OH and 0.115 mL (3.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 2 h when 0.8 mL of MeOH/g of resin were added and the resulting reaction mixture was stirred for 30 min. The final loading was found to be 0.42 mmol/g (0.09 mmol, section 5.5.3). For the peptide elongation, the standard protocols described in table 5.14 (section 5.7.1) and table 5.15 (section 5.7.2) were followed. The amounts of reagents for this synthesis are given in table 5.24. After the acidolytic treatment of the peptidyl-resin (methodology 2, section 5.5.7), 0.083 g (0.10 mmol) of crude peptide were obtained as a white solid with a 97% of chromatographic purity (22.0 min, figure 5.9). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{50}H_{54}N_4O_7^+$ 823.4; found 823.5.

Table 5.24. Amounts of reagents used for the synthesis of **22**.

Entry	**Amino acid** [mg, eq]		**HOBt·H₂O** [mg, eq]	**DIC** [mL, eq]	**Reaction time** [h]
1	Fmoc-Val-OH	100.0, 3.1	50.7, 3.5	0.044, 3.0	1
2	Fmoc-Gln(Trt)-OH	177.3, 3.0	54.9, 3.8	0.044, 3.0	1

Figure 5.9. HPLC chromatogram of 22[a]

Crude analysis[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.7.5.5. Synthesis of 8 using a new batch of Boc-Ile-OH

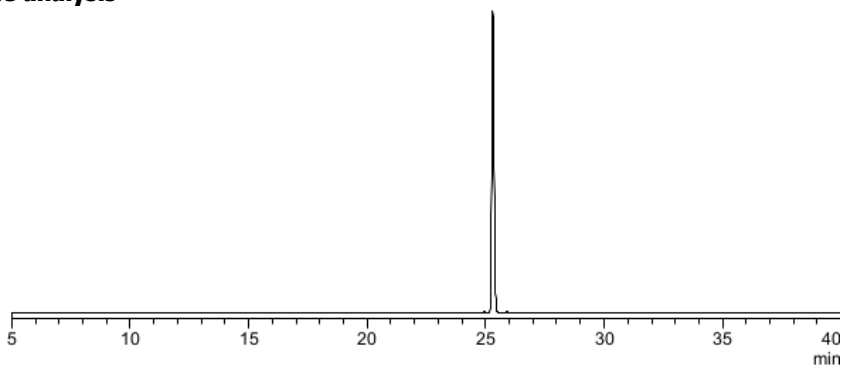
2-CTC resin (0.521 g, 1.10 mmol/g) was added into a polypropylene syringe fitted with a polyethylene filter disc. The resin was conditioned and the first coupling was performed with 311.3 mg (1.5 eq) of Fmoc-Leu-OH and 0.3 mL (3.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 2 h when 0.8 mL of MeOH/g of resin were added and the resulting reaction mixture was stirred for 30 min. The final loading was found to be 0.60 mmol/g (0.32 mmol, section 5.5.3). For the peptide elongation, the standard protocols described in table 5.14 (section 5.7.1) and table 5.15 (section 5.7.2) were followed. The amounts of reagents for this synthesis are given in table 5.25. After the acidolytic treatment of the peptidyl-resin (methodology 2, section 5.5.7), 0.389 g (0.29 mmol, 91 %) of crude peptide were obtained as a white solid with a 98% of chromatographic purity (25.3 min, figure 5.10). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{78}H_{98}N_8O_{13}^+$ 1355.7; found 1356.7.

Table 5.25. Amounts of reagents used for the synthesis of **8** using a new batch of Boc-Ile.

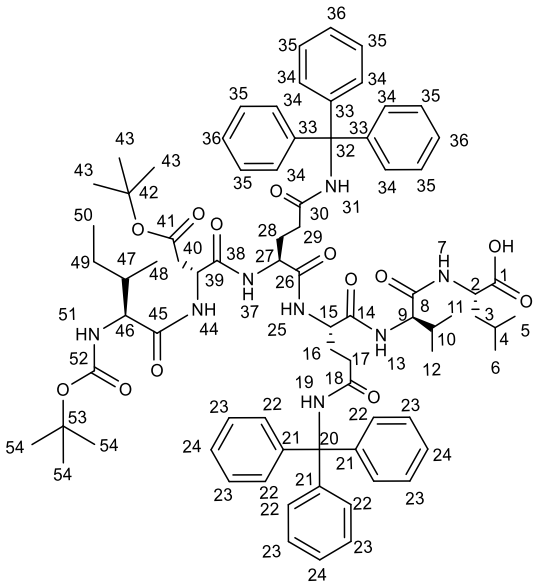
Entry	**Amino acid** [mg, eq]		**HOBt·H_2O** [mg, eq]	**DIC** [mL, eq]	**Reaction time** [h]
1	Fmoc-Val-OH	344.5, 3.2 324.0,3.0	157.3, 3.3 160.1, 3.3	0.146, 3.0	1
2	Fmoc-Gln(Trt)-OH	580.9, 3.0 599.2, 3.1 587.1, 3.1 605.0, 3.1	146.3, 3.0 179.8, 3.7 147.1, 3.0 160.2, 3.3	0.146, 3.0	1
3	Fmoc-Gln(Trt)-OH	602.3, 3.1	165.4, 3.4	0.146, 3.0	1
4	Fmoc-Asp(^{t}Bu)-OH	400.6, 3.1	154.3, 3.2	0.146, 3.0	1
5	Boc-Ile-OH	247.5, 3.4 230.4, 3.2	160.4, 3.3 168.1, 3.5	0.146, 3.0	1

Figure 5.10. HPLC chromatogram of 8 using a new batch of Boc-Ile[a]

Crude analysis[b]



NMR characterisation[c]



^{1}H NMR ($(CD_3)_2SO$, 400 MHz): δ 8.47 (s, 1H, H_{31}), 8.43 (s, 1H, H_{19}), 8.13 (d, *J* = 8.00 Hz, 2H, H_7, H_{44}), 7.94 (d, *J* = 8.70 Hz, 1H, H_{25}), 7.83 (d, *J* = 8.00 Hz, 1H, H_{37}), 7.64 (d, *J* = 8.90 Hz, 1H, H_{13}), 7.30 – 7.13 (m, 30H, H_{22}, H_{23}, H_{24}, H_{34}, H_{35}, H_{36}), 6.67 (d, *J* = 8.70 Hz, 1H, H_{51}), 4.62 (m, 1H, H_{39}), 4.29 – 4.12 (m, 4H, H_2, H_9, H_{15}, H_{27}), 3.82 (t, *J* = 8.00 Hz, H_{46}), 2.68 (dd,

Figure 5.10 (continued)

J = 4.88, 16.20 Hz, 1H, H_{40a}), 2.46 (dd, *J* = 8.52, 16.20 Hz, 1H, H_{40b}), 2.28 (m, 4H, H_{17}, H_{29}), 1.94 (sext., *J* = 6.80 Hz, 1H, H_{10}), 1.82 (m, 2H, H_{16}), 1.65 (m, 4H, H_4, H_{28}, H_{47}), 1.49 (m, 2H, H_3), 1.39 (m, 1H, H_{49a}), 1.36 (s, 9H, H_{43} or H_{54}), 1.35 (s, 9H, H_{43} or H_{54}), 1.04 (m, 1H, H_{49b}), 0.85 (d, *J* = 6.60 Hz, 3H, H_5 or H_6), 0.83 (d, *J* = 6.70 Hz, 3H, H_5 or H_6), 0.79 (d, *J* = 6.80 Hz, 3H, H_{48}), 0.78 (d, *J* = 6.40 Hz, 6H, H_{11}, H_{12}), 0.76 (t, *J* = 7.51 Hz, 3H, H_{50}). **^{13}C NMR** ($(CD_3)_2SO$, 100 MHz): δ 173.82 (C1), 171.43 (C_{18} or C_{30}), 171.39 (C_{45}), 171.28 (C_{18} or C_{30}), 170.95 (C_{26}), 170.87 (C_{14}), 170.78 (C_8), 170.13 (C_{38}), 169.24 (C_{41}), 155.34 (C_{52}), 144.86 (C_{21}, C_{33}), 128.45 (C_{22}, C_{34}), 127.40 (C_{23}, C_{35}), 126.26 (C_{24}, C_{36}), 80.19 (C_{42} or C_{53}), 78.03 (C_{42} or C_{53}), 69.14 (C_{20}, C_{32}), 58.69 (C_{46}), 57.20 (C_9), 52.27 (C_{15} or C_{27}), 52.21 (C_{15} or C_{27}), 50.02 (C_2), 49.42 (C_{39}), 39,72 (C_3), 37.34 (C_{40}), 36.58 (C_{47}), 32.72 (C_{17}, C_{29}), 30.65 (C_{10}), 28.14 (C_{16}, C_{28}), 27.63 (C_{43}), 26.80 (C_{54}), 24.29 (C_{49}), 24.18 (C_4), 22.81 (C_5 or C_6), 21.20 (C_{11} or C_{12}), 19.08 (C_5 or C_6), 17.87 (C_{11} or C_{12}), 15.30 (C_{48}), 10.97 (C_{50})

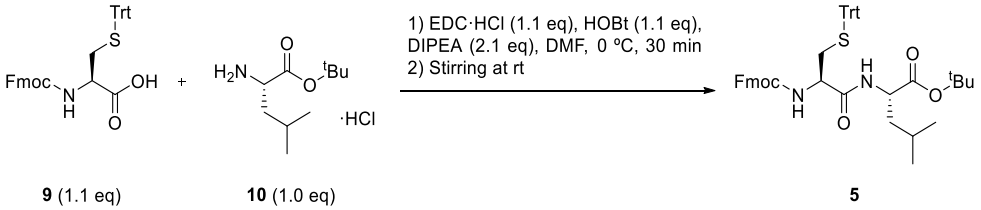
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.
(c) Varian 400 spectrometer.

5.8. Solution-phase peptide synthesis

5.8.1. Synthesis of 5

5.8.1.1. Using EDC·HCl, HOBt and DIPEA as the coupling system

5.8.1.1.1. Reaction in DMF

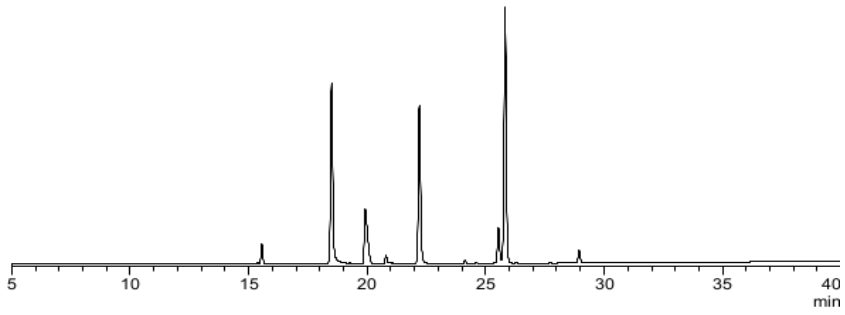


9 (1.1 eq) **10** (1.0 eq) **5**

In a Wheaton® vial, 55.0 mg (1.0 eq) of **10**, 169.8 mg (1.2 eq) of **9** and 56.9 mg (1.1 eq) of HOBt·H_2O were weighed. Then, 0.4 mL of DMF were added to the vial under N_2 atmosphere and the mixture was magnetically stirred until dissolution of reagents. Separately, 55.4 mg (1.1 eq) of EDC·HCl were weighed in a vial when 0.4 mL of DMF and 87 μL (2.0 eq) of DIPEA were added under N_2 atmosphere. The resulting solution was then added dropwise to the first solution and the reaction was monitored by HPLC-UV. After 4 h, 20 % of the starting material **9** remained in the mixture (22.2 min, figure 5.11). At that time, 50 μL (1.1 eq) of DIPEA were added and left stirring overnight. EtOAc (5 mL) was added to the reaction mixture and the resulting organic solution was washed with saturated aqueous solutions of citric acid (3 × 10 mL), $NaHCO_3$ (3 × 10 mL) and brine (1 × 10 mL). The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure to yield 0.158 g of a yellowish solid containing the dipeptide **5** with a chromatographic purity of 35 % (25.8 min, figure 5.11).

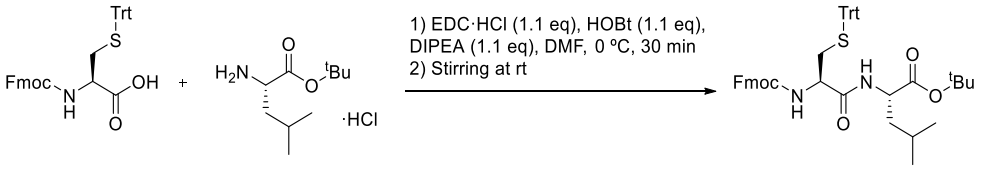
Figure 5.11. Assay using EDC·HCl, HOBt and DIPEA[a]

Crude analysis[b]**:** Synthesis in DMF



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.8.1.1.2. Reaction changing the number of equivalents

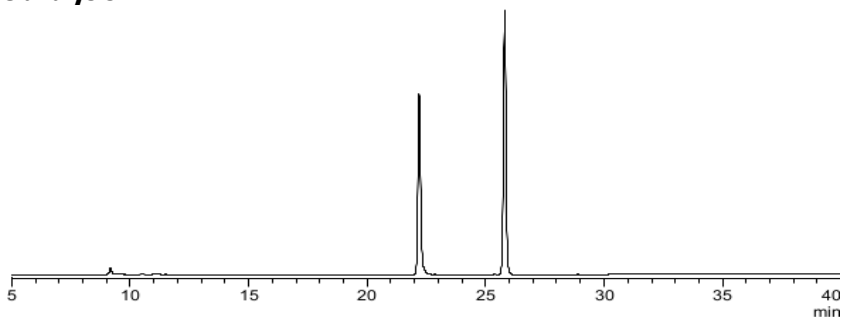


9 (1.0 eq) **10** (1.1 eq) **5**

In a Wheaton® vial, 146.4 mg (1.0 eq) of **9**, 75.4 mg (1.3 eq) of **10** and 61.5 mg (1.6 eq) of HOBt·H_2O were weighed. Then, 0.4 mL of DMF were added to the vial under N_2 atmosphere, the mixture was magnetically stirred until dissolution of reagents, and placed in an ice bath (0 °C). Separately, 53.0 mg (1.1 eq) of EDC·HCl were weighed in a vial when 0.4 mL of DMF and 48 μL (1.1 eq) of DIPEA were added under N_2 atmosphere. The resulting solution was then added dropwise to the first solution and the reaction was left stirring at 0 °C for 30 min and at rt for 17 h. EtOAc (5 mL) was added and the resulting organic solution was washed with saturated aqueous solutions of citric acid (3 × 10 mL), $NaHCO_3$ (3 × 10 mL) and brine (1 × 10 mL). The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure to yield 0.167 g of a white solid containing the dipeptide **5** with a chromatographic purity of 59 % (25.8 min, figure 5.12) and 41 % of **9** (22.2 min, figure 5.12).

Figure 5.12. Change in the equivalents[a]

Crude analysis[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

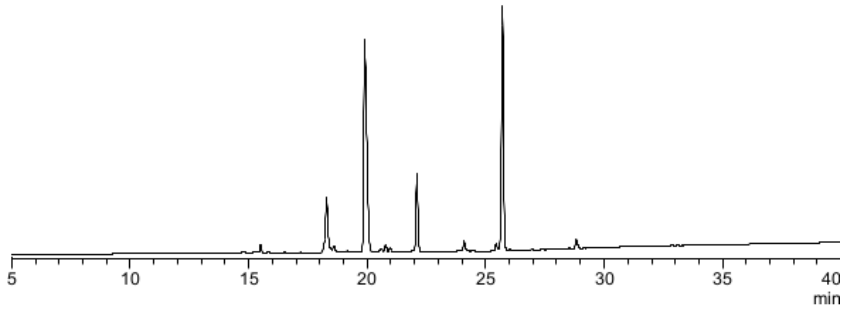
5.8.1.1.3. Assays at different reaction times

Reaction time: 24 h. 9 (146.3 mg, 1.0 eq), **10** (61.5 mg, 1.1 eq), HOBt·H_2O (42.7 mg, 1.1 eq), EDC·HCl (52.4 mg, 1.1 eq), DIPEA (48 μL, 1.1 eq) and 0.8 mL of DMF. 0.182 g of a yellowish solid containing the desired product **5** were obtained with a chromatographic purity of 30 % (25.7 min, figure 5.13).

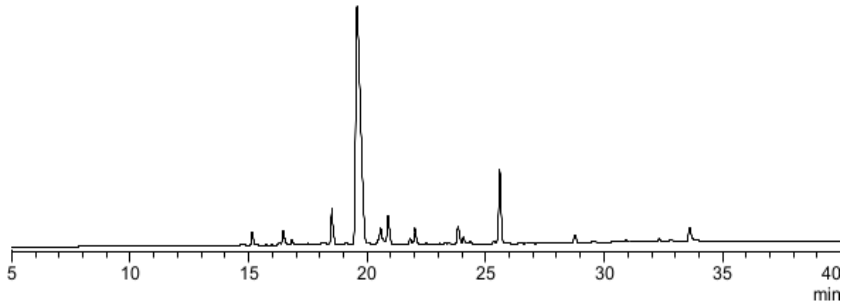
Reaction time: 48 h. 9 (146.3 mg, 1.0 eq), **10** (61.6 mg, 1.1 eq), HOBt·H_2O (43.1 mg, 1.1 eq), EDC·HCl (52.8 mg, 1.1 eq), DIPEA (48 μL, 1.1 eq) and 0.8 mL of DMF. 0.179 g of a yellowish solid containing the desired product **5** were obtained with a chromatographic purity of 10 % (25.6 min, figure 5.13).

Figure 5.13. Assays at different reaction times[a]

Crude analysis[b]**:** 24 h

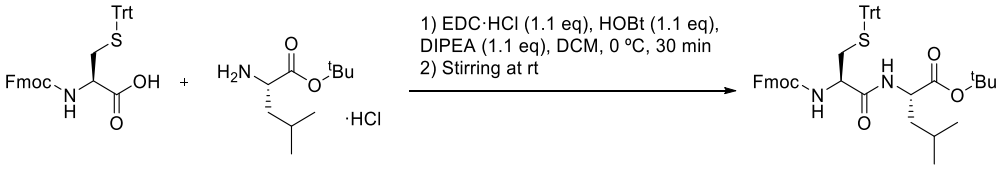


Crude analysis[b]**:** 48 h



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.8.1.1.4. Reaction in DCM

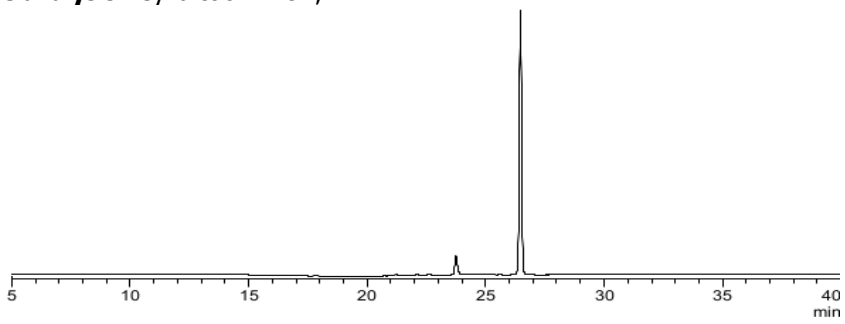


9 (1.0 eq) **10** (1.1 eq) **5**

In a Wheaton® vial, 146.6 mg (1.0 eq) of **9**, 73.9 mg (1.3 eq) of **10** and 44.0 mg (1.1 eq) of HOBt·H_2O were weighed. Then, 0.4 mL of DCM were added under N_2 atmosphere, the mixture was magnetically stirred until dissolution of reagents and placed in an ice bath (0 °C). Separately, 52.7 mg (1.1 eq) of EDC·HCl were weighed in a vial when 0.4 mL of DCM and 48 μL (1.1 eq) of DIPEA were added under N_2 atmosphere. The resulting solution was then added dropwise to the first vial and the reaction was left stirring at 0 °C for 30 min and at rt for 17 h. EtOAc (5 mL) was added and the resulting solution was washed with saturated aqueous solutions of citric acid (3 × 10 mL), $NaHCO_3$ (3 × 10 mL) and brine (1 × 10 mL). The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure to yield 0.141 g of a white solid containing the crude dipeptide **5** with a chromatographic purity of 93 % (26.4 min, figure 5.14).

Figure 5.14. Assay using EDC·HCl, HOBt and DIPEA[a]

Crude analysis[b]**:** Synthesis in DCM, 17 h



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

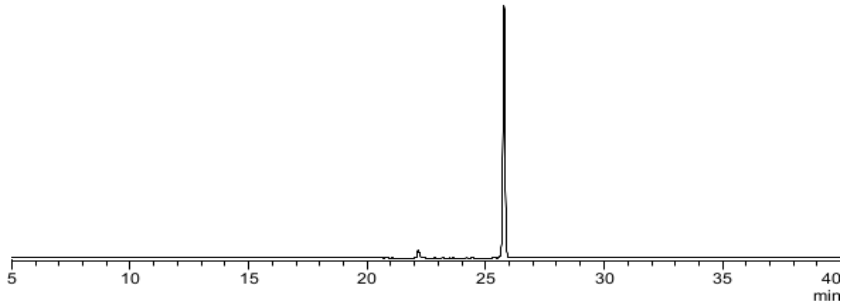
5.8.1.1.5. Assays at different reaction times

Reaction time: 24 h. 9 (146.3 mg, 1.0 eq), **10** (61.3 mg, 1.1 eq), HOBt·H_2O (42.7 mg, 1.1 eq), EDC·HCl (52.9 mg, 1.1 eq), DIPEA (48 μL, 1.1 eq) and 0.8 mL of DCM. 0.187 g of a white solid containing the desired product **5** were obtained with a chromatographic purity of 96 % (25.8 min, figure 5.15).

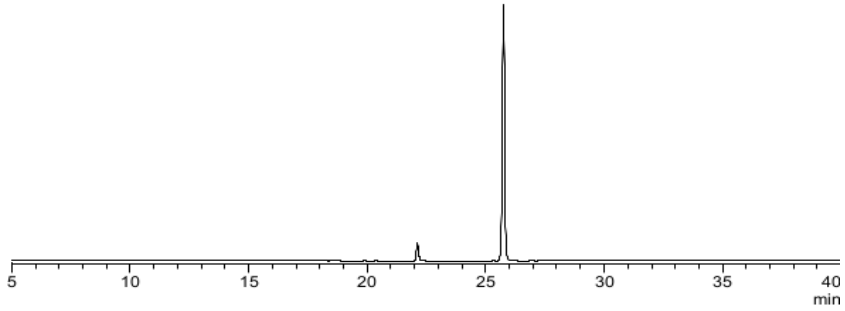
Reaction time: 48 h. 9 (146.3 mg, 1.0 eq), **10** (61.5 mg, 1.1 eq), HOBt·H_2O (42.1 mg, 1.1 eq), EDC·HCl (52.8 mg, 1.1 eq), DIPEA (48 μL, 1.1 eq) and 0.8 mL of DCM. 0.201 g of a white solid containing the desired product **5** were obtained with a chromatographic purity of 93 % (25.7 min, figure 5.15).

Figure 5.15. Assays at different reaction times[a]

Crude analysis[b]**:** 24 h



Crude analysis[b]**:** 48 h



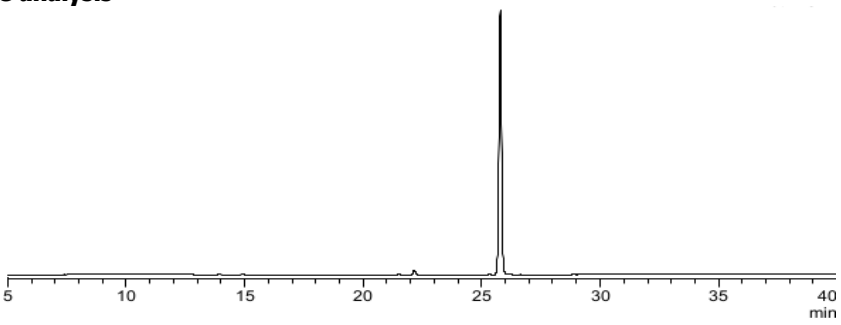
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.8.1.1.6. Batch 5A

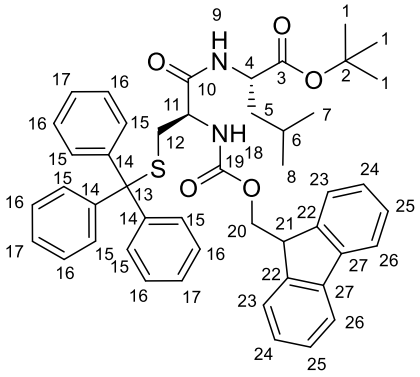
In a 50 mL round bottom flask, 3.88 g (1.0 eq) of **9**, 1.67 g (1.1 eq) of **10** and 1.17 g (1.1 eq) of HOBt·H_2O were weighed. Then, 10 mL of DCM were added under N_2 atmosphere, the resulting mixture was magnetically stirred until dissolution of reagents and placed in an ice bath (0 °C). Separately, 1.35 g (1.1 eq) of EDC·HCl were weighed in a vial when 5 mL of DCM and 1.27 mL (1.1 eq) of DIPEA were added under N_2 atmosphere. The resulting solution was then added dropwise to the round bottom flask. The vial was washed with DCM (2 × 2.5 mL) to ensure complete transfer of the reagents. The reaction was left stirring at 0 ºC for 30 min and at rt for 17 h. EtOAc (5 mL) was added and the resulting solution was washed with saturated aqueous solutions of citric acid (3 × 10 mL), $NaHCO_3$ (3 × 10 mL) and brine (1 × 10 mL). The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure to yield 5.12 g of a white solid containing the dipeptide **5** with a chromatographic purity of 98 % (25.8 min, figure 5.16). **MS (ESI)**: m/z calc. for $[M+Na]^+$ of $C_{47}H_{50}N_2O_5S$ 777.3; found 777.2.

Figure 5.16. Change in the scale[a]

Crude analysis[b]



NMR characterisation[c]



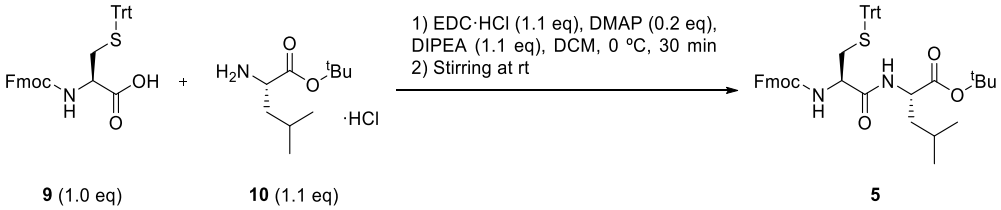
^{1}H NMR ($(CD_3)_2SO$, 400 MHz): δ 8.05 (d, *J* = 7.97 Hz, 1H, H_7), 7.96 (d, *J* = 8.01 Hz, 1H, H_{47}), 7.92 – 7.83 (m, 3H, H_{18}, H_{61}), 7.79 (d, *J* = 8.69 Hz, 1H, H_{25}), 7.69 (dd, *J* = 7.38, 3.18 Hz, 1H, H_{58}), 7.40 (t, *J* = 7.39 Hz, 2H, H_{60}), 7.36 – 7.29 (m, 3H, H_{53}, H_{59}), 6.68 (t, *J* = 5.59 Hz, 1H, H_{14}), 4.32 (m, 1H, H_{27}), 4.26 (m, 2H, H_{55}), 4.22 (m, 1H, H_9), 4.21 (m, 1H, H_{56}), 4.20 (m, 1H, H_2), 4.16 (m, 1H, H_{20}), 4.10 (m, 1H, H_{49}), 3.44 (m, 2H, H_{50}), 3.00 (d, *J* = 6.34 Hz, 2H, H_{30}), 2.91 (s, 2H, H_{44}), 2.84 (q, *J* = 6.69 Hz, 2H, H_{13}), 2.45 (s, 3H, H_{39}), 2.39 (s, 3H, H_{38}), 1.97 (s, 3H, H_{41}), 1.69-1.39 (m, 8H, H_3, H_4, H_{10} or H_{11}, H_{21}, H_{28} or H_{29}), 1.40 – 0.99 (m, 32H, H_{10} or H_{11}, H_{12}, H_{17}, H_{23}, H_{28} or H_{29}, H_{46}, H_{52}), 0.84 (d, *J* = 6.57 Hz, 3H, H_5 or H_6), 0.78 (m, 3H, H_5 or H_6), 0.77 (m, 3H, H_{22}), 0.76 (m, 3H, H_{24}). **^{13}C NMR** ($(CD_3)_2SO$, 100 MHz): δ 173.9 (C_1), 171.5 (C_8), 171.0 (C_{26}), 170.5 (C_{19}), 169.8 (C_{48}), 157.4 (C_{42}), 156.2 (C_{32}, C_{54}), 155.6 (C_{15}), 143.7 (C_{57}), 140.6 (C_{62}), 137.3 (C_{40}), 134.0 (C_{35}), 131.5 (C_{36}), 127.7 (C_{60}), 127.1 (C_{59}),125.2 (C_{58}), 124.4 (C_{43}), 120.1 (C_{61}), 116.3 (C_{37}), 86.3 (C_{45}), 77.4 (C_{16}), 72.9 (C_{51}), 65.8 (C_{55}), 61.8

Figure 5.16 (continued)

(C_{50}), 56.7 (C_{20}), 55.4 (C_{49}), 52.2 (C_9), 52.0 (C_{27}), 50.0 (C_2), 46.6 (C_{56}), 42.4 (C_{44}), 40.0 (C_{30}), 39.8 (C_3), 39.7 (C_{13}), 36.7 (C_{21}), 31.6 (C_{10} or C_{11}), 29.5 (C_{28} or C_{29}), 29.2 (C_{12}), 28.2 (C_{17}, C_{46}), 27.1 (C_{52}), 25.2 (C_{28} or C_{29}), 24.1 (C_4, C_{23}), 22.8 (C_5 or C_6), 22.4 (C_{10} or C_{11}), 21.10 (C_5 or C_6), 18.9 (C_{38}), 17.6 (C_{39}), 15.1 (C_{22}), 12.2 (C_{41}), 11.0 (C_{24})

(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.
(c) Varian 400 spectrometer.

5.8.1.2. Using EDC·HCl, DMAP and DIPEA

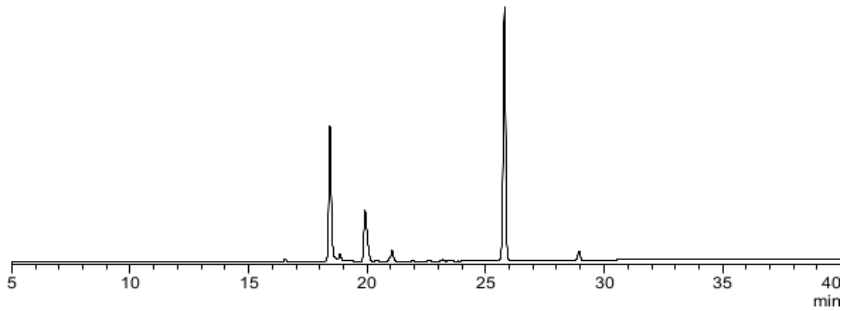


9 (1.0 eq) **10** (1.1 eq) **5**

In a Wheaton® vial, 146.0 mg (1.0 eq) of **9**, 61.8 mg (1.1eq) of **10** and 6.4 mg (0.2 eq) of DMAP were weighed. Then, 0.4 mL of DCM were added under N_2 atmosphere, the resulting mixture was magnetically stirred until dissolution of reagents and placed in an ice bath (0 °C). Separately, 54.4 mg (1.1 eq) of EDC·HCl were weighed in a vial when 0.4 mL of DCM and 65 μL (1.5 eq) of DIPEA were added under N_2 atmosphere. The resulting solution was then added dropwise to the first vial. The reaction was left stirring at 0 ºC for 30 min and at rt for 17 h. The starting material **9** was detected by HPLC-UV after 3 h when 19.30 mg (0.4 eq) of EDC·HCl were added and left stirring overnight. EtOAc (5 mL) was added and the resulting organic solution washed with saturated aqueous solutions of citric acid (3 × 10 mL), $NaHCO_3$ (3 × 10 mL) and brine (1 × 10 mL). The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure to yield 47.3 mg of a yellowish solid containing the crude dipeptide **5** with a chromatographic purity of 52 % (25.8 min, figure 5.17) and 14 % of the N-terminal deprotected dipeptide **11** (19.9 min, figure 5.17).

Figure 5.17. Using EDC·HCl, DMAP and DIPEA[a]

Crude analysis[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.8.1.2.1. Assays at different reaction times

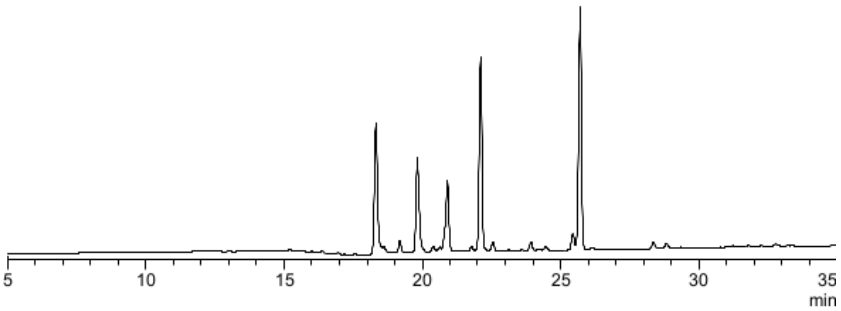
Reaction time: 17 h. 9 (146.3 mg, 1.0 eq), **10** (61.4 mg, 1.1 eq), DMAP (6.9 mg, 0.2 eq), EDC·HCl (52.5 mg, 1.1 eq), DIPEA (48 μL, 1.1 eq) and 0.8 mL of DCM. 0.229 g of a yellowish solid containing the desired product **5** were obtained with a chromatographic purity of 30 % (25.7 min, figure 5.18).

Reaction time: 24 h. 9 (146.3 mg, 1.0 eq), **10** (61.5 mg, 1.1 eq), DMAP (6.5 mg, 0.2 eq), EDC·HCl (52.9 mg, 1.1 eq), DIPEA (48 μL, 1.1 eq) and 0.8 mL of DCM. 0.143 g of a yellowish solid containing the desired product **5** were obtained with a chromatographic purity of 24 % (25.7 min, figure 5.18).

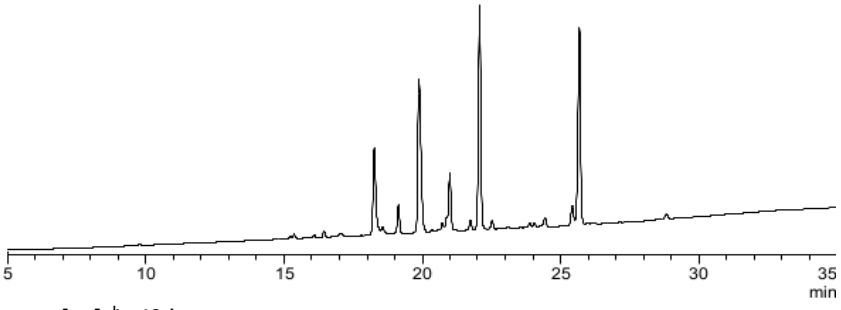
Reaction time: 48 h. 9 (146.3 mg, 1.0 eq), **10** (61.5 mg, 1.1 eq), DMAP (6.0 mg, 0.2 eq), EDC·HCl (53.1 mg, 1.1 eq), DIPEA (48 μL, 1.1 eq) and 0.8 mL of DCM. 0.154 g of a yellowish solid containing the desired product **5** were obtained with a chromatographic purity of 8 % (25.7 min, figure 5.18).

Figure 5.18. Assays at different reaction times[a]

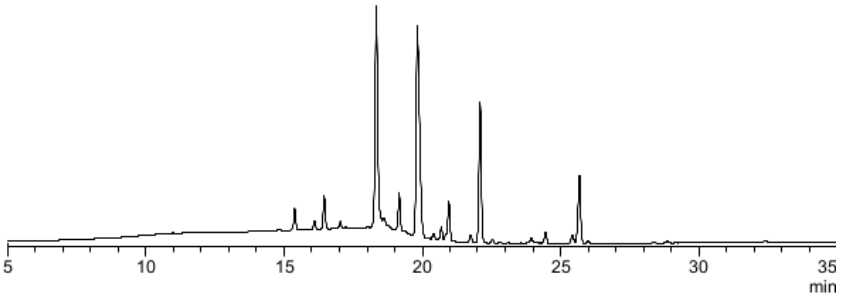
Crude analysis[b]**:** 17 h



Crude analysis[b]**:** 24 h

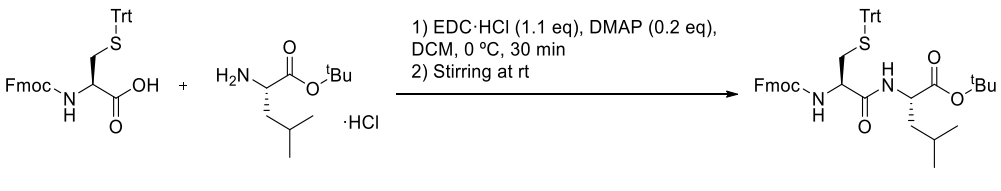


Crude analysis[b]**:** 48 h



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.8.1.3. Using EDC·HCl and DMAP

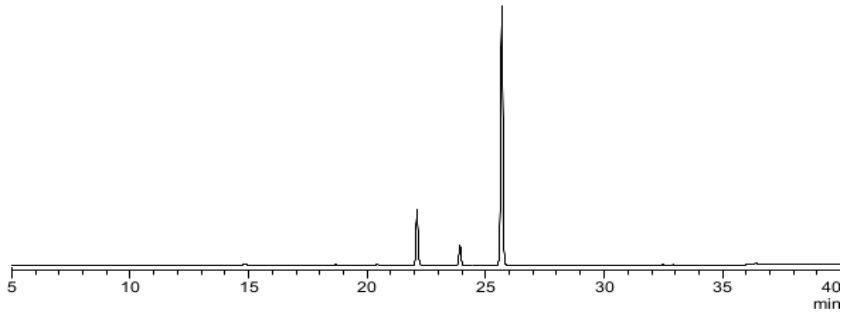


9 (1.0 eq) **10** (1.1 eq) **5**

In a Wheaton® vial, 146.3 mg (1.0 eq) of **9**, 61.4 mg (1.1 eq) of **10**, 52.9 mg (1.1 eq) of EDC·HCl and 6.0 mg (0.2 eq) of DMAP were weighed. Then, 0.8 mL of DCM were added under N_2 atmosphere, the mixture was magnetically stirred until dissolution and placed in an ice bath (0 °C). The reaction was left stirring at 0 ºC for 30 min and at rt for 17 h when EtOAc (5 mL) was added and the resulting organic solution was washed with saturated aqueous solutions of with citric acid (3 × 10 mL), $NaHCO_3$ (3 × 10 mL) and brine (1 × 10 mL). The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure to obtain 0.182 g of a white solid containing the dipeptide **5** with a chromatographic a purity of 78 % (25.7 min, figure 5.19) and 16 % of **9** (22.1 min, figure 5.19).

Figure 5.19. Using EDC·HCl and DMAP[a]

Crude analysis[b]: 17 h



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

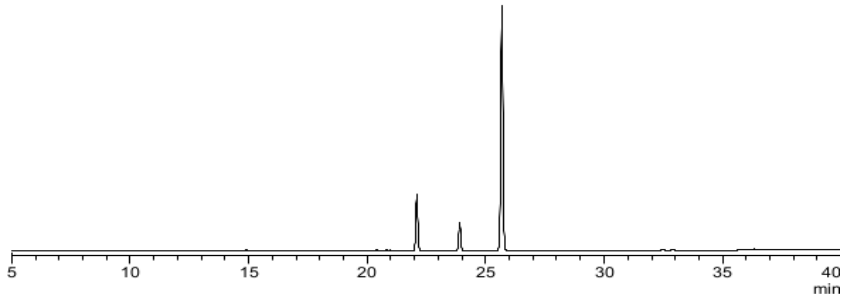
5.8.1.3.1. Assays at different reaction times

Reaction time: 24 h. 9 (146.3 mg, 1.0 eq), **10** (61.5 mg, 1.1 eq), DMAP (6.4 mg, 0.2 eq), EDC·HCl (53.1 mg, 1.1 eq) and 0.8 mL of DCM. 0.212 g of a white solid containing the desired product **5** were obtained with a chromatographic purity of 76 % (25.7 min, figure 5.20).

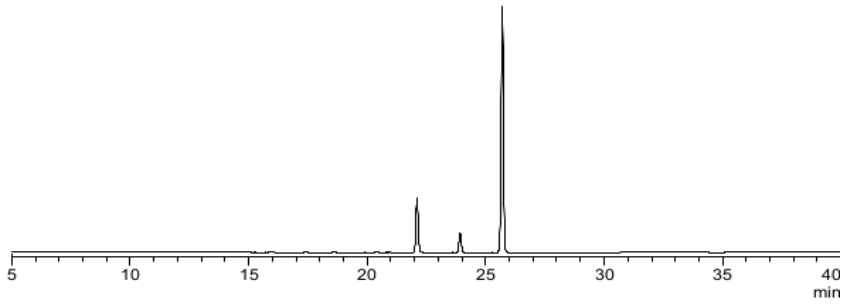
Reaction time: 48 h. 9 (146.3 mg, 1.0 eq), **10** (61.5 mg, 1.1 eq), DMAP (6.4 mg, 0.2 eq), EDC·HCl (53.1 mg, 1.1 eq) and 0.8 mL of DCM. 0.120 g of a white solid containing the desired product **5** were obtained with a chromatographic purity of 76 % (25.7 min, figure 5.20).

Figure 5.20. Assays at different reaction times[a]

Crude analysis[b]**:** 24 h

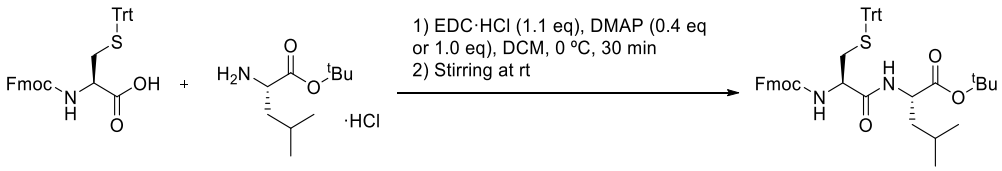


Crude analysis[b]**:** 48 h



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.8.1.3.2. Reaction changing the number of DMAP equivalents



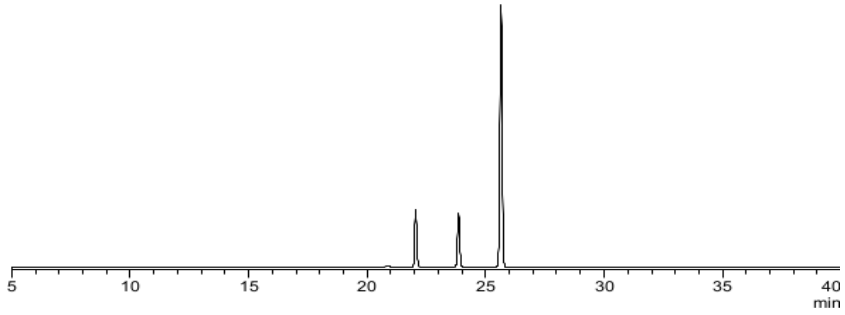
9 (1.0 eq) **10** (1.1 eq) **5**

Reaction with 0.4 eq of DMAP. A protocol similar to the described in section 5.8.1.3 was used with a reaction time of 17 h at rt. **9** (146.4 mg, 1.0 eq), **10** (62.3 mg, 1.1 eq), DMAP (13.2 mg, 0.4 eq), EDC·HCl (52.8 mg, 1.1 eq) and 0.8 mL of DCM. 0.289 g of a white solid containing the desired product **5** were obtained with a chromatographic purity of 71 % (25.7 min, figure 5.21) and 15 % of **9** (22.1 min, figure 5.21).

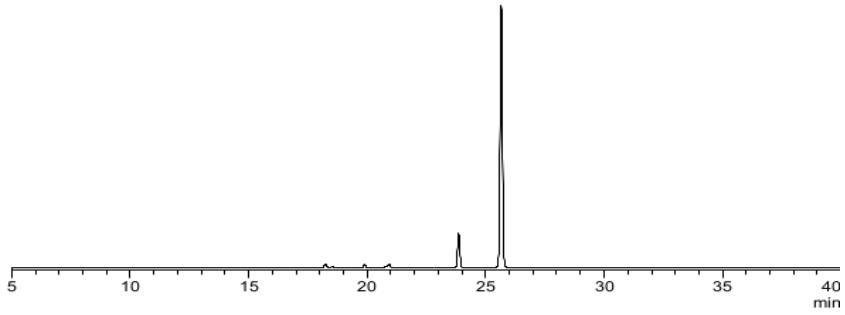
Reaction with 1.0 eq of DMAP. A protocol similar to the described in section 5.8.1.3 was used with a reaction time of 17 h at rt. **9** (146.4 mg, 1.0 eq), **10** (61.2 mg, 1.1 eq), DMAP (29.5 mg, 1.0 eq), EDC·HCl (53.7 mg, 1.1 eq) and 0.8 mL of DCM. 0.231 g of a white solid containing the desired product **5** were obtained with a chromatographic purity of 84 % (25.7 min, figure 5.21).

Figure 5.21. Change in the DMAP equivalents[a]

Crude analysis[b]**:** 0.4 eq

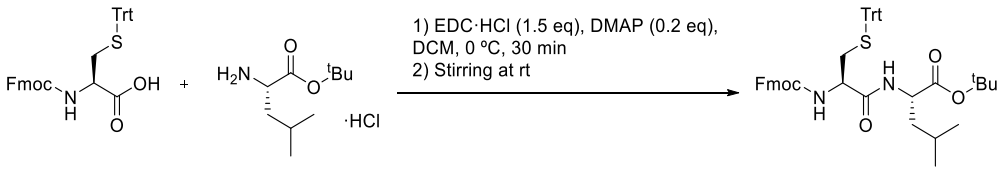


Crude analysis[b]**:** 1.0 eq



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.8.1.3.3. Reaction changing the number of the EDC·HCl equivalents



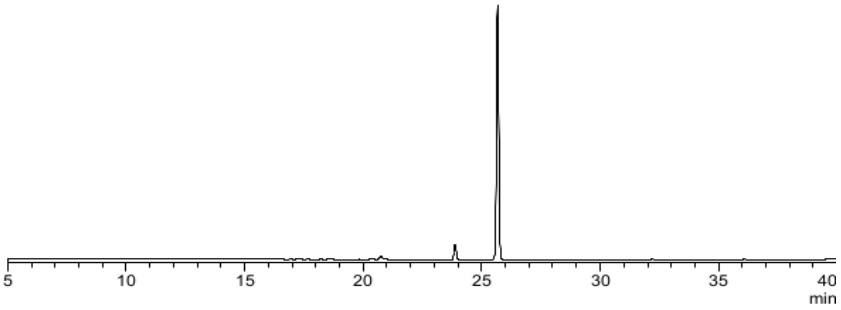
9 (1.0 eq) **10** (1.1 eq) **5**

Reaction with 1.5 eq of EDC·HCl in one batch. A protocol similar to the described in section 5.8.1.3 was used with a reaction time of 17 h at rt. **9** (147.2 mg, 1.0 eq), **10** (62.1 mg, 1.1 eq), DMAP (6.5 mg, 0.2 eq), EDC·HCl (73.1 mg, 1.5 eq) and 0.8 mL of DCM. 0.186 g of a white solid containing the desired product **5** were obtained with a chromatographic purity of 92 % (25.7 min, figure 5.22).

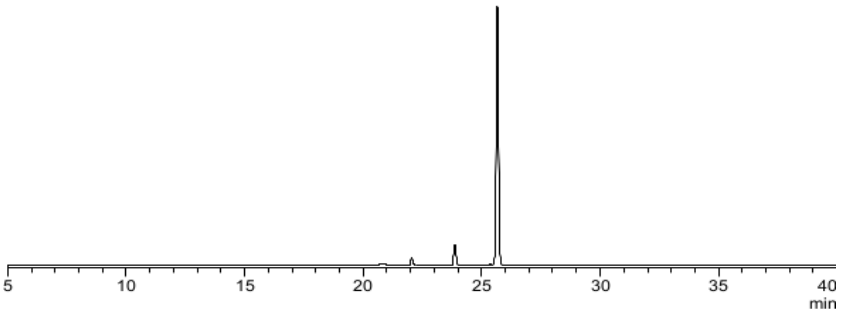
Reaction with 1.5 eq of EDC·HCl in two batches. In a Wheaton® vial, 146.6 mg (1.0 eq) of **9**, 61.7 mg (1.1 eq) of **10**, 53.7 mg (1.1 eq) of EDC·HCl and 6.1 mg (0.2 eq) of DMAP were weighed. Then, 0.8 mL of DCM were added under N_2 atmosphere, the resulting mixture was magnetically stirred until dissolution and placed in an ice bath (0 °C). The reaction was left stirring at 0 ºC for 30 min and at rt for 17 h when 20.2 mg of EDC·HCl were added and the resulting mixture was left stirring for 5 h. EtOAc (5 mL) was added and the resulting solution was washed with saturated aqueous solutions of citric acid (3 × 10 mL), $NaHCO_3$ (3 × 10 mL) and brine (1 × 10 mL). The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure to yield 0.216 g of a white solid containing the dipeptide **5** with a chromatographic a purity of 87 % (25.7 min, figure 5.22).

Figure 5.22. Change in the EDC·HCl equivalents[a]

Crude analysis[b]**:** 1.5 eq

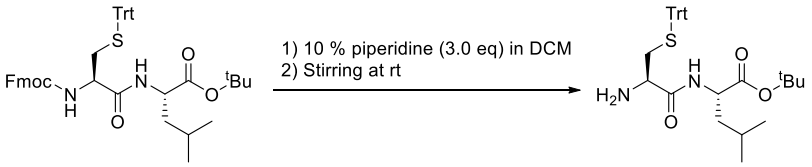


Crude analysis[b]**:** 0.4 eq after 17 h



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.8.2. Synthesis of 11



5 (1.0 eq) **11**

In a 25 mL round-bottom flask with a magnetic stir bar, **5** was mixed with a 10 % piperidine in DCM and the resulting solution was left stirring. The reaction was monitored by HPLC-UV and after 2.5 h the reaction was finished. The solvent was removed under reduced pressure and the resulting reaction crude of **11** was purified by flash column chromatography using Hexanes/EtOAc 8:2 and DCM/MeOH 9:1 as eluents (dry loading).

5.8.2.1. Batch 11A

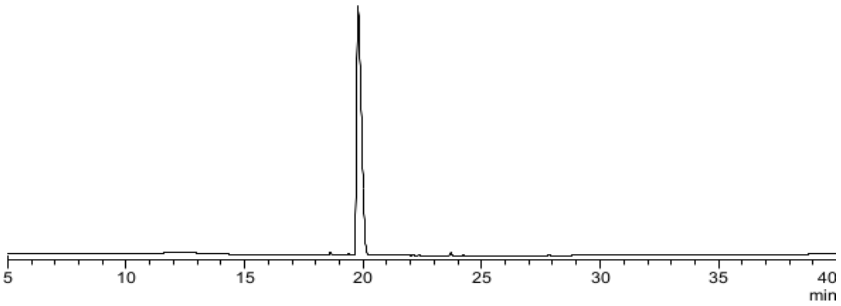
5 (1.00 g, 1.0 eq, section 5.8.1.2.2), piperidine (0.34 g, 3.0 eq) in 3.5 mL of DCM. 0.515 g (73 %) of a yellowish sticky solid of the desired product **11** were obtained with a chromatographic purity of 99 % (19.8 min, figure 5.23). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{32}H_{40}N_2O_3S$ 533.3; found 533.3.

5.8.2.2. Batch 11B

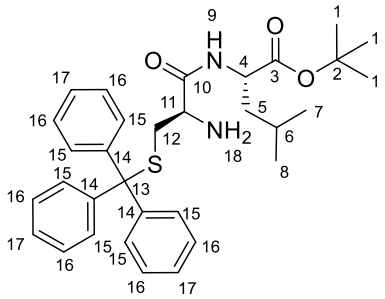
5 (3.85 g, 1.0 eq, section 5.8.1.2.2), piperidine (1.30 g, 3.0 eq) in 13.6 mL of DCM. 2.58 g (95 %) of a yellowish sticky solid of the desired product **11** were obtained with a chromatographic purity of 99 %. **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{32}H_{40}N_2O_3S$ 533.3; found 533.3.

Figure 5.23. HPLC chromatogram of 11[a]

Isolated product[b]**:** Batch 11A



NMR characterisation[c]

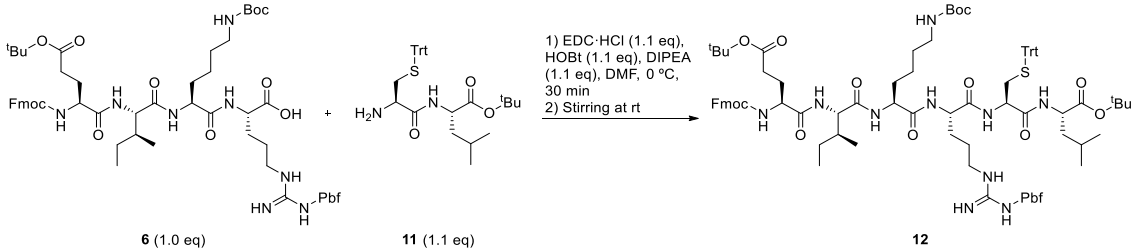


^{1}H NMR ($(CD_3)_2SO$, 400 MHz): δ 7.98 (d, *J* = 8.20 Hz, 1H, H_9), 7.38-7.30 (m, 12H, H_{15}, H_{16}), 7.27 - 7.21 (m, 3H, H_{17}), 4.14 (ddd, *J* = 9.32, 8.12, 5.54 Hz, 1H, H_4), 3.20 (dd, *J* = 7.64, 5.18 Hz, 1H, H_{11}), 2.33 (dd, *J* = 11.47, 5.20 Hz, 1H, H_{12a}), 2.19 (dd, *J* = 11.46, 7.70 Hz, 1H, H_{12b}), 1.78 (s, 2H, H_{18}), 1.64 - 1.57 (m, 1H, H_6), 1.54 - 1.42 (m, 2H, H_5), 1.32 (s, 9H, H_1), 0.86 (d, *J* = 6.57 Hz, 3H, H_7 or H_8), 0.81 (d, *J* = 6.49 Hz, 3H, H_7 or H_8). **^{13}C NMR** ($(CD_3)_2SO$, 100 MHz): δ 173.0 (C_{10}), 171.4 (C_3), 144.5 (C_{14}), 129.1 (C_{15} or C_{16}), 128.0 (C_{15} or C_{16}), 126.7 (C_{17}), 80.9 (C_2), 66.1 (C_{13}), 53.8 (C_{11}), 50.8 (C_4), 40.0 (C_5), 37.2 (C_{12}), 27.6 (C_1), 24.2 (C_6), 22.8 (C_7 or C_8), 21.6 (C_7 or C_8)

(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.
(c) Varian 400 spectrometer.

5.8.3. Synthesis of 12

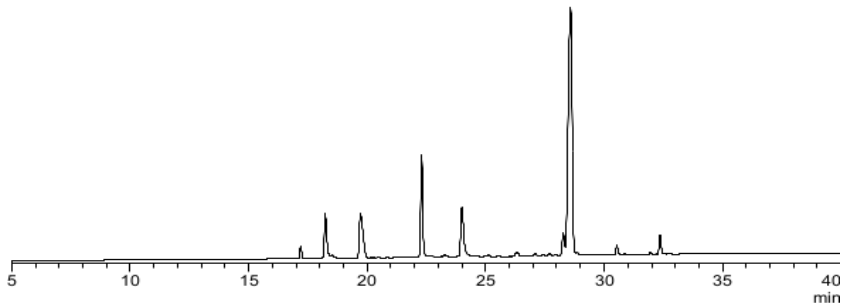
5.8.3.1. Using EDC·HCl, HOBt and DIPEA



In a 5 mL round bottom flask, 301.0 mg of the tetrapeptide **6** (1.0 eq, section 5.7.3.1), 56.2 mg (1.1 eq) of EDC·HCl and 45.9 mg (1.2 eq) of HOBt·H_2O were weighed. Then, 0.4 mL of DMF were added under N_2 atmosphere, the resulting suspension was magnetically stirred until dissolution of reagents and placed in an ice bath (0 °C). Separately, 149.8 mg of **11** (1.1 eq, section 5.8.2.2) were weighed in a vial and dissolved in 0.3 mL of DMF under N_2 atmosphere. The resulting solution and 49 μL (1.1 eq) of DIPEA were then added dropwise separately to the round bottom flask. The vial was washed with DMF (2 × 0.1 mL) to ensure complete transfer of **11**. The reaction mixture was left stirring at 0 ºC for 30 min and at rt for 8 h when the HPLC chromatogram showed that the reaction did not evolve further. The reaction crude was precipitated into a conical centrifuge tube with H_2O (30 mL) and centrifuged. The supernatant was removed and two more washings with H_2O were carried out. Finally, the precipitate was lyophilized to obtain 0.296 g of a white solid containing the hexapeptide **12** with a chromatographic purity of 56 % (28.6 min, figure 5.24). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{92}H_{124}N_{10}O_{16}S_2$ 1688.9; found 1690.0.

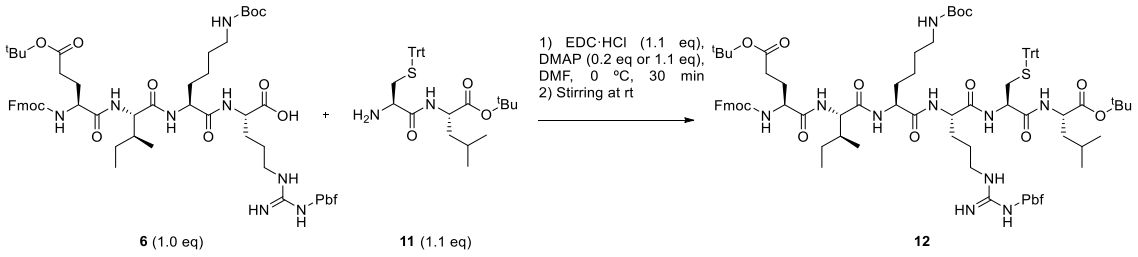
Figure 5.24. Using EDC·HCl, HOBt and DIPEA[a]

Crude analysis[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.8.3.2. Using EDC·HCl and DMAP



In a 5 mL round bottom flask, 300.7 mg of the tetrapeptide **6** (1.0 eq, section 5.7.3.1), 74.2 mg (1.5 eq) of EDC·HCl and 7.0 mg (0.2 eq) of DMAP were weighed. Then, 0.4 mL of DMF were added under N_2 atmosphere, the resulting suspension was magnetically stirred until dissolution of reagents and placed in an ice bath (0 °C). Separately, 159.5 mg of **11** (1.1 eq, section 5.8.2.2) were weighed in a vial and dissolved in 0.3 mL of DMF under N_2 atmosphere. The resulting solution was then added dropwise to the round bottom flask and the vial was washed with DMF (2 × 0.1 mL) to ensure complete transfer of **11**. The reaction was left stirring at 0 ºC for 30 min and at rt for 6 h when the HPLC chromatogram showed that the reaction did not evolve further. The reaction crude was precipitated into a conical centrifuge tube with H_2O (30 mL) and centrifuged. The supernatant was removed and two more washings with H_2O were carried out. Finally, the precipitate was lyophilized to obtain 0.382 g of a white solid containing the hexapeptide **12** with a chromatographic purity of 35 % (28.4

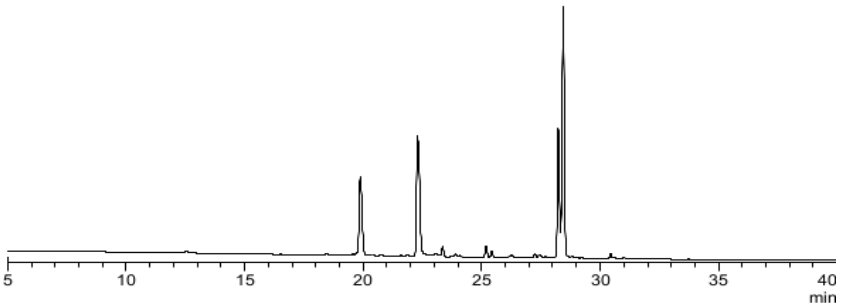
min, figure 5.25), together with a 18 % of a diastereomeric form of **12** (28.2 min, figure 5.25). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{92}H_{124}N_{10}O_{16}S_2$ 1688.9; found 1690.0.

5.8.3.2.1. Changing the number of DMAP equivalents

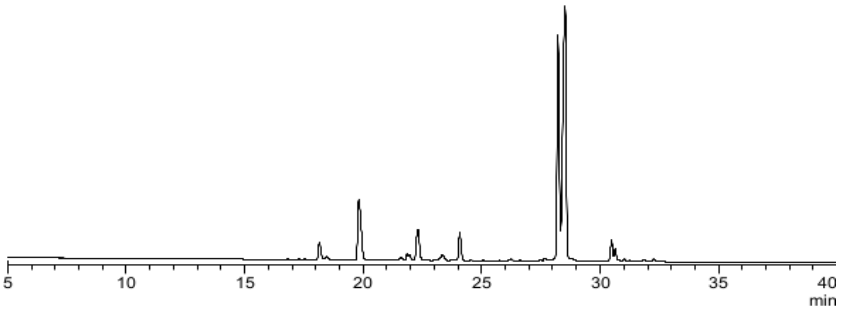
In a 5 mL round bottom flask, 298.8 mg of the tetrapeptide **6** (1.0 eq, section 5.7.3.1), 53.7 mg (1.1 eq) of EDC·HCl and 34.1 mg (1.1 eq) of DMAP were weighed. Then, 0.4 mL of DMF were added under N_2 atmosphere, the resulting suspension was magnetically stirred until dissolution of reagents and placed in an ice bath (0 °C). Separately, 156.9 mg of **11** (1.1 eq, section 5.8.2.2) were weighed in a vial and dissolved in 0.3 mL of DMF under N_2 atmosphere. The resulting solution was then added dropwise to the round bottom flask and the vial was washed with DMF (2 × 0.1 mL) to ensure complete transfer of **11**. The reaction was left stirring at 0 ºC for 30 min and at rt for 8 h when the HPLC chromatogram showed that the reaction did not evolve further. The reaction crude was precipitated into a conical centrifuge tube with H_2O (30 mL) and centrifuged. The supernatant was removed and two more washings with H_2O were carried out. Finally, the precipitate was lyophilized to afford 0.323 g of a white solid containing the hexapeptide **12** with a chromatographic purity of 44 % (28.5 min, figure 5.25), together with a 27 % of a diastereomeric form of **12** (28.1 min, figure 5.25).

Figure 5.25. Using EDC·HCl and DMAP[a]

Crude analysis[b]: 0.2 eq of DMAP



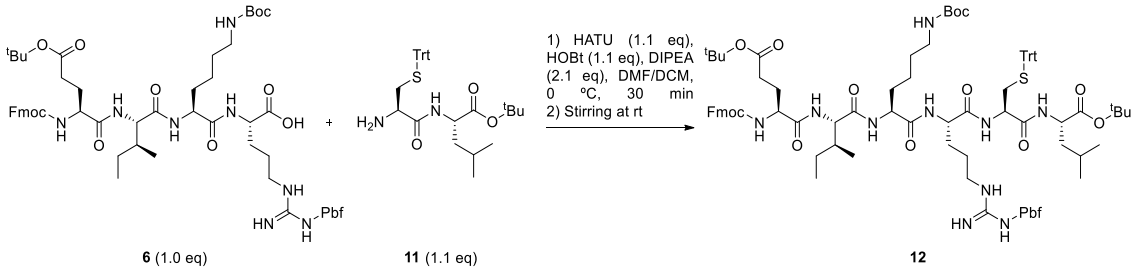
Crude analysis[b]: 1.1 eq of DMAP



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.8.3.3. Using HATU, HOBt and DIPEA

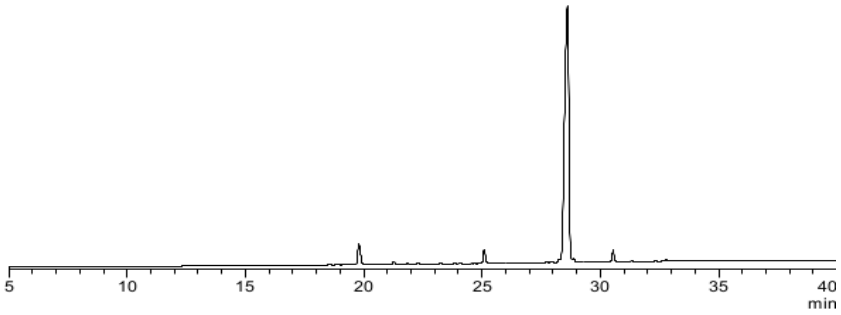
5.8.3.3.1. Batch 12A



In a 5 mL round bottom flask, 1.09 g of the tetrapeptide **6** (1.0 eq, section 5.7.3.1), 0.391 g (1.1 eq) of HATU and 0.159 g (1.1 eq) of HOBt·H_2O were weighed. Then, 1.5 mL of DMF were added under N_2 atmosphere, the resulting suspension was magnetically stirred until dissolution of reagents and placed in an ice bath (0 °C). Separately, 0.548 g of **11** (1.1 eq, section 5.8.2.2) were weighed and dissolved in 1 mL of DMF under N_2 atmosphere. The resulting solution and 343 μL (2.1 eq) of DIPEA were then added dropwise separately to the round bottom flask and the vial was washed with DMF (2 × 0.25 mL) to ensure complete transfer of **11**. The reaction was left stirring at 0 ºC for 30 min and at rt for 6 h when the HPLC chromatogram showed that the reaction did not evolve further. The reaction crude was precipitated into a conical centrifuge tube with H_2O (30 mL) and centrifuged. The supernatant was decanted and two more washings with H_2O were carried out. Finally, the precipitated was lyophilized to obtain 1.60 g of a white solid containing the hexapeptide **12** with a chromatographic purity of 88 % (28.6 min, figure 5.26). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{92}H_{124}N_{10}O_{16}S_2$ 1688.9; found 1690.0.

Figure 5.26. Using HATU, HOBt and DIPEA[a]

Crude analysis[b]**:** Batch 12A



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

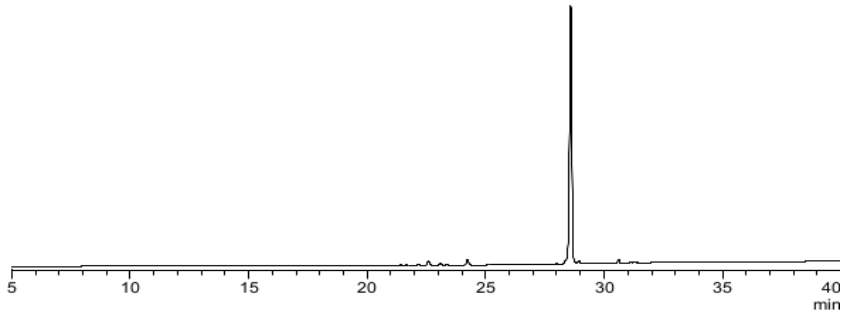
5.8.3.3.2. Batch 12B

In a 5 mL round bottom flask, 0.601 g of the tetrapeptide **6** (1.0 eq, section 5.7.3.2), 0.238 g (1.2 eq) of HATU and 0.109 g (1.4 eq) of HOBt·H_2O were weighed. Then, 1.0 mL of DCM was added under N_2 atmosphere and the resulting suspension was magnetically stirred until dissolution of reagents and placed in an ice bath (0 °C). Separately, 0.301 g of **11** (1.1 eq, section 5.8.2.1) were weighed and dissolved in 0.4 mL of DCM under N_2 atmosphere. The resulting solution and a solution of 187 μL (2.1 eq) of DIPEA in 0.2 mL of DCM were then added dropwise separately to the round bottom flask. The vial was washed with DCM (2 × 0.2 mL) to ensure complete transfer of **11**. The reaction was left stirring at 0 ºC for

30 min and at rt overnight. The reaction crude was precipitated into a conical centrifuge tube with Et_2O (10 mL) and centrifuged. The supernatant was decanted and two more washings with Et_2O were carried out. Finally, the precipitated was dried under vacuum to afford 0.908 g of a white solid containing the hexapeptide **12** with a chromatographic purity of 93 % (28.6 min, figure 5.27). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{92}H_{124}N_{10}O_{16}S_2$ 1688.9; found 1690.0.

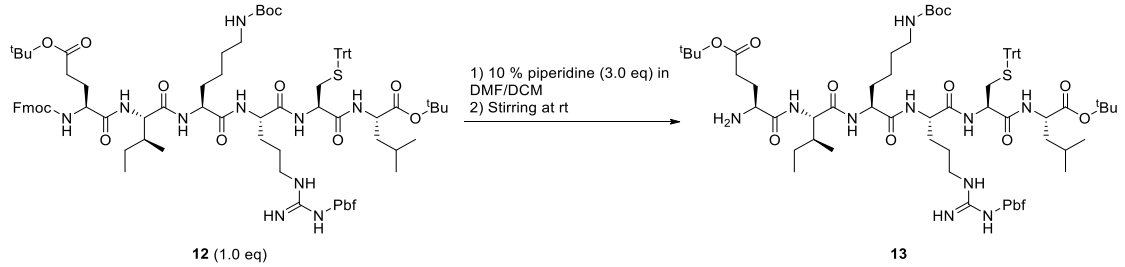
Figure 5.27. Using HATU, HOBt and DIPEA[a]

Crude analysis[b]: Batch 12B



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.8.4. Synthesis of 13

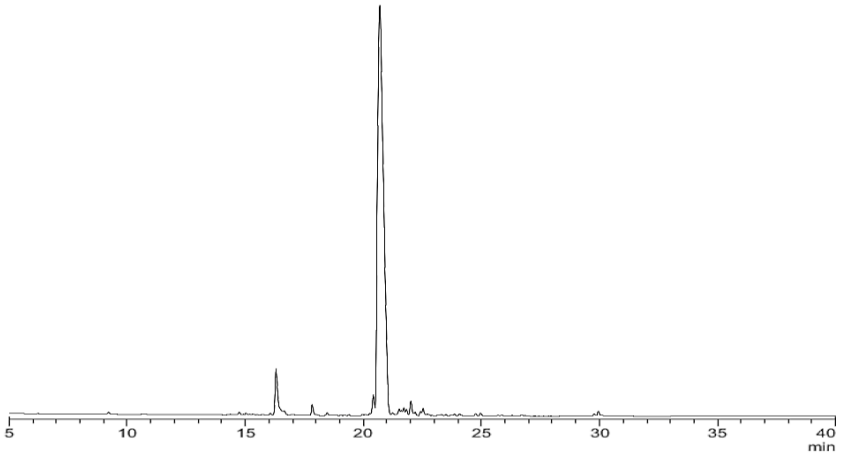


5.8.4.1. Batch 13A

In a 10 mL round bottom flask, 1.60 g of **12** (1.0 eq, section 5.8.3.3) were weighed and a solution of 0.280 mL (3.0 eq) of piperidine in 2.52 mL of DMF was added dropwise to the round bottom flask and left at rt until no starting material **12** was detected by HPLC. The reaction crude was precipitated into a conical centrifuge tube with H_2O (30 mL) and centrifuged. Then, the solvent was decanted and two more washings with H_2O were carried out. Finally, the precipitate was lyophilized to obtain 1.41 g of the crude deprotected hexapeptide **13** as a white solid. The purification of the crude by flash chromatography using Hexanes/EtOAc 8:2 and DCM/MeOH 9:1 afforded 1.09 g of a yellowish solid containing the desired **13** with a chromatographic purity of 88 % (20.6 min, figure 5.28). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{77}H_{114}N_{10}O_{14}S_2$ 1466.8; found 1469.0.

Figure 5.28. HPLC chromatogram of 13[a]

Isolated product[b]: Batch 13A



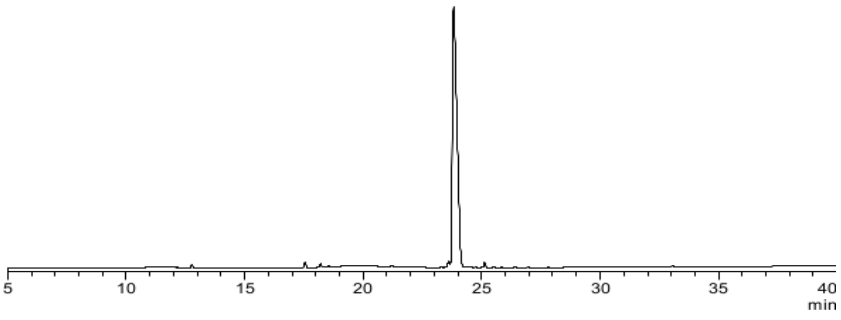
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Waters 2695, UV detection at 220 nm.

5.8.4.2. Batch 13B

In a 10 mL round bottom flask, 0.767 g of **12** (1.0 eq, section 5.8.3.3.2) were weighed and a solution of 0.134 mL (3.0 eq) of piperidine in 1.10 mL of DCM was added dropwise to the round bottom flask and left at rt until and left at rt until no starting material **12** was detected by HPLC. The solvent was evaporated under reduced pressure, Et_2O (10 mL) was added and the resulting suspension was transferred into a conical centrifuge tube for centrifugation. Then, the solvent was decanted and two more washings with Et_2O were carried out. Finally, the precipitate was dried under vacuum to afford 0.736 g of the crude of **13**. The crude (0.601 g) was purified by flash chromatography using Hexanes/EtOAc 8:2 and DCM/MeOH 9:1. **13** was obtained as a yellowish solid (0.346 g) with a chromatographic purity of 96 % (23.8 min, figure 5.29). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{77}H_{114}N_{10}O_{14}S_2$ 1466.8; found 1469.0.

Figure 5.29. HPLC chromatogram of 13[a]

Isolated product[b]: Batch 13B

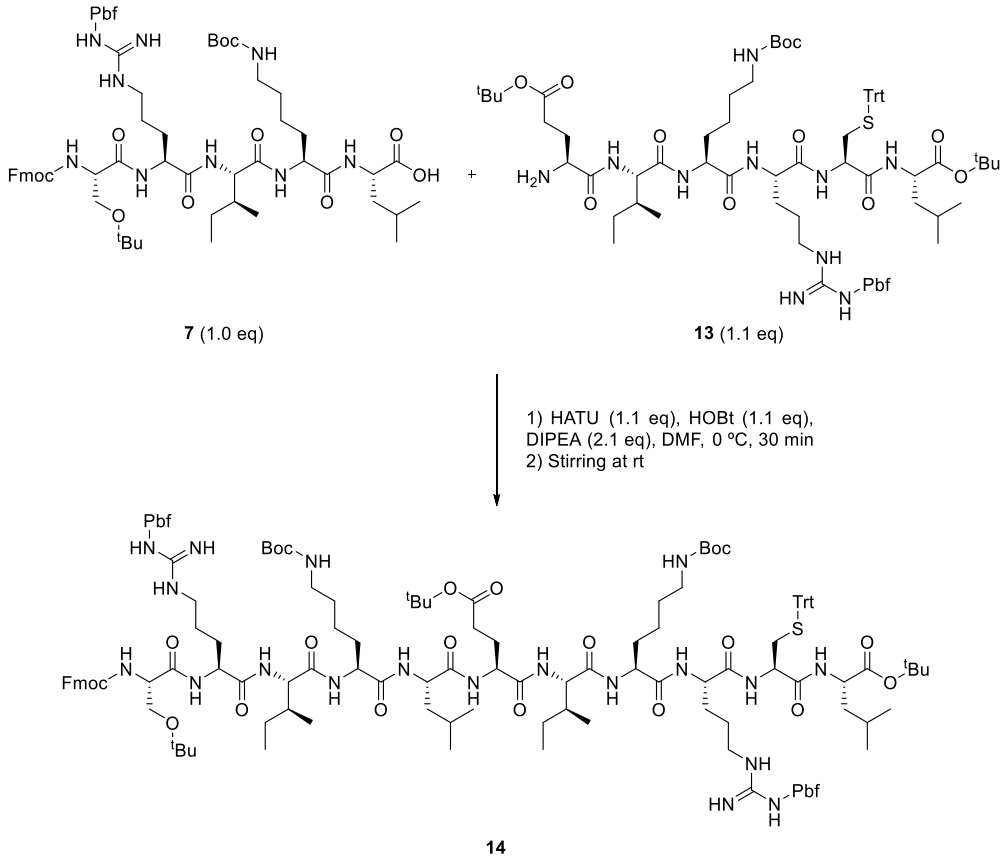


(c) Chromatographic conditions: Method B (Section 5.3.3.3).
(d) Shimadzu, UV detection at 220 nm.

5.8.5. Synthesis of 14

5.8.5.1. Using HATU, HOBt and DIPEA

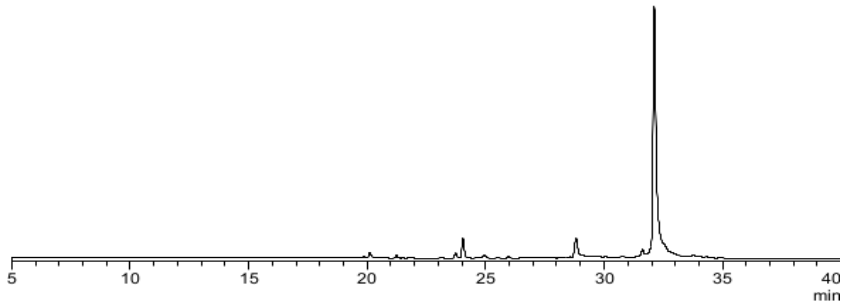
5.8.5.1.1. Batch 14A



In a 10 mL round bottomed flask, 386.9 mg of the pentapeptide **7** (1.0 eq, section 5.7.4.1), 128.7 mg (1.1 eq) of HATU and 55.8 mg (1.2 eq) of HOBt·H_2O were weighed and 1.0 mL of DMF was added under N_2 atmosphere. The mixture was magnetically stirred until dissolution of reagents and the flask was placed in an ice bath (0 ºC). Separately, 503.4 mg of **13** (1.1 eq, section 5.8.4.1) were weighed in a vial and dissolved in 3.0 mL of DMF under N_2 atmosphere. The resulting solution and 113 μL of DIPEA (2.1 eq) were then added dropwise separately to the round bottom flask. The vial was washed with DMF (2 × 0.1 mL) to ensure complete transfer of **13**. The reaction was left stirring at 0 ºC for 30 min and at rt for 4 h when the HPLC chromatogram showed that the reaction did not evolve further. The reaction mixture was precipitated into a conical centrifuge tube with H_2O (30 mL) and centrifuged. The supernatant was decanted and two more washings with H_2O were carried out. Finally, the precipitate was lyophilized to afford 0.808 g of a white solid containing the undecapeptide **14** with a chromatographic purity of 83 % (32.1 min, figure 5.30). **MS (ESI)**: m/z calc. for $[M+2H]^{2+}$ of $C_{141}H_{207}N_{19}O_{27}S_3$ 1349.2; found 1349.2.

Figure 5.30. Using HATU, HOBt and DIPEA[a]

Crude analysis[b]: Batch 14A



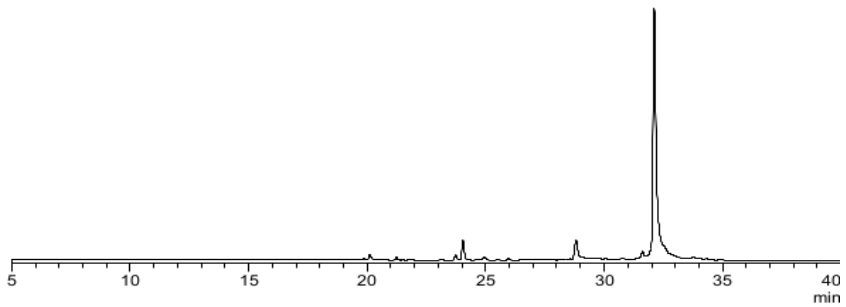
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.8.5.1.2. Batch 14B

In a 10 mL round bottomed flask, 225.9 mg of the pentapeptide **7** (1.0 eq, section 5.7.4.2), 91.2 mg (1.1 eq) of HATU and 42.9 mg (1.3 eq) of HOBt·H_2O were weighed and 1.0 mL of DMF was added under N_2 atmosphere. The mixture was magnetically stirred until dissolution of reagents and placed in an ice bath (0 ºC). Separately, 344.8 mg of **13** (1.1 eq, section 5.8.4.2) were weighed in a vial and dissolved in 2.0 mL of DMF under N_2 atmosphere. The resulting solution and 78 µL of DIPEA (2.1 eq) in 0.1 mL of DMF were then added dropwise separately to the round bottom flask. The vial was washed with DMF (2 × 0.2 mL) to ensure complete transfer of **13**. The reaction was left stirring at 0 ºC for 30 min and at rt until the HPLC chromatogram showed that the reaction did not evolve further. Then, 1.0 eq of DIPEA was added and the reaction mixture was left stirring overnight. EtOAc (5 mL) was added and the organic solution was washed with saturated aqueous solutions of citric acid (3 × 10 mL), $NaHCO_3$ (3 × 10 mL) and brine (1 × 10 mL). Formation of a precipitate was observed in the organic phase, which was filtered and dried under vacuum to obtain 0.369 g (75 %) of a white solid containing the undecapeptide **14** with a chromatographic purity of 94 % (32.6 min, figure 5.31). **MS (ESI)**: m/z calc. for $[M+2H]^{2+}$ of $C_{141}H_{207}N_{19}O_{27}S_3$ 1349.2; found 1349.2.

Figure 5.31. Using HATU, HOBt and DIPEA[a]

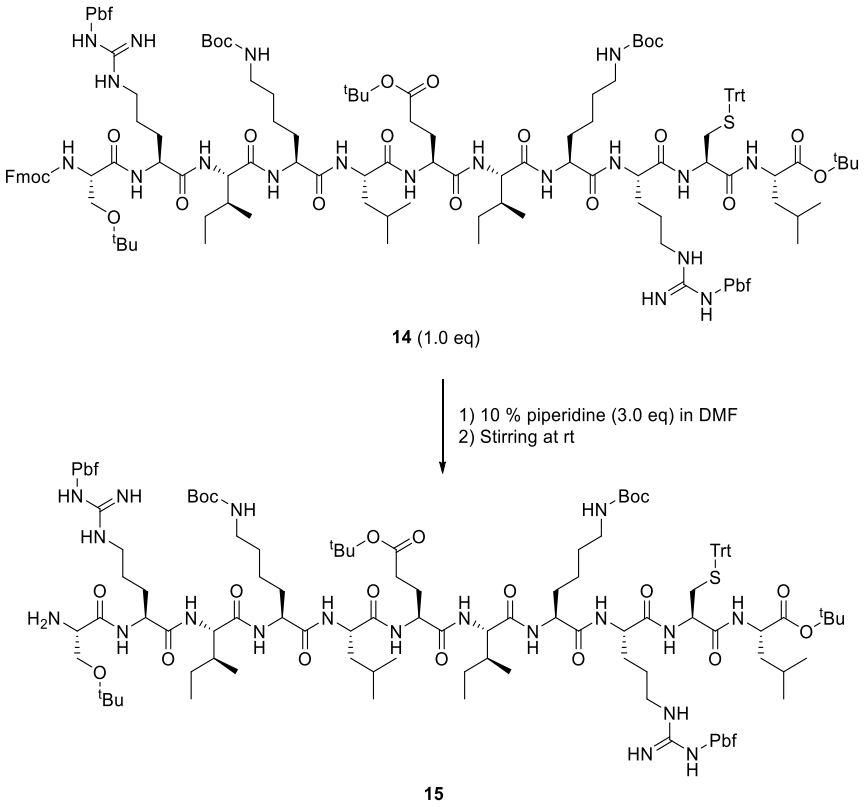
Crude analysis[b]: Batch 14B



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.8.6. Synthesis of 15

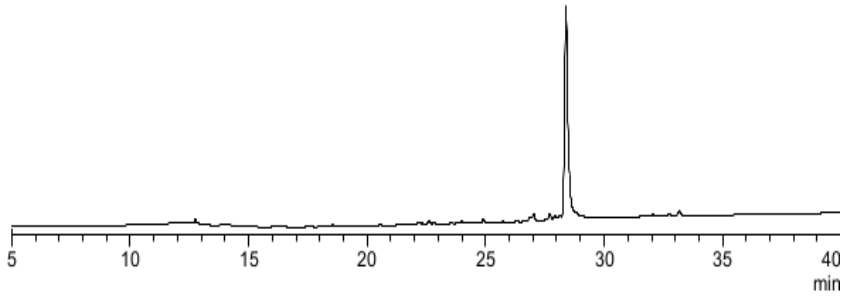
5.8.6.1. Batch 15A



In a 5 mL round-bottom flask with a magnetic stir bar containing 349.5 mg of protected undecapeptide **14** (1.0 eq, section 5.8.5.1.2), a 2 mL solution of 10 % piperidine in DMF was added and left stirring until the HPLC showed that no starting material was observed. At that time, the reaction mixture was transferred with 5 mL of DMF to a conical centrifuge tube and was placed in an ice bath (0 ºC). Then, H_2O (45 mL) was added dropwise to precipitate the product and centrifuged. The supernatant was removed and two more washings with H_2O were carried out. The precipitate was dried under vacuum, triturated with Et_2O (6 × 20 mL) and dried under vacuum to afford 331.0 mg (quantitative) of a yellowish solid containing **15** with a chromatographic purity of 91 % (28.4 min, figure 5.32). MS (ESI): m/z calc. for $[M+2H]^{2+}$ of $C_{126}H_{197}N_{19}O_{25}S_3$ 1237.2; found 1237.3.

Figure 5.32. HPLC chromatogram of 15[a]

Crude analysis[b]

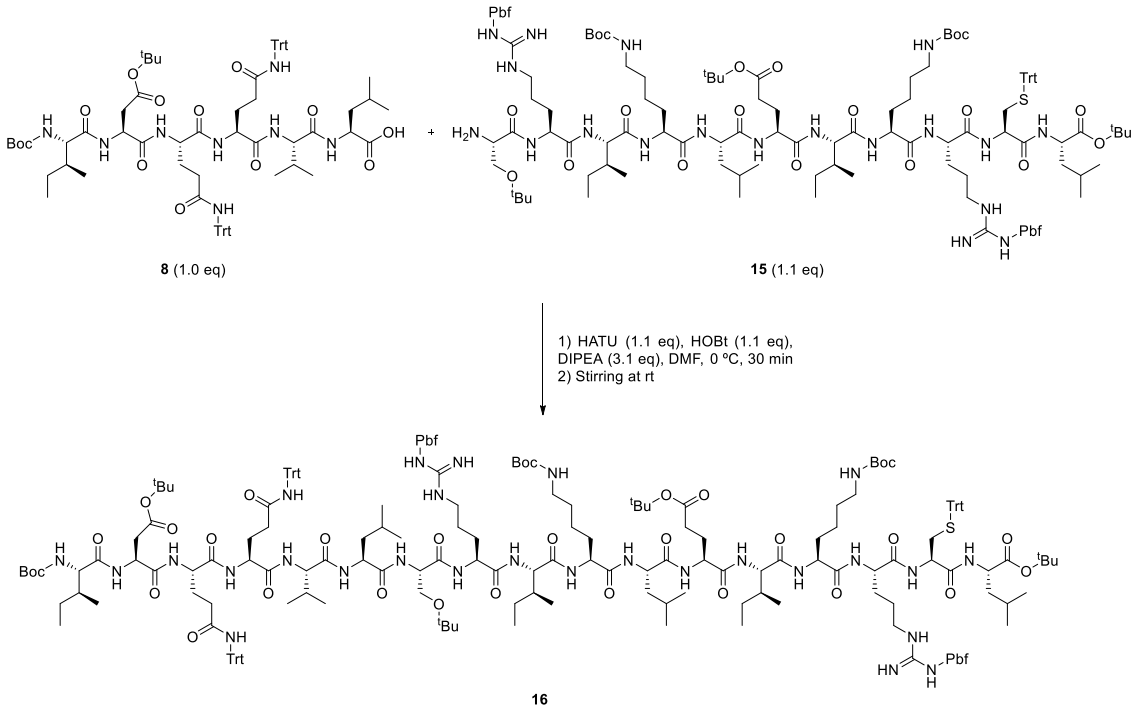


(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.8.7. Synthesis of 16

5.8.7.1. Using HATU, HOBt and DIPEA

5.8.7.1.1. Batch 16A

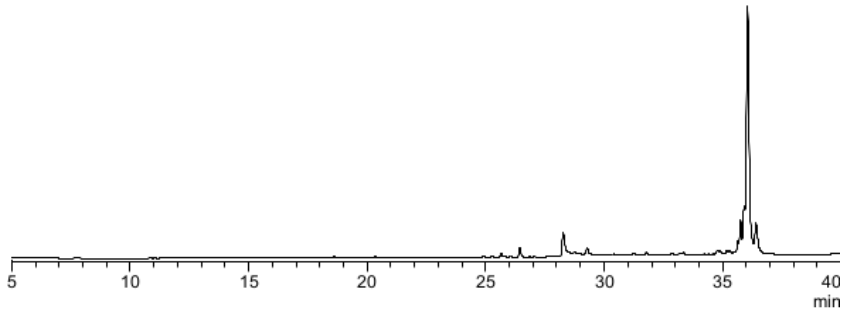


In a 10 mL round bottomed flask, 140.4 mg of the hexapeptide **8** (1.0 eq, section 5.7.5.1), 43.1 mg (1.1 eq) of HATU and 21.6 mg (1.3 eq) of HOBt·H_2O were weighed. Then, 2.0 mL of DMF were added under N_2 atmosphere, the resulting mixture was magnetically stirred until dissolution of reagents and placed in an ice bath (0 ºC). Separately, 282.4 mg of **15** (1.1 eq, section 5.8.6) were weighed in a vial and dissolved in 2.0 mL of DMF under N_2 atmosphere. The resulting solution and 56 μL of DIPEA (3.1 eq) in 0.1 mL of DMF were then added dropwise separately to the round bottom flask. The vial was washed with DMF (2 × 0.5 mL) to ensure complete transfer of **15**. The reaction was left stirring at 0 ºC for 30 min and at rt until the HPLC chromatogram showed that the reaction did not evolve further.

The reaction mixture was precipitated into a conical centrifuge tube with H_2O (30 mL) and centrifuged. The supernatant was removed and two more washings with H_2O were carried out. Finally, the precipitate was lyophilized to obtain 0.410 g of a white solid containing **16** with a chromatographic purity of 62 % (36.0 min, figure 5.33). **MS (ESI)**: m/z calc. for $[M+2H+Na]^{3+}$ of $C_{204}H_{293}N_{27}O_{37}S_3$ 1278.1; found 1279.0.

Figure 5.33. HPLC chromatogram of 16[a]

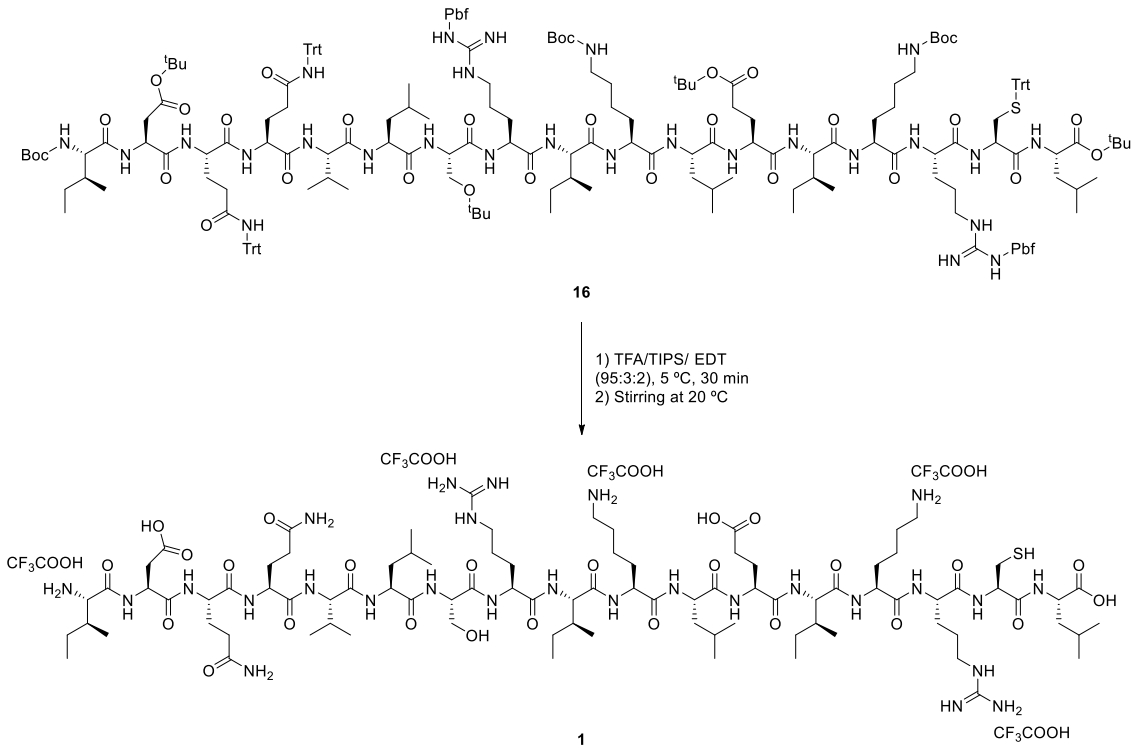
Crude analysis[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.8.8. Synthesis of 1

5.8.8.1. Batch 1B

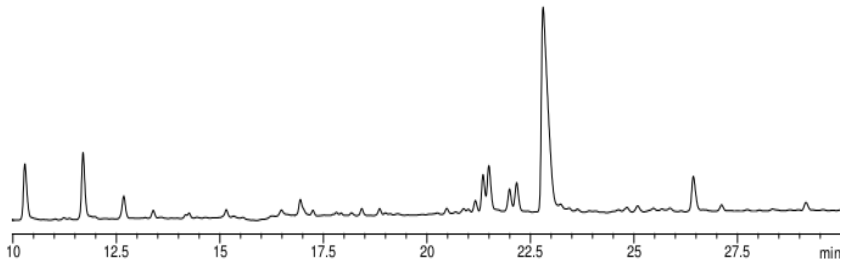


A solution (5 mL) of TFA, TIPS and EDT (95:3:2) was prepared and transferred into a 50 mL reactor vessel containing a magnetic stir bar. The temperature was set to 5 ºC with a thermostat and 0.410 g of **16** (section 5.8.7.1) were added into the reactor vessel. The reaction mixture became yellow and was left at 5 ºC for 30 min and at 20 ºC for 3.5 h. The resulting reddish mixture was cooled to 3 ºC and MTBE (28 mL) was added dropwise with an addition

funnel. A precipitate was formed which was filtered and dried under vacuum to obtain 17.5 mg of a white solid containing **1** with a chromatographic purity of 40 % (22.80 min, figure 5.34). **MS (ESI):** m/z calc. for $[M+H]^+$ of $C_{90}H_{163}N_{27}O_{25}S$ 2055.2; found 2055.2.

Figure 5.34. HPLC chromatogram of 1[a]

Crude analysis[b]



(a) Chromatographic conditions: Method F (Section 5.3.3.3).
(b) Agilent 1200 (6320 Ion Trap), UV detection at 210 nm.

Chapter 3

5.9. Solid-phase peptide synthesis protocols

5.9.1. Amino acid coupling protocols

Tables 5.26 and 5.27 summarise the protocols that were used for the solid-phase synthesis of the protected fragments. The reaction mixtures were stirred mechanically (reactor vessel) at 200 rpm. Coupling conversions were checked by the ninhydrin colorimetric test and re-couplings were carried out if required.

Table 5.26. Amino acid coupling protocol 1.

Step	Treatment	Conditions
1	Washes	DMF/NMP (2 × 1 min), DCM (2 × 1 min) and DMF/NMP (2 × 1 min)
2	Coupling	Fmoc-protected amino acid, HOBt, DIC (3:3:3) in DMF/NMP (1 h)
3	Colorimetric test	Ninhydrin test
4	Washes	DMF/NMP (2 × 1 min), DCM (2 × 1 min) and DMF/NMP (2 × 1 min)
5	Colorimetric test	Ninhydrin test

Table 5.27. Amino acid coupling protocol 2.

Step	Treatment	Conditions
1	Washes	DCM (1 × 30 min), DMF (1 × 1 min), DCM (1 × 1 min) and DMF (1 × 1 min)
2	Coupling	Fmoc-protected amino acid, HOBt, DIC (3:3.3) in DMF (2 h)
3	Colorimetric test	Ninhydrin test
4	Washes	DMF (2 × 1 min), DCM (2 × 1 min), iPrOH (2 × 1 min), DCM (2 × 1 min) and DMF (2 × 1 min)
5	Colorimetric test	Ninhydrin test

5.9.2. Fmoc removal

Tables 5.28 and 5.29 summarise the protocol that were used for the N-terminal deprotection. All treatments were performed at 25 °C and the reaction mixtures were stirred mechanically at 200 rpm.

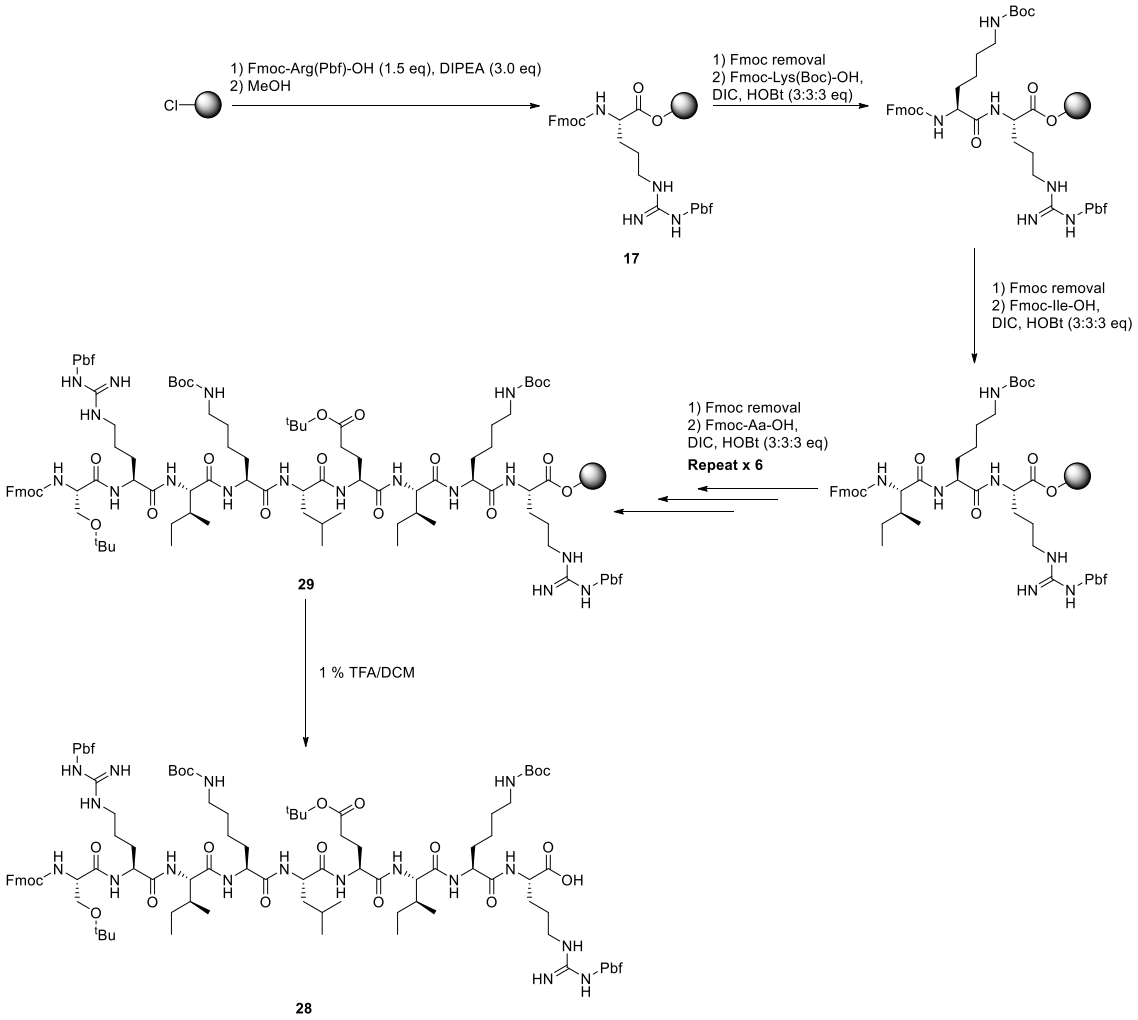
Table 5.28. Fmoc removal protocol 1.

Step	Treatment	Conditions
1	Washes	DMF (2 × 1 min), DCM (2 × 1 min) and DMF (2 × 1 min)
2	Fmoc removal	20 % piperidine in DMF (1 × 5 min and 1 × 10 min)
3	Washes	DMF (2 × 1 min), DCM (2 × 1 min) and DMF (2 × 1 min)

Table 5.29. Fmoc removal protocol 2.

Step	Treatment	Conditions
1	Fmoc removal	20 % piperidine in DMF (1 × 5 min and 1 × 2 min)
2	Washes	DMF (2 × 1 min), DCM (2 × 1 min), iPrOH (2 × 1 min), DCM (2 × 1 min) and DMF (2 × 1 min)

5.9.3. Synthesis of 28



5.9.3.1. From peptidyl-resin 17D

2-CTC resin (7.68 g, 1.40 mmol/g) was added into a 150 mL glass reactor vessel fitted with a porous glass filter disc. The resin was conditioned (section 5.5.2) and the first coupling was performed with 10.4 g (1.5 eq) of Fmoc-Arg(Pbf)-OH and 4.17 g (3.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 1.5 h when 0.8 mL of MeOH/g of resin were added and the resulting reaction mixture was stirred for 30 additional min. The final loading was found to be 0.27 mmol/g (2.51 mmol, section 5.5.3 and 5.5.4). For the peptide elongation, the standard protocols described in table 5.26 (section 5.9.1) and table 5.28 (section 5.9.2) were followed. The amounts of reagents for this synthesis are given in table 5.30. After the cleavage of the peptide from the resin (methodology 3, section 5.5.7), 480.8 mg of crude peptide (yellowish solid) were obtained with a 51 % of chromatographic

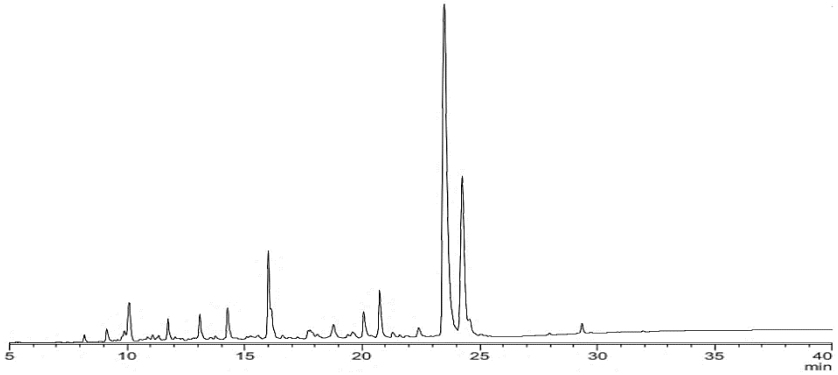
purity (23.5 min, figure 5.35). **MS (ESI)**: m/z calc. for $[M+2H]^{2+}$ of $C_{109}H_{169}N_{17}O_{25}S_2$ 1091.1; found 1091.8.

Table 5.30. Amounts of reagents used for the synthesis of **28** (batch 17D).

Entry	**Amino acid** [g, eq]		**HOBt·H_2O** [g, eq]	**DIC** [mL, eq]	**Reaction time** [h]
1	Fmoc-Lys(Boc)-OH	5.51, 4.6	1.39, 3.6	1.16, 3.0	3
2	Fmoc-Ile-OH	2.78, 3.1	2.21, 5.7	1.16, 3.0	2
3	Fmoc-Glu(^{t}Bu)-OH	3.88, 3.4	1.51, 3.9	1.16, 3.0	2
4	Fmoc-Leu-OH	2.68, 3.0	1.76, 4.6	1.16, 3.0	2
5	Fmoc-Lys(Boc)-OH	3.60, 3.0	1.70, 4.4	1.16, 3.0	2
6	Fmoc-Ile-OH	2.78, 3.1	1.69, 4.4	1.16, 3.0	2
7	Fmoc-Arg(Pbf)-OH	5.22, 3.2	1.73, 4.5	1.16, 3.0	1
8	Fmoc-Ser(^{t}Bu)-OH	3.22, 3.3	1.51, 3.9	1.16, 3.0	2

Figure 5.35. HPLC chromatogram of 28[a]

Crude analysis[b]**:** Batch 17D



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Waters 2695, UV detection at 220 nm.

5.9.3.2. From peptidyl-resin 17E

2-CTC resin (5.23 g, 1.40 mmol/g) was added into a 150 mL glass reactor vessel fitted with a porous glass filter disc. The resin was conditioned (section 5.5.2) and the first coupling was performed with 7.61 g (1.6 eq) of Fmoc-Arg(Pbf)-OH and 2.87 g (3.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 1.5 h when 0.8 mL of MeOH/g of resin were added and the resulting reaction mixture was stirred for 30 additional min. For the peptide elongation, the standard protocols described in table 5.26 (section 5.9.1) and table 5.28 (section 5.9.2) were followed. The N-terminal protecting group was removed when the resin had to be left several hours before coupling a new amino acid. The amounts of reagents for this synthesis are given in table 5.31. A final mass increment of 7.03 g (3.22 mmol) was determined after drying the resin under vacuum. After the cleavage of the peptide from the resin (methodology 3, section 5.5.7), 5.04 g (2.31 mmol) of crude peptide (yellowish solid) were obtained with an 88 % of chromatographic purity (26.6 min, figure 5.36). **MS (ESI)**: m/z calc. for $[M+2H]^{2+}$ $C_{109}H_{169}N_{17}O_{25}S_2$ 1091.1; found 1091.9.

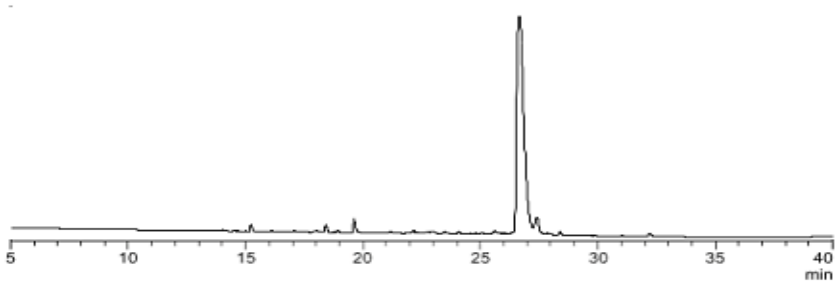
Table 5.31. Amounts of reagents used for the synthesis of **28** (batch 17E).

Entry	Amino acid [g, eq]		HOBt·H_2O [g, eq]	DIC [mL, eq]	Reaction time [h]
1	Fmoc-Lys(Boc)-OH	10.6, 3.0	3.61, 3.2	3.44, 3.0	4
2	Fmoc-Ile-OH	8.57, 3.2 8.10, 3.1	3.26, 2.8 3.34, 2.9	3.44, 3.0	1 1
3	Fmoc-Glu(^{t}Bu)-OH	10.1, 3.1	3.61, 3.2	3.44, 3.0	2
4	Fmoc-Leu-OH	7.97, 3.0	4.18, 3.7	3.44, 3.0	2
5	Fmoc-Lys(Boc)-OH	11.0, 3.2 11.0, 3.2 11.0, 3.2	4.06, 3.5 4.07, 3.5 4.07, 3.5	3.44, 3.0	1 1 1
6	Fmoc-Ile-OH	7.82, 3.0 7.55, 2.8	3.54, 3.1 3.77, 3.3	3.44, 3.0	2 2
7	Fmoc-Arg(Pbf)-OH	14.4, 3.0 14.3, 3.0 14.3, 3.0	3.79, 3.3 3.56, 3.4 4.16, 3.6	3.44, 3.0	2 2 o/n[a]
8	Fmoc-Ser(^{t}Bu)-OH	9.26, 3.3 9.93, 3.5	3.63, 3.2 3.76, 3.3	3.44, 3.0	3 4

(a) o/n: overnight

Figure 5.36. HPLC chromatogram of 28[a]

Crude analysis[b]: Batch 17E



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.9.3.3. From peptidyl-resin 17F

2-CTC resin (4.98 g, 1.40 mmol/g) was added into a 150 mL glass reactor vessel fitted with a porous glass filter disc. The resin was conditioned (section 5.5.2) and the first coupling was performed with 6.61 g (1.5 eq) of Fmoc-Arg(Pbf)-OH and 2.77 g (3.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 1.5 h when 0.80 mL of MeOH/g of resin were added and the resulting reaction mixture was stirred for 30 additional min. The final loading was found to be 0.55 mmol/g (4.15 mmol, sections 5.5.3 and 5.5.4). For the peptide elongation, the standard protocols described in table 5.27 (section 5.9.1) and table 5.29 (section 5.9.2) were followed. All coupling treatments were performed at -5 °C for the 15 initial min of the reaction and then, temperature was raised to 25 °C linearly during 20 min. The N-terminal protecting group was removed when the resin had to be left several hours before coupling a new amino acid. The amounts of reagents for this synthesis are given in table 5.32. After the cleavage of the peptide from the resin (methodology 4, section 5.5.7), 5.25 g (2.41 mmol) of crude peptide (yellowish solid) were obtained with a 79 % of

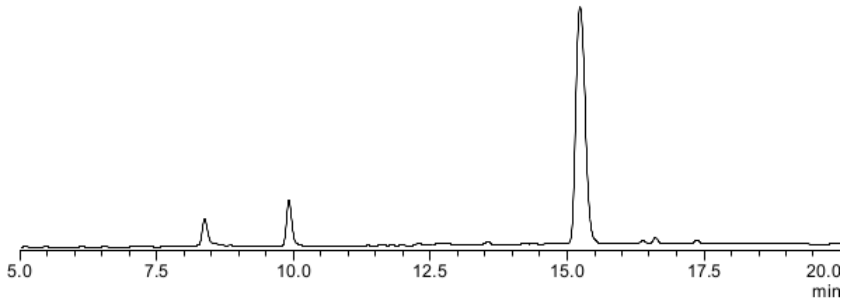
chromatographic purity (15.2 min, figure 5.37). **MS (ESI)**: m/z calc. for $[M+2H]^{2+}$ of $C_{109}H_{169}N_{17}O_{25}S_2$ 1091.1; found 1092.0.

Table 5.32. Amounts of reagents used for the synthesis of **28** (batch 17F).

Entry	**Amino acid** [g, eq]		**HOBt·H_2O** [g, eq]	**DIC** [g, eq]	**Reaction time** [h]
1	Fmoc-Lys(Boc)-OH	6.03, 3.0	1.98, 3.0	1.68, 3.1	3
2	Fmoc-Ile-OH	4.63, 3.0	2.37, 3.6	1.86, 3.4	3
		5.26, 3.5	1.90, 2.9	1.77, 3.3	3
3	Fmoc-Glu(^{t}Bu)-OH	6.11, 3.4	2.93, 4.5	1.79, 3.3	3
		5.87, 3.2	2.33, 3.5	1.65, 3.0	3
4	Fmoc-Leu-OH	5.17, 3.4	2.33, 3.4	1.84, 3.4	4
5	Fmoc-Lys(Boc)-OH	6.15, 3.0	1.92, 2.8	1.86, 3.4	3
		5.59, 2.8	2.03, 3.0	1.99, 3.7	3
6	Fmoc-Ile-OH	4.75, 3.1	2.15, 3.3	1.64, 3.0	4
7	Fmoc-Arg(Pbf)-OH	8.46, 3.0	2.50, 3.8	1.72, 3.2	4
8	Fmoc-Ser(^{t}Bu)-OH	4.88, 3.0	1.96, 3.0	1.96, 3.6	3

Figure 5.37. HPLC chromatogram of 28[a]

Crude analysis[b]**:** Batch 17F

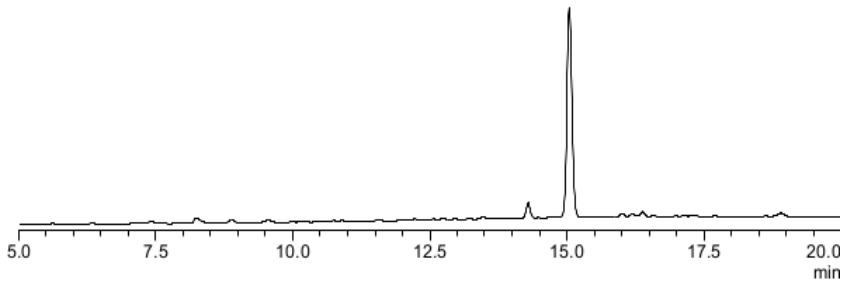


(a) Chromatographic conditions: Method D (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

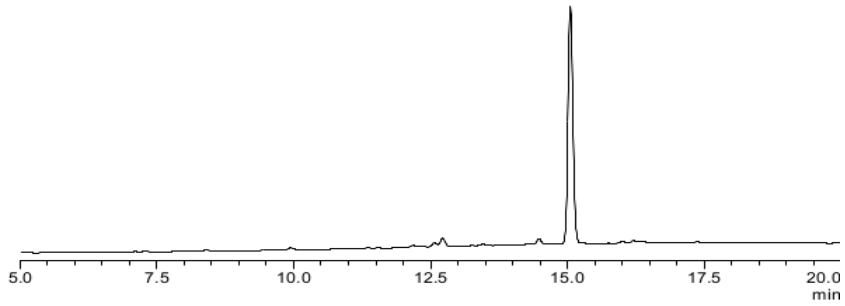
The target peptide **28** (157.6 mg) was purified by flash column chromatography with DCM/MeOH (9:1) containing 1 % of formic acid as eluents (dry loading). Two fractions were collected and solvents were removed by concentrating under reduced pressure. In the first fraction, 42.2 mg of peptide were obtained with a chromatographic purity of 80 % (15.0 min, figure 5.38) and 69.0 mg of peptide were obtained in the second one, with a chromatographic purity of 87 % (15.1 min, figure 5.38).

Figure 5.38. Purified by flash column chromatography[a]

Isolated product[b]**:** First fraction



Isolated product[b]**:** Second fraction



(a) Chromatographic conditions: Method D (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.9.3.4. From peptidyl-resin 17G

2-CTC resin (5.02 g, 1.40 mmol/g) was added into a 150 mL glass reactor vessel fitted with a porous glass filter disc. The resin was conditioned (section 5.5.2) and the first coupling was performed with 6.92 g (1.5 eq) of Fmoc-Arg(Pbf)-OH and 2.75 g (3.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 1.5 h when 0.80 mL of MeOH/g of resin were added and the resulting reaction mixture was stirred for 30 additional min. The final loading was found to be 0.45 mmol/g (3.80 mmol, sections 5.5.3 and 5.5.4). The standard protocols described in table 5.27 (section 5.9.1) and table 5.29 (section 5.9.2) were followed for the peptide elongation except for the amino acids corresponding to entries 5, 6 and 7 (table 5.33) for which the number of eq were increased. All coupling reactions were performed at 5 °C for the 15 initial min of the reaction and then, temperature was raised to 25 °C linearly during 20 min. The N-terminal protecting group was removed when the resin had to be left several hours before coupling a new amino acid. The amounts of reagents for this synthesis are given in table 5.33. After the cleavage of the peptide from the resin (section 5.9.3.4.1), 4.81 g (2.20 mmol, 58 %) of crude peptide (white solid) were obtained with a chromatographic purity between 90 % and 95 % (26.5 min, figure 5.39 and 5.40). **MS (ESI)**: m/z calc. for $[M+2H]^{2+}$ of $C_{109}H_{169}N_{17}O_{25}S_2$ 1091.1; found 1091.9.

Table 5.33. Amounts of reagents used for the synthesis of **28** (batch 17G).

Entry	Amino acid [g, eq]		HOBt·H_2O [g, eq]	DIC [g, eq]	Reaction time [h]
1	Fmoc-Lys(Boc)-OH	5.69, 3.1	1.93, 3.2	1.53, 3.1	5
2	Fmoc-Ile-OH	6.02, 3.3	2.57, 3.2	2.08, 3.2	3
3	Fmoc-Glu(^{t}Bu)-OH	6.66, 3.0	3.53, 4.4	2.07, 3.1	4
4	Fmoc-Leu-OH	4.74, 3.4	2.14, 3.5	1.52, 3.0	4
5	Fmoc-Lys(Boc)-OH	7.56, 4.1	2.60, 4.3	2.06, 4.2	4
6	Fmoc-Ile-OH	5.87, 4.2	2.17, 3.6	2.06, 4.2	3
7	Fmoc-Arg(Pbf)-OH	10.8, 4.2 9.72, 3.8	2.64, 3.9 2.09, 3.5	2.01, 4.1 2.07, 4.2	3 3
8	Fmoc-Ser(^{t}Bu)-OH	5.00, 3.3 4.33, 2.9	2.02, 3.4 2.22, 3.7	1.60, 3.2 1.72, 3.5	4 2

5.9.3.4.1. Study of a new peptidyl-resin cleavage methodology

Assay 1. In a polypropylene syringe fitted with a polyethylene filter disc, 1.125 g of peptidyl-resin from batch 17G, were weighed and washed with DCM (3 × 10 mL). Then, 10 mL of a 1 % TFA/DCM solution were added and stirred manually for 5 min. At that time, the solution was filtered to a round-bottom flask that contained 40 mL of Et_2O and pyridine (1.2 eq relative to TFA) and the formation of a precipitate was observed. The procedure was repeated two more times and the resin was washed with 20 mL of DCM that were collected by suction in the round-bottom flask. The suspension was filtered, washed with H_2O (3 × 100 mL) and Et_2O (4 × 50 mL), and dried under reduced pressure. The desired product (0.519 g) was obtained with a chromatographic purity of 94 % (figure 5.39).

Assay 2. In a polypropylene syringe fitted with a polyethylene filter disc, 1.227 g of peptidyl-resin from batch 17G, were weighed and washed with DCM (3 × 10 mL). Then, 10 mL of a 1 % TFA/DCM solution were added and stirred manually for 5 min. At that time, the solution was filtered to a round-bottom flask that contained 40 mL of Et_2O and TEA (1.2 eq relative to TFA) and the formation of a precipitate was observed. The procedure was repeated two more times and the resin was washed with 20 mL of DCM that were collected by suction in the round-bottom flask. The suspension was filtered, washed with H_2O (3 × 100 mL) and Et_2O (4 × 50 mL), and dried under reduced pressure. The desired product (0.612 g) was obtained with a chromatographic purity of 95 % (figure 5.39).

Assay 3. In a polypropylene syringe fitted with a polyethylene filter disc, 1.052 g of peptidyl-resin from batch 17G, were weighed and washed with DCM (3 × 10 mL). Then, 10 mL of a 1 % TFA/DCM solution were added and stirred manually for 5 min. At that time, the solution was filtered to a round-bottom flask that contained 40 mL of MTBE and TEA (1.2 eq relative to TFA) and the formation of a precipitate was observed. The procedure was repeated two more times and the resin was washed with 20 mL of DCM that were collected by suction in the round-bottom flask. The suspension was filtered, washed with H_2O (3 × 100 mL) and MTBE (4 × 50 mL), and dried under reduced pressure. The desired product (0.534 g) was obtained with a chromatographic purity of 94 % (figure 5.39).

Assay 4. In a polypropylene syringe fitted with a polyethylene filter disc, 1.038 g of peptidyl-resin from batch 17G, were weighed and washed with DCM (3 × 10 mL). Then, 10 mL of a 1 % TFA/DCM solution were added and stirred manually for 5 min. At that time, the solution was filtered to a round-bottom flask that contained 40 mL of diisopropyl ether and TEA (1.2 eq relative to the TFA) and the formation of a precipitate was observed. The

procedure was repeated two more times and the resin was washed with 20 mL of DCM that were collected by suction in the round-bottom flask. The suspension was filtered, washed with H_2O (3 × 100 mL) and diisopropyl ether (4 × 50 mL), and dried under reduced pressure. The desired product (0.676 g) was obtained with a chromatographic purity of 91 % (figure 5.39).

Assay 5. In a polypropylene syringe fitted with a polyethylene filter disc, 1.183 g of peptidyl-resin from batch 17G, were weighed and washed with DCM (3 × 10 mL). Then, 10 mL of a 1 % TFA/DCM solution were added and stirred manually for 5 min. At that time, the solution was filtered to a round-bottom flask that contained 40 mL of DCM and TEA (1.2 eq relative to TFA). The procedure was repeated two more times and the resin was washed with 20 mL of DCM that were collected by suction in the round-bottom flask. The solution was concentrated under reduced pressure until a volume of 40 mL when Et_2O (150 mL) was added dropwise with an addition funnel. The resulting suspension was filtered, washed with H_2O (3 × 100 mL) and Et_2O (4 × 50 mL), and dried under reduced pressure. The desired product (0.599 g) was obtained with a chromatographic purity of 92 % (figure 5.39).

Assay 6. In a polypropylene syringe fitted with a polyethylene filter disc, 1.042 g of peptidyl-resin from batch 17G, were weighed and washed with DCM (3 × 10 mL). Then, 10 mL of a 1 % TFA/DCM solution were added and stirred manually for 5 min. At that time, the solution was filtered to a round-bottom flask that contained 40 mL of THF and TEA (1.2 eq relative to TFA). The procedure was repeated two more times and the resin was washed with 20 mL of DCM that were collected by suction in the round-bottom flask. The solution was concentrated under reduced pressure until a volume of 40 mL when H_2O (80 mL) was added dropwise with an addition funnel. The resulting suspension was filtered, washed with Et_2O (50 mL × 4), and dried under reduced pressure. The desired product (0.335 g) was obtained with a chromatographic purity of 90 % (figure 5.39).

Figure 5.39. Study of a new cleavage methodology[a]

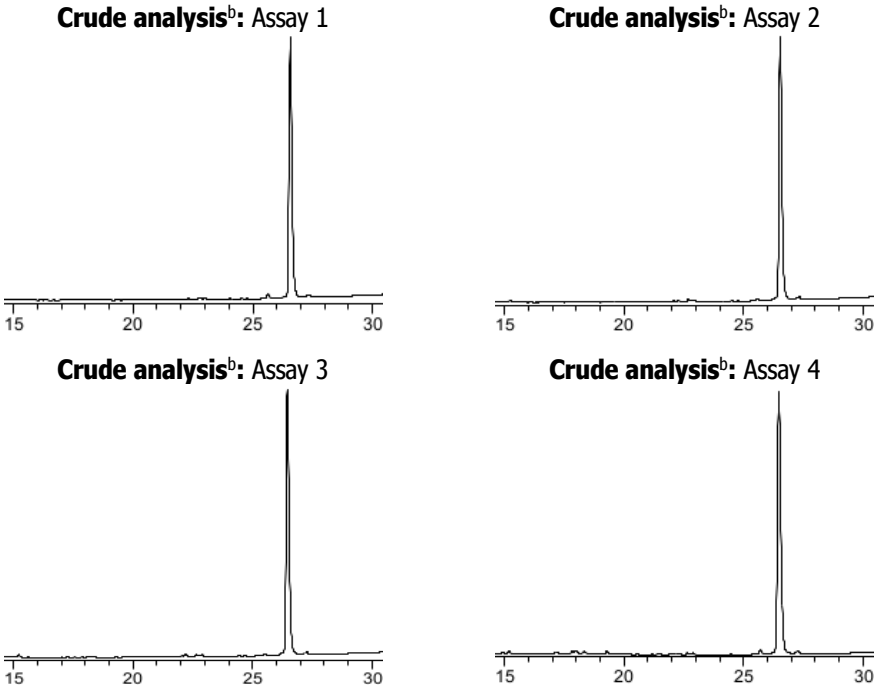
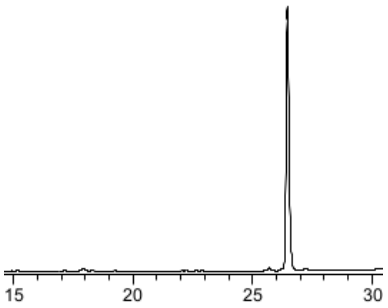
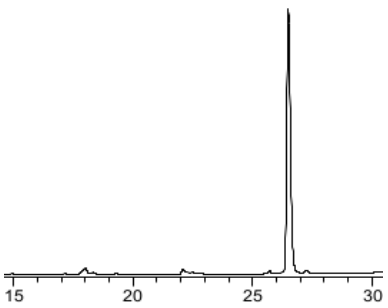


Figure 5.39 (continued)

Crude analysis[b]: Assay 5



Crude analysis[b]: Assay 6



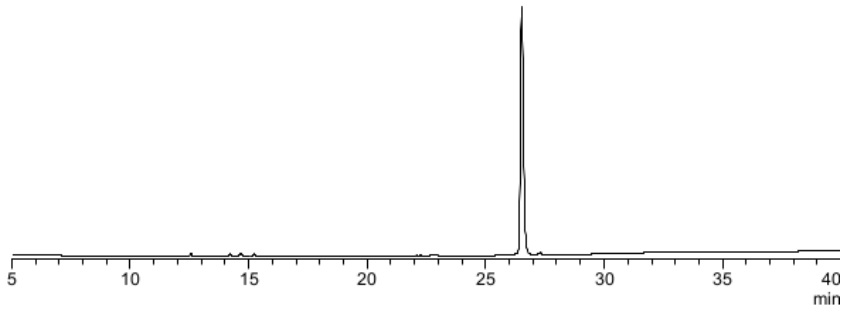
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

Other syntheses:

Cleavage under conditions of assay 2 (section 5.9.3.4.1). In a polypropylene syringe fitted with a polyethylene filter disc, 2.946 g of peptidyl-resin from batch 17G were weighed and washed with DCM (3 × 10 mL). Then, 30 mL of a 1 % TFA/DCM solution were added and stirred manually for 5 min. At that time, the solution was filtered to a round-bottom flask that contained 120 mL of Et_2O and TEA (1.2 eq relative to TFA) and the formation of a precipitate was observed. The procedure was repeated two more times and the resin was washed with 20 mL of DCM that were collected by suction in the round-bottom flask. The suspension was filtered, washed with H_2O (3 × 100 mL) and Et_2O (4 × 50 mL), and dried under reduced pressure. The desired product (1.533 g) was obtained with a chromatographic purity of 94 % (figure 5.40).

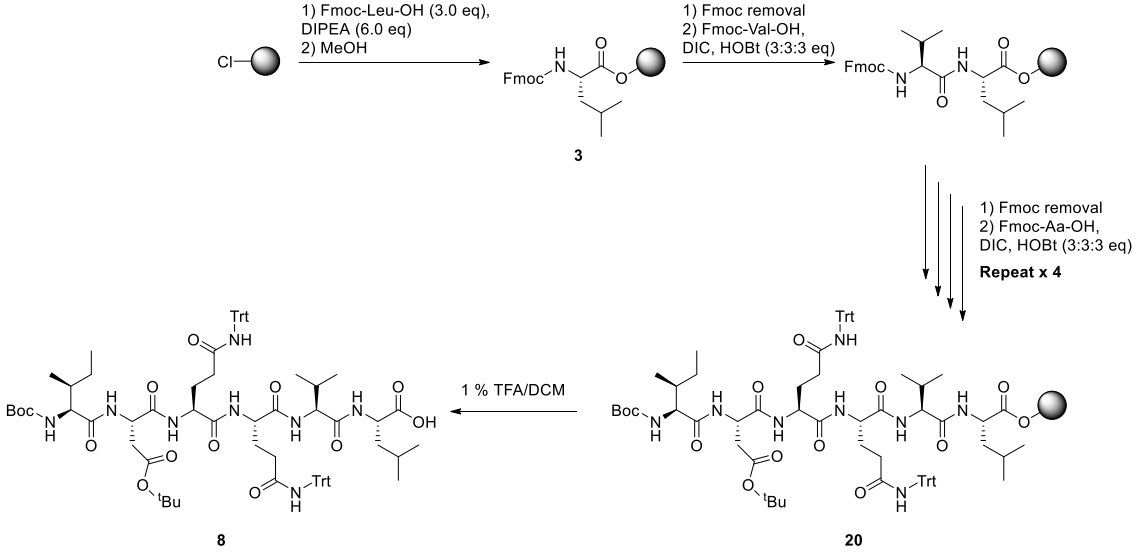
Figure 5.40. Cleavage under conditions of assay 2[a]

Crude analysis[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.9.4. Synthesis of 8



5.9.4.1. From peptidyl-resin 3E

2-CTC resin (10.2 g, 1.40 mmol/g) was added into a 150 mL glass reactor vessel fitted with a porous glass filter disc. The resin was conditioned (section 5.5.2) and the first coupling was performed with 7.96 g (1.57 eq) of Fmoc-Leu-OH and 5.58 g (3.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 1.5 h when 0.80 mL of MeOH/g of resin were added and the resulting reaction mixture was stirred for 30 additional min. The final loading was found to be 0.72 mmol/g (9.53 mmol, sections 5.5.3 and 5.5.4). The standard protocols described in table 5.26 (section 5.9.1) and table 5.28 (section 5.9.2) were followed for the peptide elongation except for the last two amino acids (table 5.34, entries 4 and 5) for which the coupling reagent was changed to HATU. The amounts of reagents for this synthesis are given in table 5.34. After the cleavage of the peptide from the resin (methodology 3, section 5.5.7), 5.65 g (4.16 mmol) of crude peptide was obtained as a white solid with a chromatographic purity of 85 % (25.0 min, figure 5.41). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{78}H_{98}N_8O_{13}$ 1355.7; found 1356.1.

Table 5.34. Amounts of reagents used for the synthesis of **8** (batch 3E).

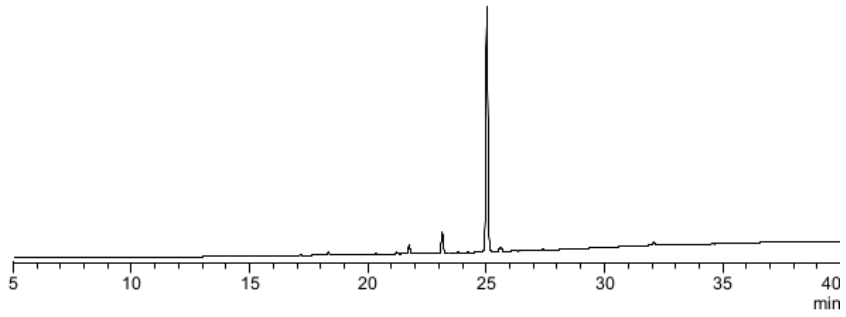
Entry	**Amino acid** [g, eq]		**HOBt·H₂O** [g, eq]	**DIC** [g, eq]	**Reaction time** [h]
1	Fmoc-Val-OH	9.91, 3.0	4.53, 3.0	3.90, 3.2	4
2	Fmoc-Gln(Trt)-OH	18.0, 3.0 16.9, 2.8	4.58, 3.0 4.51, 3.0	3.83, 3.1 3.61, 2.9	4 3
3	Fmoc-Gln(Trt)-OH	17.9, 3.0 17.0, 2.9	4.75, 3.2 4.81, 3.2	3.87, 3.1 4.18, 3.4	4 4
Entry	**Amino acid** [g, eq]		**HATU** [g, eq]	**DIPEA** [g, eq]	**Reaction time** [h]
3	Fmoc-Gln(Trt)-OH	16.4, 2.8	11.3, 3.0	3.90, 3.1	4
4	Fmoc-Asp(^{t}Bu)-OH	12.4, 3.1 12.1, 3.0	11.2, 3.0 11.6, 3.1	3.61, 2.8 3.72, 2.9	4 2

Table 5.34 (continued)

5	Boc-Ile-OH	6.72, 3.0	11.3, 3.0	3.97, 3.1	3
		7.51, 3.4	14.3, 3.9	4.01, 3.2	2
		6.92, 3.1	12.3, 3.3	3.52, 2.8	4
		6.50, 2.9	11.2, 3.0	4.06, 3.2	2

Figure 5.41. HPLC chromatogram of 8[a]

Crude analysis[b]: Batch 3E



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.9.4.2. From peptidyl-resin 3F

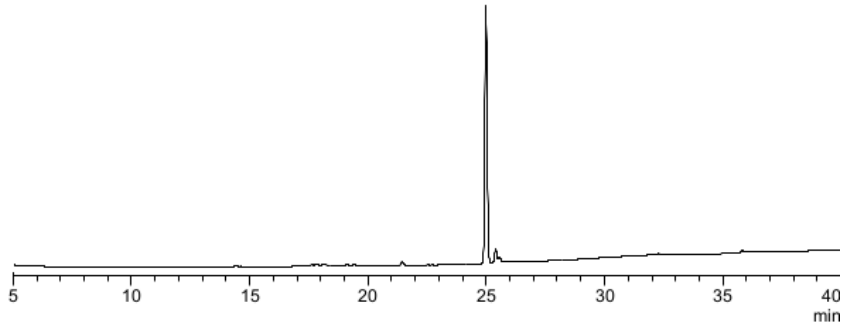
2-CTC resin (5.39 g, 1.40 mmol/g) was added into a 150 mL glass reactor vessel fitted with a porous glass filter disc. The resin was conditioned (section 5.5.2) and the first coupling was performed with 4.14 g (1.50 eq) of Fmoc-Leu-OH and 2.95 g (3.0 eq) of DIPEA in DCM. The reaction mixture was stirred for 1.5 h when 0.80 mL of MeOH/g of resin were added and the resulting reaction was stirred for 30 additional min. The final loading was found to be 0.59 mmol/g (3.91 mmol, sections 5.5.3 and 5.5.4). The standard protocols described in table 5.27 (section 5.9.1) and table 5.29 (section 5.9.2) were followed for the peptide elongation. All coupling reactions were performed at 5 °C for the 15 initial min of the reaction and then, temperature was raised to 25 °C linearly during 20 min. The N-terminal protecting group was removed when the resin had to be left several hours before coupling a new amino acid. The amounts of reagents for this synthesis are given in table 5.35. After the cleavage of the peptide from the resin (assay 5, section 5.9.3.4.1), 5.23 g (3.85 mmol, 98 %) of crude peptide was obtained as a white solid with a chromatographic purity of 91 % (25.0 min, figure 5.42). **MS (ESI)**: m/z calc. for $[(M+H)]^+$ of $C_{78}H_{98}N_8O_{13}$ 1355.7; found 1356.0.

Table 5.35. Amounts of reagents used for the synthesis of **8** (batch 3F).

Entry	**Amino acid** [g, eq]		**HOBt·H_2O** [g, eq]	**DIC** [g, eq]	**Reaction time** [h]
1	Fmoc-Val-OH	4.05, 2.9	2.08, 3.4	1.76, 3.4	3
2	Fmoc-Gln(Trt)-OH	7.54, 3.1	3.30, 5.5	1.74, 3.4	2
3	Fmoc-Gln(Trt)-OH	7.14, 2.9	1.83, 2.8	1.78, 3.5	3
4	Fmoc-Asp(^{t}Bu)-OH	4.99, 3.0	2.08, 3.4	1.71, 3.3	2
5	Boc-Ile-OH	2.76, 2.9	2.08, 3.4	1.67, 3.3	3

Figure 5.42. HPLC chromatogram of 8[a]

Crude analysis[b]**:** Batch 3F



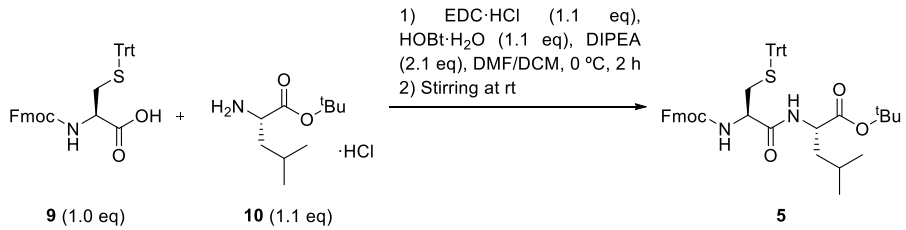
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.9.4.2.1. Slurry of 8 with an aqueous solution of 2.0 N of NaH_2PO_4 pH = 3

In a reactor vessel of 100 mL provided with a stir bar were mixed 541.3 mg of the hexapeptide **8** (from batch 3F) in 50 mL of 2-MeTHF. Then, 35 mL of a 2.0 N aq NaH_2PO_4 solution (pH 3) were added and left stirring for 10 min. The aqueous phase was separated and the procedure was repeated twice. The organic phase was washed twice with 20 mL of H_2O and the organic solvent was reduced under vacuum. Precipitation was achieved with the dropwise addition of 55 mL of H_2O. The suspension was filtered and dried under vacuum to afford 492.4 mg of the desired product was obtained.

5.10. Solution-phase peptide synthesis

5.10.1. Synthesis of 5



9 (1.0 eq) **10** (1.1 eq) **5**

In a 25 mL round-bottom flask with a magnetic stir bar, **9** and **10** were mixed in 10 mL of DMF or DCM until achieving a clear solution and placed in an ice bath. After 10 min, HOBt·H_2O was added followed by the addition of EDC·HCl. Then, DIPEA was added dropwise with an automatic injector during 105 min. The solution was left stirring on the ice bath for 15 additional min and the ice bath was removed. The reaction was monitored by HPLC-UV and after 4 h, the reaction mixture was transferred to a 250 mL separating funnel containing EtOAc (30 mL) and washed with saturated aqueous solution of citric acid (3 × 30 mL), saturated aqueous solution of $NaHCO_3$ (3 × 30 mL) and H_2O (3 × 30 mL). The organic fraction was dried with anhydrous $MgSO_4$, filtered and concentrated under reduced pressure.

5.10.1.1. Batch 5B

9 (3.863 g, 1.0 eq), **10** (1.646 g, 1.1 eq), HOBt·H_2O (1.206g, 1.2 eq), EDC·HCl (1.439 g, 1.3 eq), DIPEA (1.836 g, 2.2 eq) and 10 mL of DMF. 4.979 g (99 %) of the desired product were obtained as a white solid with a chromatographic purity of 96 % (25.5 min, figure 5.43). **MS (ESI)**: m/z calc. for $[M+Na]^+$ of $C_{47}H_{50}N_2O_5S$ 777.3; found 777.4.

5.10.1.2. Batch 5C

9 (3.871 g, 1.0 eq), **10** (1.626 g, 1.1 eq), HOBt·H_2O (1.144g, 1.1 eq), EDC·HCl (1.420 g, 1.1 eq), DIPEA (1.844 g, 2.2 eq) and 10 mL of DCM. 4.655 g of the desired product were obtained as a white solid with a chromatographic purity of 87 % (25.5 min, figure 5.43), containing 5 % of starting material **9** (21.9 min, figure 5.43). **MS (ESI)**: m/z calc. for $[M+Na]^+$ of $C_{47}H_{50}N_2O_5S$ 777.3; found 777.4.

Figure 5.43. HPLC chromatograms of 5[a]

Crude analysis[b]: Batch 5B

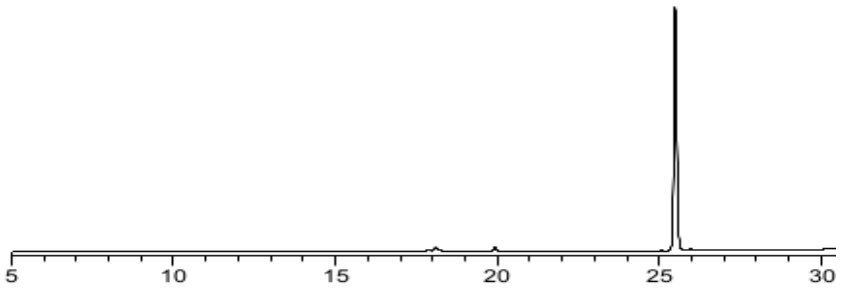
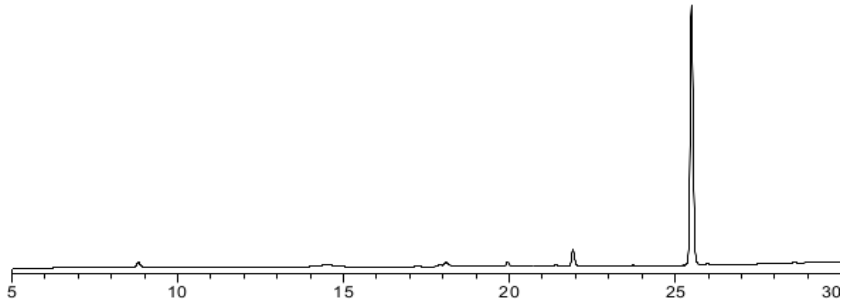


Figure 5.43 (continued)

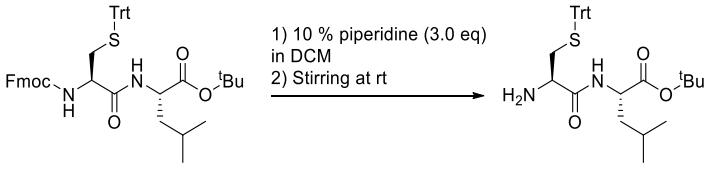
Crude analysis[b]: Batch 5C



(c) Chromatographic conditions: Method B (Section 5.3.3.3).
(d) Shimadzu, UV detection at 220 nm.

5.10.2. Synthesis of 11

5.10.2.1. Batch 11C

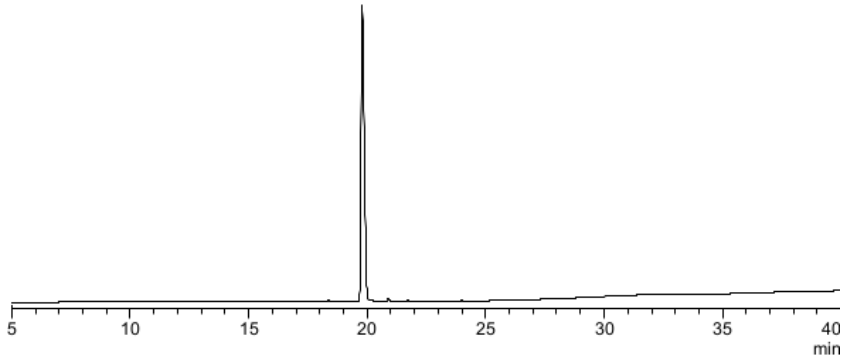


5 (1.0 eq) **11**

In a 25 mL round-bottom flask with a magnetic stir bar, **5** (0.823 g, 1.0 eq) was mixed with a solution of 10 % piperidine (0.291 g, 3.1 eq) in DCM (2.7 mL) and left stirring. The reaction was monitored by HPLC and after 2.5 h the reaction was finished. The reaction mixture was then transferred to a 250 mL separating funnel containing 10 mL of DCM/g of staring material and washed three times with 10 mL of H_2O/g of starting material. The organic fraction was dried with anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The crude of **11** was purified by flash column chromatography with Hexanes/EtOAc 8:2 and DCM/MeOH 9:1 as eluents (dry loading). **11** was isolated by concentrating the purified fractions under reduced pressure. 0.490 g (84 %) of the desired **11** were obtained as a yellowish solid with a chromatographic purity of 99 % (19.8 min, figure 5.44). **MS (ESI)**: m/z calc. for $[M+H]^+$ of $C_{32}H_{40}N_2O_3S$ 533.3; found 533.3.

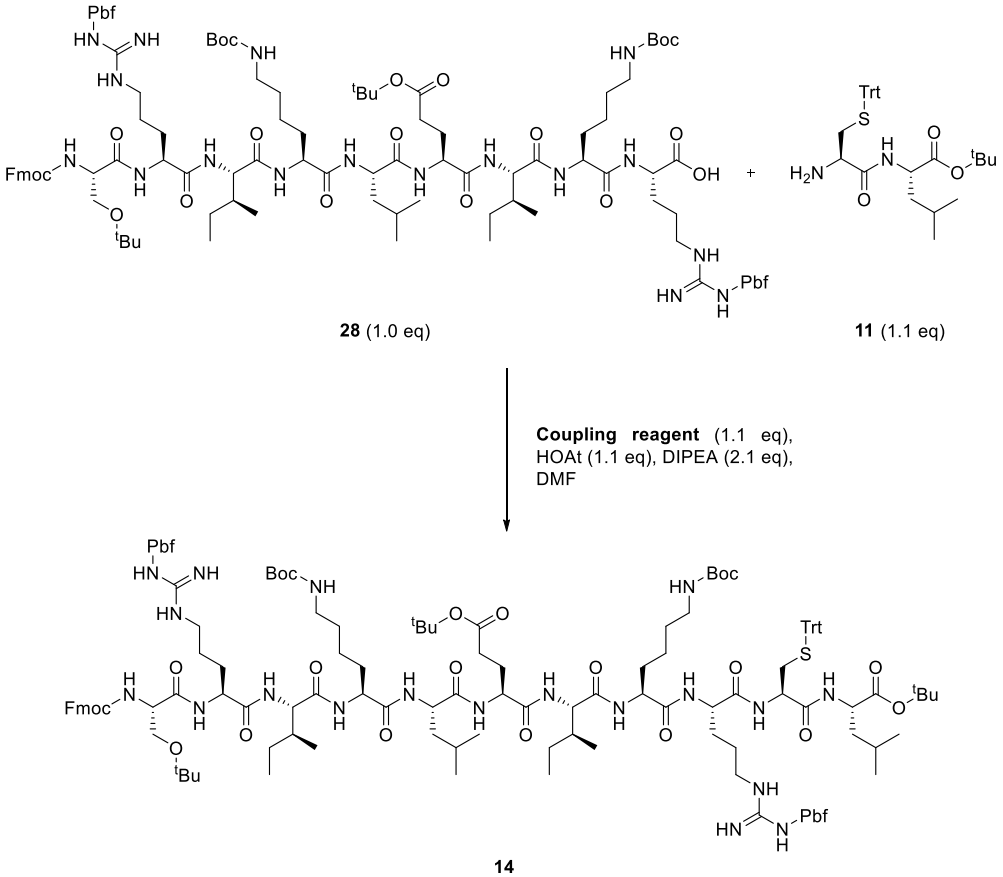
Figure 5.44. HPLC chromatogram of 11[a]

Isolated product[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.10.3. Synthesis of 14



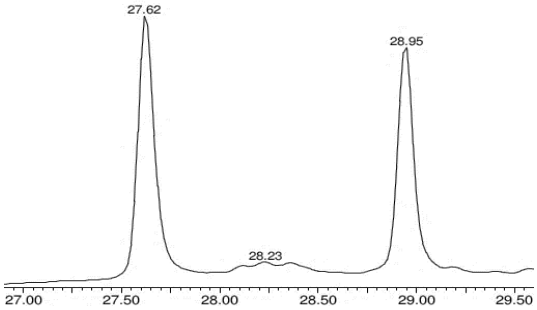
5.10.3.1. Test of epimerisation during fragment condensation

In a 10 mL round-bottom flask with a magnetic stir bar were mixed in 4.5 mL of DMF the protected nonapeptide **28** (295.0 mg, 1.0 eq), HOAt (21.2 mg, 1.1 eq) and HATU (65.4 mg, 1.3 eq). Once the solution was clear, DIPEA (38.7 mg, 2.2 eq) was weighted over 0.5 mL of DMF and added to the solution at rt. The resulting mixture was left for 16 h at room temperature under magnetic stirring when **11** (102.9 mg, 1.4 eq) was added and the resulting mixture was left for 2 h. Then, the crude reaction was transferred to a 250 mL round-bottom

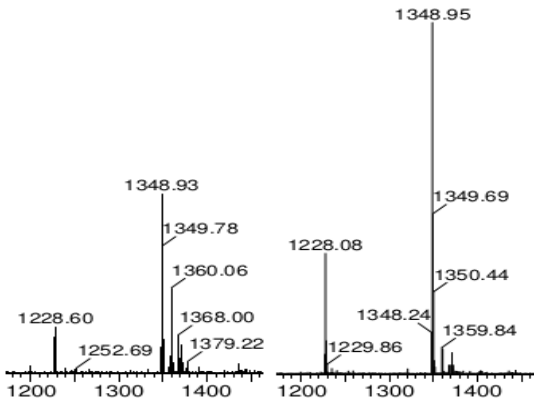
flask containing a magnetic stir bar and 5 mL of DMF, and placed in an ice bath where the product was precipitated by adding dropwise H_2O (90 mL) with an addition funnel. The white solid was filtered, washed with Et_2O (3 × 20 mL) and dried under vacuum. 251.6 mg (69 %) of a diastereomeric mixture of **14** (55:45) were obtained (figure 5.45). **MS (ESI)**: m/z calc. for $[M+2H]^{2+}$ of $C_{141}H_{207}N_{19}O_{27}S_3$ 1349.2; found 1348.9.

Figure 5.45. Forcing epimerisation[a]

Isolated product[b]



MS spectra[b]



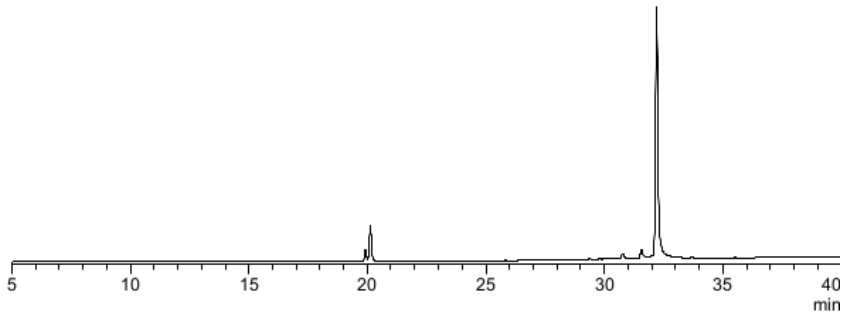
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Waters 2695, UV detection at 220 nm.

5.10.3.2. Using EDC·HCl, HOAt and DIPEA

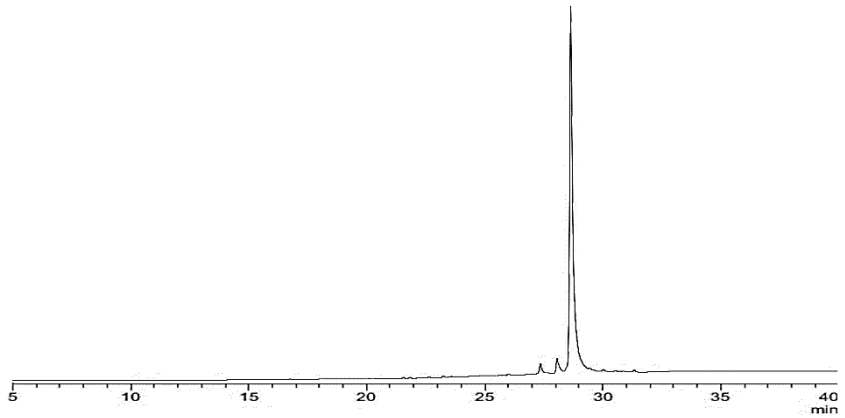
In a 10 mL round-bottom flask with a magnetic stir bar were mixed in 4.5 mL of DMF the protected nonapeptide **28** (368.5 mg, 1.0 eq), **11** (103.6 mg, 1.1 eq) and HOAt (27.5 mg, 1.1 eq). Once the solution was clear, it was placed in a MeOH/ice bath (-20 ºC) and EDC·HCl (37.7 mg, 1.1 eq) was added after 10 min. Then, DIPEA (26.5 mg, 1.1 eq) was weighted over 0.5 mL of DMF and added dropwise with an automatic injector (30 min). The reaction was monitored by HPLC-UV and no starting material **28** was observed after 3 h of reaction time. At that time, the reaction mixture was transferred to a 250 mL round-bottom flask containing a magnetic stir bar and 5 mL of DMF, and placed in an ice bath where the product was precipitated by adding dropwise H_2O (90 mL) with an addition funnel. The white solid was filtered, washed with Et_2O (3 × 20 mL) and dried under vacuum. 403.6 mg (89 %) of the desired **14** were obtained with a chromatographic purity of 94 % (28.7 min, figure 5.46). The diastereomeric ratio measured at λ = 220 nm was 98.2:1.8. **MS (ESI)**: m/z calc. for $[M+2H]^{2+}$ of $C_{141}H_{207}N_{19}O_{27}S_3$ 1349.2; found 1349.5.

Figure 5.46. Using EDC·HCl, HOAt and DIPEA[a]

In control process[b] (t = 3 h)



Crude analysis[c]



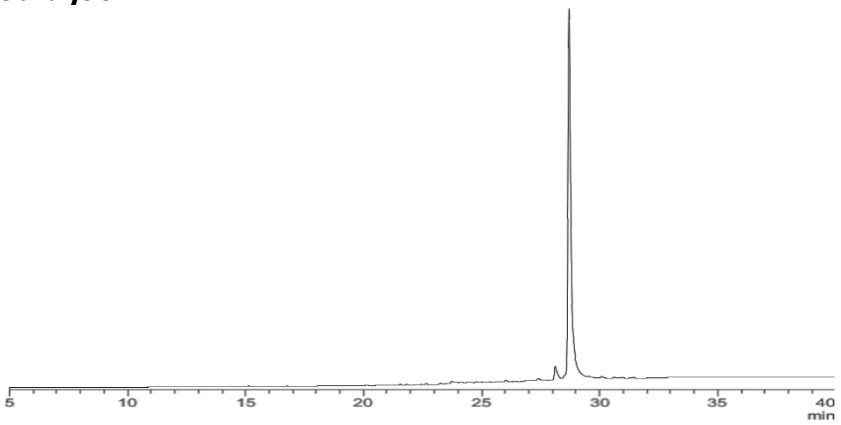
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.
(c) Waters 2795, UV detection at 220 nm.

5.10.3.3. Using PyAOP, HOAt and DIPEA

In a 10 mL round-bottom flask with a magnetic stir bar were mixed in 4.5 mL of DMF the protected nonapeptide **28** (381.4 mg, 1.0 eq) and **11** (103.1 mg, 1.1 eq). Once the solution was clear, it was placed in a MeOH/ice bath (-20 ºC) and PyAOP (102.6 mg, 1.1 eq) was added after 10 min. Then, DIPEA (51.3 mg, 2.1 eq) was weighted over 0.5 mL of DMF and added dropwise with an automatic injector (30 min). The reaction was monitored by HPLC-UV and no starting material **28** was observed after 1 h. At that time, the reaction mixture was transferred to a 250 mL round-bottom flask containing a magnetic stir bar and 5 mL of DMF, and placed in an ice bath where the product was precipitated by adding dropwise H_2O (90 mL) with an addition funnel. The white solid was filtered, washed with Et_2O (3 × 20 mL) and dried under vacuum. 408.0 mg (87 %) of the desired **14** were obtained with a chromatographic purity of 95 % (28.7 min, figure 5.47). The diastereomeric ratio measured at λ = 220 nm was 99.5:0.5. **MS (ESI)**: m/z calc. for $[M+2H]^{2+}$ of $C_{141}H_{207}N_{19}O_{27}S_3$ 1349.2; found 1349.0.

Figure 5.47. Using PyAOP, HOAt and DIPEA[a]

Crude analysis[b]



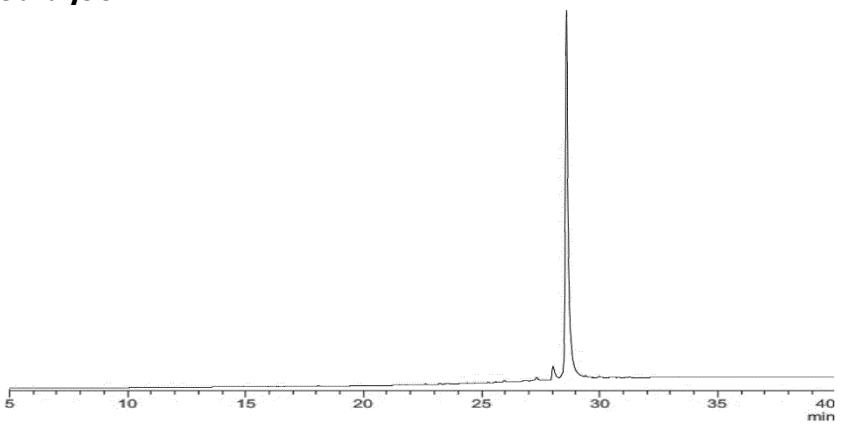
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Waters 2795, UV detection at 220 nm.

5.10.3.4. Using HCTU, HOAt and DIPEA

In a 10 mL round-bottom flask with a magnetic stir bar were mixed in 4.5 mL of DMF the protected nonapeptide **28** (369.4 mg, 1.0 eq), **11** (105.3 mg, 1.1 eq) and HOAt (27.3 mg, 1.1 eq). Once the solution was clear, it was placed in a MeOH/ice bath (-20 ºC) and HCTU (81.2 mg, 1.1 eq) was added after 10 min. Then, DIPEA (48.2 mg, 2.1 eq) was weighted over 0.5 mL of DMF and added dropwise with an automatic injector (30 min). The reaction was monitored by HPLC-UV no starting material **28** was observed after 2 h. At that time, the reaction mixture was transferred to a 250 mL round-bottom flask containing a magnetic stir bar and 5 mL of DMF, and placed in an ice bath where the product was precipitated by adding dropwise H_2O (90 mL) with an addition funnel. The white solid was filtered, washed with Et_2O (3 × 20 mL) and dried under vacuum. 396.4 mg (87 %) of the desired **14** were obtained with a chromatographic purity of 96 % (28.6 min, figure 5.48). The diastereomeric ratio measured at $\lambda = 220$ nm was 99.3:0.7. **MS (ESI)**: m/z calc. for $[M+2H^{2+}$ of $C_{141}H_{207}N_{19}O_{27}S_3$ 1349.2; found 1349.4.

Figure 5.48. Using HCTU, HOAt and DIPEA[a]

Crude analysis[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Waters 2795, UV detection at 220 nm.

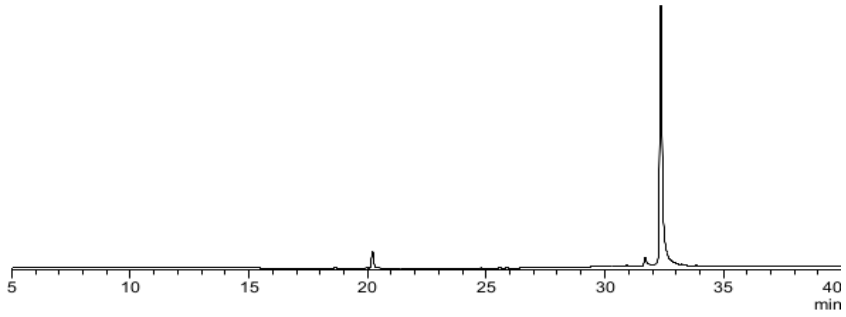
5.10.3.5. Using HATU, HOAt and DIPEA

5.10.3.5.1. Batch 14C: Addition of DIPEA in 0.5 mL of DMF

In a 10 mL round-bottom flask with a magnetic stir bar were mixed in 4.5 mL of DMF the protected nonapeptide **28** (369.1 mg, 1.0 eq), **11** (105.1 mg, 1.2 eq) and HOAt (28.2 mg, 1.2 eq). Once the solution was clear, it was placed in a MeOH/ice bath (-20 ºC) and HATU (72.9 mg, 1.1 eq) was added after 10 min. Then, DIPEA (48.3 mg, 2.2 eq) was weighted over 0.5 mL of DMF and added dropwise with an automatic injector during 30 min. The reaction was monitored by HPLC-UV and no starting material **28** was observed after 1 h. At that time, the reaction mixture was transferred to a 250 mL round-bottom flask containing a magnetic stir bar and 5 mL of DMF, and placed in an ice bath where the product was precipitated by adding H_2O (90 mL) with an addition funnel. The white solid was filtered, washed with Et_2O (3 × 20 mL) and dried under vacuum. 399.5 mg (88 %) of the desired **14** were obtained with a chromatographic purity of 95 % (32.4 min, figure 5.52). The diastereomeric ratio measured at λ = 220 nm was 99.7:0.3. **MS (ESI):** m/z calc. for $[M+2H]^{2+}$ of $C_{141}H_{207}N_{19}O_{27}S_3$ 1349.2; found 1349.3.

Figure 5.52. Addition of DIPEA in 0.5 mL of DMF[a]

Crude analysis[b]



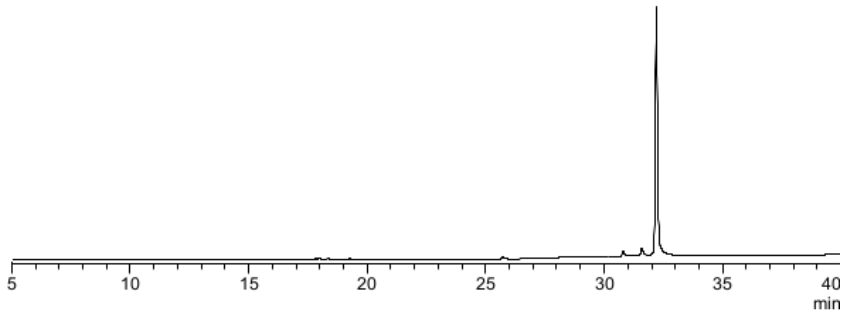
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.10.3.5.2. Batch 14D: Addition of 11 after HATU

In a 10 mL round-bottom flask with a magnetic stir bar were mixed in 4 mL of DMF the protected nonapeptide **28** (369.3 mg, 1.0 eq), HOAt (25.6 mg, 1.1 eq) and HATU (70.6 mg, 1.1 eq). Once the solution was clear, it was placed in a MeOH/ice bath (-20 ºC). In a vial, **11** (107.6 mg, 1.2 eq) and DIPEA (46,9 mg, 2.1 eq) were weighed and dissolved in 1 mL of DMF and added dropwise to the cold solution with an automatic injector during 30 min. The reaction was monitored by HPLC-UV and no starting material **28** was observed after 1 h. At that time, the reaction mixture was transferred to a 250 mL round-bottom flask containing a magnetic stir bar and 5 mL of DMF, and placed in an ice bath where the product was precipitated by adding dropwise H_2O (90 mL) with an addition funnel. The white solid was filtered, washed with Et_2O (3 × 20 mL) and dried under vacuum. 400.30 mg (88 %) of the desired **14** were obtained with a chromatographic purity of 92 % (32.2 min, figure 5.49). The diastereomeric ratio measured at λ = 220 nm was 97.9:2.1. **MS (ESI):** m/z calc. for $[M+2H]^{2+}$ of $C_{141}H_{207}N_{19}O_{27}S_3$ 1349.2; found 1349.1.

Figure 5.49. Addition of 11 before HATU[a]

Crude analysis[b]



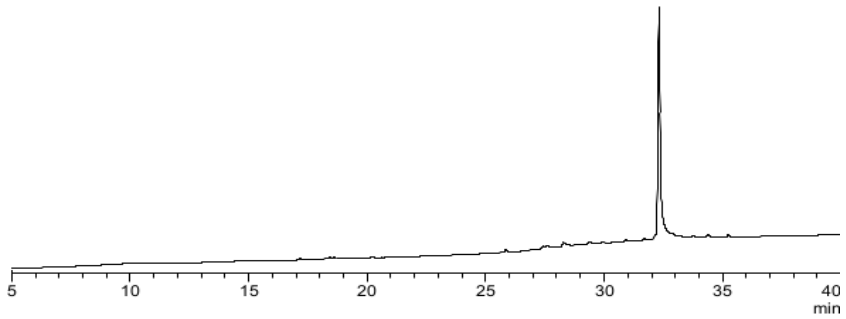
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.10.3.5.3. Batch 14E: Addition of neat DIPEA

In a 10 mL round-bottom flask with a magnetic stir bar were mixed in 5 mL of DMF the protected nonapeptide **28** (370.0 mg, 1.0 eq), **11** (104.3 mg, 1.1 eq) and HOAt (26.0 mg, 1.1 eq). Once the solution was clear, it was placed in a MeOH/ice bath (-20 ºC) and HATU (71.4 mg, 1.1 eq) was added after 10 min. Then, DIPEA (48.3 mg, 2.2 eq) was weighed and added dropwise. The reaction was monitored by HPLC-UV and no starting material **28** was observed after 1 h. At that time, the reaction mixture was transferred to a 250 mL round-bottom flask containing a magnetic stir bar and 5 mL of DMF, and placed in an ice bath where the product was precipitated by adding dropwise H_2O (90 mL) with an addition funnel. The white solid was filtered, washed with Et_2O (3 × 20 mL) and dried under vacuum. 340.6 mg (74 %) of the desired **14** were obtained with a chromatographic purity of 91 % (32.3 min, figure 5.51). The diastereomeric ratio measured at λ = 220 nm was 98.9:1.1. **MS (ESI):** m/z calc. for $[M+2H]^{2+}$ of $C_{141}H_{207}N_{19}O_{27}S_3$ 1349.2; found 1348.8.

Figure 5.51. Addition of neat DIPEA[a]

Crude analysis[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

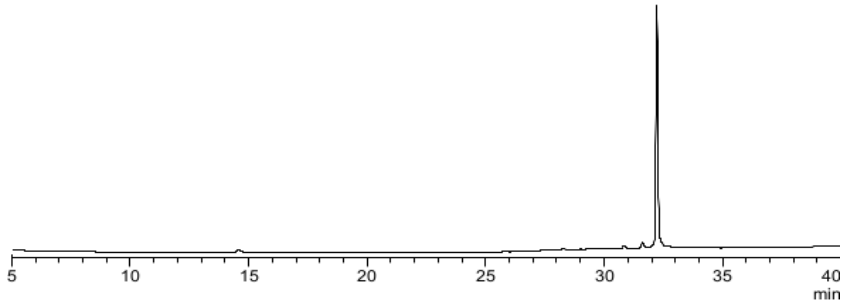
5.10.3.5.4. Batch 14F: Addition of DIPEA in 1 mL of DMF

In a 10 mL round-bottom flask with a magnetic stir bar were mixed in 4 mL of DMF the protected nonapeptide **28** (369.5 mg, 1.0 eq), **11** (105.9 mg, 1.2 eq) and HOAt (25.7 mg, 1.1 eq). Once the solution was clear, it was placed in a MeOH/ice bath (-20 ºC) and HATU (71.4 mg, 1.1 eq) was added after 10 min. Then, DIPEA (52.8 mg, 2.4 eq) was weighted over 1 mL of DMF and added dropwise with an automatic injector for 30 min. The reaction was monitored by HPLC-UV and no starting material **28** was observed after 1 h. At that time,

the reaction mixture was transferred to a 250 mL round-bottom flask containing a magnetic stir bar and 5 mL of DMF, and then placed in an ice bath where the product was precipitated by adding dropwise H_2O (90 mL) with an addition funnel. The white solid was filtered, washed with Et_2O (3 × 20 mL) and dried under vacuum. 372.4 mg (82 %) of the desired **14** were obtained with a chromatographic purity of 92 % (32.2 min, figure 5.53). The diastereomeric ratio measured at λ = 220 nm was 98.7:1.3. **MS (ESI):** m/z calc. for $[M+2H]^{2+}$ of $C_{141}H_{207}N_{19}O_{27}S_3$ 1349.2; found 1349.2.

Figure 5.53. Addition of DIPEA in 1 mL of DMF[a]

Crude analysis[b]



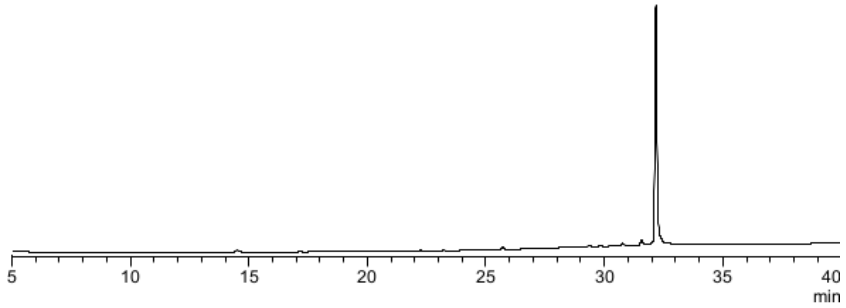
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.10.3.5.5. Batch 14G: Lowering the temperature

In a 10 mL round-bottom flask with a magnetic stir bar were mixed in 4 mL of DMF the protected nonapeptide **28** (372.3 mg, 1.0 eq), **11** (107.1 mg, 1.2 eq) and HOAt (26.4 mg, 1.1 eq). Once the solution was clear, it was placed in an ACN/N_2(l) bath (-40 ºC) and HATU (74.3 mg, 1.1 eq) was added after 10 min. Then, DIPEA (48.4 mg, 2.2 eq) was weighted over 1 mL of DMF and added dropwise with an automatic injector for 30 min. The reaction was monitored by HPLC-UV and no starting material **28** was observed after 1 h. At that time, the reaction mixture was transferred to a 250 mL round-bottom flask containing a magnetic stir bar and 5 mL of DMF, and then placed in an ice bath where the product was precipitated by adding dropwise H_2O (90 mL) with an addition funnel. The white solid was filtered, washed with Et_2O (3 × 20 mL) and dried under vacuum. 384.8 mg (84 %) of the desired **14** were obtained with a chromatographic purity of 93 % (32.17 min, figure 5.54). The diastereomeric ratio measured at λ = 220 nm was 98.8:1.2. **MS (ESI):** m/z calc. for $C_{141}H_{207}N_{19}O_{27}S_3^+$ $[M+2H]^{2+}$ 1349.2; found 1349.1.

Figure 5.54. Lowering the temperature[a]

Crude analysis[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

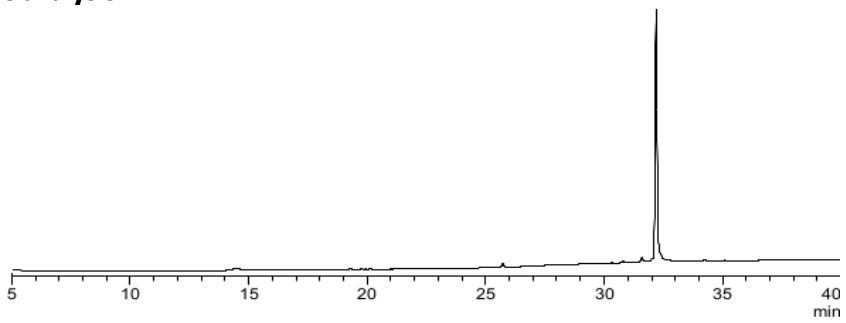
5.10.3.5.6. Batch 14H and 14J: Reaction with change in the dilution of reagents

In a 25 mL round-bottom flask with a magnetic stir bar were mixed in 8 mL of DMF the protected nonapeptide **28** (367.8 mg, 1.0 eq), **11** (110.3 mg, 1.2 eq) and HOAt (28.4 mg, 1.2 eq). Once the solution was clear, it was placed in a MeOH/ice bath (-20 ºC) and HATU (71.9 mg, 1.1 eq) was added after 10 min. Then, DIPEA (48.0 mg, 2.2 eq) was weighted over 2 mL of DMF and added dropwise with an automatic injector for 30 min. The reaction was monitored by HPLC-UV and no starting material **28** was observed after 1 h of reaction time. At that time, the reaction mixture was transferred to a 250 mL round-bottom flask containing a magnetic stir bar and 10 mL of DMF, and then placed in an ice bath where the product was precipitated by adding dropwise H_2O (180 mL) with an addition funnel. The white solid was filtered, washed with Et_2O (3 × 20 mL) and dried under vacuum. 381.5 mg (84 %) of the desired **14** were obtained with a chromatographic purity of 93 % (32.2 min, figure 5.55). The diastereomeric ratio measured at λ = 220 nm was 99.3:0.7. **MS (ESI):** m/z calc. for $[M+2H]^{2+}$ of $C_{141}H_{207}N_{19}O_{27}S_3$ 1349.2; found 1348.9.

The synthesis was repeated **(Batch 14H-2)** with 371.8 mg (1.0 eq) of the protected nonapeptide **28**, 102.0 mg (1.1 eq) of **11**, 26.4 mg (1.1 eq) of HOAt, 70.9 mg (1.1 eq) of HATU and 53.7 mg (2.4 eq) of DIPEA. 459.5 mg (99 %) of the desired product were obtained with a chromatographic purity of 94 %. The diastereomeric ratio measured at λ = 220 nm was 99.1:0.9.

Figure 5.55. Change in the dilution[a]

Crude analysis[b]



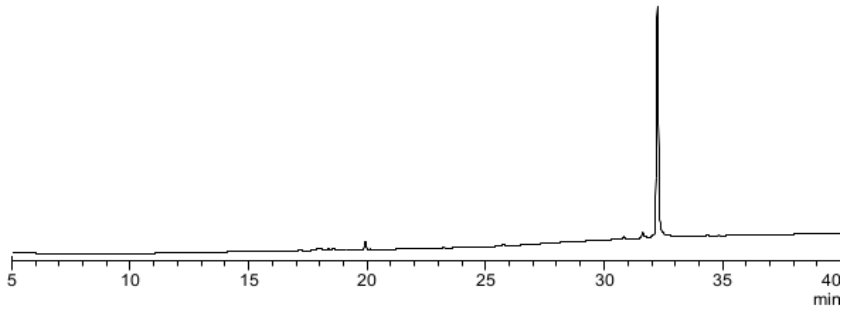
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

Other syntheses:

Batch 14J: Increasing the scale. In a 50 mL round-bottom flask with a magnetic stir bar were mixed in 18 mL of DMF the protected nonapeptide **28** (0.884 g, 1.0 eq), **11** (0.241 g, 1.1 eq) and HOAt (70.8 mg, 1.3 eq). Once the solution was clear, it was placed in a MeOH/ice bath (-20 ºC) and HATU (173.1 mg, 1.1 eq) was added after 10 min. Then, DIPEA (114.8 mg, 2.8 eq) was weighted over 6 mL of DMF and added dropwise to the cold solution with an automatic injector for 30 min. The reaction was monitored by HPLC-UV and no starting material **28** was observed after 1 h. At that time, the reaction mixture was transferred to a 500 mL round-bottom flask containing a magnetic stir bar and 12 mL of DMF, and then placed in an ice bath where the product was precipitated by adding dropwise H_2O (325 mL) with an addition funnel. The white solid was filtered, washed with Et_2O (3 × 20 mL) and dried under vacuum. 0.955 g (87 %) of the desired **14** were obtained with a chromatographic purity of 93 % (figure 5.56). The diastereomeric ratio measured at λ = 220 nm was 98.9:1.1.

Figure 5.56. Increasing the scale[a]

Crude analysis[b]



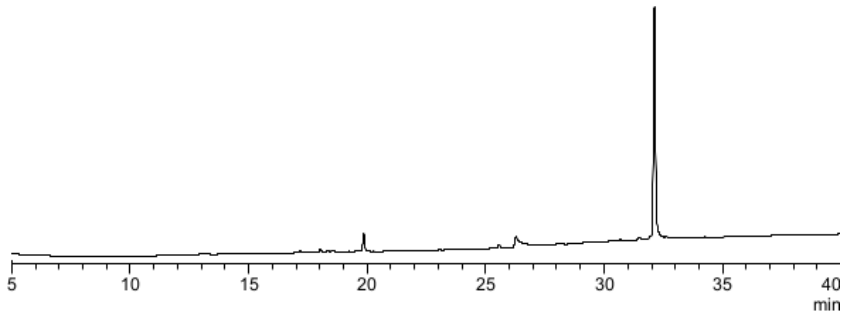
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.10.3.5.7. Batch 14I: Addition of HATU with DIPEA

In a 25 mL round-bottom flask with a magnetic stir bar were mixed in 8 mL of DMF the protected nonapeptide **28** (370.3 mg, 1.0 eq), **11** (102.5 mg, 1.1 eq) and HOAt (38.3 mg, 1.6 eq). Once the solution was clear, it was placed in a MeOH/ice bath (-20 ºC). In a vial, HATU (72.5 mg, 1.1 eq) was weighted and dissolved in 2 mL of DMF. Then, DIPEA (49.2 mg, 2.2 eq) was weighed and added to the vial. The HATU/DIPEA solution was added dropwise to the cold solution with an automatic injector for 30 min and the reaction was monitored by HPLC-UV. Starting materials **28** and **11** were detected in a 9.5 and 5.4 % respectively after 3 h of reaction time. At that time, the reaction mixture was transferred to a 250 mL round-bottom flask containing a magnetic stir bar and 10 mL of DMF, and placed in an ice bath where the product was precipitated by adding dropwise H_2O (180 mL) with an addition funnel. The white solid was filtered, washed with Et_2O (3 × 20 mL) and dried under vacuum. 381.1 mg of crude peptide (83 %) were obtained with a chromatographic purity of 86 % (32.1 min, figure 5.50). The diastereomeric ratio measured at λ = 220 nm was 99.3:0.7.

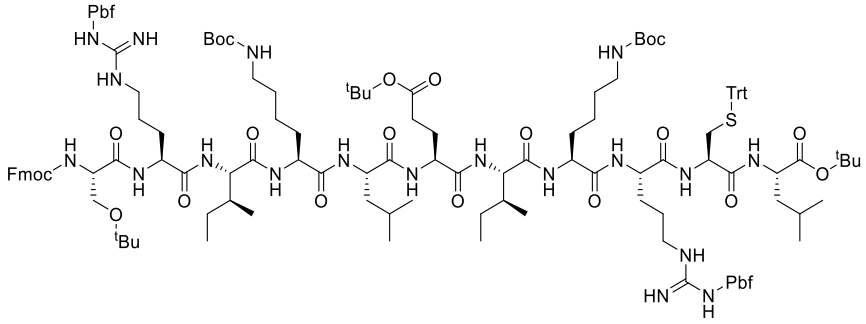
Figure 5.50. Addition of HATU with DIPEA[a]

Crude analysis[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.10.4. Synthesis of 15



5.10.4.1. Fmoc removal using a 2 % piperidine solution

In a 5 mL round-bottom flask with a magnetic stir bar containing protected undecapeptide **14**, a solution of 2 % piperidine in DMF was added and left stirring. The reaction was monitored by HPLC-UV and no starting material was observed after 1 h. At that time, the reaction mixture was transferred with 10 mL of DMF to a 250 mL round bottom flask containing a magnetic stir bar and was placed in an ice bath. With an addition funnel, H_2O (180 mL) was added dropwise to precipitate the product. The yellowish solid was filtered, triturated with Et_2O (6 × 20 mL) and dried under vacuum.

5.10.4.1.1. Batch 15B

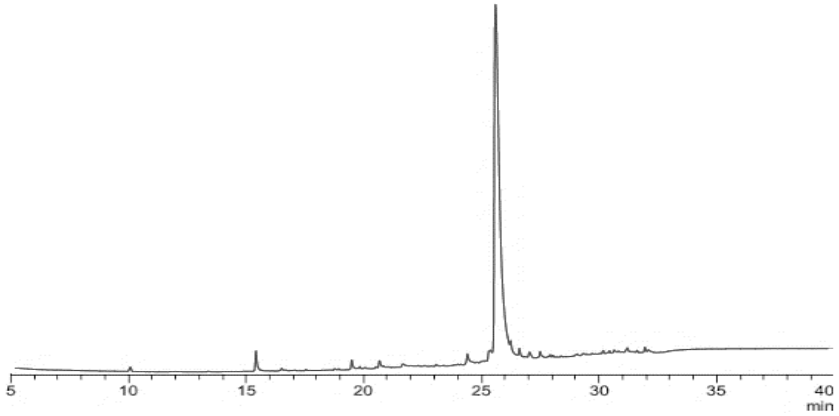
Protected undecapeptide **14** (1.326 g, 1.0 eq, from batches 14D, 14E, 14F,14G), piperidine (0.125 g, 3.0 eq) and DMF (6.75 g). 0.972 g of the desired **15** were obtained with a chromatographic purity of 86 % (25.6 min, figure 5.57). **MS (ESI):** m/z calc. for $[M+2H]^{2+}$ of $C_{126}H_{197}N_{19}O_{25}S_3$ 1237.6; found 1237.9.

5.10.4.1.2. Batch 15C

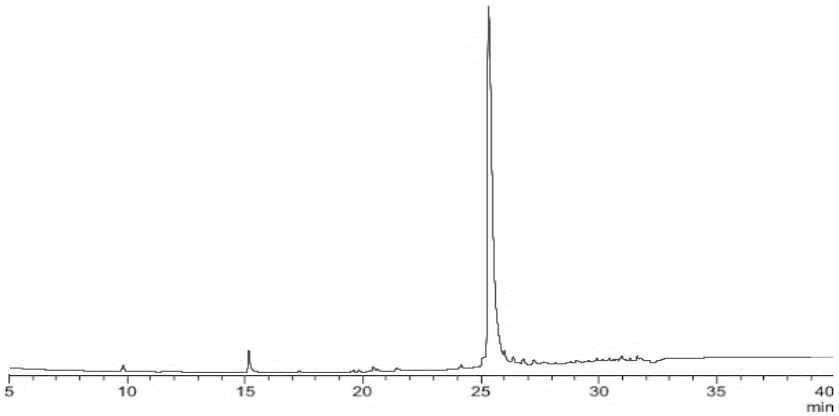
Protected undecapeptide **14** (0.932 g, 1.0 eq, from batch 14J), piperidine (86.7 mg, 3.0 eq) and DMF (4.78 g). 0.593 g of the desired product **15** were obtained with a chromatographic purity of 88 % (25.4 min, figure 5.57). **MS (ESI):** m/z calc. for $[M+2H]^{2+}$ of $C_{126}H_{197}N_{19}O_{25}S_3$ 1237.6; found 1238.0.

Figure 5.57. HPLC chromatograms of 15[a]

Crude analysis[b]**:** Batch 15B



Crude analysis[b]**:** Batch 15C



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Agilent 1100, UV detection at 220 nm.

5.10.4.2. Modification of the work-up methodology due to filtration problems

In a 5 mL round-bottom flask with a magnetic stir bar containing protected undecapeptide **14**, a solution of 2 % piperidine in DMF was added and left stirring. The reaction was monitored by HPLC-UV and no starting material was observed after 1 h. At that time, the reaction mixture was transferred with 3 mL of DMF to a 250 mL round-bottom flask containing a magnetic stir bar and was placed in an ice bath. With an addition funnel, H_2O

(60 mL) were added dropwise to precipitate the product and the resulting suspension was transferred into a conical centrifuge tube and centrifuged. H_2O was removed and the humid solid was lyophilised. The solid was triturated with Et_2O (6 × 20 mL) and dried under vacuum.

5.10.4.2.1. Batch 15D

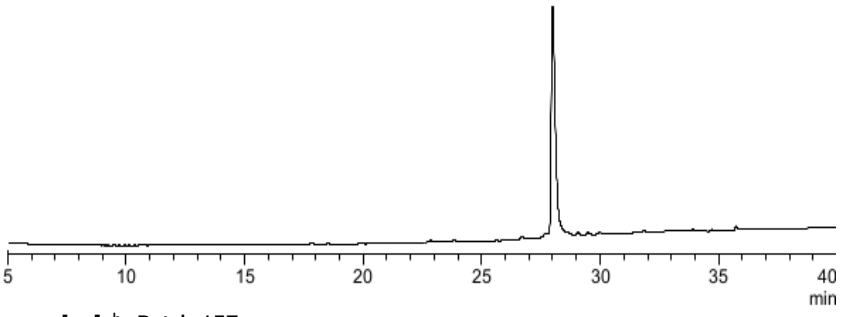
Protected undecapeptide **14** (0.374 g, 1.0 eq, from batch 14H-2), piperidine (46.1 mg, 3.9 eq) and DMF (1.98 g). 0.335 g (97 %) of the desired **15** were obtained with a chromatographic purity of 92 % (28.0 min, figure 5.58). **MS (ESI):** m/z calc. for $[M+2H]^{2+}$ of $C_{126}H_{197}N_{19}O_{25}S_3$ 1237.6; found 1237.9.

5.10.4.2.2. Batch 15E

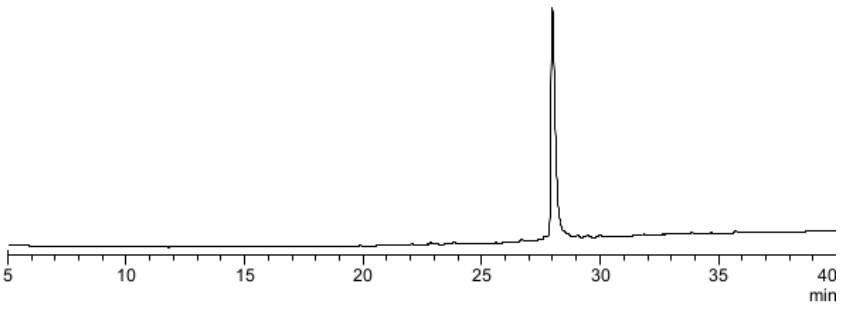
Protected undecapeptide **14** (0.316 g, 1.0 eq, from batch 14H), piperidine (37.8 mg, 3.8 eq) and DMF (1.98 g). 0.257 g (89 %) of the desired product **15** were obtained with a chromatographic purity of 94 % (28.0 min, figure 5.58).

Figure 5.58. HPLC chromatograms of 15[a]

Crude analysis[b]**:** Batch 15D



Crude analysis[b]**:** Batch 15E



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.10.4.3. Batch 15F: Slurry with water

In a 50 mL reactor vessel with a magnetic stir bar were mixed 0.276 g of N-terminal deprotected undecapeptide **15** (batch 15D) in 6 mL of H_2O for 5 h. The suspension was filtered and dried under vacuum. 0.179 g of the desired product were obtained.

5.10.4.4. Batch 15G: Reprecipitation with an aqueous solution of 2.5 % citric acid

In a 50 mL reactor vessel with a magnetic stir bar were mixed 150.3 mg of N-terminal deprotected undecapeptide **15** (batch 15B) in 6 mL of THF. Then, 3 mL of 2.5 % aq. citric acid were added and left stirring for 10 min. The aqueous phase was separated and the procedure was repeated. The organic phase was washed twice with H_2O and precipitated by adding dropwise H_2O (120 mL) with an addition funnel. The suspension was filtered and the resulting solid was dried under vacuum to afford 69.48 mg of the desired product.

5.10.4.5. Batch 15H: Reprecipitation with a 2.0 N aqueous solution of NaH_2PO_4 at pH 3

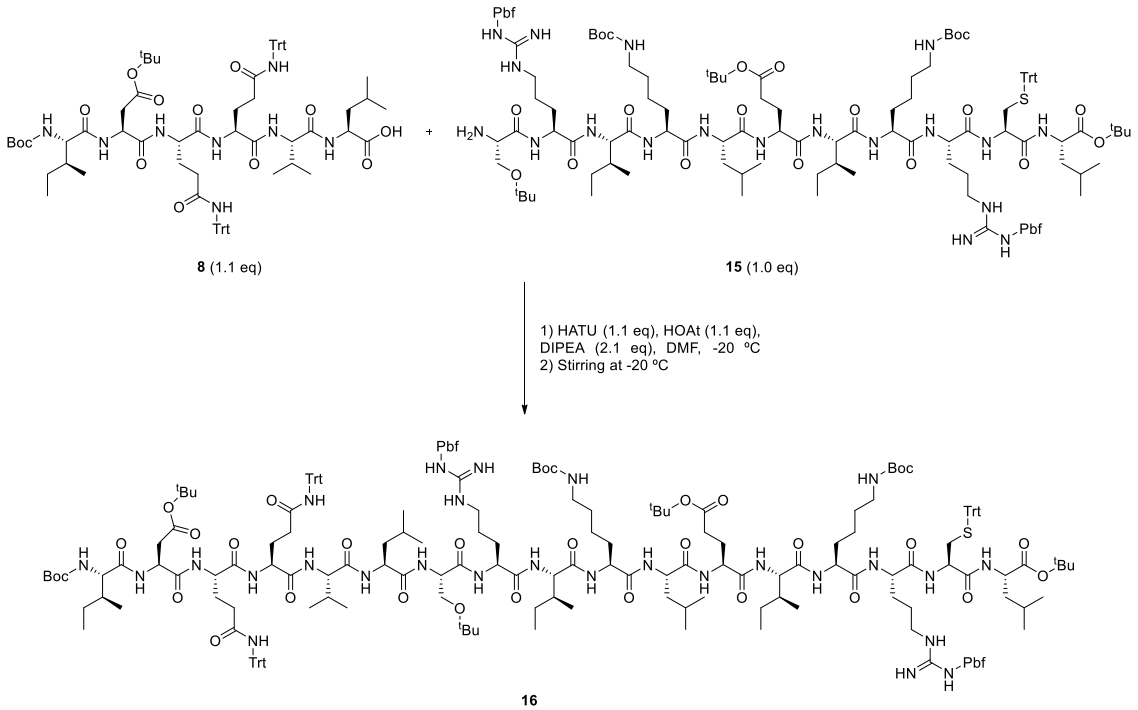
5.10.4.5.1. Batch 15H-1

In a 50 mL reactor vessel with a magnetic stir bar were mixed 200.6 mg of N-terminal deprotected undecapeptide **15** (batch 15B) in 30 mL of 2-MeTHF. Then, 20 mL of 2.0 N aq. NaH_2PO_4 were added and left stirring for 10 min. The aqueous phase was separated, and the procedure was repeated twice. The organic phase was washed with H_2O (2 × 20 mL) and the organic solvent was reduced under vacuum and precipitated with the addition of H_2O (30 mL) with an addition funnel. The suspension was filtered and dried under vacuum to afford 0.140 g of the desired product.

5.10.4.5.2. Batch 15H-2

The procedure was repeated with 350.9 mg of N-terminal deprotected undecapeptide **15** (batch 15C) obtaining 315.8 mg of the desired product.

5.10.5. Synthesis of 16

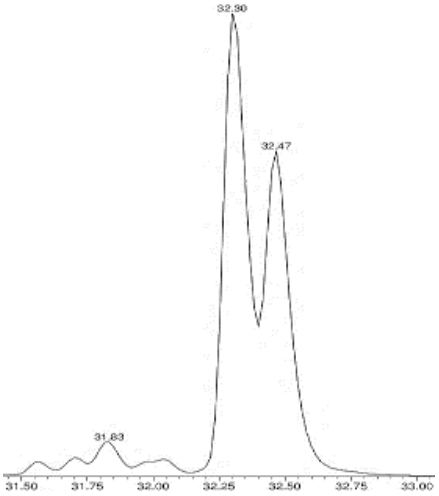


5.10.5.1. Test of epimerisation during fragment condensation

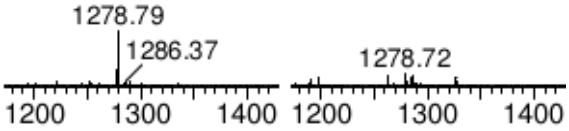
In a 10 mL round-bottom flask with a magnetic stir bar were mixed in 4.5 mL of DMF the protected hexapeptide **8** (93.6 mg, 1.0 eq), HOAt (10.5 mg, 1.1 eq) and HATU (29.6 mg, 1.1 eq). Once the solution was clear, DIPEA was weighted (20.5 mg, 2.3 eq) over 0.5 mL of DMF and added to the solution at room temperature. The resulting mixture was left for 16 h at rt under magnetic stirring when the N-terminal deprotected undecapeptide **15** (172.9 mg, 1.0 eq) was added. The reaction mixture was transferred after 2 h to a 250 mL round-bottom flask containing a magnetic stir bar and 5 mL of DMF, and placed in an ice bath where the product was precipitated by adding dropwise H_2O (90 mL) with an addition funnel. The sticky yellowish solid was filtered, washed with Et_2O (3 × 20 mL) and dried under vacuum. 149.3 mg (56 %) of a diastereomeric mixture of **16** (64:36) were obtained (figure 5.59). **MS (ESI)**: m/z calc. for $[M+2H+Na]^{3+}$ of $C_{204}H_{293}N_{27}O_{37}S_3$ 1278.1; found 1279.3.

Figure 5.59. Forcing epimerisation[a]

Crude analysis[b]



MS spectra[b]



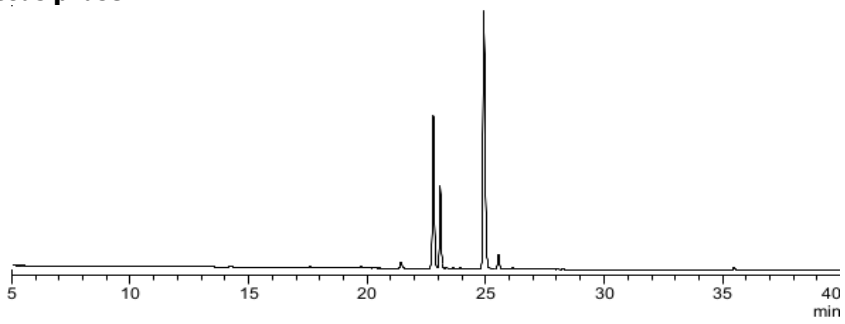
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Waters 2695, UV detection at 220 nm.

5.10.5.2. Assay of a new work-up for removing the excess of 8

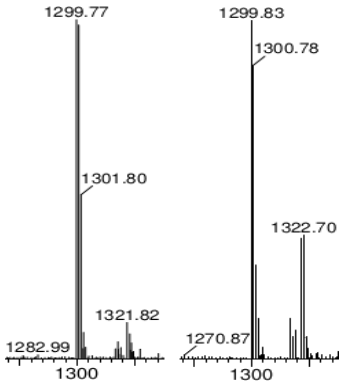
In a 25 mL round-bottom flask with a magnetic stir bar were mixed in 1 mL of DMF the protected hexapeptide **8** (105.8 mg, 1.0 eq) and DIPEA (43.10 mg, 5.0 eq). The mixture was left for 1 h and 9 mL of H_2O were added. Formation of a precipitate was not observed and the reaction mixture was placed in an ice bath where 9 additional mL of H_2O were added but precipitation was not observed. Analysis by HPLC and HPLC-MS (figure 5.60) showed the presence of **8** (24.9 min, 54 % of chromatographic purity) and two more products at retention times of 22.8 min (26 %) and 23.1 min (14 %), with masses of $[M+H]^+$ 1299.7 and 1299.8.

Figure 5.60. Assay of a new work-up[a]

Aqueous phase[b]



MS spectra[c]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.
(c) Waters 2695.

5.10.5.3. Using HATU, HOAt and DIPEA

5.10.5.3.1. Batch 16B

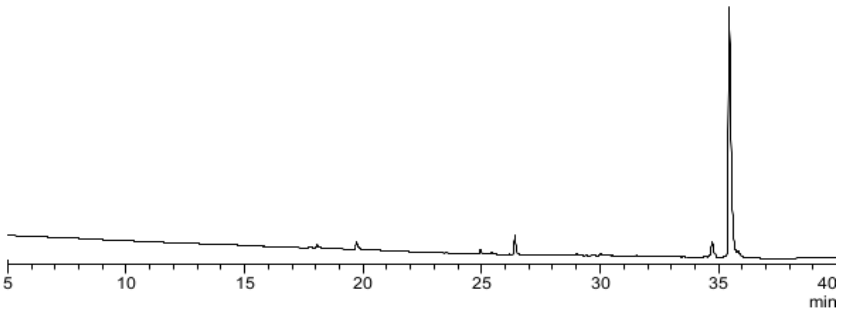
In a 25 mL round-bottom flask with a magnetic stir bar were mixed in 8 mL of DMF the protected hexapeptide **8** (103.9 mg, 1.1 eq), N-terminal deprotected undecapeptide **15** (170.7 mg, 1.0 eq) and HOAt (10.2 mg, 1.1 eq). Once the solution was clear, it was placed in a MeOH/ice bath (-20 ºC) and HATU (29.0 mg, 1.1 eq) was added after 10 min. Then, DIPEA (17.6 mg, 2.1 eq) was weighted over 2 mL of DMF and added to the cold solution dropwise with an automatic injector for 30 min. The reaction was monitored by HPLC-UV and after 4.5 h, less than 1 % of starting material **15** remained. At that time, DIPEA (2.9 mg, 1.2 eq relative to **8**) was added to the reaction mixture and left for 30 min. The reaction mixture was then transferred to a 250 mL round-bottom flask containing a magnetic stir bar and 5 mL of DMF and placed in an ice bath where the product was precipitated by adding dropwise H_2O (140 mL) with an addition funnel. The suspension was transferred into a conical centrifuge tube, centrifuged and the supernatant was separated. Lyophilisation of the humid solid afforded 259.4 mg of a white solid containing the desired product with a chromatographic purity of 74 % (35.4 min) and a 2 % of the starting material **8**.

The solid was dissolved in 2 mL of DMF and transferred into a 50 mL round-bottom flask. Then, DIPEA (43,0 mg, 4.5 eq relative to **8**) was added and the resulting mixture was left stirring for 30 min. The product was precipitated by the addition of H_2O (45 mL) and the suspension was transferred into a conical centrifuge tube and centrifuged. The supernatant

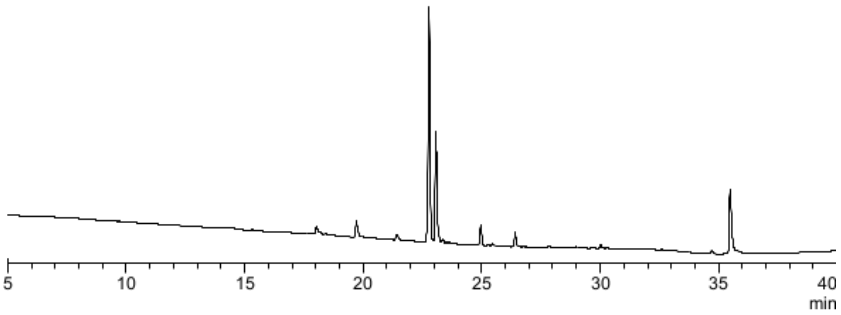
was separated and the humid solid was lyophilised to afford 195.0 g of a crude product containing **16** with a chromatographic purity of 82 % (35.4 min, figure 5.61), a 0.4 % of starting material **8** and a 4 % of the piperidide **38** (m/z of $[M+H]^+$ 1422.8, 26.4 min). **MS (ESI)**: m/z calc. for $[M+2H+Na]^{3+}$ of $C_{204}H_{293}N_{27}O_{37}S_3$ 1278.1; found 1279.3.

Figure 5.61. Batch 16B[a]

Crude analysis[b]



Aqueous filtrates analysis[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Shimadzu, UV detection at 220 nm.

5.10.5.3.2. Batches 16C and 16D: Reaction with batch 15B

Batch 16C: In a 100 mL reactor vessel with a magnetic stir bar were mixed in 8 mL of DMF the protected hexapeptide **8** (103.2 mg, 1.1 eq), the N-terminal deprotected undecapeptide **15** (169.7 mg, 1.0 eq, section 5.10.4.1) and HOBt·H_2O (11.9 mg, 1.1 eq). Once the solution was clear, the temperature was set at -20 ºC with a thermostat and HATU (29.6 mg, 1.1 eq) was added after 10 min. Then, DIPEA (28.0 mg, 3.1 eq) was weighted over 2 mL of DMF and was added dropwise with an automatic injector for 30 min. After 4.5 h the temperature was raised to 5 ºC and the crude was precipitated by adding dropwise H_2O (90 mL) with an addition funnel. The resulting suspension was filtered and dried under vacuum to afford 0.184 g of a white solid containing the desired product with a chromatographic purity of 56 % (32.6 min, figure 5.62), the piperidide **38** (17 %, 24.0 min) and an unknown impurity (9 %, 25.9 min). **MS (ESI):** m/z calc. for $[M+2H]^{2+}$ of $C_{204}H_{293}N_{27}O_{37}S_3$ 1905.6; found 1906.6.

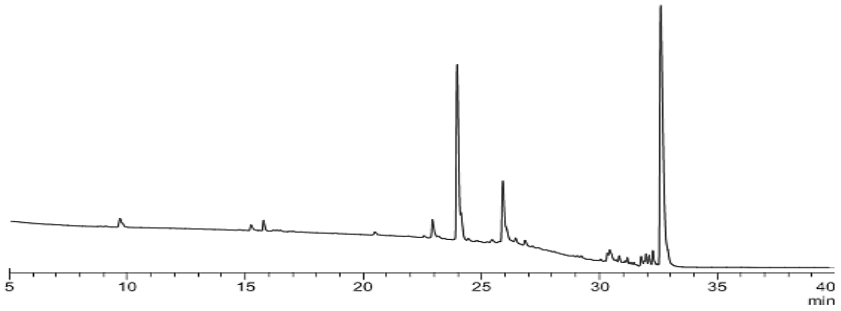
Other syntheses:

Batch 16D: The protected hexapeptide **8** (101.9 mg, 1.1 eq), the N-terminal deprotected undecapeptide **15** (169.7 mg, 1.0 eq, section 5.10.4.1), HOAt (14.6 mg, 1.5 eq), HATU (29.4 mg, 1.1 eq) and DIPEA (28.2 mg, 3.1 eq) were mixed in 10 mL of DMF at -20 ºC. The reaction was monitored by HPLC-UV and after 2 h, no starting material **8** was observed. 0.224 g of a white solid were obtained containing the desired **16** with a chromatographic purity of 54 % (figure 5.62), the piperidide **38** (14 %, 23.9), starting material **15** (4 %,

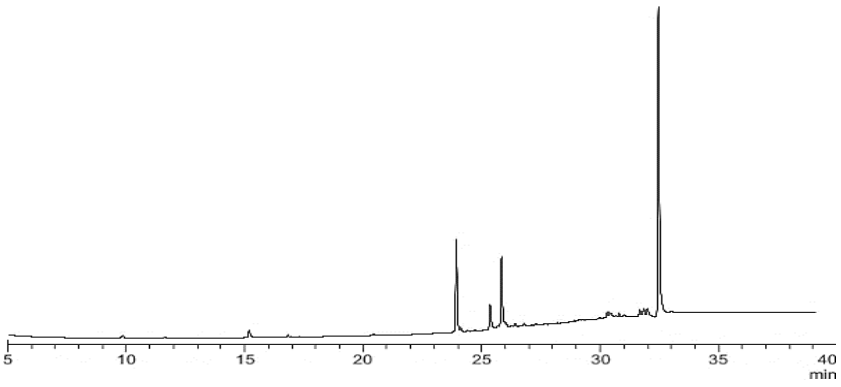
25.4 min) and an unknown impurity (12 %, 25.8 min). **MS (ESI):** m/z calc. for $[M+2H]^{2+}$ of $C_{204}H_{293}N_{27}O_{37}S_3$ 1905.6; found 1906.6.

Figure 5.62. Change in the batch of 15[a]

Crude analysis[b]**:** Batch 16C

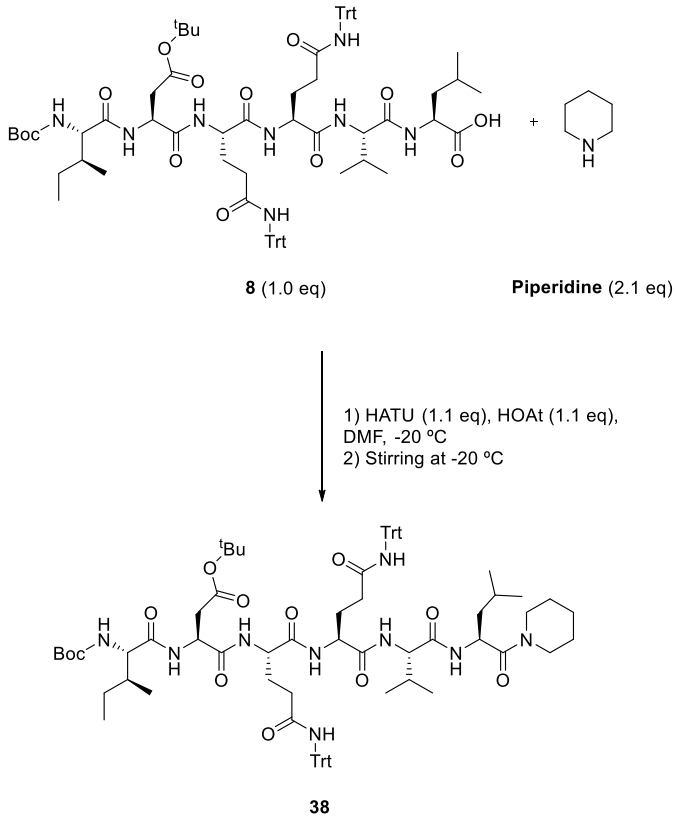


Crude analysis[c]**:** Batch 16D



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Agilent 1100, UV detection at 220 nm.
(c) Agilent 1260, UV detection at 220 nm.

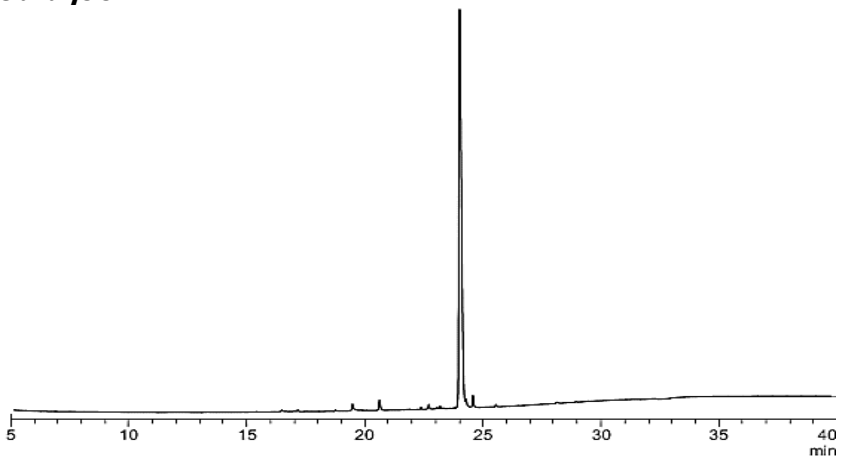
5.10.5.3.3. Synthesis of the piperidide 38



In a 100 mL reactor vessel with a magnetic stir bar were mixed in 40 mL of DMF the protected hexapeptide **8** (797.2 mg, 1.0 eq) and HOAt (96.1 mg, 1.2 eq). Once the solution was clear, the temperature was set at -20 ºC with a thermostat and HATU (267.0 mg, 1.2 eq) was added after 10 min. Then, piperidine (127.5 mg, 2.5 eq) was weighted over 10 mL of DMF and was added dropwise with an automatic injector for 30 min. The reaction was monitored by HPLC-UV and after 1 h, only 0.3 % of starting material **8** was observed. At that time, the reaction mixture was transferred to a 500 mL reactor vessel with a magnetic stir bar and the temperature was set to 5 ºC. The product was precipitated by adding dropwise H_2O (350 mL) with an addition funnel. The suspension was filtered and dried under vacuum to afford 0.7292 g (87 %) of the desired **38** with a chromatographic purity of 92 % (24.0 min, figure 5.63). **MS (ESI):** m/z calc. for $[M+H]^+$ of $C_{83}H_{107}N_9O_{12}$ 1422.8; found 1422.8.

Figure 5.63. Synthesis of the piperidide 38[a]

Crude analysis[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Agilent 1100, UV detection at 220 nm.

5.10.5.3.4. Solubility studies of the piperidide 38

The piperidide **38** (around 40 mg) was weighed in 15 different test tubes. To each tube, 120 µL of different solvents were added (table 5.36), the temperature was set to 40 ºC and solubility was checked every 10 min (1.5 h in total). After this time, the temperature was lowered to 25 ºC, the mixtures were left overnight, and solubility was again checked. Finally, the temperature was set to 5 ºC and the solubility was checked after 30 min and after 2 h. The samples in which the piperidide was soluble were tested with a solid of **16** that contained the piperidide **38**.

Table 5.36. Solubility results of the piperidide **38**.

	mg	Solvent	r.t	Time (min)							
				–	10	30	60	90	o. n.	30	120
				40 ºC					25 ºC	5 ºC	
1	40.71	MeOH	×	×	×	×[b]	×[b]	×	×	×	×
2	39.46	EtOH	✓	✓	✓	✓	✓	✓	×	×	×
3	39.68	iPrOH	✓	✓	✓	✓	✓	✓	×	×	×
4	42.84	ACN	×	×	×	×[b]	×[b]	×	×	×	×
5	39.31	Acetone	×	×	×	×[b]	×[b]	×	×	×	×
6	42.10	THF	✓	✓	✓	✓	✓	✓	✓	(-)	(-)
7	39.98	Toluene	×	Gel	Gel	Gel[b]	Gel[b]	Gel	×	×	×
8	40.82	DMF	✓	✓	✓	✓	✓	✓	✓	×	×
9	41.75	MeOH/H_2O[a]	×	×	×	×	×	×	×	×	×
10	39.39	EtOH/H_2O[a]	✓	✓	×	×	×[c]	×	×	×	×
11	39.68	iPrOH/H_2O[a]	✓	✓	✓	✓	✓	✓	×	×	×
12	40.16	ACN/H_2O[a]	×	×	×	×	×	×	×	×	×
13	42.43	Acetone/H_2O[a]	✓	✓	✓	×	×[b]	×	×	×	×
14	40.07	THF/H_2O[a]	×	×	×	×	×[b]	✓	(-)	(-)	(-)
15	41.09	DMF/H_2O[a]	✓	✓	✓	✓	✓	✓	✓	×	×

(a) 9:1 (v/v), ✓: Yes; ×: No; (-): Cloudy.
(b) Addition of 60 µL.
(c) Addition of 120 µL.

5.10.5.3.5. Solubility test of the piperidide 38 present in a batch of 16

Samples of **16** (batch 16G, section 5.10.5.3.7) that contained a 7 % of the piperidide **38** (36.98, 37.26 and 39.84 mg) were weighed in three test tubes and 120 μL of iPrOH, EtOH and THF were added, respectively. The temperature was set at 40 ºC and the mixture was left stirring for 4 h. Then, the suspensions obtained in iPrOH and EtOH were filtered and dried under vacuum. The THF solution was left overnight at 25 ºC but formation of a precipitate was not observed. 16.95 mg (45 %) and 38.39 mg (quantitative) of **16** were obtained from the iPrOH and EtOH suspensions, respectively. The solid obtained from the assay with iPrOH contained a 2 % of the piperidide **38** and the one obtained from the assay with EtOH contained a 0.6 % of the piperidide form and a chromatographic purity of 84 %.

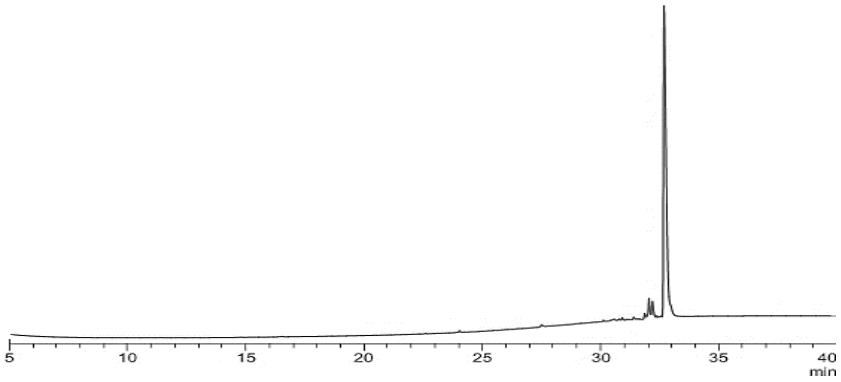
The experiment with EtOH (300 μL) was repeated with 76.79 mg of **16** that contained 7 % of the piperidide **38**. The temperature was set to 40 ºC and the mixture was left stirring for 4 h. At that time, the resulting suspension was filtered and dried under vacuum. 26.81 mg (35 %) of the desired product were obtained with a chromatographic purity of 80 %, and 0.8 % of the piperidide **38**.

5.10.5.3.6. Test of purification of 16 with EtOH

In a test tube, **16** (118.5 mg, batch 16B, section 5.10.5.3.1) was mixed with 1.5 mL of EtOH using a magnetic stir bar. The temperature was set to 40 ºC and the mixture was left stirring for 4 h when the suspension was filtered and dried under vacuum. 70.1 mg (15 %) of the desired product were obtained with a chromatographic purity of 90 % (figure 5.64). **MS (ESI):** m/z calc. for $[M+2H]^{2+}$ of $C_{204}H_{293}N_{27}O_{37}S_3$ 1905.6; found 1906.6.

Figure 5.64. Purification with EtOH[a]

Isolated product[b]



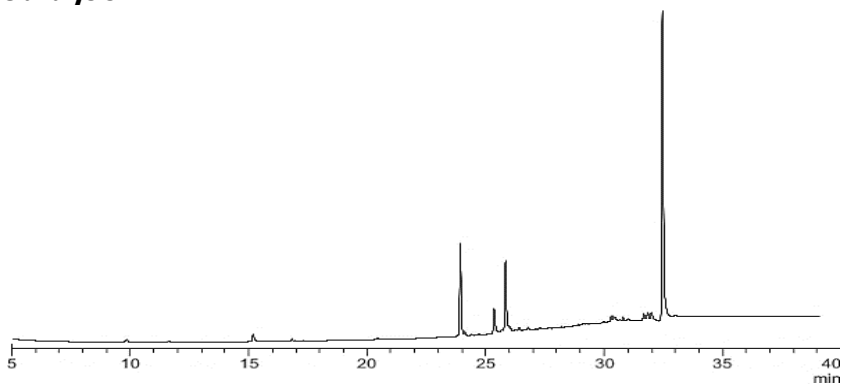
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Agilent 1100, UV detection at 220 nm.

Other syntheses:

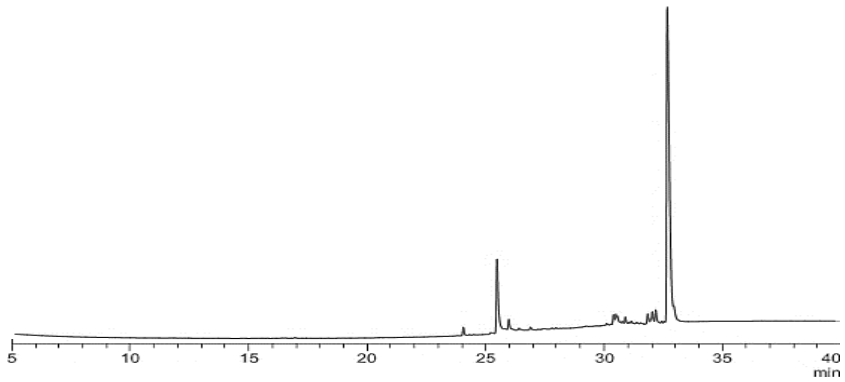
16 (201.8 mg, batch 16D, section 5.10.5.3.2) and 500 μL of EtOH. 30.8 mg of a white solid containing the desired product were obtained with a chromatographic purity of 69 % (figure 5.65), and a 12 % of starting material **15**.

Figure 5.65. Test of purification with EtOH[a]

Crude analysis[b]



Isolated product[c]



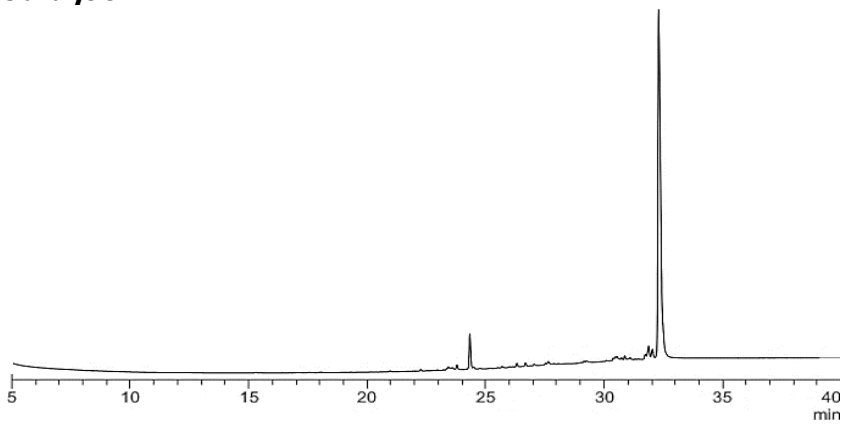
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Agilent 1260, UV detection at 220 nm.
(c) Agilent 1100, UV detection at 220 nm.

5.10.5.3.7. Batch 16E: Reaction with batch 15E

In a 100 mL reactor vessel with a magnetic stir bar were mixed in 8 mL of DMF the protected hexapeptide **8** (135.0 mg, 1.1 eq), the N-terminal deprotected undecapeptide **15** (230.0 mg, 1.0 eq, section 4.10.4.2.2) and HOAt (21.0 mg, 1.6 eq). Once the solution was clear, the temperature was set at -20 ºC with a thermostat and HATU (48.0 mg, 1.3 eq) was added after 10 min. Then, DIPEA (31.0 mg, 2.6 eq) was weighted over 2 mL of DMF and added dropwise with an automatic injector for 30 min. The reaction was monitored by HPLC-UV and no starting material **8** was observed after 2 h. At this time, the temperature was then set to 5 ºC and the product was precipitated by adding dropwise H_2O (90 mL) with an addition funnel. The suspension was filtered and dried under vacuum to afford 0.316 g of a white solid containing the desired **16** with a chromatographic purity of 78 % (32.3 min, figure 5.66), and 6 % of the piperidide **38**.

Figure 5.66. Change in the batch of 15[a]

Crude analysis[b]



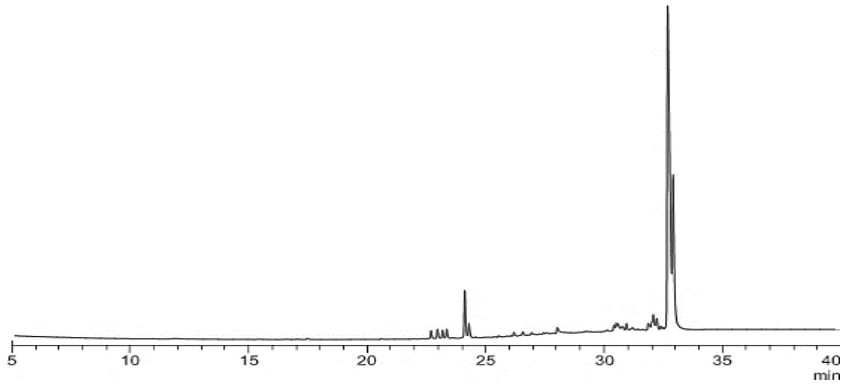
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Agilent 1200, UV detection at 220 nm.

5.10.5.3.8. Batch 16F: Preliminary assay with batch 15D

Protected hexapeptide **8** (10.9 mg, 1.1 eq), the N-terminal deprotected undecapeptide **15** (17.8 mg, 1.0 eq, section 5.10.4.2.1), HOAt (1.14 mg, 1.1 eq), HATU (3.29 mg, 1.1 eq) and DIPEA (3.71 mg, 3.9 eq) were mixed in 1 mL of DMF with a magnetic stir bar. Temperature was not controlled and the reaction was monitored by HPLC-UV after 1 h. The desired product was not isolated in this reaction but the piperidide **38** was detected in a 6 % (figure 5.67).

Figure 5.67. Change in the batch of 15[a]

In process control[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Agilent 1100, UV detection at 220 nm.

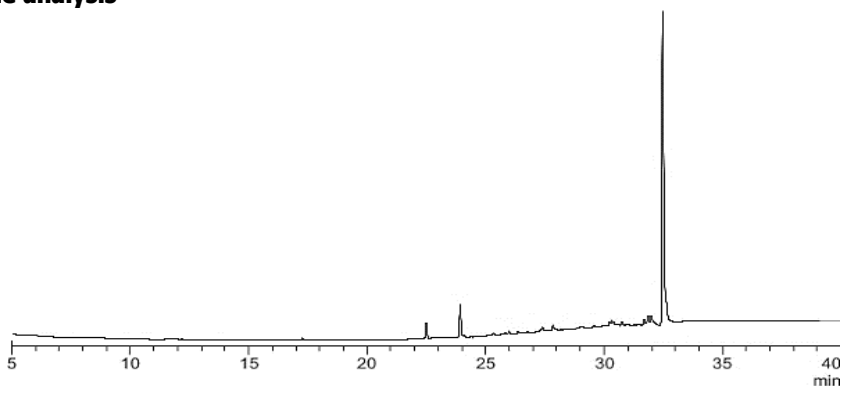
5.10.5.3.9. Batch 16G: Reaction with batch 15F

In a 100 mL reactor vessel with a magnetic stir bar were mixed in 8 mL of DMF the protected hexapeptide **8** (104.1 mg, 1.1 eq), the N-terminal deprotected undecapeptide **15** (169.3 mg, 1.0 eq, section 5.10.4.3) and HOAt (12.4 mg, 1.3 eq). Once the solution was clear, the temperature was set at -20 ºC with a thermostat and HATU (31.6 mg, 1.2 eq) was added after 10 min. Then, DIPEA (30.6 mg, 3.4 eq) was weighted over 2 mL of DMF and added dropwise with an automatic injector for 30 min. The reaction was monitored by HPLC-UV and after 1 h a 7 % of the piperidide **38** was detected. The temperature was raised to 5 ºC

after 3 h and the product was precipitated by adding dropwise H_2O (90 mL) with an addition funnel. The suspension was filtered and dried under vacuum to obtain 0.283 g of a white solid containing the desired **16** with a chromatographic purity of 73 % (32.5 min, figure 5.68), 7 % of the piperidide **38** and 3 % of staring material **8**.

Figure 5.68. Change in the batch of 15[a]

Crude analysis[b]



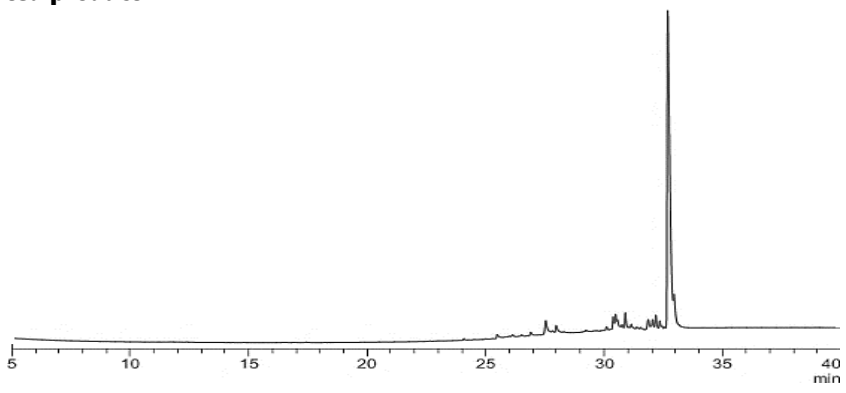
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Agilent 1260, UV detection at 220 nm.

5.10.5.3.10. Purification of batch 16E and batch 16G using EtOH

63.1 mg of **16** (batch 16E and batch 16G, section 5.10.5.3.5 and 5.10.5.3.7) were mixed with a magnetic stir bar with 500 μL of EtOH in a test tube. The temperature was set to 40 ºC and the mixture was left stirring for 4 h when the suspension was filtered and dried under vacuum. 40.9 mg of the desired product were obtained with a chromatographic purity of 74 % (figure 5.69).

Figure 5.69. Purification using EtOH[a]

Isolated product[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Agilent 1100, UV detection at 220 nm.

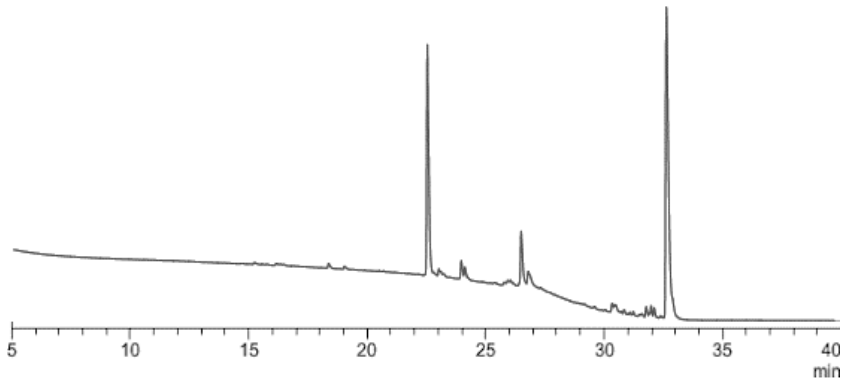
5.10.5.3.11. Batch 16H: Reaction with batch 15G

In a 50 mL reactor vessel with a magnetic stir bar were mixed in 2 mL of DMF the protected hexapeptide **8** (30.3 mg, 1.1 eq), the N-terminal deprotected undecapeptide **15** (47.9 mg, 1.0 eq, section 5.10.4.4) and HOAt (4.28 mg, 1.6 eq). Once the solution was clear, the temperature was set at -20 ºC with a thermostat and HATU (8.96 mg, 1.2 eq) was added after

10 min. Then, DIPEA (4.13 mg, 2.0 eq) was weighted over 2 mL of DMF and added dropwise with an automatic injector for 30 min. The reaction was monitored by HPLC-UV and after 4 h there was starting material **8**. The temperature was raised to 5 ºC and the product was precipitated by adding dropwise H_2O (15 mL) with an addition funnel. The suspension was filtered and dried under vacuum to obtain a white solid with a chromatographic purity of 50 % (32.6 min, figure 5.70), containing 26 % of the starting material **8**, 2 % of the piperidide **38** and 7 % of an unknown impurity. **MS (ESI):** m/z calc. for $[M+2H]^{2+}$ of $C_{204}H_{293}N_{27}O_{37}S_3$ 1905.6; found 1906.6.

Figure 5.70. 15 from batch 15G[a]

Crude analysis[b]



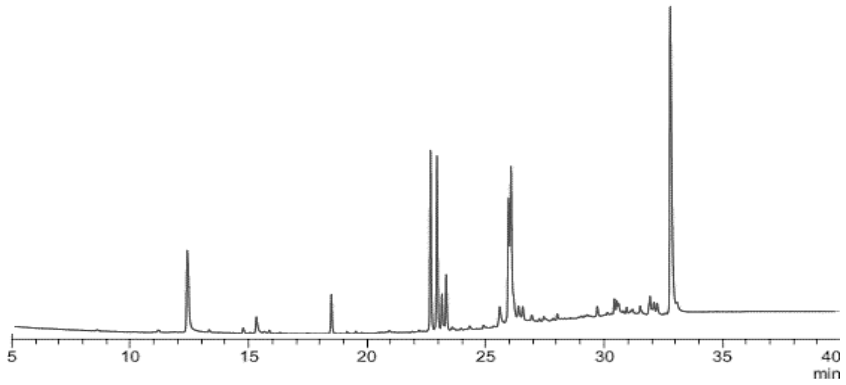
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Agilent 1100, UV detection at 220 nm.

5.10.5.3.12. Batch 16I: Preliminary assay of reaction with batch 15H-1

Protected hexapeptide **8** (13.0 mg, 1.5 eq), the N-terminal deprotected undecapeptide **15** (16.0 mg, 1.0 eq, section 5.10.4.5.), HOAt (2.80 mg, 3.1 eq), HATU (8.01 mg, 3.1 eq) and DIPEA (2.50 mg, 3.1 eq) were mixed in 1 mL of DMF with a magnetic stir bar. Temperature was not controlled, and the reaction was monitored by HPLC-UV after 1 h. The piperidide **38** was not detected but two new impurities were detected at 23.1 min (2 %, m/z of $[M+H]^+$ 1368.7, figure 5.71) and 23.3 min (3 %, m/z of $[M+H]^+$ 1382.7, figure 5.71).

Figure 5.71. Preliminary assay with 15 from batch 15H-1[a]

In process control[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Agilent 1100, UV detection at 220 nm.

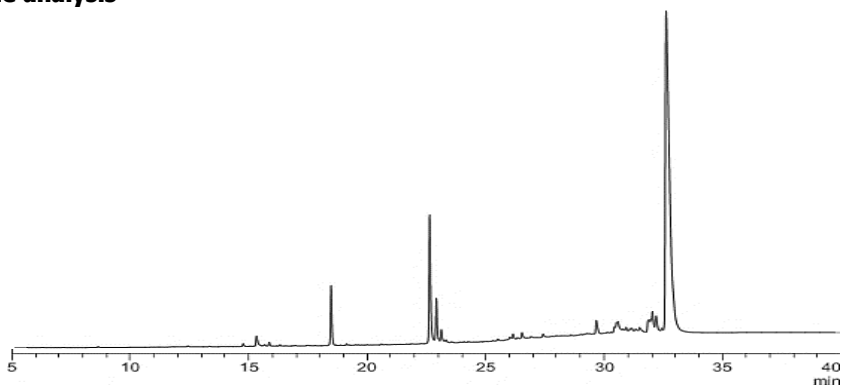
5.10.5.3.13. Batch 16J: Reaction with batch 15H-1

In a 50 mL reactor vessel with a magnetic stir bar were mixed in 4 mL of DMF the protected hexapeptide **8** (48.1 mg, 1.1 eq), the N-terminal deprotected undecapeptide **15** (78.9 mg, 1.0 eq, section 5.10.4.5) and HOAt (4.67 mg, 1.1 eq). Once the solution was clear, the temperature was set at -20 ºC with a thermostat and HATU (15.5 mg, 1.2 eq) was added after 10 min. Then, DIPEA (9.12 mg, 2.2 eq) was weighted over 1 mL of DMF and added dropwise with an automatic injector for 30 min. The reaction was monitored by HPLC-UV and after 3 h, 1 % of starting material **15** remained and the impurities with a $[M+H]^+$ of 1368.7 and 1382.7 were detected in a 3 % and 0.4 % respectively. The temperature was then set to 5 ºC and precipitation was achieved by adding dropwise H_2O (10 mL) with an addition funnel. The resulting suspension was filtered and dried under vacuum to afford 0.1125 g of a white solid containing **16** with a chromatographic purity of 67 % (32.6 min, figure 5.72), and 10 % of starting material **8**.

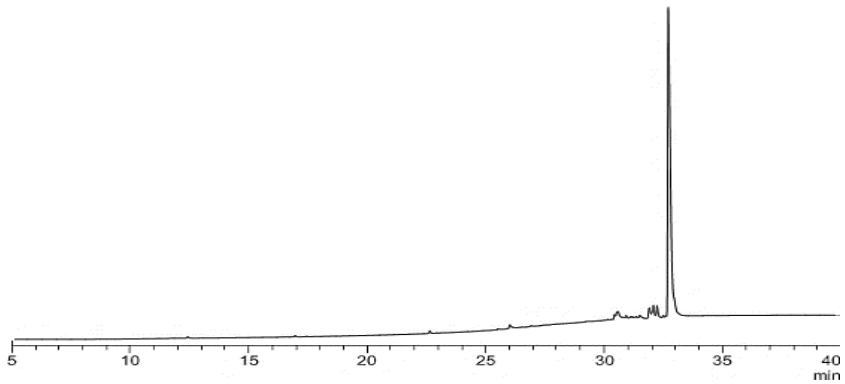
To purify the desired product, in a 50 mL round-bottom flask with a magnetic stir bar were mixed 94.3 mg the protected peptide **16** with 500 μL of EtOH. The solution became clear and additional EtOH (1.5 mL) and few drops of H_2O were added to precipitate the product. The temperature was then set to 40 ºC and the mixture was left stirring for 3 h when the resulting suspension was filtered and dried under vacuum. 38.2 mg of a white solid were obtained containing the desired product with a chromatographic purity of 84 % (figure 5.72), and a 0.5 % of starting material **8**. **MS (ESI):** m/z calc. for $[M+2H]^{2+}$ of $C_{204}H_{293}N_{27}O_{37}S_3$ 1905.6; found 1906.6.

Figure 5.72. 15 from batch 15H-1[a]

Crude analysis[b]



Isolated product[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Agilent 1100, UV detection at 220 nm.

5.10.5.3.14. Preliminary assay of reaction with a different batch of 8 (section 5.9.4.2.1)

The protected hexapeptide **8** (14.2 mg, 1.8 eq), the N-terminal deprotected undecapeptide **15** (14.1 mg, 1.0 eq, section 5.10.4.5), HOAt (2.23 mg, 2.8 eq), HATU (3.40 mg, 1.5 eq) and DIPEA (1.63 mg, 2.2 eq) were mixed in 1 mL of DMF with a magnetic stir bar. Temperature was not controlled, and the reaction was monitored by HPLC. After 1 h, 2 % of the impurity at 23.1 min and 2.5 % of the impurity at 23.3 min were detected (figure 5.73).

5.10.5.3.15. Preliminary assay of reaction using DMF from a different quality grade

The protected hexapeptide **8** (10.3 mg, 1.3 eq, section 5.9.4.2.1), the N-terminal deprotected undecapeptide **15** (13.8 mg, 1.0 eq, section 5.10.4.5), HOAt (1.76 mg, 2.3 eq), HATU (6.09 mg, 2.8 eq) and DIPEA (6.09 mg, 8.4 eq) were mixed in 1 mL of DMF-GC quality with a magnetic stir bar. Temperature was not controlled and the reaction was monitored by HPLC. After 1 h, 6 % of the impurity at 23.1 min and 4 % of the impurity at 23.3 min were detected (figure 5.73).

5.10.5.3.16. Preliminary assay of reaction using DIPEA from a different bottle

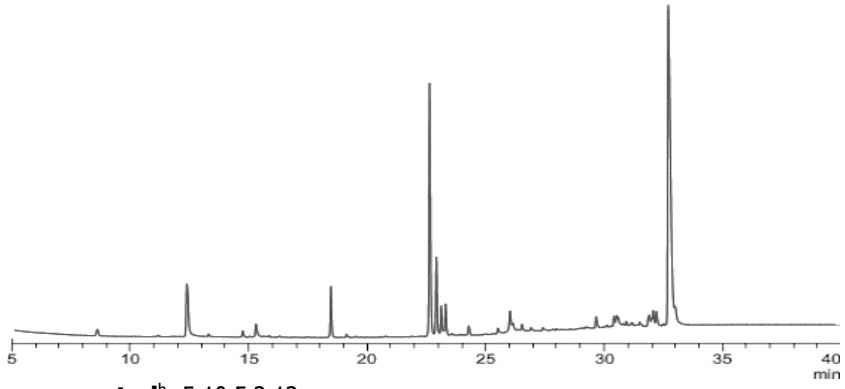
The protected hexapeptide **8** (9.75 mg, 1.3 eq, section 5.9.4.2.1), the N-terminal deprotected undecapeptide **15** (13.8 mg, 1.0 eq, section 5.10.4.5), HOAt (1.91 mg, 2.5 eq), HATU (4.50 mg, 2.1 eq) and DIPEA (2.9 mg, 2.1 eq) were mixed in 1 mL of DMF with a magnetic stir bar. Temperature was not controlled and the reaction was monitored by HPLC. After 1 h, 4.5 % of the impurity at 23.1 min and 3 % of the impurity at 23.3 min were detected (figure 5.73).

5.10.5.3.17. Preliminary assay of reaction using THF

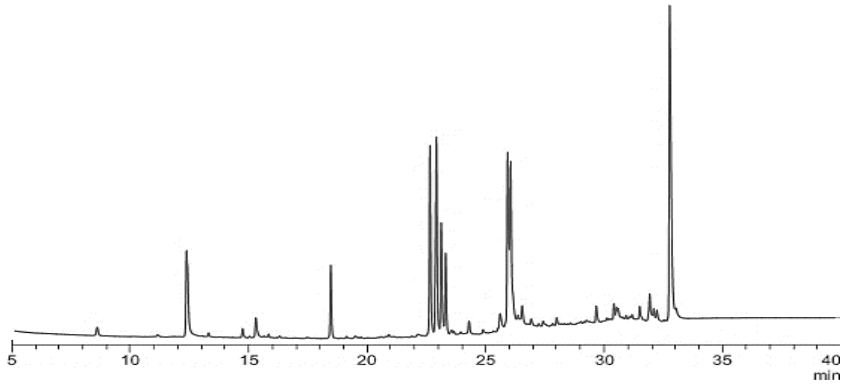
The protected hexapeptide **8** (8.84 mg, 1.3 eq, section 5.9.4.2.1), the N-terminal deprotected undecapeptide **15** (12.8 mg, 1.0 eq, section 5.10.4.5), HOAt (1.24 mg, 1.8 eq), HATU (7.28 mg, 3.7 eq) and DIPEA (2.12 mg, 3.1 eq) were mixed in 1 mL of THF with a magnetic stir bar but the reaction mixture did not become clear. Temperature was not controlled and the reaction was monitored by HPLC. The analytical sample had to be diluted NMP to dissolve all products. The impurities at 23.1 min and 23.3 min were not detected (figure 5.73).

Figure 5.73. Preliminary assays of different reaction conditions[a]

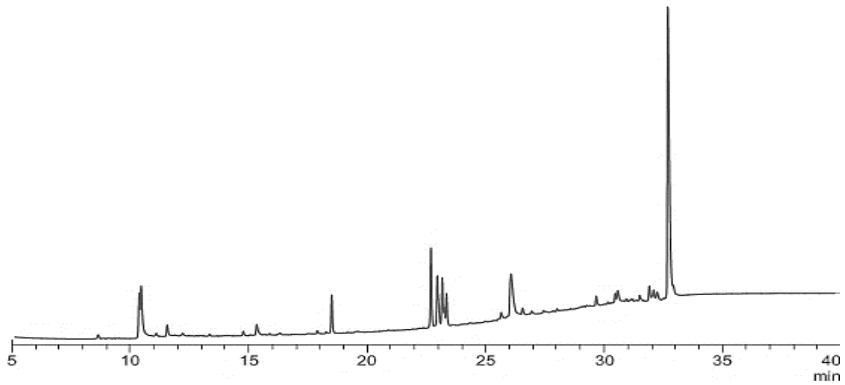
In process control[b]**:** 5.10.5.3.12.



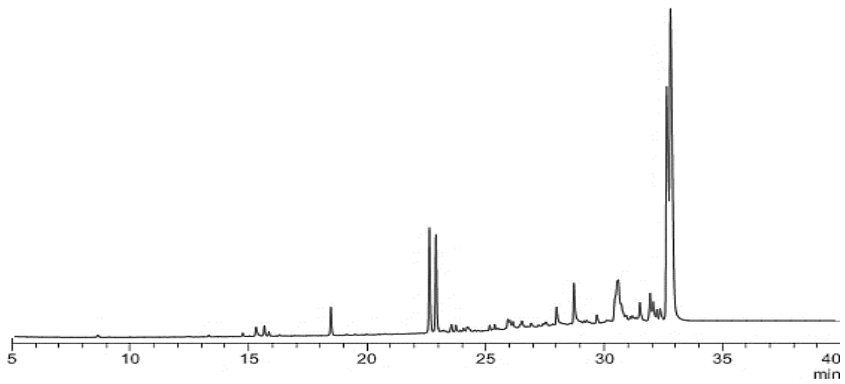
In process control[b]**:** 5.10.5.3.13.



In process control[b]**:** 5.10.5.3.14.



In process control[b]**:** 5.10.5.3.15.



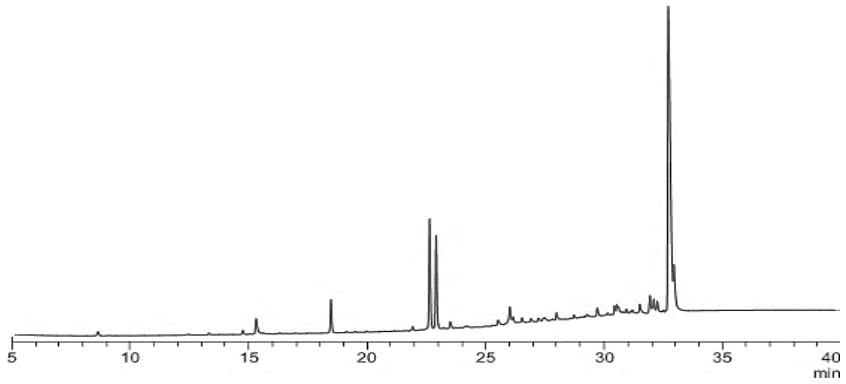
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Agilent 1100, UV detection at 220 nm.

5.10.5.3.18. Preliminary assay of reaction using NMP

The protected hexapeptide **8** (8.80 mg, 1.2 eq, section 5.9.4.2.1), the N-terminal deprotected undecapeptide **15** (13.2 mg, 1.0 eq, section 5.10.4.5), HOAt (0.78 mg, 1.1 eq), HATU (2.47 mg, 1.2 eq) and DIPEA (2.82 mg, 4.1 eq) were mixed in 1 mL of NMP with a magnetic stir bar. Temperature was not controlled and the reaction was monitored by HPLC. After 1 h, the impurities at 23.1 min and 23.3 min were less than 0.3 % (figure 5.74).

Figure 5.74. Preliminary assay using NMP[a]

In process control[b]



(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Agilent 1100, UV detection at 220 nm.

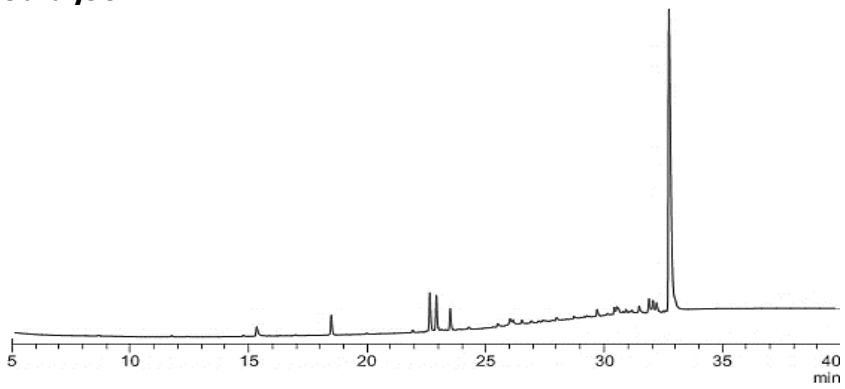
5.10.5.3.19. Batch 16K: Reaction using NMP

In a 100 mL reactor vessel with a magnetic stir bar were mixed in 12 mL of NMP the protected hexapeptide **8** (157.7 mg, 1.1 eq), the N-terminal deprotected undecapeptide **15** (261.8 mg, 1.0 eq, batch 15H-2, section 5.10.4.5) and HOAt (15.1 mg, 1.1 eq). Once the solution was clear, the temperature was set at -20 ºC with a thermostat and HATU (59.8 mg, 1.5 eq) was added after 10 min. Then, DIPEA (29.8 mg, 2.2 eq) was weighted over 3 mL of NMP and added dropwise with an automatic injector for 30 min. The reaction was monitored by HPLC-UV and after 4 h, there was a 1 % of starting material **15**. Then, the temperature was set to 5 ºC and the peptide was precipitated by adding dropwise H_2O (120 mL) with an addition funnel. The suspension was filtered and dried under vacuum to afford 0.335 g of a white solid containing the desired product with a chromatographic purity of 66 % (32.7 min, figure 5.75), and 9 % of starting material **8**.

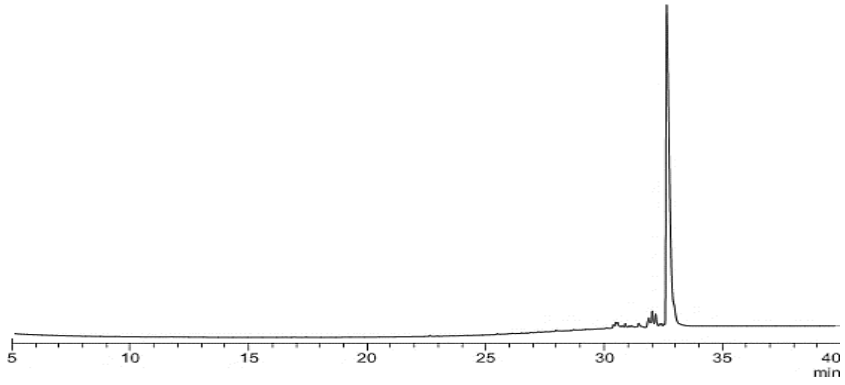
The desired product was purified in two batches. In the first batch (**A**), 500 μL of EtOH and **16** (92.9 mg) were mixed with a magnetic stir bar in a test tube at 40 ºC for 4 h. The suspension was filtered and dried under vacuum to afford 64.2 mg of a white solid that contained the desired **16** with a chromatographic purity of 82 %. In the second batch (**B**), 500 μL of EtOH and **16** (169.4 mg) were mixed with a magnetic stir bar in a test tube at 40 ºC for 4 h. The suspension was filtered and dried under vacuum to obtain 95.3 mg of a white solid containing the desired **16** with a chromatographic purity of 88 % (figure 5.75). **MS (ESI):** m/z calc. for $[M+2H]^{2+}$ of $C_{204}H_{293}N_{27}O_{37}S_3$ 1905.6; found 1906.6.

Figure 5.75. Reaction using NMP[a]

Crude analysis[b]

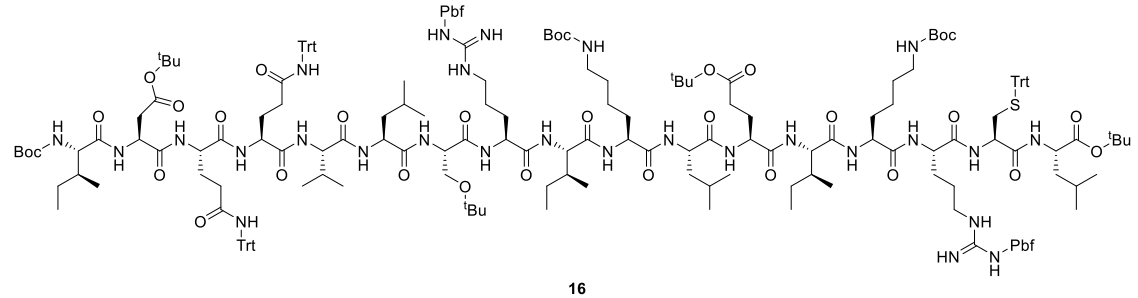


Isolated product[b]



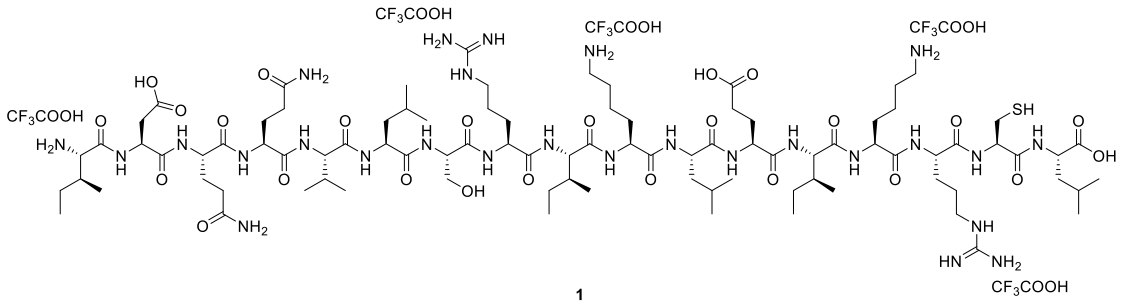
(a) Chromatographic conditions: Method B (Section 5.3.3.3).
(b) Agilent 1100, UV detection at 220 nm.

5.10.6. Synthesis of 1



16

1) TFA/TIPS/ EDT
(95:3:2), 5 ºC, 30 min
2) Stirring at 20 ºC



1

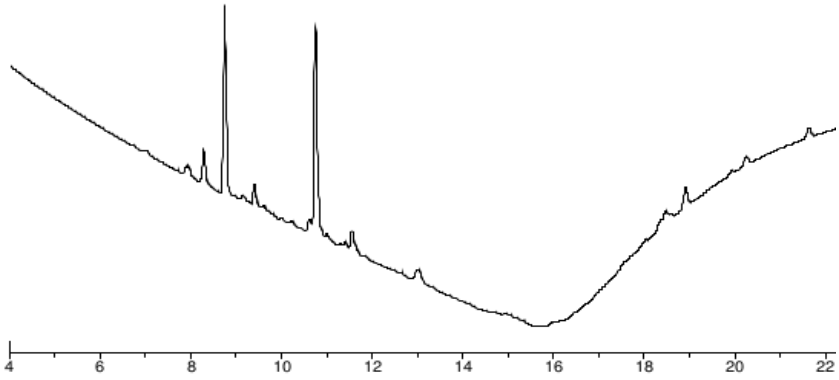
A solution of TFA, TIPS and EDT (95:3:2) was prepared and transferred into a 50 mL reactor vessel containing a magnetic stir bar. The temperature was set to 5 ºC with a thermostat and **16** was added into the reactor vessel. The reaction mixture became yellow. The temperature was at 5 ºC for 30 min. After this time, the temperature was increased to 20 ºC and the reaction mixture was left stirring for 3.5 h. The solution became reddish and after this time, the temperature was set to 3 ºC and MTBE was added dropwise with an addition funnel. A precipitate was formed. The suspension was filtered and dried under vacuum.

5.10.6.1. Batch 1C

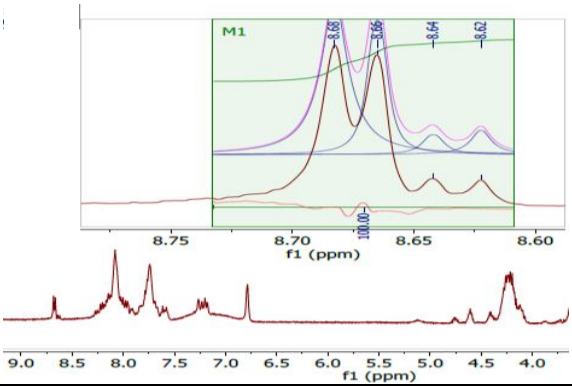
0.177 g of **16** (batch 16C) and 4 mL of the TFA solution. 0.1396 g of the desired **1** were obtained with a chromatographic purity of 50 % (figure 5.76), together with the unprotected piperidide **43** as the main impurity. ^{1}H-NMR deconvolution studies were performed to calculate the real percentage of the piperidide impurity (12 %). **MS (ESI):** m/z calc. for $[M+H]^+$ of $C_{90}H_{163}N_{27}O_{25}S$ 2055.2; found 2055.2.

Figure 5.76. HPLC chromatogram of 1[a]

Crude analysis[b]**:** Batch 1C



^{1}H-NMR deconvolution study[c]



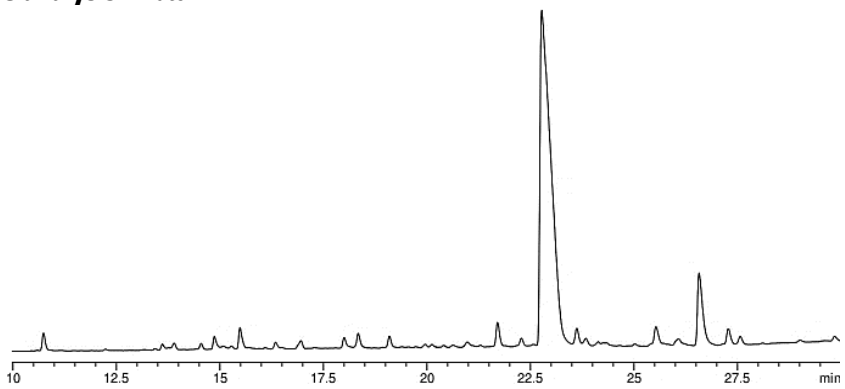
(a) Chromatographic conditions: Method E (Section 5.3.3.3).
(b) Agilent 1200 (QTOF), UV detection at 220 nm.
(c) Varian 400 spectrometer.

5.10.6.2. Batch 1D

238.6 mg of **16** (purified batches of 16B, 16J, 16K) and 6 mL of the TFA solution. 0.1381 g of the desired **1** were obtained with a chromatographic purity of 71 % (22.8 min, figure 5.77). **MS (ESI):** m/z calc. for $[M+H]^+$ of $C_{90}H_{163}N_{27}O_{25}S$ 2055.2; found 2055.2.

Figure 5.77. HPLC chromatogram of 1[a]

Crude analysis[b]**:** Batch 1D



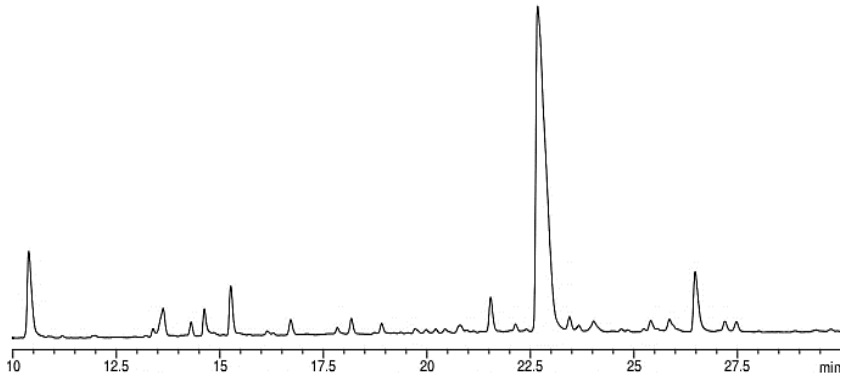
(a) Chromatographic conditions: Method F (Section 5.3.3.3).
(b) Agilent 1200 (6320 Ion Trap), UV detection at 210 nm.

5.10.6.3. Batch 1E

75.12 mg of **16** (purified batches of 16D, 16E and 16G) and 3 mL of the TFA solution. 34.53 mg of the desired **1** were obtained with a chromatographic purity of 57 % (22.7 min, figure 5.78). **MS (ESI):** m/z calc. for $[M+H]^+$ of $C_{90}H_{163}N_{27}O_{25}S$ 2055.2; found 2055.2.

Figure 5.78. HPLC chromatogram of 1[a]

Crude analysis[b]**:** Batch 1E



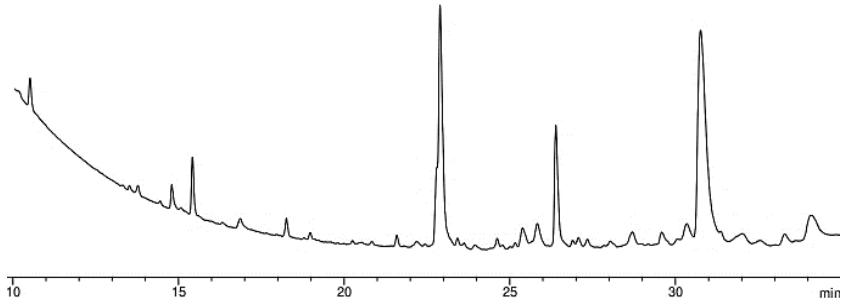
(a) Chromatographic conditions: Method F (Section 5.3.3.3).
(b) Agilent 1200 (6320 Ion Trap), UV detection at 210 nm.

5.10.6.4. Purification by precipitation at pH 9.5

35.88 mg of **1** (batch 1E) were added to a 25 mL two-necked round bottom flask that contained 8 mL of a 10 mM aq KH_2PO_4 buffer (pH = 2.0) and dissolved using a magnetic stir bar. An aqueous solution of 1.5 % NH_3 was added dropwise until pH 9.5 and the solution became cloudy. At this point, the mixture was left stirring for 2 h, the formation of a white solid was observed and the resulting suspension was filtered. The solid obtained was transferred into a 50 mL reactor vessel that contained 2 mL of TFA and left stirring for 30 min. The product was precipitated by adding dropwise MTBE (10 mL) with an addition funnel. The suspension was filtered, and the white solid was dried under vacuum. 5.68 mg of the desired **1** were obtained with a chromatographic purity of 22 % (22.9 min, figure 5.79), and 34 % of the dimeric form of **1** (30.8 min, figure 5.79). **MS (ESI):** m/z calc. for $[M+H]^+$ of $C_{90}H_{163}N_{27}O_{25}S$ 2055.2; found 2055.2.

Figure 5.79. Purification by precipitation at pH 9.5[a]

Crude analysis[b]



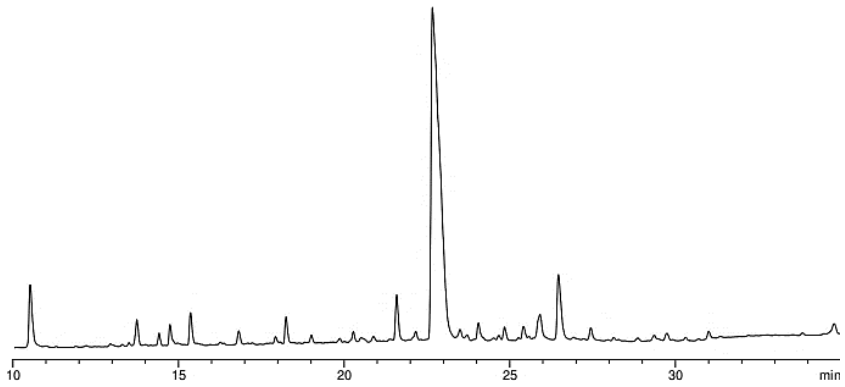
(a) Chromatographic conditions: Method F (Section 5.3.3.3).
(b) Agilent 1200 (6320 Ion Trap), UV detection at 210 nm.

5.10.6.5. Disulfide bond reduction using TCEP·HCl

17.11 mg of TCEP·HCl were dissolved in 10 mL of 0.045 % TFA/H_2O to obtain a 1.7 mg/mL solution of TCEP·HCl. 5.68 mg of **1** (section 5.10.6.4) were dissolved in 4 mL of this solution and left stirring. The reaction was monitored by HPLC-UV and after 4 h there was less than 0.5 % of the dimeric form of **1**. The chromatographic purity of **1** was of 61 % (22.7 min, figure 5.80).

Figure 5.80. Disulfide bond reduction using TCEP·HCl[a]

In process control[b] (t = 4 h)



(a) Chromatographic conditions: Method F (Section 5.3.3.3).
(b) Agilent 1200 (6320 Ion Trap), UV detection at 210 nm.

5.10.6.6. Purification by precipitation at pH 8.7: assay 1

40.97 mg of TCEP·HCl were dissolved in 10 mL of H_2O to obtain a 4.1 mg/mL solution of TCEP·HCl. Then, 30.24 mg of **1** (batch 1D) were weighed on a 50 mL round bottom flask and dissolved with the TCEP·HCl solution. An aqueous solution of 1.5 % NH_3 was added dropwise to the peptide solution until pH 8.7 and the solution became cloudy. The mixture was left stirring for 3 h and the white solid formed was filtered. The solid obtained was transferred intro a 50 mL reactor vessel that contained 2 mL of TFA/TIPS/EDT (95:3:2) and the resulting mixture was left stirring for 30 min. The product was precipitated by adding dropwise MTBE (10 mL) with an addition funnel. The suspension was filtered and dried under vacuum to afford 1.20 mg of the desired **1** with a chromatographic purity of 80 % (22.6 min, figure 5.81). **MS (ESI):** m/z calc. for $C_{90}H_{163}N_{27}O_{25}S^+$ $[M+H]^+$ 2055.2; found 2055.2.

The pH of the aqueous filtrates was readjusted to 8.7 and left stirring for 2 h. No precipitate was observed and the pH was adjusted to 9.5, when the solution became cloudy. At this point, the mixture was left stirring for 24 h and the resulting suspension was filtered. The solid was transferred intro a 25 mL round bottom flask that contained 1 mL of TFA/TIPS/EDT (95:3:2) and the mixture was left stirring for 30 min. The product was precipitated by adding dropwise MTBE (5 mL) with an addition funnel. The suspension was filtered and dried under vacuum to obtain 0.90 mg of a white solid where **1** had a chromatographic purity of 52 % (figure 5.82).

Figure 5.81. Purification by precipitation at pH 8.7[a]

Isolated product[b]

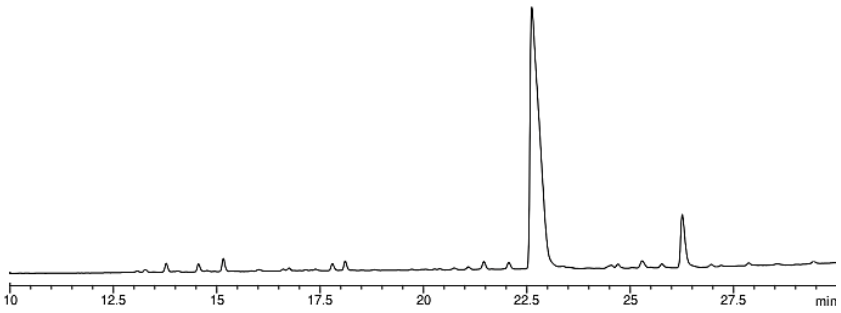
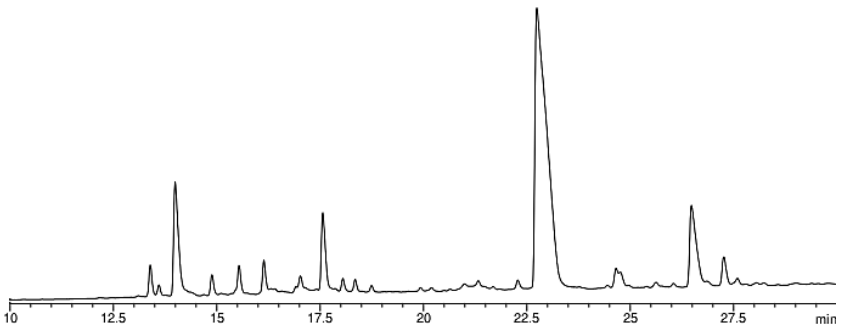


Figure 5.82. Filtrates at pH 9.5[a]

Isolated product[b]



(a) Chromatographic conditions: Method F (Section 5.3.3.3).
(b) Agilent 1200 (6320 Ion Trap), UV detection at 210 nm.

5.10.6.7. Purification by precipitation at pH 8.7: assay 2

156.02 mg of TCEP·HCl were dissolved in 20 mL of H_2O to obtain a 7.8 mg/mL solution of TCEP·HCl. Then, 67.70 mg of **1** (batch 1D) were weighed on a 50 mL round bottom flask and dissolved with 18 mL of the TCEP·HCl solution. An aqueous solution of 2.25 % NH_3 was added dropwise until pH 8.7 and the solution became cloudy. The mixture was left stirring for 6 h and the white solid formed was filtered. The obtained solid was transferred into a 50 mL reactor vessel that contained 2 mL of TFA/TIPS/EDT (95:3:2) and the resulting mixture was left stirring for 30 min. The product was precipitated by adding dropwise MTBE (10 mL) with an addition funnel. The suspension was filtered and dried under vacuum to afford 17.04 mg of the desired **1** with a chromatographic purity of 80 % (22.7 min, figure 5.83). **MS (ESI):** m/z calc. for $C_{90}H_{163}N_{27}O_{25}S^+$ $[M+H]^+$ 2055.2; found 2055.2.

The pH of the aqueous filtrates was readjusted to 8.7 and the resulting solution was left stirring for 2 h. No precipitate was observed and the pH was adjusted to 9.5 when the

solution became cloudy. At this point, the mixture was left stirring for 24 h and the suspension formed was filtered. The solid was transferred into a 25 mL round bottom flask that contained 1 mL of TFA/TIPS/EDT (95:3:2) and the resulting mixture was left stirring for 30 min. The product was precipitated by adding dropwise MTBE (5 mL) with an addition funnel. The resulting suspension was filtered and dried under vacuum to obtain 0.36 mg of a white solid containing **1** with a chromatographic purity 54 % (figure 5.84).

Figure 5.83. Purification by precipitation at pH 8.7[a]

Isolated product[b]

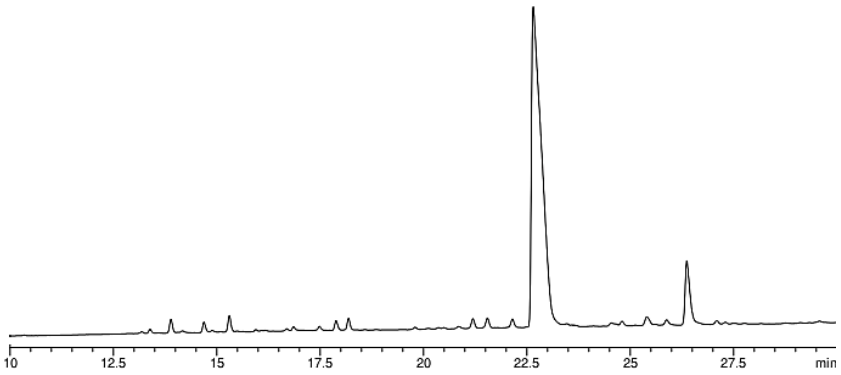
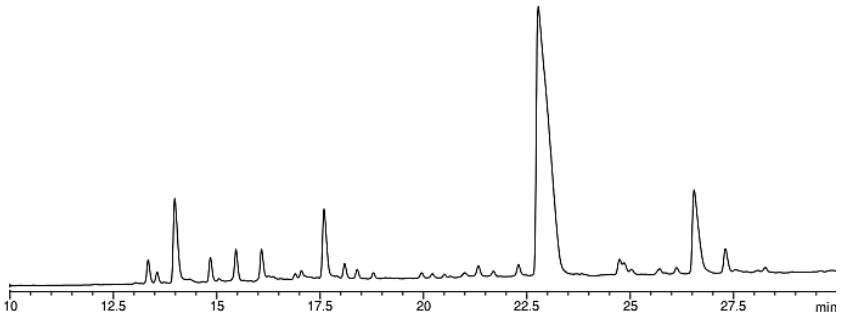


Figure 5.84. Filtrates at pH 9.5[a]

Isolated product[b]



(a) Chromatographic conditions: Method F (Section 5.3.3.3).
(b) Agilent 1200 (6320 Ion Trap), UV detection at 210 nm.

Chapter 4

5.11. Preliminary studies for the development of a new methodology to monitor reactions in SPPS

5.11.1. Incorporation of the first amino acid

5.11.1.1. Preliminary assay

5.30 g of 2-CTC resin (1.40 mmol/g) were weighed, transferred into a 150 mL reactor vessel and washed with DMF, DCM, DMF, DCM and DMF (1 × 50 mL). Then, 3.98 g of Fmoc-Leu-OH (1.5 eq) were weighed and dissolved with 9.45 g of DMF. This solution was transferred to the reactor vessel and stirred for 1 min. An aliquot of the supernatant was weighed in a 100 mL volumetric flask and diluted with ACN. Then, 2.96 g of DIPEA (3.0 eq) were weighed, added to the mixture and mechanically stirred. An aliquot of the supernatant was weighed at different reaction times in a 100 mL volumetric flask and diluted with ACN. These solutions were analysed by HPLC.

The solution containing the aliquot taken before the addition of the base was considered as a control solution and the amino acid concentration in that solution was calculated from the amount of amino acid in the volumetric flask, which was determined as shown in entry 1, table 5.37. That allowed to determine the ratio Aa concentration to peak area by HPLC. The other aliquots (entries 2, 3 and 4; table 5.38) were taken after the addition of the base, that is, once the reaction had started. The amino acid concentration in the volumetric flask of these samples was calculated taking in consideration the peak area of the sample and the ratio amino acid concentration to peak area of the control solution. Finally, the total amount of non-reacted amino acid in the reaction mixture was determined from the amount of amino acid in the volumetric flask (entry 2, table 5.37) and, thus, the amount of amino acid that was incorporated into the resin could be determined.

Table 5.37. Determination of the amount of Aa in the volumetric flask.

Entry	Before the addition of DIPEA
1	$\text{g of aliquot}\dfrac{\text{g of Aa in the reaction}}{\text{g of Aa in the reaction + g of DMF}} = \text{g of Aa in the volumetric flask}$
Entry	**After the addition of DIPEA**
2	$\text{g of aliquot}\dfrac{\text{g of Aa in the reaction}}{\text{g of Aa in the reaction + g of DMF + g of DIPEA}} = \text{g of Aa in the volumetric flask}$

Table 5.38. Results obtained for the HPLC quantification of Aa in the supernatant.

Entry	Time [h]	g of aliquot	HPLC area	mg/mL volumetric flask	g of Aa in the volumetric flask	g of Aa in the reaction mixture
1	0	0.114	13067111	0.338[a]	0.0338	3.98
2	1	0.237	16364053	0.423[b]	0.0423	2.69
3	2	0.240	17479765	0.452[b]	0.0452	2.88
4	3	0.230	14287058	0.369[b]	0.0369	2.37

(a) Calculated with experimental data.
(b) Calculated by HPLC.

As shown in table 5.38, the amount of amino acid decreased from 3.98 g (entry 1) to 2.37 g after 3 h (entry 4). Therefore, 1.61 g of amino acid was incorporated to the resin (0.66 mmol/g).

5.11.1.2. Reproducibility of the methodology

5.31 g of 2-CTC resin (1.40 mmol/g) were weighed, transferred into a 150 mL reactor vessel and washed with DMF, DCM, DMF, DCM and DMF (1 × 50 mL). Then, 3.97 g of Fmoc-Leu-OH (1.5 eq) were weighed and dissolved with 18.90 g of DMF. This mixture was transferred to the reactor vessel and stirred for 1 min when an aliquot of the supernatant was weighed in a 50 mL volumetric flask and diluted with ACN. Then, 9.62 g of DIPEA (10.0 eq) were weighed, added to the resulting suspension and stirred. Two aliquots of the supernatant were taken at each sampling reaction time, weighed in a 50 mL volumetric flask and diluted with ACN. After 3 h of reaction, 0.8 mL/g of MeOH were added and the mixture was stirred for an 1 h. At that time, the suspension was filtered by suction and the resin was washed with DMF (3 × 50 mL) and MeOH (2 × 50 mL).

The solutions prepared from the samples were analysed by HPLC and a protocol similar to that explained in section 5.11.1.1 was followed to determine the amounts of amino acid that reacted. Table 5.39 shows the results obtained in these experiments.

Table 5.39. Results obtained in the HPLC quantification of Aa in the supernatant.

Entry		**Time** [h]						
		0	1[c]	1[d]	2[c]	2[d]	3[c]	3[d]
1	g of aliquot	0.133	0.166	0.165	0.125	0.141	0.172	0.169
2	HPLC area	17730350	11013494	12066114	7590006	8541107	9995543	10201567
3	mg/mL in the volumetric flask	0.461[a]	0.286[b]	0.314[b]	0.197[b]	0.222[b]	0.259[b]	0.265[b]
4	g of Aa in the volumetric flask	0.023	0.014	0.016	0.009	0.011	0.013	0.013
5	g of Aa in the reaction mixture	3.97	2.69	2.99	2.44	2.44	2.31	2.43

(a) Calculated with experimental data.
(b) Calculated by HPLC.
(c) First sampling.
(d) Second sampling.

The amount of amino acid that was incorporated to the resin after 3 h of reaction was contrasted with that obtained by weighing the resin before and after coupling the amino acid. Thus, the HPLC analysis gave a loading between 0.64 mmol/g and 0.67 mmol/g while a loading of 0.57 mmol/g was determined by weight.

5.11.1.3. HPLC quantification vs Fmoc /by weight quantification

4.97 g of 2-CTC resin (1.40 mmol/g) were weighed, transferred into a 150 mL reactor vessel and washed with DMF, DCM, DMF, DCM and DMF (1 × 50 mL). Then, 3.69 g of Fmoc-Leu-OH (1.5 eq) were weighed and dissolved with 18.72 g of DMF. This mixture was transferred to the reactor vessel and the resulting suspension was stirred for 1 min. At that time, an aliquot of the supernatant was weighed in a 100 mL volumetric flask and diluted with ACN. Then, 9.04 g of DIPEA (10.0 eq) were weighed, added to the reactor vessel and stirred. An aliquot of the supernatant was taken at different reaction times, weighed in a 100 mL volumetric flask and diluted with ACN. After 3 h of reaction, 0.8 mL/g of MeOH were

added and stirred for 1 h. Then, the suspension was filtered by suction and the resin was washed with DMF (3 × 50 mL) and MeOH (2 × 50 mL).

The solutions prepared from the samples were analysed by HPLC and a protocol similar to that explained in section 5.11.1.1 was followed to determine the amounts of amino acid that reacted. Table 5.40 shows the results obtained in these experiments.

Table 5.40. Results obtained for the HPLC quantification of Aa in the supernatant.

Entry		Time [h]				
		0	1	2	3	4
1	g of aliquot	0.201	0.216	0.200	0.200	0.210
2	HPLC area	12711573	6202497	5318555	5101910	5226806
3	mg/mL in the volumetric flask	0.331[a]	0.162[b]	0.138[b]	0.133[b]	0.136[b]
4	g of Aa in the volumetric flask	0.033	0.016	0.014	0.0133	0.014
5	g of Aa in the reaction mixture	3.69	2.24	2.06	1.97	1.92

(a) Calculated with experimental data.
(b) Calculated by HPLC.

The amount of amino acid that was incorporated to the resin after 3 h of reaction was contrasted with that obtained by Fmoc quantification (UV-Vis, section 5.5.3) and by weight. The loading was found to be 0.74 mmol/g (HPLC), 0.67 mmol/g (UV-Vis) and 0.50 mmol/g (weight).

5.11.1.4. Assay of the HPLC methodology when increasing the scale

9.77 g of 2-CTC resin (1.40 mmol/g) were weighed, transferred into a 150 mL reactor vessel and washed with DMF, DCM, DMF, DCM and DMF (1 × 100 mL). Then, 7.69 g of Fmoc-Leu-OH (1.5 eq) were weighed and dissolved with 41.18 g of DMF. The mixture was transferred to the reactor vessel and stirred for 1 min when an aliquot of the supernatant was weighed in a 100 mL volumetric flask and diluted with ACN. Then, 17.92 g of DIPEA (10.1 eq) were weighed, added to the mixture and the resulting suspension was stirred. An aliquot of the supernatant was taken at different reaction times, weighed in a 100 mL volumetric flask and diluted with ACN. After 3 h of reaction, 0.8 mL/g of MeOH were added and stirred for 1 h. Then, the suspension was filtered by suction and the resin was washed with DMF (3 × 100 mL) and MeOH (2 × 100 mL).

The solutions prepared from the samples were analysed by HPLC and a protocol similar to that explained in section 5.11.1.1 was followed to determine the amounts of amino acid that reacted. Table 5.41 shows the results obtained in these experiments.

Table 5.41. Results obtained for the HPLC quantification of Aa in the supernatant.

Entry		Time [h]				
		0	1	2	3	4
1	g of aliquot	0.204	0.207	0.214	0.227	0.206
2	HPLC area	11466047	6173030	6186822	56413423	5847361
3	mg/mL in the volumetric flask	0.321[a]	0.173[b]	0.173[b]	0.179[b]	0.164[b]
4	g of Aa in the volumetric flask	0.032	0.017	0.017	0.018	0.016
5	g of Aa in the reaction mixture	7.69	5.38	5.20	5.07	5.10

(a) Calculated with experimental data.
(b) Calculated by HPLC.

After 3 h of reaction, the loadings were determined: 0.59 mmol/g (HPLC), 0.64 mmol/g (UV-Vis) and 0.58 mmol/g (weight).

5.11.1.5. Analysis of a standard solution by the HPLC methodology

10.08 g of 2-CTC resin (1.40 mmol/g) were weighed, transferred into a 150 mL reactor vessel and washed with DMF, DCM, DMF, DCM and DMF (1 × 100 mL). Then, 7.50 g of Fmoc-Leu-OH (1.5 eq) were weighed and dissolved with 32.45 g of DMF. This mixture was transferred to the reactor vessel and stirred for 1 min. An aliquot of the supernatant was weighed in a 100 mL volumetric flask and diluted with ACN. Then, 5.64 g of DIPEA (3.1 eq) were weighed, added to the mixture and stirred. An aliquot of the supernatant was taken at different reaction times, weighed in a 100 mL volumetric flask and diluted with ACN. After 3 h of reaction, 0.8 mL/g of MeOH were added and stirred for 1 h. Then, the suspension was filtered by suction and the resin was washed with DMF (3 × 100 mL) and MeOH (2 × 100 mL).

The solutions prepared from the samples were analysed by HPLC and a protocol similar to that explained in section 5.11.1.1 was followed to determine the amounts of amino acid that reacted. Table 5.42 shows the results obtained in these experiments.

Table 5.42. Results obtained in the HPLC quantification of Aa in the supernatant using the control solution as reference.

Entry		Time [h]			
		0	1	2	3
1	g of aliquot	0.211	0.209	0.233	0.206
2	HPLC area	12919413	8485403	8949788	7810109
3	mg/mL in the volumetric flask	0.396[a]	0.260[b]	0.274[b]	0.239[b]
4	g of Aa in the volumetric flask	0.039	0.026	0.027	0.024
5	g of Aa in the reaction mixture	7.50	5.41	5.08	5.00

(a) Calculated with experimental data.
(b) Calculated by HPLC.

To prepare the standard solution, 87.3 mg of Fmoc-Leu-OH were weighed in a 100 mL volumetric flask and diluted with ACN. Then, 5 mL of this solution were transferred to a 25 mL volumetric flask with a volumetric pipette, diluted with ACN and analysed by HPLC to

determine the ratio amino acid concentration to peak area. The amino acid concentration of the solutions prepared from the samples taken at different reaction times was calculated taking in consideration the peak area of the sample and the ratio mentioned before. Finally, the total amount of non-reacted amino acid in the reaction mixture was calculated and thus, the amount of amino acid that was incorporated into the resin could be determined. Table 5.43 shows the results that were obtained using the standard solution as reference.

Table 5.43. Results obtained in HPLC the quantification of Aa in the supernatant using the standard solution as reference.

Entry		**Time** [h]			
		Standard solution	1	2	3
1	g of aliquot	0.08729	0.209	0.233	0.206
2	HPLC area	7981072	8485403	8949788	7810109
3	mg/mL in the volumetric flask	0.175[a]	0.185[b]	0.198[b]	0.173[b]
4	g of Aa in the volumetric flask	0.017	0.018	0.019	0.017
5	g of Aa in the reaction mixture	-	3.71	3.53	3.48

(a) Calculated with experimental data.
(b) Calculated by HPLC.

The amino acid loadings were 0.56 mmol/g and 0.80 mmol/g when the control and standard solutions were used, respectively. The amino acid loading determined by Fmoc quantification (section 5.5.3) was 0.63 mmol/g.

5.11.2. Fmoc removal

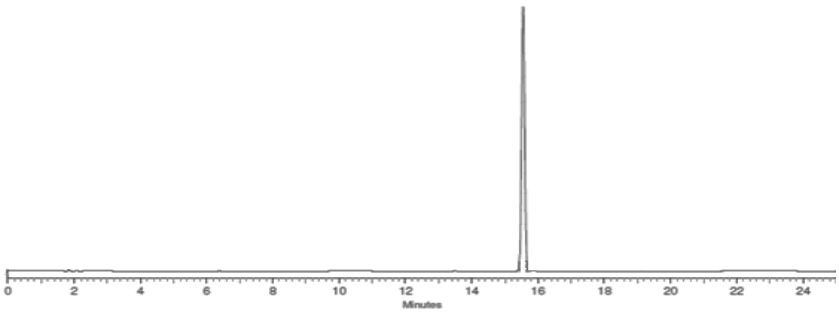
5.11.2.1. Using 1,8-diazabicycloundec-7-ene (DBU)

5.11.2.1.1. Synthesis of DBF

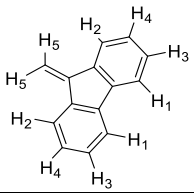
DBF was prepared from the treatment with a 5 % DBU in DMF of an Fmoc protected aminoacyl resin. The reaction mixture was filtered by suction and the filtrate was taken up with EtOAc. Extractions with H_2O (3 × 100 mL) were performed and the organic phase was dried with Na_2SO_4 and concentrated under reduced pressure to yield 0.378 g of the target product as a white solid. Figure 5.85 shows the ^{1}H-RMN and HPLC analysis (15.5 min) of the product.

Figure 5.85. HPLC chromatogram of DBF[a]

Isolated product[b]



^{1}H-NMR[c]



($CDCl_3$, 400 MHz): δ 7.73 (ddd, *J* = 7.50, 1.20, 0.80 Hz, 2H, H_1), 7.69 (ddd, *J* = 7.50, 0.80 Hz, 2H, H_2), 7.37 (td, *J* = 7.50, 1.20 Hz, 2H, H_3), 7.30 (td, *J* = 7.40, 1.20 Hz, 2H, H_4), 6.09 (s, 2H, H_5)

(a) Chromatographic conditions: Method C (Section 5.3.3.3).
(b) Lachrom Elite, UV detection at 265 nm.
(c) Varian 400 spectrometer.

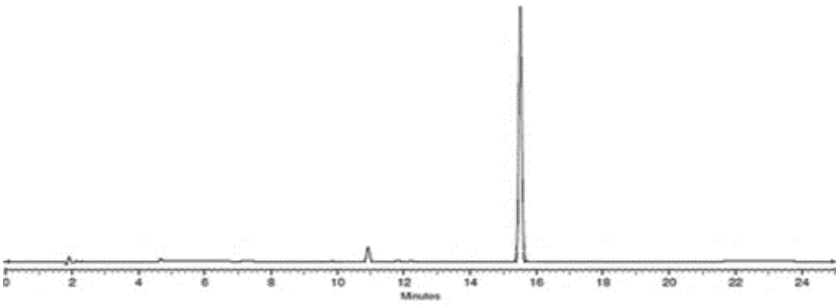
5.11.2.1.2. HPLC monitoring with a DBF external standard

A 5 % DBU in DMF solution (47.16 g) was added to an aminoacyl resin with a loading of 0.41 mmol/g, determined by UV-Vis (section 5.5.3) and stirred for 20 min at rt. An aliquot of the supernatant was taken every 10 min after the addition of the DBU solution, weighed in a 100 mL volumetric flask and diluted with ACN.

An external DBF standard solution was prepared in a 25 mL volumetric flask, diluting 2.78 mg of DBF in DMF. An aliquot of 5 mL of this solution was then transferred to a 10 mL volumetric flask with a volumetric pipette, diluted with ACN and analysed by HPLC to determine the ratio DBF concentration to peak area. However, this ratio could not be evaluated because of the unexpected presence of impurities in the DBF solution when analysed prior to its use and the poor solubility of the product (figure 5.86).

Figure 5.86. HPLC chromatogram of DBF standard solution[a]

In process control[b]



(a) Chromatographic conditions: Method C (Section 5.3.3.3).
(b) Lachrom Elite, UV detection at 265 nm.

5.11.2.1.3. HPLC monitoring of two consecutive DBU treatments

A 5 % DBU in DMF solution (49.47 g) was added to an aminoacyl resin (6.74 g) and the resulting suspension was stirred for 20 min. An aliquot of the supernatant was taken every 10 min after the addition of the DBU solution, weighed in a 100 mL volumetric flask and diluted with ACN. After 20 min, the reaction mixture was filtered by suction and the procedure was repeated. The supernatant solutions were analysed by HPLC and DBF peak areas were determined. Table 5.44 summarises the results obtained in this assay.

Table 5.44. Results obtained of the HPLC monitoring (Fmoc removal using DBU).

Entry	**Time** [min]	**mg of aliquot**	**HPLC area**	**HPLC area/mg**
First DBU treatment				
1	10	206	45483234	220792
2	20	216	47184950	218449
Second DBU treatment				
3	10	431	51148683	118674
4	20	431	51687246	119924

5.11.2.1.4. HPLC monitoring of two DBU treatments with washings in between

A 5 % DBU in DMF solution (93.90 g) was added to an aminoacyl resin (12.28 g) and the resulting suspension was stirred for 20 min. An aliquot of the supernatant was taken every 10 min after the addition of the DBU solution, weighed in a 100 mL volumetric flask and diluted with ACN. After 20 min, the reaction mixture was filtered by suction, the resin was washed with DMF (3 × 100 mL) and the procedure was repeated. The supernatant solutions were analysed by HPLC and DBF peak areas were determined. Table 5.45 summarises the results obtained in this assay.

Table 5.45. Results obtained of the HPLC monitoring with washings in between (Fmoc removal using two DBU treatments).

Entry	**Time** [min]	**mg of aliquot**	**HPLC area**	**HPLC area/mg**
First DBU treatment				
1	10	149	36553163	245323
2	20	164	37759505	230240
Second DBU treatment				
3	10	152	1878799	12360
4	20	168	2149270	12793

5.11.2.1.5. HPLC monitoring of three DBU treatments with washings in between

A 5 % DBU in DMF solution (93.92 g) was added to a peptidyl-resin (12.97 g) and the resulting solution was stirred for 20 min. An aliquot of the supernatant was taken every 10 min after the addition of the DBU solution, weighed in a 100 mL volumetric flask and diluted with ACN. After 20 min, the reaction mixture was filtered by suction, the resin was washed with DMF (3 × 100 mL) and the procedure was repeated twice. The supernatant solutions were analysed by HPLC and DBF peak areas were determined. Table 5.46 summarises the results obtained in this assay.

Table 5.46. Results obtained of the HPLC monitoring with washings in between (Fmoc removal using three DBU treatments).

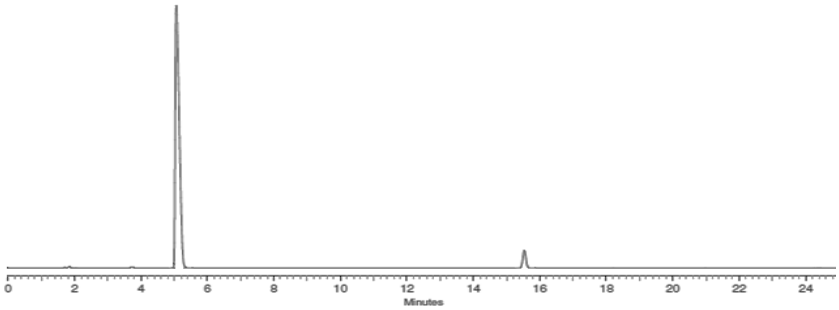
Entry	Time [min]	mg of aliquot	HPLC area	HPLC area/mg
First DBU treatment				
1	10	222	35168935	158419
2	20	212	32975892	155547
Second DBU treatment				
3	10	207	276919	1338
4	20	213	288539	1355
Third DBU treatment				
5	10	204	0	0
6	20	207	0	0

5.11.2.2. Using piperidine

A 20 % piperidine in DMF solution was added to an Fmoc protected aminoacyl resin and the resulting suspension was stirred for 2 h. An aliquot of supernatant was taken every 10 min, weighed in a 100 mL volumetric flask and diluted with ACN. These solutions were analysed by HPLC (figure 5.87) and, after 2 h of reaction time, a 4 % of DBF (15.5 min) and a 96 % of the piperidine-adduct (5.1 min) were observed.

Figure 5.87. HPLC chromatogram of Fmoc removal using piperidine[a]

In process control[b]**:**



(a) Chromatographic conditions: Method C (Section 5.3.3.3).
(b) Lachrom Elite, UV detection at 265 nm.

5.11.2.2.1. HPLC monitoring of three piperidine treatments with washings in between

A 20 % piperidine in DMF solution (100 mL) was added to an aminoacyl resin (14.26 g) and the resulting solution was stirred for 5 min. An aliquot of the supernatant was taken, weighed in a 100 mL volumetric flask and diluted with ACN. Then, the reaction mixture was filtered by suction and the resin was washed with DMF (3 × 100 mL). The procedure was repeated twice and the supernatant solutions were analysed by HPLC. Table 5.47 summarises the results obtained in this assay.

Table 5.47. Results obtained of the HPLC monitoring (Fmoc removal using piperidine).

Entry	Time [min]	mg of aliquot	HPLC area	HPLC area/mg
First piperidine treatment				
1	5	211	27037114	128137
Second piperidine treatment				
2	2	211	228385	1082
Third piperidine treatment				
3	2	208	0	0

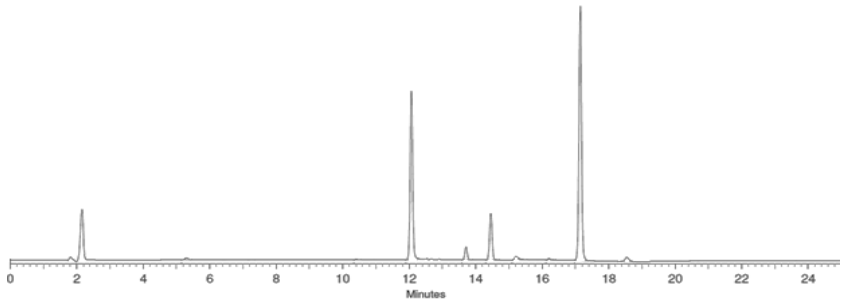
5.11.3. Elongation of the peptide chain

5.11.3.1. Preliminary assay

4.29 g (3.1 eq) of Fmoc-Val-OH and 1.86 g (3.0 eq) of HOBt were dissolved in 22.27 g of DMF and transferred to a reactor vessel containing the aminoacyl resin (6.65 g). An aliquot of supernatant (0.338 g) was weighed in a 100 mL volumetric flask and diluted with ACN. Then, 1.54 g (3.0 eq) of DIC were added and the resulting suspension was stirred 1.5 h when an aliquot (0.253 g) of supernatant was weighed in a 50 mL volumetric flask and diluted with ACN. These solutions were analysed by HPLC to determine the total amount of non-reacted amino acid in the supernatant, but that amount was not quantified due to the presence of more than one peak in the chromatogram (figure 5.88). The peak with a retention time of 12.1 min belongs to Fmoc-Val-OH but the peaks with retention time of 14.5 min and 17.2 min might correspond to the intermediates generated during the coupling reaction.

Figure 5.88. HPLC monitoring of the coupling reaction[a]

In process control[b]:



(a) Chromatographic conditions: Method C (Section 5.3.3.3).
(b) Lachrom Elite, UV detection at 265 nm.

5.11.3.2. Assay using 0.1 % NH_3 in ACN as HPLC sample diluent

10.97 g (3.1 eq) of Fmoc-Val-OH and 4.82 g (3.0 eq) of HOBt were dissolved in 36.70 g of DMF and transferred to a reactor vessel containing the aminoacyl resin (14.10 g). An aliquot of supernatant (0.220 g) was weighed in a 100 mL volumetric flask and diluted with 0.1 % NH_3 in ACN. Then, 3.99 g (3.0 eq) of DIC were added with 7.64 g of DMF and the resulting mixture was stirred for 1 h when an aliquot (0.223 g) of supernatant was weighed in a 100 mL volumetric flask and diluted with 0.1 % NH_3 in ACN.

The aliquot taken before the addition of the carbodiimide was considered as a control solution and the amino acid concentration in that solution was determined as described in section 5.11.1.1 The aliquot taken after addition of the carbodiimide was analysed by HPLC

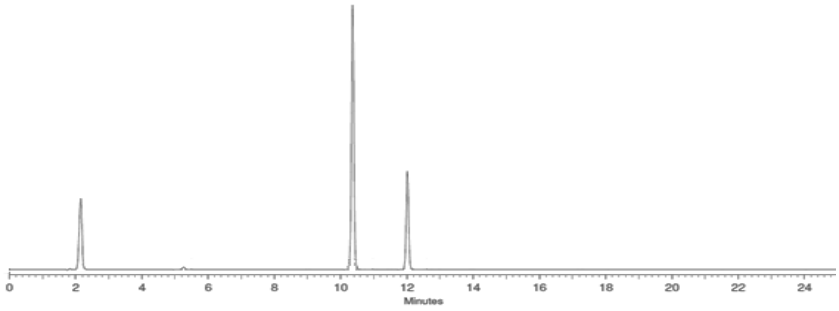
and a protocol similar to that explained in this section was followed to determine the amount of amino acid that was reacted.

A standard solution was prepared weighing 63.2 mg of Fmoc-Val-OH in a 100 mL volumetric flask and diluting with 0.1 % NH_3 in ACN. Then, 1 mL of this solution was transferred to a 2 mL volumetric flask with a volumetric pipette, diluted with 0.1 % NH_3 in ACN and was analysed by HPLC. The amino acid concentration in the volumetric flask of the sample taken after 1 h of reaction was calculated taking in consideration the peak area of the amino acid and the ratio amino acid concentration to peak area determined for the standard solution. Thus, the total amount of non-reacted amino acid in the reaction mixture could be calculated.

The chromatographic profile of the sample taken after 1 h of reaction showed two peaks with retention times of 12.1 min and 10.4 min corresponding to Fmoc-Val-OH and Fmoc-Val-NH_2, respectively (figure 5.89).

Figure 5.89. HPLC chromatogram with 0.1 % NH_3 in ACN as diluent[a]

In process control[b]:



(a) Chromatographic conditions: Method C (Section 5.3.3.3).
(b) Lachrom Elite, UV detection at 265 nm.

Table 5.48 and table 5.49 summarise the results obtained in this assay.

Table 5.48. Results obtained in the HPLC quantification of Fmoc-Val-OH in the supernatant using the control solution as reference.

Entry		**Time** [h]	
		0	1
1	g of aliquot	0.220	0.223
2	HPLC area	21254455	10389563
3	mg/mL in the volumetric flask	0.459[a]	0.224[b]
4	g of Aa in the volumetric flask	0.046	0.022
5	g of Aa in the reaction mixture	10.97	5.95
6	eq of Aa in the reaction mixture	3.1	1.68

(a) Calculated with experimental data.
(b) Calculated by HPLC.

Table 5.49. Results obtained in the HPLC quantification of Fmoc-Val-OH in the supernatant using the standard solution as reference.

Entry		Time [h]	
		External standard	1
1	g of aliquot	0.06316	0.223
2	HPLC area	14827967	10389563
3	mg/mL in the volumetric flask	0.316[a]	0.221[b]
4	g of Aa in the volumetric flask	-	0.022
5	g of Aa in the reaction mixture	-	5.85
6	eq of Aa in the reaction mixture	-	1.65

(a) Calculated with experimental data.
(b) Calculated by HPLC.

After 1 h of reaction, 5.95 g (1.68 eq) of non-reacted amino acid remained in the reaction mixture when the control solution was used for quantification, and 5.85 g (1.65 eq) of unreacted amino acid resulted from quantification the standard solution. In both cases, the ninhydrin test gave a positive result.

5.11.3.3. Evaluation and simulation of a coupling reaction

Fmoc-Val-OH (6.25 g, 3.0 eq) and HOBt (2.65 g, 2.8 eq) were dissolved in 9.44 g of DMF. The solution was transferred to the reactor that contained 4.42 g of resin and the resulting suspension was stirred for 3 h. An aliquot of the supernatant was weighed at different reaction times in a 100 mL volumetric flask and diluted with ACN. Then, 1 mL of this solution was transferred to a 10 mL volumetric flask with a volumetric pipette and diluted with ACN for further analysis by HPLC.

A solution of 31.83 mg of Fmoc-Val-OH in 100 mL of ACN was prepared in a volumetric flask to be used as the standard solution to determine the amount of amino acid in the supernatant by HPLC.

Table 5.50 summarises the results obtained in this assay. An amount of amino acid in the reaction mixture of 4.06 g on average was determined by HPLC, while the amount of 6.22 g (average) resulted when considering the total amount of amino acid used in the assay.

Table 5.50. Results obtained in the simulation of a coupling reaction.

mg/mL standard solution	0.3183	**HPLC area standard solution**		17241106	
Entry	**g of supernatant**	**HPLC area**	**mg/mL volumetric flask[a]**	**g of Aa in the supernatant[a]**	**g of Aa in the supernatant**
1	0.457	6280734	0.116	4.11	6.20
2	0.461	6292229	0.116	4.07	6.23
3	0.447	6072228	0.112	4.04	6.23
4	0.466	6322688	0.117	4.04	6.23

(a) Calculated by HPLC.