UNIVERSITAT DE BARCELONA

Linkers for Bioconjugation

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Programa de Doctorado de Química Orgánica

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Linkers for bioconjugation

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A veces sentimos que lo que hacemos es tan solo una gota en el mar,
pero el mar sería menos si le faltara una gota.
Madre Teresa de Calcuta (*escrito de un sobrecito de azúcar*)

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Abbreviations and Acronyms

AA	Amino acid
Ac	Acetyl
ADC	Antibody drug conjugate
BSA	bovine serum albumin protein
Bzl	Benzyl
CDI	*N,N'*-carbonyldiimidazole
Chx	Ciclohexyl
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DDS	Drug delivery systems
dil.	diluted
DKP	Diketopiperazine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDC·HCl	*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride
ESI	Electrospray ionization
FITC	Fluoresceinisothiocyanate
GSH	Reduced glutathione
GSSG	Oxidized glutathione
h	Hours
HBSS	Hank's Balanced Salt solution
HPLC	High performance liquid chromatography
J	Coupling constant
m	Minutes
m/z	Mass charge relation

mAb	Monoclonal antibody
MCRs	Multicomponent reactions
MeCN	Acetonitrile
MES	2-(*N*-morpholino)ethanesulfonic acid
MFI	Mean fluorescence intensity
PABC	*para*-aminobenzyl carbamate
PBS	Phosphate buffer saline
PEG	Polyethyleneglycol
PG	Protecting group
PMB	*para*-methoxybenzyl
PSA	Prostate specific antigen
PSMA	Prostate-specific membrane antigen
s	Seconds
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide electrophoresis
SPPS	Solid-phase peptide synthesis
TBMB	1,3,5-tris(bromomethyl)benzene
TCEP	tris(2-carboxyethyl)phosphine (TCEP)
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Thp	Tetrahydropyranyl
TMB	2,4,6-trimethoxybenzyl
t_R	Retention time
U-4CR	Ugi four component reaction
UV	Ultraviolet

Thesis Overview

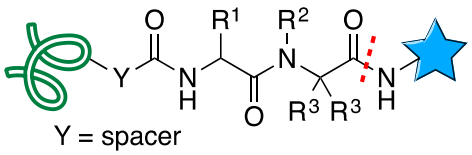
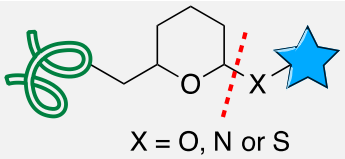
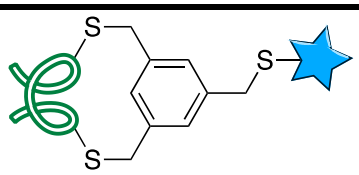
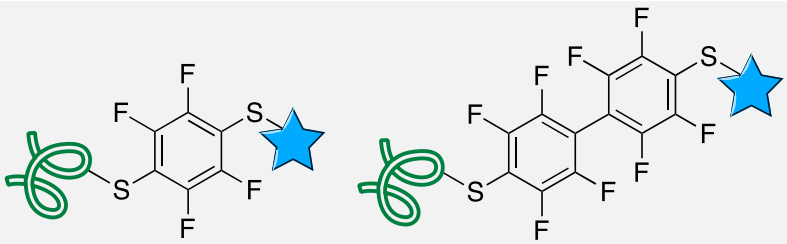
Cancer is a heterogeneous disease that represents one of the principal causes of mortality in developed countries. Due to the social and economic implications of this pathology, tremendous efforts have been making over the past decades to improve available therapeutic options to tackle this illness. Although a large number of potent chemotherapeutic agents have been identified and successfully used in clinical practice, the development of new anticancer chemotherapeutic drugs with higher antitumor efficacy and less toxicity remains as a research challenge.

Current advances in the mechanistic understanding of the molecular drivers of malignancy have led to many anticancer drugs, which are targeted directly at the cancerous cells and not the neighbouring healthy cells. Thus, in order to enhance the efficacy of existing anticancer agents, several drug delivery approaches have been developed. Appropriately, ADCs strategy consists on bind determinate cell-killing drug to a monoclonal antibody (mAb) through a specific linker. Due to their high-binding specificity for tumor-specific antigens, mAb can be used as vehicles to target cell-killing payloads to tumor cells.[1] Accordingly, two kinds of linkers are suitable to attach the payload to the antibody. On one hand, **non-cleavable linkers** afford and stable binding between the drug and the antibody, and alternatively, the drug could be released in the proximity or even inside of affected cells using **cleavable linkers**.

Based on the potential of ADCs as prodrugs,[2–4] our main goal is develop linkers, which could promote the drug release close to the affected tumor cells. Taking into account that connection between the antibody and the cytotoxic agent has significant effects on the selectivity, pharmacokinetics and therapeutic index of the ADC,[5] an efficient binding is needed. Therefore, the properties of a successful linker can be split up into different modules that have been combined to form an effective bridge between the cytotoxic payload and the carrier antibody.

In the present thesis, we focus on the development of new linkers for bioconjugation and more specifically for Antibody–Drug conjugates (ADCs). With this purpose in mind, a conscious design of the linkers for ADCs has to be done. In the present thesis, different cleavable (Chapter 1) and non-cleavable (Chapter 2) linkers are studied and it will be explained in detail along the thesis (**Table 1**).

Table 1. Linkers for bioconjugation studied in the present thesis.

Cleavable Linkers	
Ugi Adducts α,α-*N*-alkylated dialkylglycines (1,4-Dicarbonylic systems)	
Tetrahydropyranyl (Thp) (Acetal bond)	
Non-Cleavable Linkers	
Tris(methylbenzene) (Benzylic thioether linkage)	
Perfluoroarylated compounds (Aromatic thioether linkage)	

As a consequence, the design has to be focused on linkers stables in systemic circulation. Additionally, cleavable linkers should be unstable in tumor cells environment, such as high concentration of glutathione, highest acidic concentration, and enzymatic cleavage, among others as above-mentioned. These conditions should allow, chemically or enzymatically, the release of the drug from the carrier (antibody-linker).

Introduction

Linkers for bioconjugation

General introduction

In chemical and biological sciences, bioconjugation is considered the linkage between two molecules where at least one is considered a biomolecule. In this regard, a biomolecule could be considered any molecule, which is present in living organisms and is mainly based on carbon, hydrogen, oxygen and nitrogen. To date, a wide range of biomolecules has been isolated, synthetized and/or successfully characterized.[6] Regarding bioconjugates, the most common used biomolecules are amino acids, peptides, as well as hormones, proteins, nucleic acids and antibodies, among others. In order to achieve the mentioned bioconjugation, several chemical tools are needed. In the present thesis, several methods to perform bioconjugation in peptides, proteins and antibodies have been developed in order to contribute to this challenging field.

In the recent years, the field of drug discovery drives to find out efficient, selective, stable and biocompatible new drugs, also looking for avoiding side effects. In this regard, the fruitfully use of peptide- proteins- or antibodies-based therapies became a choice for the treatment of a huge range of diseases, due to the biocompatibility that this macromolecular systems presents.[7,8] For an efficient development of biomedicines, several factors such as physicochemical and biological behaviour, physiological stability, immunogenicity as well as pharmacokinetic properties are mandatory to consider.[9] In this sense, the lack of stability in front of enzymes or reductive environments is part of their weaknesses as potential efficient drugs. For that, tremendous efforts have done to date in order to improve the lack of stability by chemical modifications of peptides and proteins, increasing their bioavailability.[10–12]

Every chemical modification or conjugation in proteins, usually involves the reaction between two functional groups, generating a new covalent bond between both molecules or, as it is said in the bioconjugation field, a crosslinking between a protein and the conjugated molecule. Accordingly, the crosslinking in bioconjugation is achieved through a variety of reactive groups such as amines, thiols, alcohols or even carboxylic acids,[11] functional groups that are present in the structure of peptides and proteins and they will be discussed below. In some cases, the innovative application of relatively old organic reactions are now used to solve new bioconjugation problems resulting in significant advances in the field.[13]

Linkers for Bioconjugation

The studies carried out in the present thesis are based on mentioned crosslinking between two molecules where at least one is a biomolecule. Of the repertoire of the existing linkers for bioconjugation we will focused on homobifunctionals crosslinkers in which the reaction between two molecules occurs through the same functional group (e.g. conjugation between two amines or two sulfhydryl groups) and, on the other hand, on the denominated heterobifunctional crosslinkers where the conjugation take place through two different functional groups (e.g. conjugation between an amine and a thiol groups) (**Figure 1**).

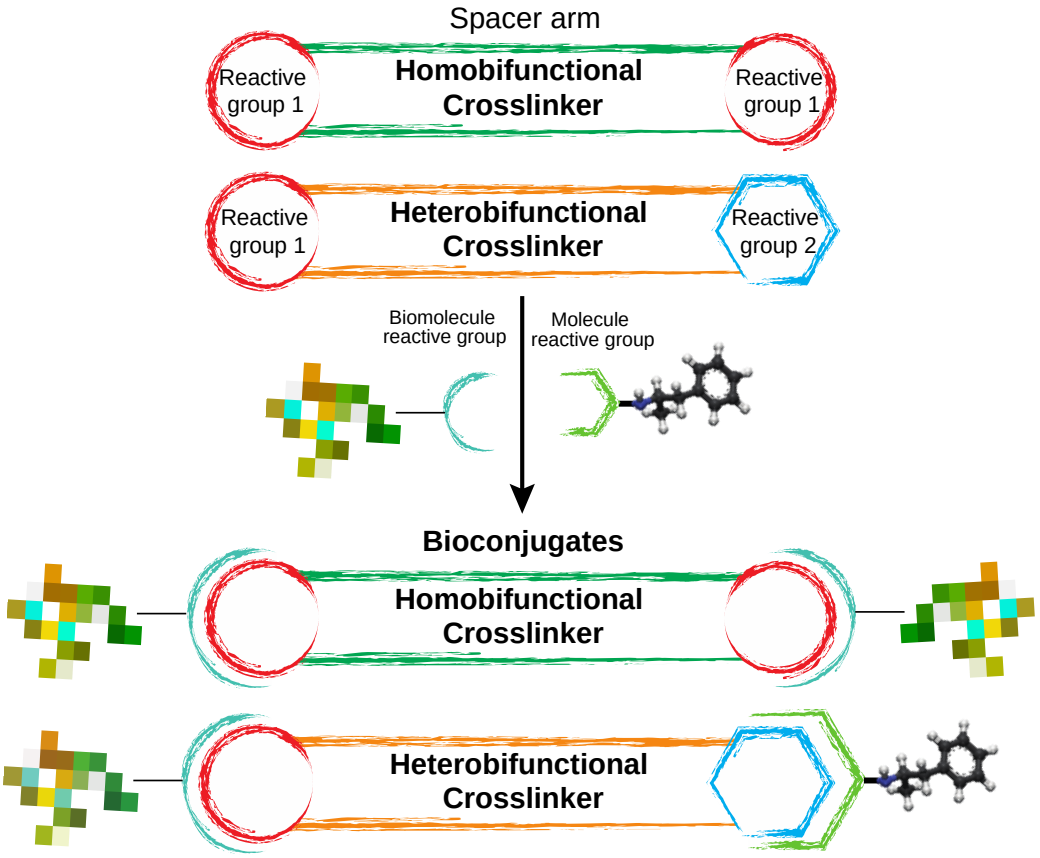


Figure 1. Bioconjugation with homo- and hetero-bifunctional crosslinkers.

The first homobifunctional linker used for conjugation of macromolecules consisted of diimido esters and Hartmann and Wold used for bifunctionalization of bovine pancreatic ribonuclease A through reactive amines.[14] The major drawbacks of the use of homobifunctional conjugation are polymerization and intramolecular crosslinking. In addition, this kind of linkers has been broadly used for proteins or peptide macrocyclization. The therapeutic activity of proteins and peptides is highly dependent on their conformational structure, and for that reason tremendous efforts have been done in order to fix through

chemical transformations their structure, which adopt the optimal conformational structure for the interaction with their therapeutic target and, moreover to enhance their stability profile.[15,16]

The above-mentioned chemical transformations include peptide stapling or cyclization. Head-to-tail, head-to-side chain, side chain-to-side chain and side chain-to-tail are the strategies to follow in order to perform peptide cyclization (**Figure 2**).[17,18] The chemical tools available for cyclization include amide bond formation, as well as ring-closing metathesis,[19,20] also disulfide bond formation[21] or cysteine alkylating agents[22] among others.

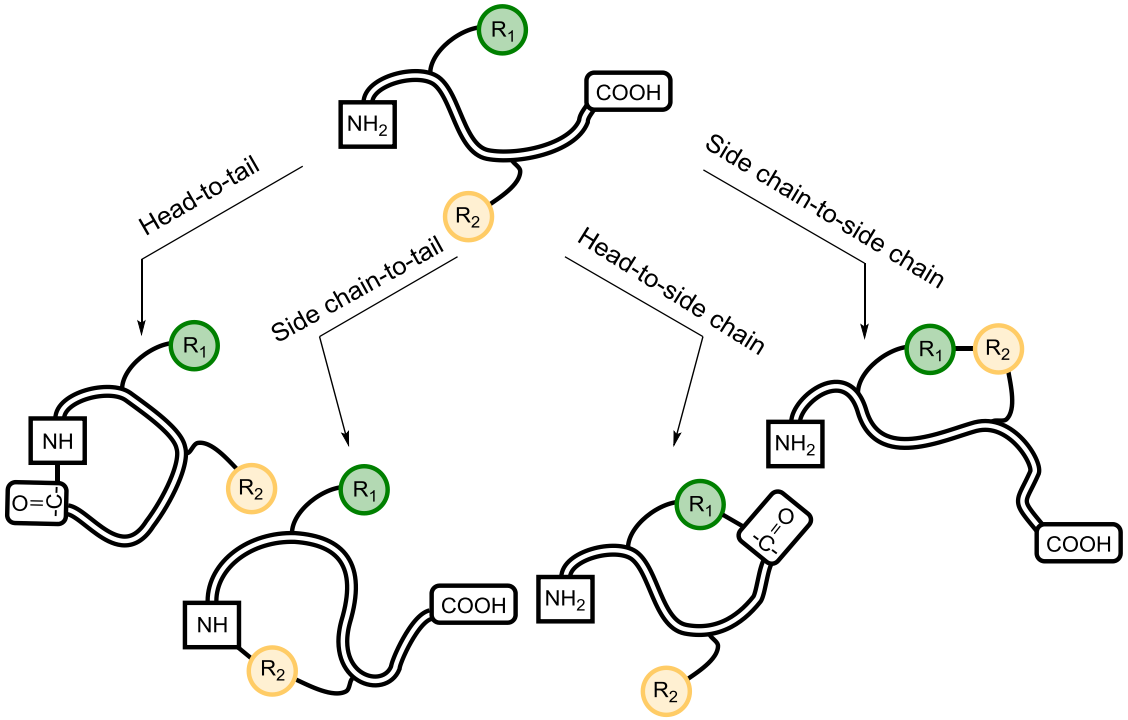


Figure 2. Peptide cyclization strategies.

Conversely, one of the first heterobifunctional crosslinkers used for bioconjugation was the *N*-acetyl homocysteine thiolactone, which reacts with primary amines in a ring-opening reaction to create free sulfhydryl group. Initially it was used for thiolation of proteins[23] but shortly after was applied by Eldjarn and Jellum[24] to link a dimeric organomercurial compound to polysaccharides for their use for purification of sulfhydryl-containing proteins. Nevertheless, Traut's reagent[25,26] which is based on the same principle (thiolactone ring opening by an amine and a thiol group releasing) nowadays is more used for introduction of a thiol group into the macromolecule.

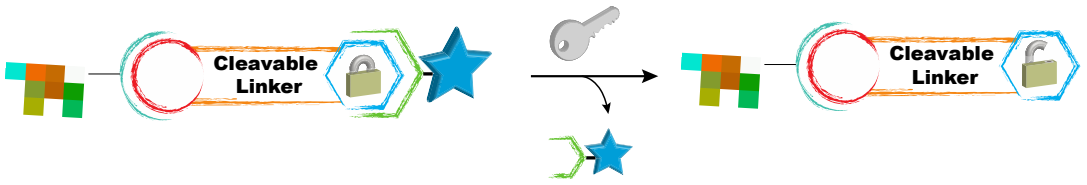
Linker properties

Regarding linkers for bioconjugation, a really important point to mention is the physico-chemical properties that these entities may have to be effective. Concerning its chemical properties, linkers for conjugation are mainly classified in two types as the present thesis chapters: **cleavable and non-cleavable linkers.** Due to the significant and quick progress that drug delivery systems (DDS) have experienced, a vast number of these systems have been developed. Drug delivery systems aim to administer an active drug to achieve a therapeutic effect and to improve the product's efficacy and safety, compared to the drug alone. Biopolymers,[27] nanoparticles,[28] proteins[9] or antibodies[29] conjugates are trending compounds in this competitive field, which allow the controlled release of a specific drug into a specific target.[30]

- **Cleavable linkers**

Cleavable linkers have the ability to break one of its chemical bond that binds the two molecular entities, allowing the release of the compound attached to this bond, which is generally a cytotoxic drug. These linkers are required to be stable during the synthesis steps as well as during *in vivo* circulation of the conjugate, and are eventually cleaved into specific conditions to release the drug. Accordingly, the release has to be under specific conditions at the surrounding of the target cell or tissue.[31]

In addition, the development of new cleavable linkers have to satisfy various challenging constraints such as mild cleavage conditions, high bioconjugation yields, bioortogonality, and ease excess of reagents and by-products removal.[32] Following the mentioned points that a cleavable linker has to own intrinsically, several cleavable linkers at specific biological conditions has been developed. Some of them (linkers and environment to release) are following described and depicted (**Figure 3**).



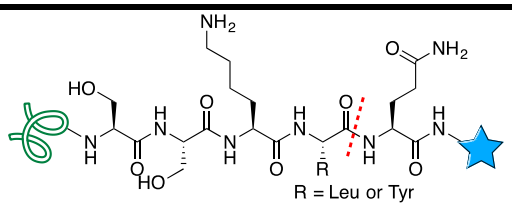
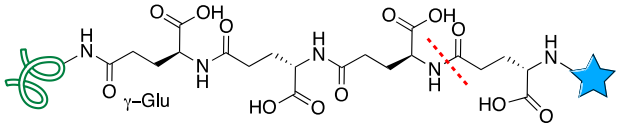
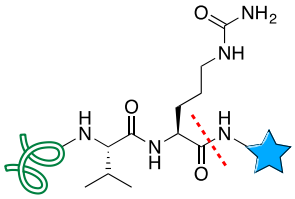
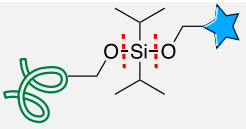
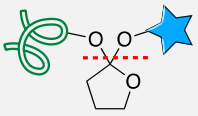
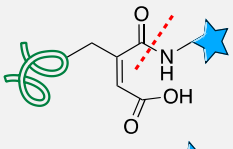
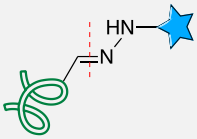
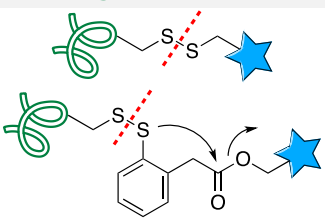
Linker structure	**Cleavage mediator (kind)**	**Linker Name**[Reference]
	PSA (Enzymatic)	SSKL/YQ[33–35]
	PSMA (Enzymatic)	Poly-γ-Glutamated[36]
	Cathepsin B (Enzymatic)	Val-Cit[37–39]
	H^+ (Acidic)	silyl ether[40,41]
		orthoester[42]
		ketal/Thp[43–45]
		cis-aconityl[46]
		hydrazone[47,48]
	Gluthathione (Disulfide reduction)	disulfide bonds[49–52]
		self-immolative disulfide[53]

Figure 3. Cleavable linkers for bioconjugation and its cleavage mediators.

Enzymatic cleavage

Understand, and selectively use the enzymatic cleavage become a milestone in the development of cleavable linkers, and only a limited number of enzymes have been proven to successfully activate prodrugs till now. Generally, the enzyme should be specifically located in tumours, so that only the prodrug in the target tumor cells can be cleaved to release the active agent, leading to minimal side effects. Proteases are able to cleave amide bonds into a specific peptide sequence. Enzymes such as prostate specific antigen (PSA),[33–35] which specifically cleave the peptide at the carboxyl group of tyrosine or leucine into the peptide sequence Ser-Ser-Lys-Leu/Tyr-Gln; as well as prostate-specific membrane antigen (PSMA)[36] which catalyses the hydrolysis of *N*-acetylaspartylglutamate to Glutamate and *N*-acetylaspartate peptides; or also cathepsin B, a lysosomal enzyme that selectively cleave the bond between the peptide with the termini valine-citrulline and the *para*-aminobenzyl carbamate (PABC)[37–39] have been successfully used.

Acidic cleavage

Several cleavable linkers have been developed for their use in cancer therapies. For these cases, the pH responsive systems are of significant interest because of an acidic pH has found in the microenvironment of tumor cells and tissues, resulting from the excessive generation of lactic acid derived from glucose metabolims.[54–56] In the literature, we found a sort of examples of acid-sensitive linkers for drug delivery such as bifunctional silyl ether cross-linkers,[40,41] orthoester,[42] acetal,[43–45] cis-aconityl[46] and hydrazone,[47,48] among others.[57]

Glutathione-mediated cleavage

When the cleavable linker contains a disulphide bond as a hydrolytic point, the release of the payload is attributed to the high intracellular concentration of reduced glutathione (GSH), which has found increased in tumor cells.[58–60] Thus, the significant differences in intracellular (cytosol 1-10 mM) and extracellular (0.2 μM) levels of GSH make disulfide bond linkers an interesting choice for drug delivery applications, because in absence or low concentrations of GSH, disulphide bonds remain stable. Therefore disulfide bond-mediated cleavable linkers developed to date, include from simple disulfide bonds[49–52] to fashionable disulfide self-immolative linkers[53] which trap one of the liberated thiol.

– **Non-cleavable linkers**

On contrary, non-cleavable linkers are considered as the spacers which it principal function is to maintain linked both molecular entities. In this regard, the stability of non-cleavable linkers allows an effective delivery to the target cell without losing the compound attached to the macromolecule.

Similarly to the cleavable linkers, non-cleavable linkers may have some characteristics to be useful in bioconjugation. High conjugation yield, bioortogonality, ease non-reacted reagents removal and the most important, they should remain stable under physiological conditions. Nevertheless it was demonstrated that antibody drug conjugates (ADC´s) despite of wearing a non-cleavable linker, cytotoxic effects have been observed due to the enzymatic cleavage of the inmunoconjugates into the cells.[61] Accordingly, as a huge number of drugs, the non-cleavable linkers finally tend suffer degradation to simplest metabolites in order to be excreted from our body.

Due to the vast number of physiologically stable linkers, handles or spacers, only the most used linkages will be mentioned above. It is well known that in our body, several enzymes manage the degradation of a sort of compounds such as esters, amides, disulphides, among others. Consequently alkyls (C-C bond), thioethers (C-S bond), ethers (C-O bond) or amines (C-N bond) contain suitable bonds for their uses as stable linkers in bioconjugation. In this regard, several stable linkers has been described for bioconjugation[13,62] but however, the key point remain into the selectivity to perform the mentioned linkages more than the way to be obtained. Some examples will be further discussed.

Site-selective bioconjugation

Despite all the advances done in the field, regioselectivity in bioconjugation suppose one of the biggest challenges for scientists due to the vast number of functional groups present in biomolecules.

- Amino acids

Bioconjugation takes place mainly through the functional groups present in the side chains of the amino acids, which are distributed along peptides and proteins sequence. Therefore, the amino acids those contain aliphatic side chains lack of interest for bioconjugation due to the absence of reactivity. Nevertheless they play a key role in the structural conformation, reactivity and function of the polyamino acid biomolecule. In contrast, bioconjugation has

focused on the natural amino acids, which contains potential reactive functional groups (**Figure 4**).

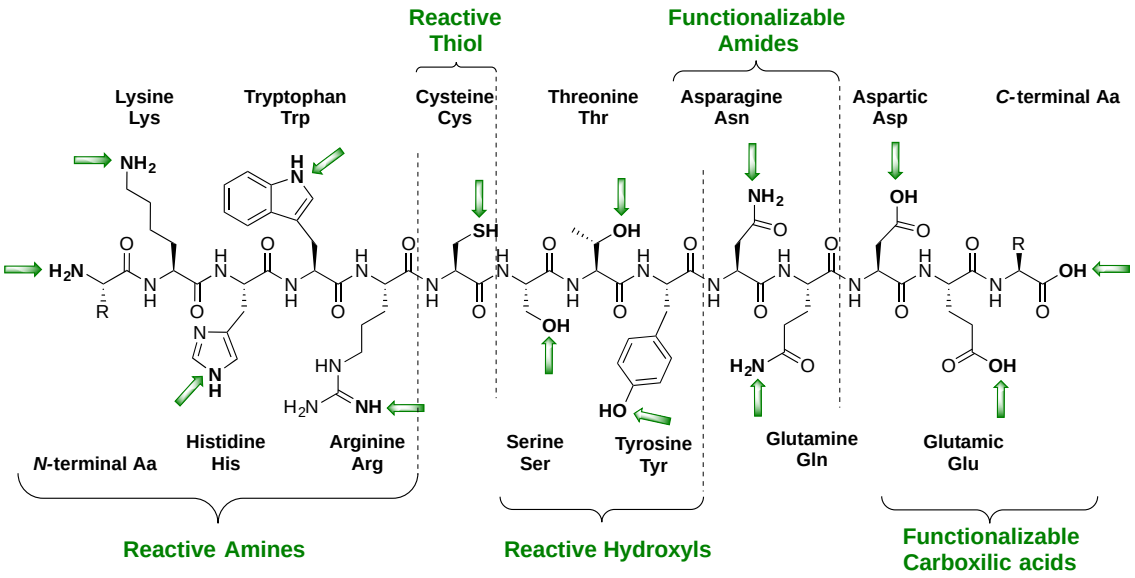


Figure 4. Amino acid reactive sites.

- Reactive amines

Most of the amine-containing amino acids in its side chain (except Trp) and on the *N*-termini confer an overall net positive charge to peptides and proteins, which is essential for its *in vivo* solubility properties. These residues are generally used in bioconjugation due to its ability to suffer alkylation or acylation, in front of activated alkyl or acyl groups respectively. Thus both alkylating and acylating reagents are broadly used for bioconjugation. Accordingly, lysine (Lys) is the most popular choice for conjugation, due to the number of successful reactions that can be applied to it highly nucleophilic ε-amino group.[63] Lys residues, which are abundant in native proteins and peptides, are usually found randomly distributed into proteins and peptide structure.

Therefore, conventional Lys conjugation protocols are useful when the regioselectivity and the homogeneity of the conjugates are perceived not to be important.[11] Preferential conjugation with amines can be achieved through use a sort of electrophiles such as activated *N*-hydroxysuccinimide esters,[64] as well as isothiocyanates[65,66] or aldehydes through the well-known sodium cyanoborohydride-mediated reductive alkylation.[67] Although the mentioned methodologies yields heterogeneous conjugates, due to the improvements done in the field, nowadays the site-selective targeted Lys conjugation under kinetically controlled modification conditions is possible.[68]

- Reactive Thiols

Cysteine (Cys), which contain a sulfhydryl group dangling from it side chain, usually it found as a disulfide bond. Accordingly, the disulfide bonds confer an additional stability to the structure and conformation of peptides and proteins.[7,9,16,69] The reduced form lacks of reactivity, however after reduction the thiol reactive group is liberated. While the natural way to reduced disulfide bonds is through the action of glutathione,[70] other synthetic reducing agents have been developed. The most using reducing agents are the water-soluble phosphine tris(2-carboxyethyl)phosphine (TCEP),[71,72] and the dithiol-based dithiothreitol (DTT).[73–75]

Correspondingly, thiol group of Cys is considered as the most robust nucleophile of the 20 canonical amino acids. Thus, it sulfhydryl group offers an specific reactive handle within proteins as it is exploited extensively in nature.[76] The remarkable selectivity of thiol groups under controlled pH conditions, over other nucleophilic residues such as Lys and histidine (His),[77] highlight Cys as one of the most attractive amino acids for bioconjugation despite its low abundance in peptides, proteins and antibodies (<2%). Cys has been successfully used to promote regioselective bioconjugation in peptides, proteins and antibodies[78–80] obtaining site-selective and homogeneous conjugates.[81]

The most used methodologies for Cys conjugation takes places through nucleophilic substitution to α-haloacetyl derivatives as well as alkyl or aryl halides.[62,82] Furthermore, disulfide linkage is contemplated as a good strategy for the introduction of a cleavable site in presence of reduced glutathione.[52,83] Nevertheless, the most powerful tool used for decades is the thiol alkylation via Michael addition to a double bond of a maleimide ring.[84,85] Accordingly, it commercial availability, ease of use and synthesis of maleimide derivatives have led to widespread use.[78,86–90] Although *S*-maleimide bond is considered irreversible, yet it has been suggested that other thiols could compete with this reactive site releasing the introduced maleimide.[91,92] In this regard, bromomaleimides improve the mentioned inconveniences and moreover improve the bioconjugation abilities.[78,93–97]

Additionally, more fashionable methodologies have been described, such as the effective oxidative elimination of Cys to dehydroalanine (Dha) and the subsequent Michael addition of a thiol group allowing the bioconjugation,[98] with the lack of stereospecificity for the Michael addition reactions as the main drawback, and leading the diastereomeric mixture of the modified Cys. Besides, the thiol-ene[99,100] and thiol-yne[101] connection were highlighted as efficient “click” reactions between a thiol group and an alkene or alkyne respectively, to

obtain a thioether bond through a radical mechanism. Moreover, based on Cys bis-alkylation procedures[102,103] it is possible to assemble antibodies through cross-linking agents with dyes or drugs changing or improving the biomacromolecule activity.

Th**e** above-mentioned methodologies used for Cys-mediated conjugation are depicted in the following figure (**Figure 5**).

- Reactive hydroxyls

In a large number of biomolecules, amino acids such as serine (Ser), threonine (Thr) or tyrosine (Tyr) are considered as the linking point to carbohydrates or phosphoryl groups.[104] Similarly to amine reactive groups, alkylation or acylation could take place with hydroxyl reactive groups from Ser, Thr and Tyr side chains yielding the corresponding ethers or ester respectively.[13] However, due to the lack of site-specificity of this strategy, hydroxyl conjugation has fallen into disused. While it is true that site-specific bioconjugation is possible through *N*-terminal Ser/Thr conjugation utilising salicylaldehyde ester.[105–107]

On contrary, tyrosine not only may be targeted by it hydroxyl group. Tyr also may be chemically modified through electrophilic substitution reactions on the *ortho* position to the –OH group. Accordingly, different methodologies have emerged for this site-specific bioconjugation[108,109] being pioneered Francis *et al*.[110,111]

- Functionalizable amides

Similarly to Ser, Thr and Tyr, asparagine (Asn) has mainly been found covalently linked to oligosaccharides and it modification becomes a challenge. However, carbohydrates are attached to Asn through a *N*-glycosidic bond at its carboxamide nitrogen.[112] From a chemical point of view, the modification of an amide bond involved harsh chemical conditions that may affect to the integrity of the protein. Nevertheless, this modification is possible through the enzymatic action of transglutaminase as Clarke *et al* introduced in 1959.[113] In their native role, transglutaminases catalyse the cross-linking of proteins, by mediating a transamidation reaction between the side-chains of peptide-bound Gln and Lys residues.[114] Taking advantage of this fact, it has been used to conjugate biodegradable polymers[115] as well as for site-specific protein labelling[116,117] or the preparation of homogeneous antibody drug conjugates (ADCs).[118]

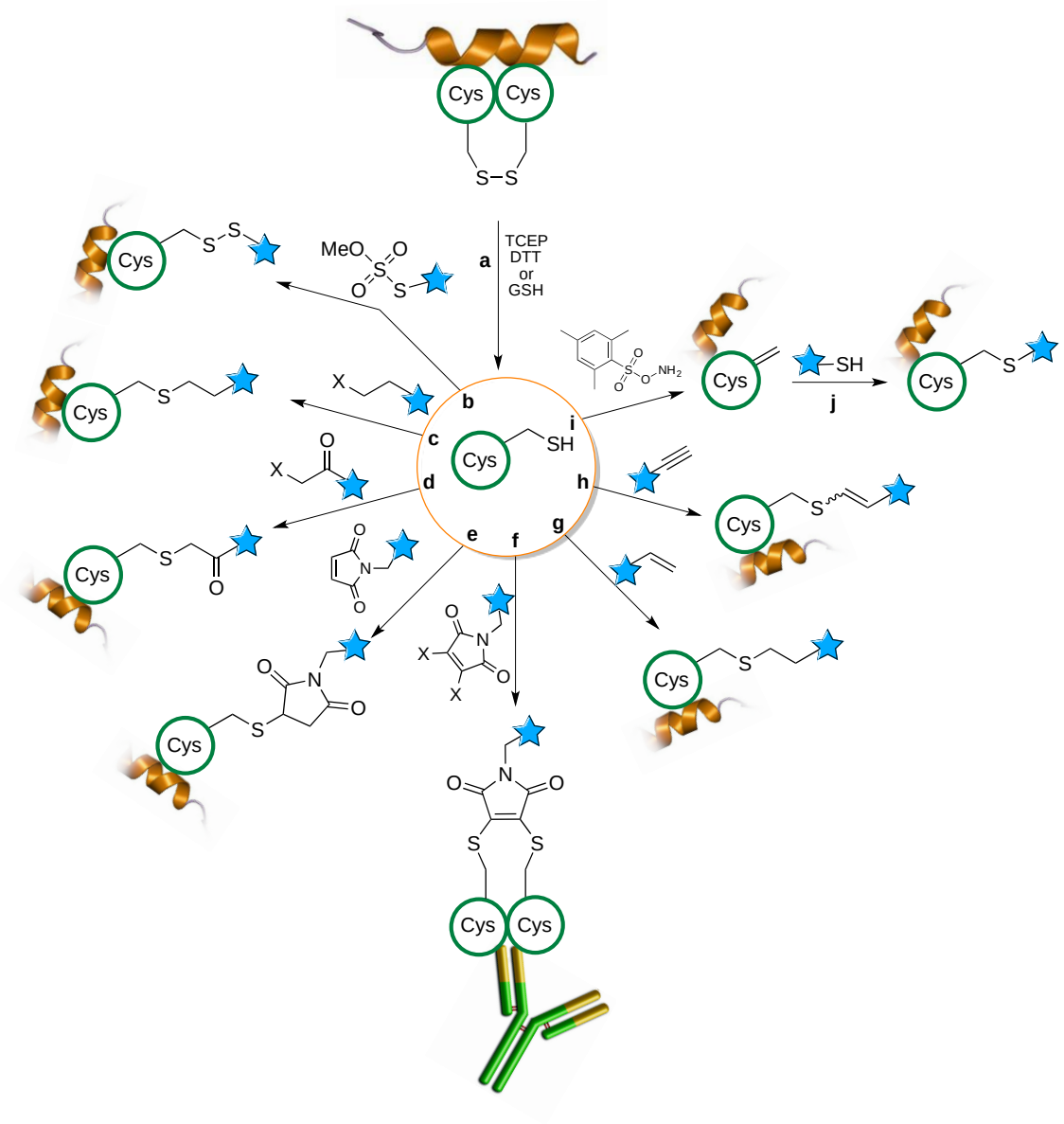


Figure 5. Cysteine-mediated conjugation. a) disulfide bond reduction, b) disulfide bond formation[119] c) haloalkylation[120] d) α-haloacetyl alkylation[121] e) maleimide alkylation[78,86–90] f) bromomaleimide bialkylation[78,122] g) "Click" thiol-ene[99,100] and h) thiol-yne[101] reactions i) dehydroalanine formation with *O*-mesitylenesulfonyl-hydroxylamine followed by j) thio-Michael addition alkylation.[98,123–125]

- Functionalizable carboxylic acids

Aspartic, glutamic or C-terminal acids may be used for bioconjugation through amide bond formation. Accordingly, reactive amines may be covalently attached to the active form of a carboxylic acid present into the biomolecule surface. For that, several methods which render an amide bond has been described.[126] However, mild water-soluble activating agents as the soluble carbodiimide *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride

(EDC·HCl),[127,128] as well as *N,N'*-carbonyldiimidazole (CDI)[129] are needed to achieve the amide bond formation at bioconjugation conditions.[118]

- Introduction of new reactive groups

As previously explained, bioconjugation could achieve by directly linking a molecule to the functional groups present in the biomolecule. Nevertheless, is possible to transform or introduce other functional groups through chemical reactions, thus improving the regioselectivity in bioconjugation. For example, Traut's reagent (2-iminotiolane)[25,26] can react with amines, releasing a thiol reactive group while the amine charge is maintained. Moreover, cystamine has been used for introduction of a masked thiol group into carboxylic acid-containing biomolecules by carbodiimide-mediated coupling reactions.[130] Furthermore, the well-known "click" reaction between an azide group and an alkyne to yield a triazole have successfully achieved by the introduction of an azide or alkyne to the previously mentioned reactive amino acid side chains.[63]

Collaborative project, MarinMab

The present thesis are involved in a collaborative project title MarinMab for the development of new therapeutic agents base on Antibody drug conjugates. In this multidisciplinary project participate the following institutions: PharmaMar Company, Universidad Autónoma de Madrid, CSIC, Universitat de Barcelona and Institut de Recerca Biomèdica.

Our participation in the mentioned project funded by the Ministerio de Economía y Competitividad (MINECO), are based on the development of new linkers for their use in antibodies drug conjugates. Due to a confidentiality agreement with the biopharmaceutical company PharmaMar is not possible to explain in detail the entire procedures or chemical structures studied in the present thesis.

Research group precedents

Our research group, with its longstanding experience in the field of peptides, has developed a sort of methodologies, coupling agents, linkers and protecting groups for solid phase peptide synthesis (SPPS). With the present thesis project, our group seek to amplify the experience to the bioconjugation field and moreover, contribute to the development of new therapeutic agents (as ADCs), with a tremendous pharmaceutical interest for the treatment of cancer disease.

Objectives

Chapter 1. Cleavable linkers

I. Study Ugi adducts for their use as cleavable linkers for bioconjugation.
   - Functionalization
   - Stability
   - Lability
   - Bioortogonality in bioconjugation
   - Preparation and characterization of bioconjugates
   - ADCs characterization

II.1. Study tetrahydropyran (Thp) as cleavable linker for bioconjugation.
   - Functionalization
   - Stability
   - Lability

II.2. Study tetrahydropyran (Thp) as Cysteine protecting group in SPPS
   - Cys functionalization
   - Stability
   - Lability studies
   - Cys(Thp)-containing peptide synthesis and cleavage

Chapter 2. Non-Cleavable linkers

I. Study the thiol monoalkylation of 1,3,5-tris(bromomethyl)benzene (TBMB) for bioconjugation.
   - Stability
   - Bioortogonality in bioconjugation
   - Preparation and characterization of bioconjugates
   - ADCs characterization

II. Study the thiol monoalkylation of decafluorobiphenyl and pentafluorobenzene for bioconjugation.
   - Monoalkylated thioether preparation
   - Stability
   - Bioortogonality in bioconjugation
   - Preparation and characterization of bioconjugates, (ADCs)

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Chapter I.

Cleavable linkers for bioconjugation

An extensive part of the results present in this chapter are considered for further publication, the manuscripts are under preparation.

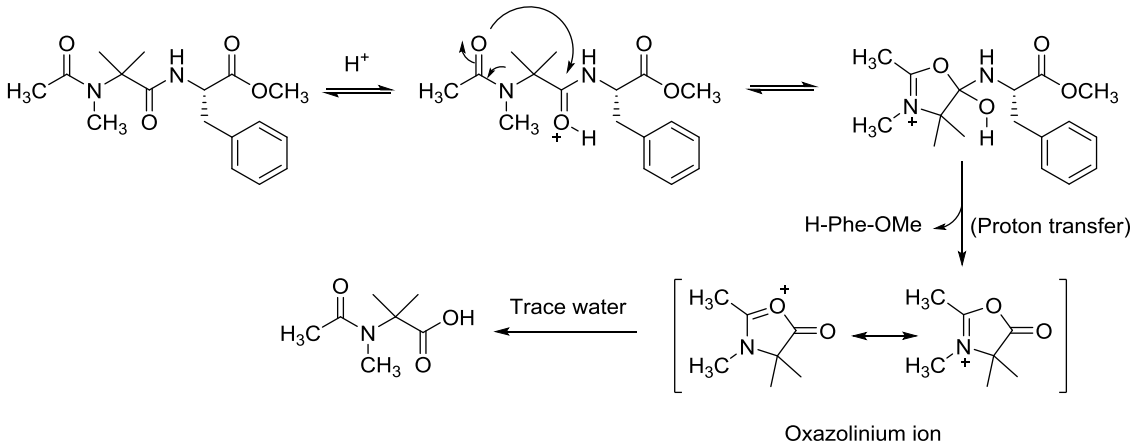
Chapter I. Cleavable linkers

1. Ugi linkers: *N*-alkylated α,α-trialkylglycines

2.1. Introduction

Generally, chemists tend not to publish negative or undesired results as these are non-conclusive or definitive. This is one of the principal reasons because there are not publications only with negative results or uncompleted reactions described as difficult or impossible.[1] Despite of not been described, over the years chemical side reactions suppose a rich source of interesting chemical procedures or compounds that the society make benefit of it.

In concordance with the purposes of this chapter section for the development of new cleavable linkers for bioconjugation, one of the most commonly side reactions is the unexpected cleavage or disconnection of functional groups, which prevents furnish the desired final products. For example, in peptide science several undesirable side reactions occur during the peptide synthesis or the final protecting group elimination. Alkylation, racemization, amino acid deletions or amide cleavage are some examples of the mentioned side reactions which jeopardize the peptide synthesis at different stages.[2–8] A clear example is the unusual peptide bond cleavage that Spencer *et al*.[9] described for the amide cleavage into *C*-termini of sterically hindered *N*-methylated amino acid residues such as aminoisobutyric (Aib) or 1-aminocyclopentane-carboxylic (Ac^5c) acids afterward the acidolytic TFA treatment. Shortly after, the same research group has found a reasonable mechanisms than explain the acidolytic cleavage of certain *N*-methylated α,α-dialkylglycines containing-peptides (**Scheme 1**).[10]



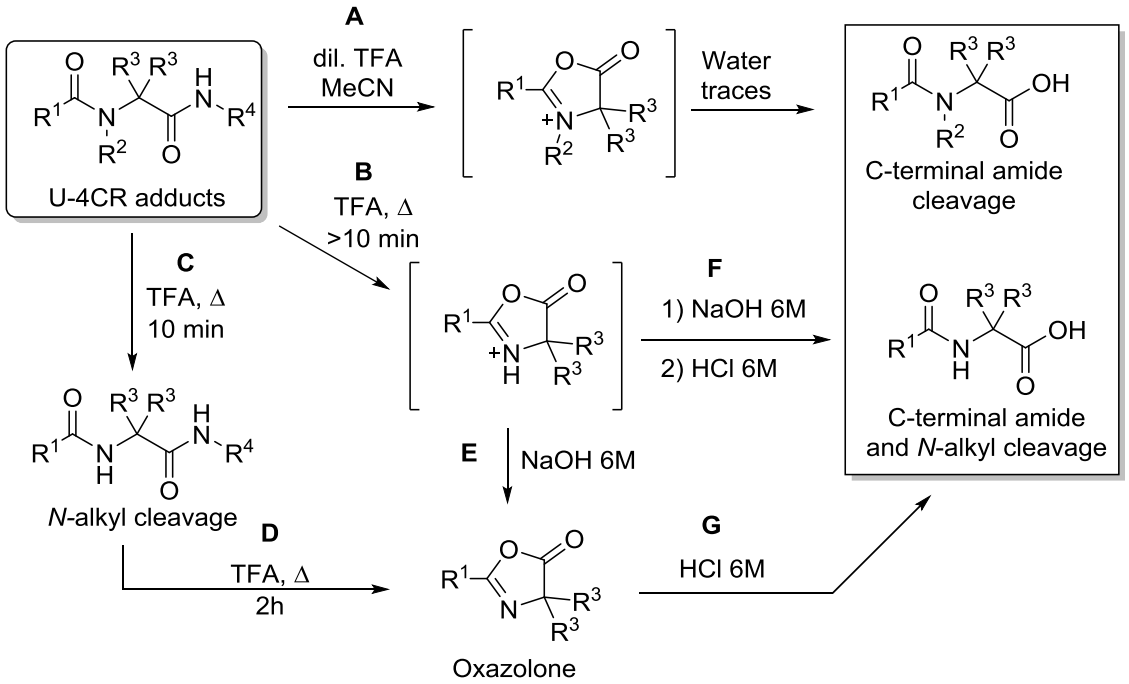
Scheme 1. *C*-terminal amide cleavage mechanisms for *N*-methyl α,α-dimethyl glycine *via* oxazolone ring intermediate.

Accordingly, the carbonyl oxygen of the acetyl group acts as a nucleophile and attacks the adjacent carbonyl group to form a five-membered oxazolinium intermediate, releasing the corresponding amine.

More recently, other research groups deal with this kind of amide cleavage via the oxazolone ring intermediate. Aldrich and collaborators[11] looking for minimize this side reaction during the synthesis of [NMePhe[1]]arodyn analogues, described the optimal conditions to avoid this unwanted acidolytic cleavage, in order to improve the yields of peptides which contain an *N*-terminal Ac-*N*-methylamino acid, and also to improve the synthesis of stable analogues that not undergo this mentioned collateral reaction. However, not necessarily only *N*-methylated peptides suffer this acidolytic cleavage. As Rubini *et al*.[12] find out an unusual amide bond scission for the pipecolic acid, which is a sort of cyclic *N*-alkylated amino acid, again under acidic conditions. Their studies demonstrated that under standard TFA cleavage/deprotection conditions, peptides containing consecutive pipecolic acid residues undergo facile hydrolysis at the amide bond adjacent to the C-terminus of the activable pipecolic residue.[12]

Additionally, emphasizing that *N*-acyl *N*,α,α-trialkyl glycine amides undergo selective *C*-terminal amide cleavage under TFA, Maia research group tackled the selective amide cleavage[13,14] as well as studied the acidolysis mechanisms[15–17] for an special kind of trialkyl glycines obtained through the Ugi four-component reaction (U-4CR). The studies carried out by Maia and co-workers[13–17] not only corroborated the selective C-terminal amide cleavage using diluted acid concentration (**Scheme 2A**), they also elegantly described the complete cleavage (**Scheme 2** follow route **B** and **C)** or, including the C-terminal amide and the alkyl group attached to the central nitrogen (**Scheme 2C**). The acidolysis mechanism takes place *via* an oxazolinium ion intermediate which leads to the cleaved *C*-terminal amide (**Scheme 2**).

By this means, the acid-cleavable characteristics of the 1,4-dicarbonylic compounds based on *N*-alkylated α,α-dialkyl glycine caught our attention to use as a possible cleavable linker for bioconjugation. Thus the first step involves the synthesis of the mentioned trialkyl glycine derivatives, but some aspects regarding the synthesis are following considerate.



Scheme 2. Proposed acidolytic routes for U-4CR adducts treated with TFA. Adapted from reference 13.

Synthesis of *N*-alkylated α,α-dialkyl glycine

During the 1960's various authors such as Faust and Lange,[18] Diehl and Young,[19] and McGahren and Goodman;[20] were pioneering for the synthesis of peptides containing residues of α,α-dialkylglycines, however, the most representative work was carried out by Jones and collaborators,[21] who concentrated mainly on developing methodologies to deal with these "sterically hindered" amino acids.

Usually, the synthesis starts with the preparation of the modified alkylglycines which are not commercially available amino acids, with the exception of dimethyl and diethyl glycine. Unfortunately, its incorporation into peptides becomes problematic, because of the steric hindrance that amino acid side chains larger than methyl present. In fact, steric crowding and conformational restriction not only make the synthetic reactions very slow, but also they tend to modify their course and lead to undesired products, which hinder purification and decrease yields considerably.[14] In concordance, due to the low reactivity of the α-amino and carboxyl group, which is associated with steric hindrance of dialkyl amino acids, the classical coupling methodologies found inefficient to yield α,α-dialkylglycines. Thus, alternatives to classical peptides methodologies are needed. In this sense, multicomponent reactions (MCRs) have

found to be highly convergent processes that yield complex molecules with great efficiency and atom economy.[22–24]

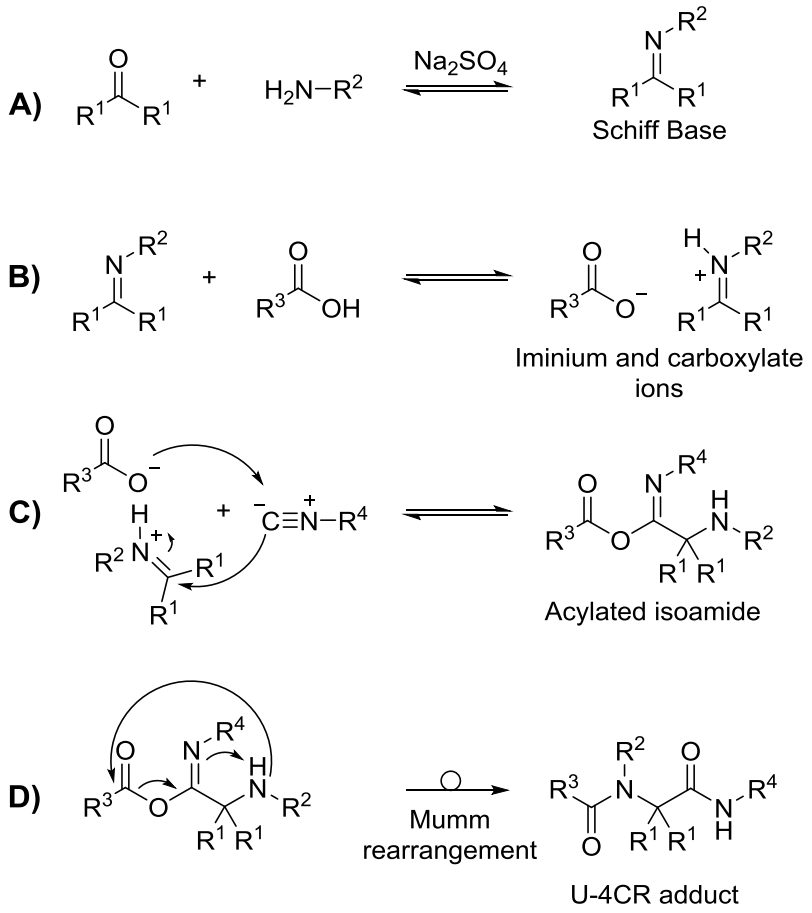
An attractive alternative for its preparation, which avoid the mentioned drawbacks, was initiated in 1959 by Ivar Ugi *et al*. with the four components reaction (U-4CR).[25] A few years later, the use of a four-component reaction is contemplated as a complement to classical peptide synthesis with common amino acids, due to the synthetic simplifications offered by this reaction.[26] Nevertheless, the use of the Ugi reaction restricts its use for peptide synthesis due to two inherent drawbacks: 1) the high tendency of racemization by amino acid and peptide isonitriles required for the reaction and moreover 2) the need to cleave the *N*-alkyl group to maintain the peptidic scaffold intact.

The U-4CR, which yield the desired *N*-alkylated α,α-dialkylglycines, is based on a MCR where a carboxylic acid, a ketone, a primary amine and a isocyanide fuse into a single product as many other multicomponent reactions.[27] The reaction mechanisms was elucidated in 1967 by the Bayer AG company using one of the first commercially available computer at that time.[28]

Following different individual reaction steps, Ugi reaction involves the initial imine formation by the condensation between the corresponding ketone (or aldehyde) and an amine in presence of a dehydrating agent (generally Na_2SO_4) (**Scheme 3A**). Then the nitrogen atom from the Schiff base is protonated by the carboxylic acid obtaining the corresponding iminium and carboxylate ions (**Scheme 3B**). Then the isocyanide carbon reacts with activated imino carbon followed by the carboxylate oxygen atom addition to the isocyanide (**Scheme 3C**). Finally, the resulting acylated isoamide rearranges by acyl transfer, following the described Mumm rearrangement,[29–31] to yield the mentioned *N*-alkylated α,α-dialkylglycine (**Scheme 3D**).

It is noteworthy that U-4CR could yield a vast number of molecules due to the huge variety of starting materials that is possible to choose. Taking into account that Scifinder® has indexed millions of commercially available primary amines, ketones, carboxylic acids and isonitriles, it is possible to prepare an unthinkable number of *N*-alkylated α,α-dialkylglycine using the outstanding U-4CR. Therefore Ugi reaction is one of the first reactions to be exploited explicitly to develop chemical libraries using the principles of combinatorial

chemistry for a rapid and easy synthesis in one reaction of compounds with biological or pharmaceutical interest.



Scheme 3. Ugi four-component reaction (U-4CR) mechanisms.

In addition, Ugi reaction has an enormous impact in organic synthesis as demonstrated by a huge number of publications in the field citing the Ugi multicomponent reactions over the years, since it was first developed in 1959. Academic labs and pharmaceutical companies in search of biologically active molecules have also harnessed the power of MCRs to explore, in a synthetically inexpensive way, various biological targets. Examples of the use of the Ugi reaction include the synthesis of a schistosomiasis drug Praziquantel[32] as well as antimalarial agents, among others.[33] Furthermore, taking advantage of the unusual acid lability of the C-terminal amide bond that Ugi derivatives present, Pereira-Lima's group adapt this synthetic methodology to solid phase synthesis to simplify purification processes.[34]

Considering that our principal goal in the present thesis is the development of both kind of linkers for bioconjugation, cleavable and non-cleavable, and also that an ideal linker has to obtain in high reaction yields, as well as it has to be ease to modify its structure to satisfy

different conjugation manners, we considered that the above mentioned Ugi adducts has a potential for being used as a new cleavable linker due to its facility to hydrolyse the C-terminal amide under acidic conditions. Accordingly, *N*-alkylated α,α-dialkylglycines mostly satisfy these assumptions, so this chapter section focussed intensely on the study of Ugi adducts as potential cleavable linkers for bioconjugation.

2.2. Objectives

- Study Ugi adducts preparation and acidolysis for their use as cleavable linkers.
- Improve C-terminal amide cleavage rate of the synthesized Ugi adducts.
- Find optimal conditions for C- and N-terminal functionalization and activation for their further bioconjugation.
- Use *N*-alkylated α,α-dialkylglycine compounds for conjugate to different complex biomolecules.
- Study the antibody binding-affinity of the synthetized conjugates.

2.3. Results and discussion

In order to find an efficient acid-cleavable linker for ADCs, we synthetized an small library of 1,4-dicarbonilic compounds based on α,α-dialkylglycines (Ugi adducts). The syntheses of each scaffold are discussed further below.

***N*-alkylated α,α-dialkylglycine**

The synthesis of the N-alkylated α,α-dialkylglycines were carry out by the condensation of a primary amine, a ketone, a carboxylic acid and cyclohexyl isocyanide through the previously described Ugi multicomponent reaction (**Figure 6**). This outstanding methodology allows easily the synthesis of complex 1,4-dicarbonilics compounds from short (hours) to long (weeks) reaction times. The reactions were carried out using an excess of the starting ketone, which also act as a solvent. For high boiling point or solid ketones, 2 mL of MeOH was added to the reaction. All starting amines were commercially available except 4-MeO-PEG-benzylamine, which was previously prepared by the ether formation between the 4-cyanophenol and the bromoPEGytaled derivative; following by the nitrile reduction to yield the 4-MeO-PEG-benzylamine (two steps, 58%, see experimental section). As Costa *et al*. suggested,[35] no special isonitrile is required to ensure selective cleavage at the C-terminus of the generated amino acid. Thus, cyclohexyl isocyanide was our choice based only on the availability and price of this starting material allowing to obtain a library of 15 α,α-dialkylglycines.

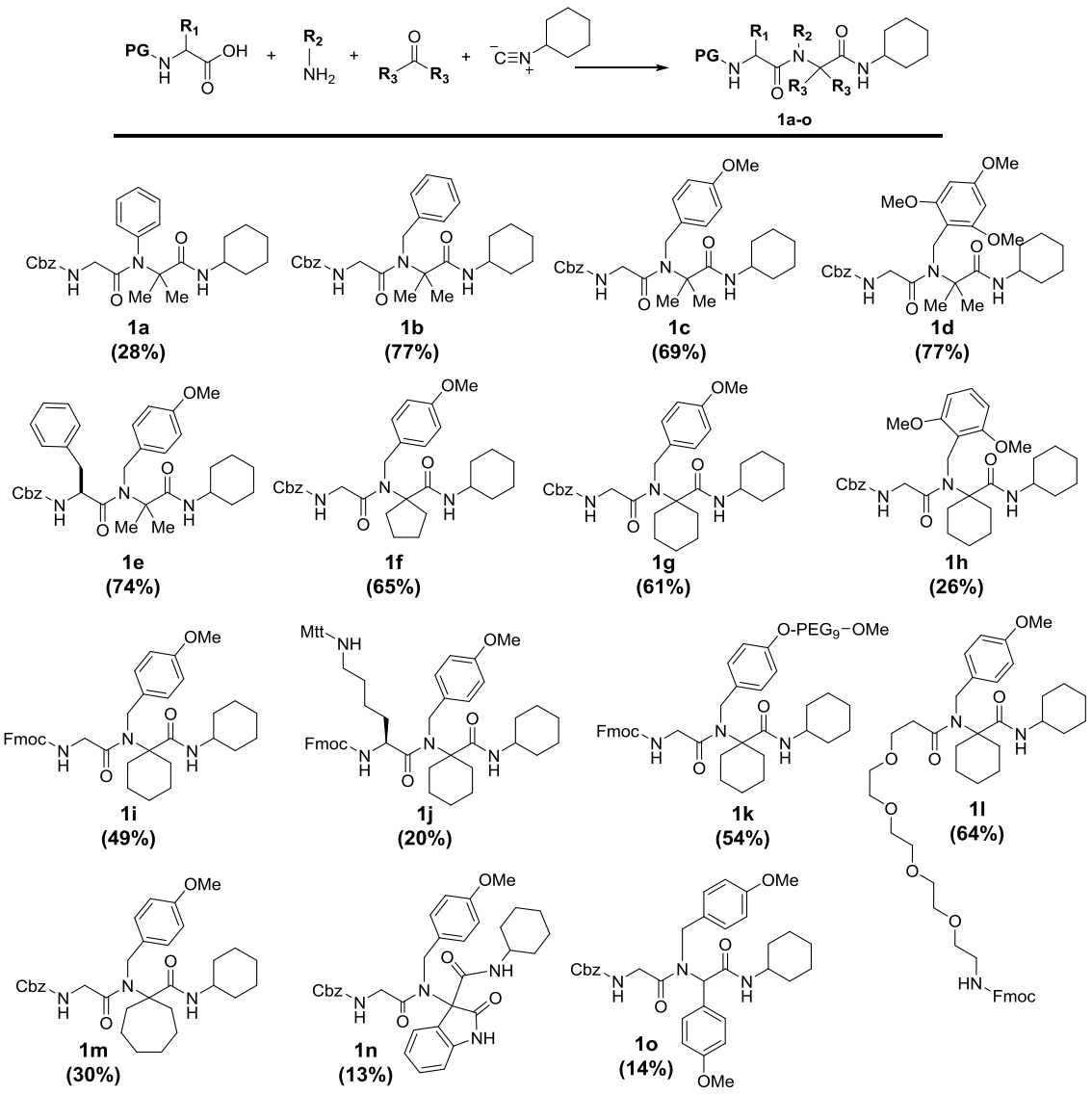


Figure 6. Chemical reaction, structures and yields (in brackets) of α,α-dialkylglycines synthetized by Ugi reaction.

All the reaction tested furnishes the products (**1a-o**) from moderate to high yields and without evidence of amino acid racemization for compounds **1e** and **1j**. The design of the α,α-dialkylglycines library was made studying the acidolysis rate of the synthetized compounds and modifying conscientiously the functional groups involved into the acidolysis mechanisms. First of all, different starting amines were tested in order to observe how affect the electro-donating effect into the oxazolone intermediate, which give rise to the amine release. The α,α-dialkylglycines derived from aniline, benzyl-, 4-metoxybenzyl- and 2,4,6-trimethoxybenzylamine were synthetized (**1a-e**). As expected, the low nucleophilicity of aniline results in lower reaction yield compared to benzylic amines. Cbz- or Fmoc- protected

glycine as well as Fmoc-Lys(Mtt)-OH (Mtt = 4-methyltrityl) or Fmoc-d_4PEG-OH are some examples of the starting carboxylic acids, which also were tested for the UGI reaction.

As the acidolysis mechanism via oxazolone ring in 1,4-dicarbonilyc compounds indicates, the first carbonyl group attack to the C-terminal amide releasing the *C*-terminal amine, then the proximity between the both carbonyl groups will determine the reaction rate. Accordingly, acyclic and cyclic ketones were used for the synthesis of the Ugi adducts, in order to restrict the conformation of the dialkylglycine and thus increase the amide acidolytic cleavage rate. When isatin was used as starting ketone for the Ugi reaction, compound **1n**, and **1o** was isolated. Apparently, the isatin core suffered a formamide elimination and the consequently addition of a methoxy group from the MeOH used as solvent. We also test the acidolysis for the obtained side product.

In order to assess the selectivity of the amide bond cleavage in acidic media, the Ugi derivatives 1a-o were treated with different acid concentrations until complete amine release (**Figure 7**). Accordingly, the lack of solubility of the protected dipeptides difficult the acidolysis study in aqueous solvents. For that reason a previous study had done using acetonitrile (MeCN) in order to solubilize the protected dipeptides and thus determine the compounds with higher acidolysis rate. The experiments were performed treating a solution of the amidated compounds 1a-o in MeCN (1 mg/mL) with 1% of trifluoroacetic acid (TFA) then the acidolysis reactions were monitored directly by HPLC analysis at different time points, and the % of the chromatographic peak area in front of time was represented (**Figure 8**). Moreover the main acidolysis product (carboxylic acid derivatives) was identified through mass spectrometry. Furthermore we have further evaluated the effect in the acidolysis rate of the substituents at the N-terminus, and also at the carbon atom of the fully substituted amino acid.

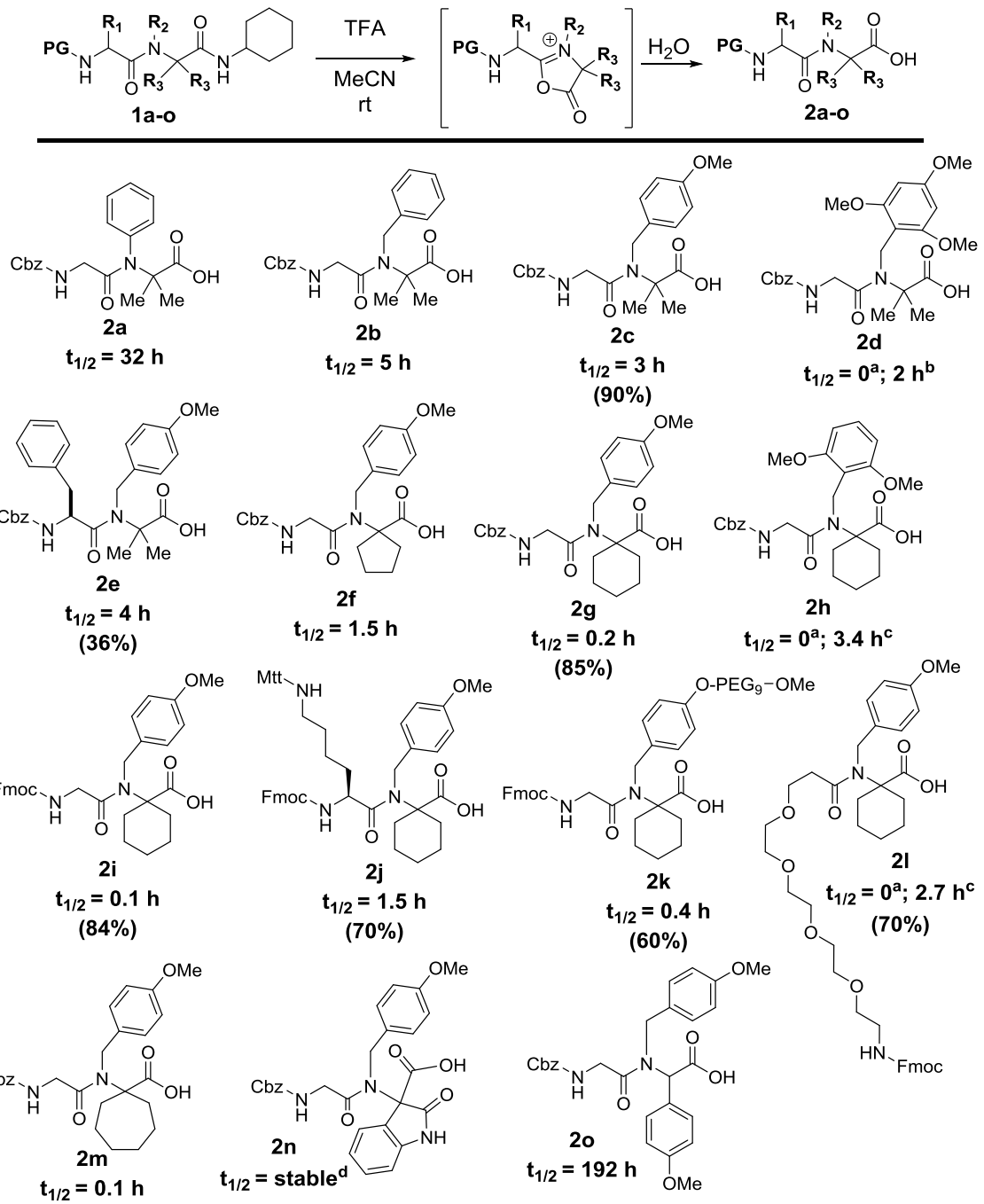


Figure 7. Acidolysis study and the resulting carboxylic acids. $t_{1/2}$ was determined by the linearization of the acidolysis curve. [a] Complete product decomposition. Generally acidolysis was performed using 1% TFA in MeCN except: [b] Acidolysis with 0.1% TFA in MeCN. [c] Acidolysis with 1% TFA in H_2O/MeCN (1:1). [d] Compound 1n was stable to 1% TFA in MeCN.

As it appreciate, almost all the studied compounds suffered *C*-terminal amide acidolysis obtaining the corresponding carboxylic acid from high (**1o** or **1a**) to extremely low (**1d, 1i** and **1m**) reaction times (**Figure 8A**). As expected, an excellent linear relationship between substrate concentration and HPLC peak areas was found, which allowed the $t_{1/2}$ calculation directly from peak areas. All reactions exhibited pseudo-first order behaviour with respect to

amide derivative, which is shown by the linear representation of ln A, being A HPLC peak area, in front of time (**Figure 8B**).

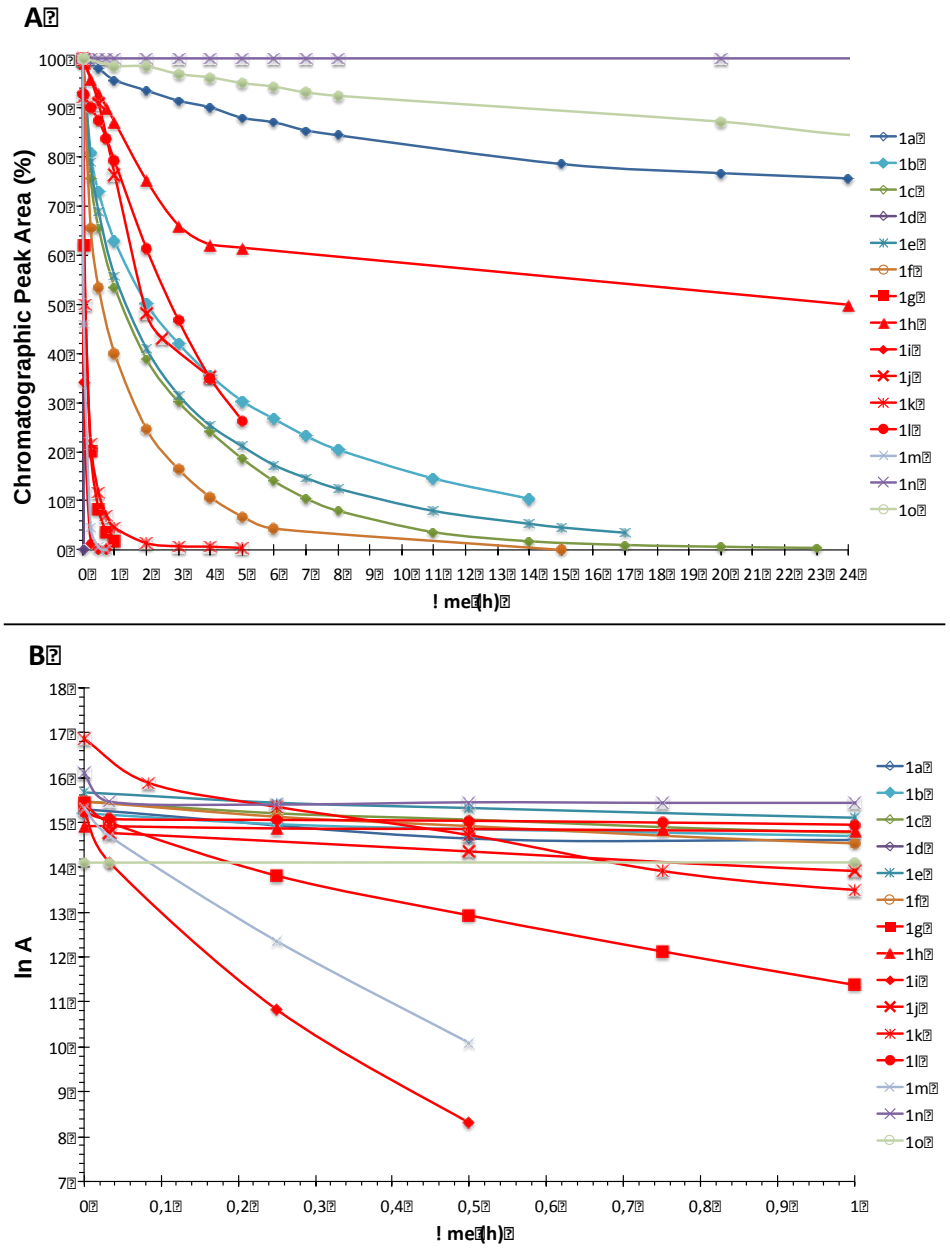


Figure 8. Acidolysis study for compounds 1a-o at 1 mg/mL in TFA/MeCN (1:99). A) Chromatographic peak area (%) corresponds to the amide degradation at the indicated time and B) linearization of the acidolysis curve, A correspond to the chromatographic peak area of the amides 1a-o at the indicated time of treatment.

The acidolytic study carried out contributes to understand which functional groups are involved into the amine release. Accordingly, the central *N*-alkyl group and the dialkylic scaffold, increase the acidolysis rate in the studied 1,4-dicarbonilic systems. Comparing compounds **1a-d**, where the starting amine used was different for each compound, an increment in the acidolysis rate was observed as more electrodonating groups are attached to

the starting amine. In this sense, compound **1d**, which contains trimethoxy-benzyl group, presented the higher amide acidolysis than **1b** and **1c**, which contain benzyl and *p*-methoxybenzyl groups, respectively. However, the **1d** reactivity in front of TFA in MeCN leads to complete compound decomposition. The addition of water into the reaction mixture decreases the acidolysis rate as it was previously described into the literature for others examples of amide scission.[10]

Regarding the acidolysis rate study, we also observed that the *N*-terminal amino acid side chain affect to the acidolysis rate (see compound **1c** *vs* **1e** and **1i** *vs* **1j**). Accordingly, the flexibility that glycine confers to the system, facilitate the oxazolone ring intermediate formation allowing the *C*-terminal amide cleavage.

Going a step further, the introduction of bulky groups in the α-carbon of the dialkylglycine has found to favour drastically the amide acidolysis. Hence, the use of cyclic ketones which yield cyclic dialkylglycines affect directly to the oxazolone formation increasing the amide cleavage rate. The decreasing differences in terms of acidolysis rate observed between the acyclic derivative (**1c**, $t_{1/2}$ = 3h) and the corresponding penta-, hexa- and heptacyclic derivatives (**1f**, **1g** and **1m** $t_{1/2}$ = 1.5, 0.2 and 0.1 h, respectively) depict the cyclic dialkylglycine derivatives as better hydrolysable compounds to used as a cleavable linkers. Correspondingly, the introduction of cyclic compounds into the dipeptidic scaffold force the approximation of the central carbonyl group to the *C*-terminal amide by an angle reduction, and consequently the acidolysis will take place faster than with the acyclic compounds. Surprisingly, the synthetized isatin derivative, which similar performance to cyclic derivatives was expected, it has found stable into the acidolytic conditions tested.

As the introduction of a large PEG chain into the protected dipeptide (**1k**) has improved the water solubility of the fully protected dipeptides, the acidolysis of compound **1k** was also tested using milder acid conditions in aqueous media. For that, compound **1k** was treated with an acidolytic cocktail that is usually used to mimic the inner of lysosomes. The mixture contains 2-(*N*-morpholino)ethanesulfonic acid (MES) (0.1 M), NaCl (137 mM) and KCl (2.7 mM) at pH = 4.8 and compound **1k** was incubated in the mentioned buffer at 37 ºC during 24 h. Unfortunately, compound **1k** remain stable under this conditions. This fact could jeopardize the use of this kind of 1,4-dicarbonilic systems as cleavable linkers for

bioconjugation because it remains stable at the lower pH conditions (4.8), which is found in the systemic circulation.

Although the heptacyclic derivative (**1m**) presented higher acidolysis rate than penta- or hexacyclic derivatives, hexacyclic compounds were studied deeply due to the higher isolation yield as well as better solubility. Therefore compounds **2g-l**, which contains the central cyclohexane ring, present similar acidolysis rates for the conditions tested. Generally cyclohomoleucine-containing compounds (**1g-e**), have shown elevated acidolysis rate in comparison to the acyclic Ugi adducts (**1c, 1e** and **1o**), and then is important to take into accounts the use of this kind of scaffold as cleavable linkers for bioconjugation.

***C*-terminal functionalization:**

Taking advantage of the lability of the amide bond at the *C*-terminus of the α,α-dialkyl glycine unit, further studies with some hydrolysed compounds for *C*-terminal amine incorporation were carried out. For that, aliphatic and aromatic amines were successfully incorporated into the free *C*-terminal carboxylic acid using an excess of the soluble carbodiimide (EDC·HCl) as coupling agent, in presence of Oxyma Pure® as an additive furnishing the corresponding amides from moderate to high reaction yields after purification (**Figure 9**).

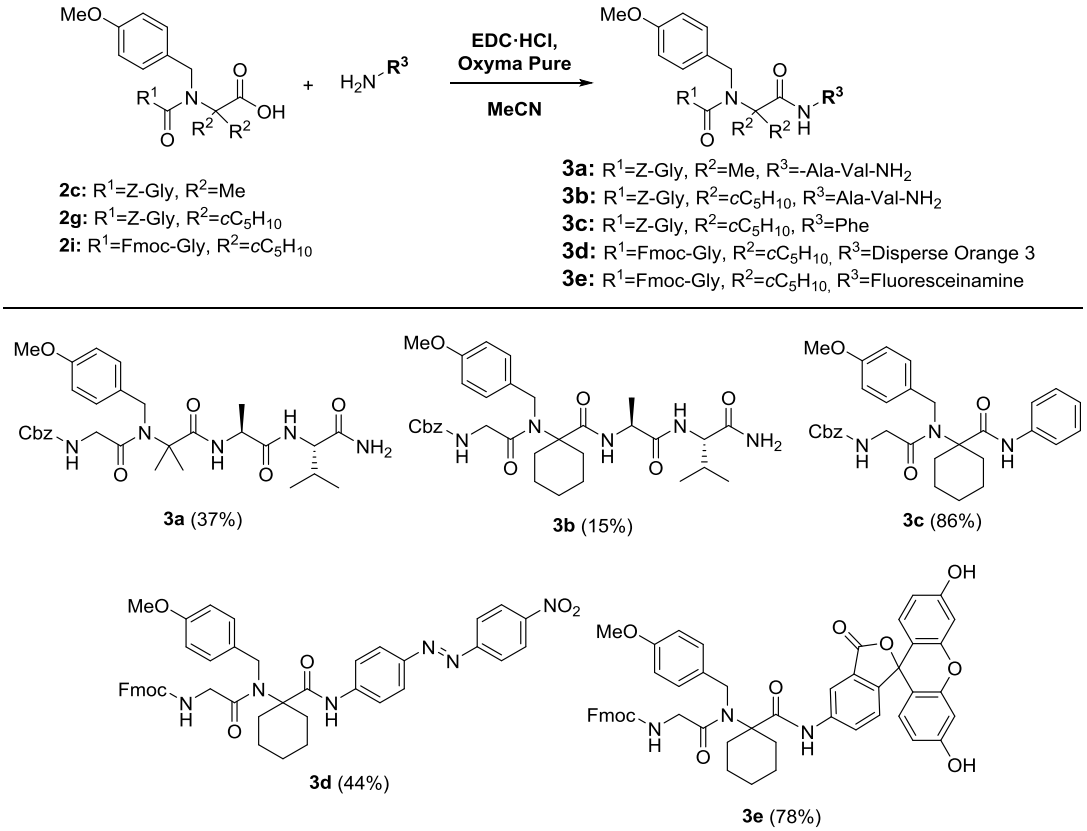


Figure 9. Products and yields for the Ugi adducts *C*-terminal functionalization**.**

The introduction of different kind of amines, confer to the system an additional benefit for the possibility to modulate by introducing amines of interest. After its incorporation, the amide cleavage of compounds **3b** and **3c** were carried out using the previously described acidolysis conditions. Once again these substrates undergo acidolysis, which proceeds via an oxazolone derivative to yield the corresponding open-chain *N*-acyl-*N*-α,α-trialkylglycine. The acidolysis study for each compound has shown in the experimental section.

Simultaneously to the *C*-terminal preparation, some linkers prepared with this strategy were sent to PharmaMar Company in order to prepare antibody drug conjugates (ADCs) with cytotoxic payloads anchored to monoclonal antibodies.

***N*-terminal functionalization:**

First of all, in order to functionalize the *N*-terminal dipeptidic compounds, the protecting groups have to be removed. On one hand, as a first approach for Cbz protecting group, palladium-catalysed hydrogenolysis has been done to yield the corresponding amines (**Figure 10**) for its further functionalization. Generally, the deprotected amines were obtained with high yields. Nevertheless, when the Cbz group was eliminated, an intramolecular cyclization could occur yielding the diketopiperazine as side product and consequently decreasing the reaction yield (**4e**).

At this point, the obtained amines **4a-d** could be directly anchored to carboxylic acids present in a macromolecule via amide bond. However, the dipeptidic scaffold favours the DKP formation, which affects the bioconjugation due to the amine deactivation.

From the macromolecule point of view, amine bioconjugation has found more efficient than the carboxylic acid conjugation. Hence, to the studied systems after Cbz or Fmoc elimination, amine functionalization is needed in order to anchor to the amines present in the macromolecule. In this regard, the most convenient way to anchor to the reactive amines from peptides or proteins is via amide bond formation. For that, to our dipeptidic scaffolds, previous introduction of a carboxylic group into the *N*-terminal dipeptide is needed.

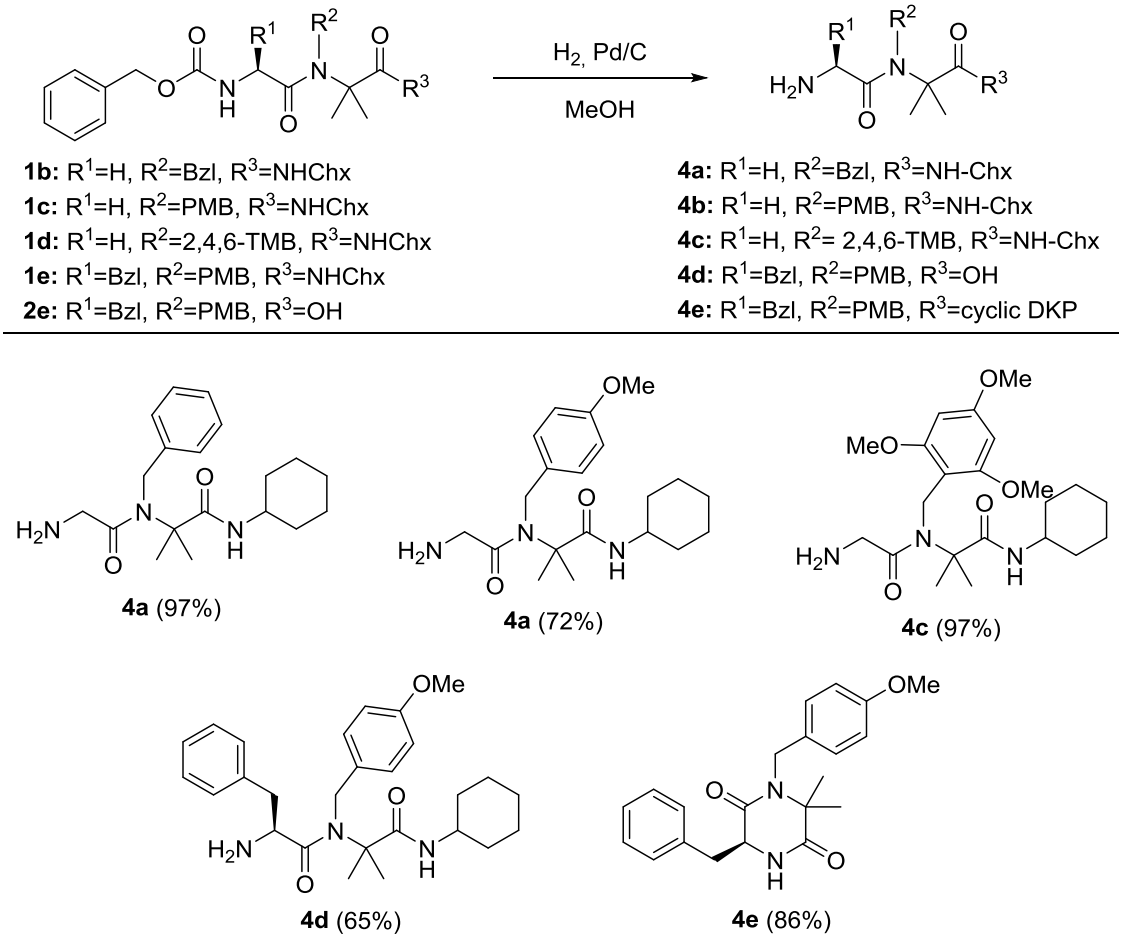
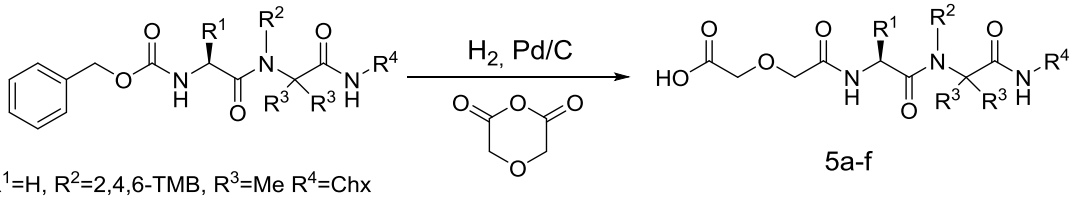


Figure 10. Cbz group elimination by Pd-catalysed hydrogenolysis.

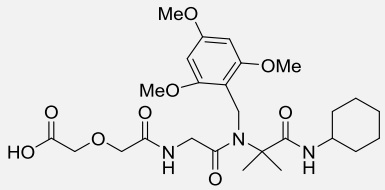
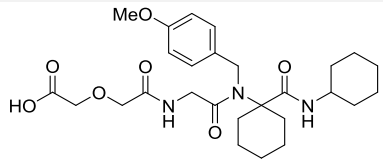
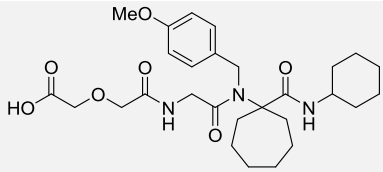
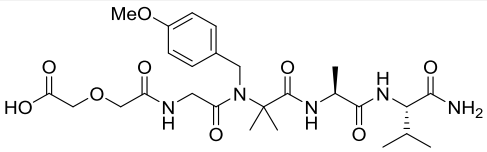
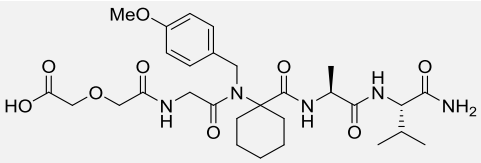
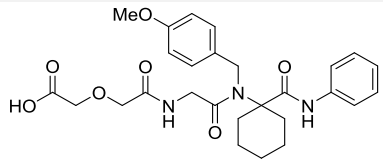
Accordingly the obtained amines **4a-d** could be functionalized by amide formation but however, in order to reduce the reaction steps with symmetric anhydride-mediated acetylation, reaction optimization was performed by the Cbz hydrogenolysis followed by the *in situ* amine acylation using diglycolic anhydride (**Scheme 4**). As a result the amine protecting group elimination and its acylation was performed in one pot reaction with excellent yields and without further crude purification (**Scheme 4, Table 2**).



1d: R^1=H, R^2=2,4,6-TMB, R^3=Me R^4=Chx
1g: R^1=H, R^2=PMB, R^3=cC_5H_{10}, R^4=Chx
1m: R^1=H, R^2=PMB, R^3=cC_6H_{10}, R^4=Chx
3a: R^1=H, R^2=2,4,6-TMB, R^3=Me R^4=Ala-Val-NH_2
3b: R^1=H, R^2=PMB, R^3=cC_5H_{10}, R^4=Ala-Val-NH_2
3c: R^1=H, R^2=PMB, R^3=cC_5H_{10}, R^4=Phe

Scheme 4. One pot Cbz elimination and diglycolic incorporation.

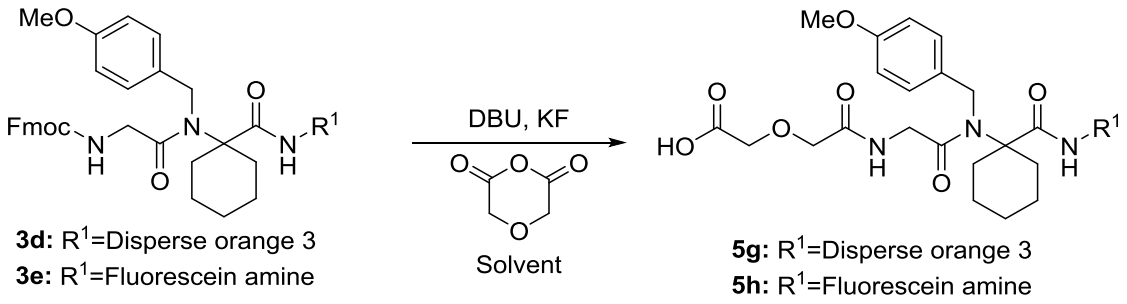
Table 2. One pot Cbz elimination and acylation obtained products, yields and crude purity.

Entry	Protected compound	Obtained compound		Yield (%)	Purity (%)[a]
1	1d	5a		97	96
2	1g	5b		98	97
3	1m	5c		90	94
4	3a	5d		97	83
5	3b	5e		99	93
6	3c	5f		97	98

[a] Determined by the HPLC peak area of the isolated product without purification.

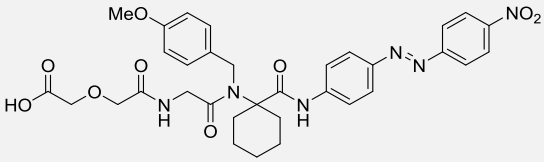
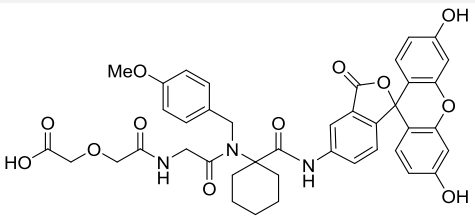
Furthermore, the Fmoc protected dipeptides (**3d** and **3e**) were functionalized with the diglycolic moiety (**Scheme 5**). For that purpose, the simultaneous protecting group elimination and acylation was tackled. For Fmoc substrates, the *N*-terminal protecting group

was removed using a strong base (DBU) and in the same reaction mixture, potassium fluoride and diglycolic anhydride was added in order to be trapped by the liberated amine, allowing the simultaneous Fmoc elimination and the liberated amine acylation. Although the reaction yields were low compared with the Cbz analogues, the use of Fmoc group is crucial when the attached amine to the C-terminal is hydrogen sensitive (**Scheme 5; Table 3**).



Scheme 5. Simultaneous Fmoc group elimination and amine acylation.

Table 3. One pot Fmoc elimination and acylation obtained products and yields.

Entry	Protected compound	Solvent	Obtained compound	Yield (%)
1	3d	THF	5g 	9
2	3e	DMF	5h 	30

The diglycolic acid incorporation improved the solubility in aqueous media of some of the synthetized products. Hence, the amide acid cleavage for compounds **5a-c** was tested in aqueous media and also in the previously described acidolysis conditions (1% TFA in MeCN). Once again these substrates suffer acidolytic cleavage under 1% TFA acid conditions with similar $t_{1/2}$ than its Cbz protected precursors. However, no significant effects were observed when the mentioned compounds at 1mg/mL were treated in aqueous media using 0.1 M of MES buffer at pH = 4.8. Although no significant amide cleavage was observed in the prepared linkers using mild acidic conditions, PharmaMar Company prepared two drug

linkers to be attached into a mAb. Accordingly, they functionalize the *N*-terminal linkers with a maleimide function for the conjugation via thiol groups from the antibody.

After protecting group removal and diglycolic acid incorporation, compounds **5a**, **5b** and **5c** become water-soluble. First of all, compound **5b** stability in human serum was tested. Fort that, compound **5b** at 1 mg/mL was incubated in male Human serum type AB and Hank's Balanced Salt solution (HBSS) (9:1) at 37 ºC for 24 h. The degradation reaction was monitored by HPLC from 30 min to 24 h of incubation (**Figure 11**). After 24 h of treatment no presence of degradation of compound **5b** has found, interestingly indicating that the linker is stable enough to human serum conditions.

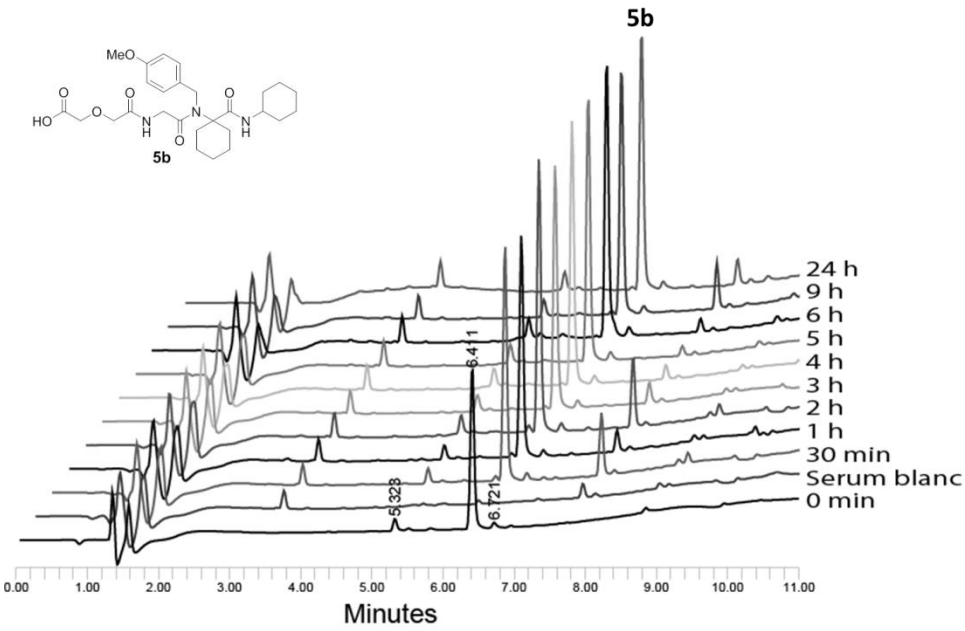


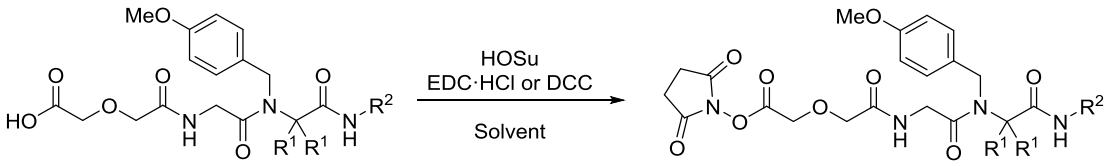
Figure 11. HPLC chromatograms of human serum stability study for compound 5b.

Furthermore the mentioned compounds **5a-c** were treated with milder acidolytic aqueous mixture in order to observed if the prepared system hydrolysed under the studied conditions. Unfortunately, compounds **5a-c** did not suffer *C*-terminal amide cleavage using MES 0.1 M at pH = 4.8. Though compounds **5a-c** amides were not hydrolysed using mild acidic conditions, we decided to test its bioconjugation to different complex biomolecules as last opportunity to test its acidolysis cleavage.

Activation for bioconjugation:

In order to achieve the bioconjugation through lysine amino group of peptides, proteins and antibodies, some of the obtained carboxylic acids (**5b**, **5d, 5f, 5g** and **5h**) were activated

through *N*-hydroxysuccinimide esterification (**Scheme 6**). A first attempt was performed using EDC·HCl as a coupling agent, but unfortunately the obtained HOSu ester was hydrolysed during the work-up. Accordingly, the optimal conditions which avoid the presence of water for the carboxylic acid activation was found out. The HOSu activation was carried out by treating the free carboxylic acid in presence of equimolecular quantity of *N*-hydroxysuccinimide and slight excess of dicyclohexyl carbodiimide as a coupling agent in a mixture of AcOEt:dioxane (1:1) as solvents. The mixture of solvent promoted the dicyclohexyl urea precipitation and ease it elimination from the reaction crude by filtration and yield the activated HOSu ester in a quantitative yield (**Scheme 6, Table 4**).



5b: R^1=*c*-C_5H_{10}, R^2=Chx
5d: R^1=CH_3, R^2=Ala-Val-NH_2
5f: R^1=*c*-C_5H_{10}, R^2=Phe
5g: R^1=*c*-C_5H_{10}, R^2=Disperse Orange 3
5h: R^1=*c*-C_5H_{10}, R^2=Fluorescein

6a: R^1=*c*-C_5H_{10}, R^2=Chx
6b: R^1 =CH_3, R^2=Ala-Val-NH_2
6c: R^1 =*c*-C_5H_{10}, R^2=Phe
6d: R^1=*c*-C_5H_{10}, R^2=Disperse Orange 3
6e: R^1=*c*-C_5H_{10}, R^2=Fluorescein

Scheme 6. Ugi linkers HOSu activation**.**

Table 4. Ugi linkers HOSu reaction conditions and yield.

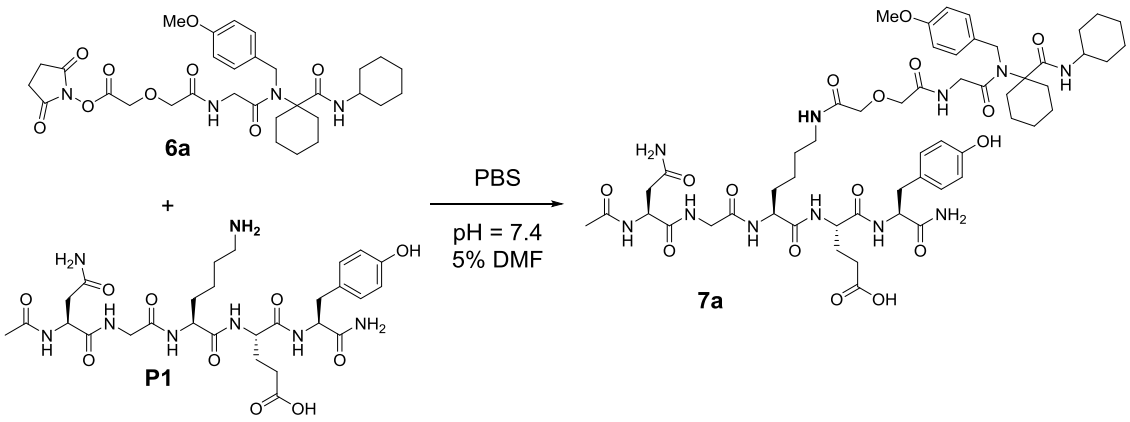
Entry	Carboxylic Acid	Coupling agent	Solvent	Obtained compound	Yield (%)
1	5b	EDC·HCl	MeCN	6a	0
2		DCC	AcOEt:Dioxane (1:1)		Quantitative
3	5d		THF	6b	0
4	5f		AcOEt:Dioxane (1:1)	6c	Quantitative
5	5g			6d	Quantitative
6	5h			6e	Quantitative

Bioconjugation:

With the activated *N*-hydroxysuccinic esters in hand, the bioconjugation of a peptide, a protein and a monoclonal antibody was tackled. First of all, peptide bioconjugation was carried out and studied. A lysine-containing pentapeptide amide was manually synthetized by solid phase peptide synthesis (SPPS) using the Fmoc/tBu strategy, DIC/Oxyma Pure® as coupling agent and additive; and Rink amide as solid support. The selected pentapeptide Ac-

Asn-Gly-Lys-Glu-Tyr-NH_2 **(P1)** is a sequence which is present in anti-Her2 (Trastuzumab a humanized IgG1 monoclonal antibody).

The bioconjugation was performed by adding the activated carboxylic acid dissolved in DMF to a solution of the synthetized peptide in PBS at pH = 7.4 and room temperature for 17 h (**Scheme 7**).



Scheme 7. Lysine-mediated peptide bioconjugation.

After 17 hours, the analysis by HPLC reveals that the conjugate resulting from the attachment of the functionalized Ugi adduct **6a** to lysine from peptide **P1**, was obtained (**Figure 12**). Accordingly, using 1.1 eq of the succinic ester the conjugation reaction was not complete due to the deactivation of the mentioned ester by aqueous hydrolysis. Thus, more equivalents are needed in order to achieve the complete lysine mediated bioconjugation.

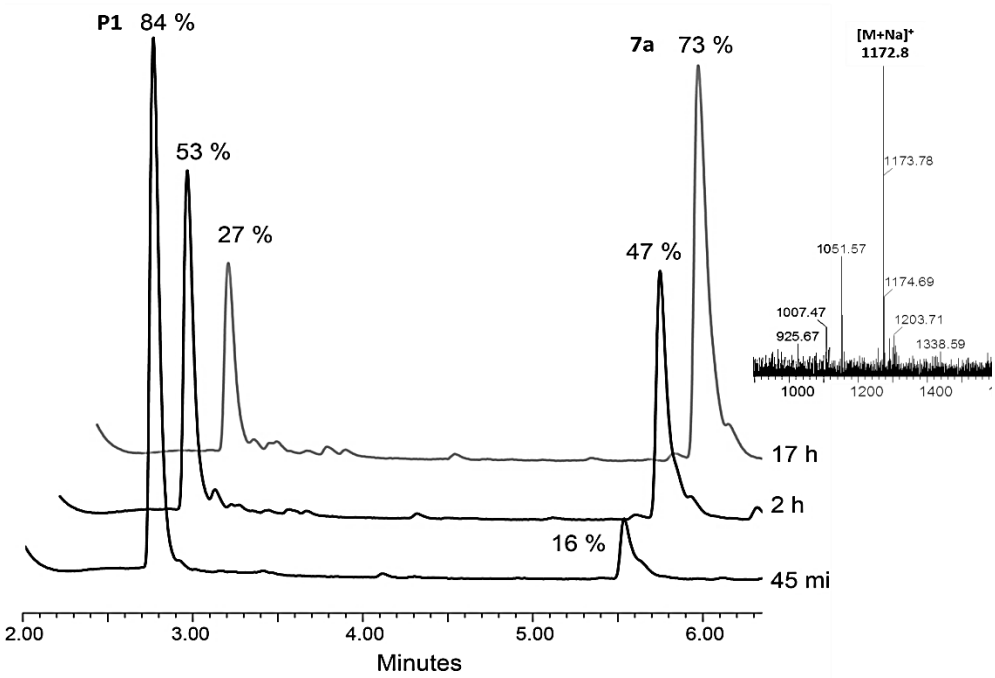
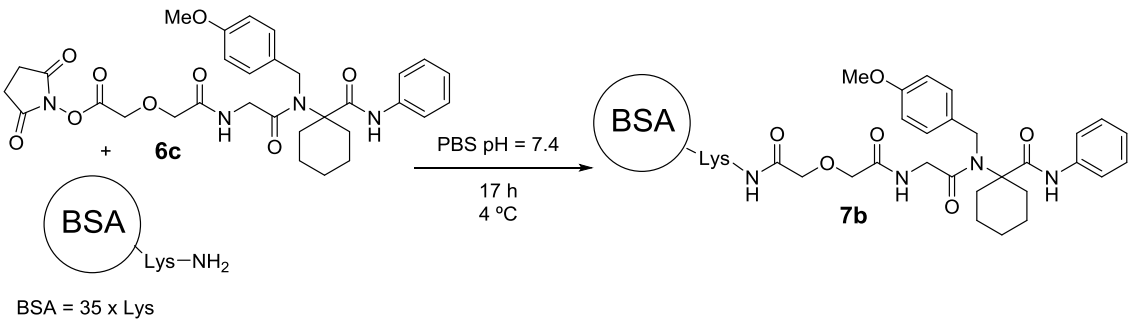


Figure 12. HPLC chromatograms and ESI analysis of the Lys-mediated bioconjugation at 45 min, 2 and 17 h, respectively.

After observed that lysine bioconjugation was possible into a peptidic sequence, we ventured to the bioconjugation with the same methodology into more complex system. For this purpose, bioconjugation to bovine serum albumin protein (BSA) was performed (**Scheme 8**). Following the same above mentioned strategy, to a solution of BSA (11 mg/mL) in PBS at pH = 7.4, a solution of the previously activated compound **6c** (1.7 eq. respect to one BSA lysine) in DMSO (5%) was added, and the reaction was stirred at 4 ºC during 17 h. Then, the excess of compound **6c** or its hydrolysed analogue was removed using a size exclusion column. The resulting BSA-conjugate **7b** was conveniently characterized by HPLC, UV, and mass spectrometry (**Figure 13**).



Scheme 8. BSA Lys-mediated conjugation with HOSu activated Ugi adduct **6c.**

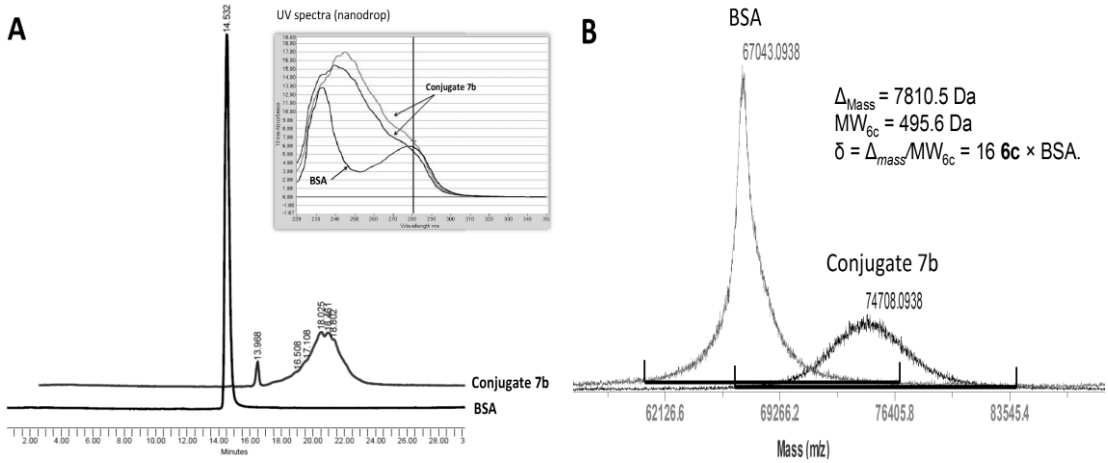
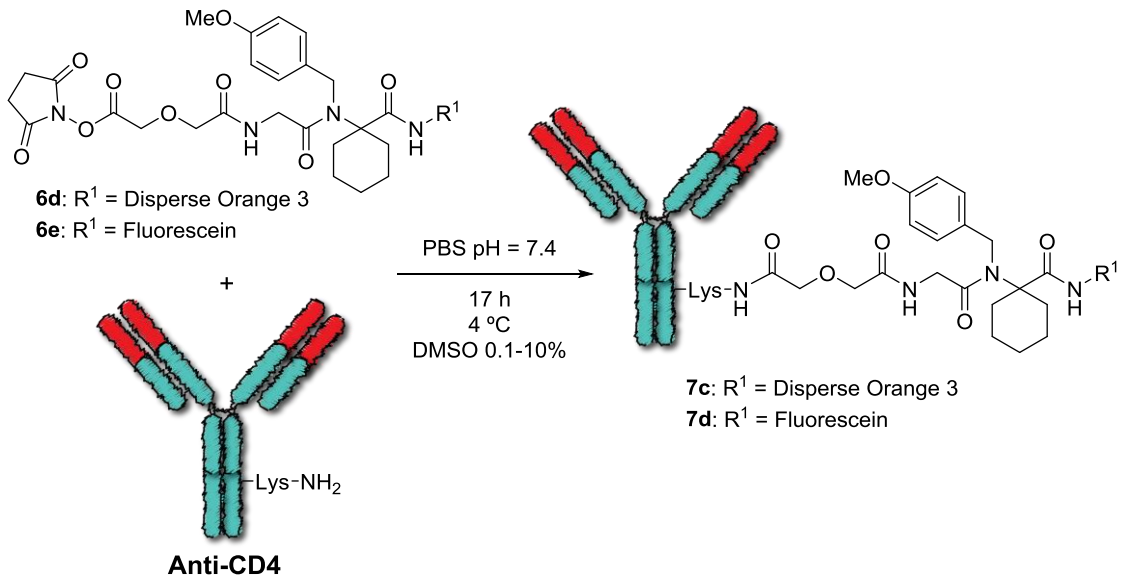


Figure 13. BSA and conjugate **7b A**) HPLC and UV nanodrop analysis and **B)** MALDI analysis.

The analysis carried out reveals that the conjugation occurs but however as it was expected for lysine-mediated bioconjugation, the broad HPLC peak observed corresponding to conjugate **7b** reveals that a heterogeneous conjugation to BSA took place. Moreover, the conjugation has found efficient enough because the mass analysis determined that 16 molecules of the compound **6c** were attached to the BSA surface using 1.7 eq. of compound **6c** × one BSA. The obtained BSA conjugated **7b** was treated with a MES 0.1 M buffer at pH = 4.8 at 37 ºC for 24 h, but one more time, not appreciable hydrolysis was observed.

After observed that the conjugation took place successfully in peptides and BSA protein, we tested the bioconjugation to antibodies (**Scheme 9, Table 5**). A solution of the activated compounds **6d** and **6e** in DMSO were added separately to a solution of anti-CD4 (1.5 and 1.0 mg/mL for **6d** and **6e** respectively) in PBS at pH 7.4 and it was left to react for 17 h at 4 ºC. After that, the excess of linker was removed using size exclusion column and the obtained fractions were conveniently characterized.



Scheme 9. Anti-CD4 Lys-mediated conjugation with the HOSu activated compounds **6d and 6e.**

Table 5. Conjugation conditions for anti-CD4 and compounds **6d** and **6e.**

Entry	Conjugated compound (Eq.)	DMSO (%)	Resulting Conjugate	Conjugation density (δ)
1	6d (3.7)	2	7c	2
2	6e (4.2)	9	7d	2/3

The UV spectra analysis of both conjugates (**Figure 14A**) demonstrated that the conjugation occurs. In the UV spectra is possible to observe a slightly variation at 375 and 490 nm for compound **7c** and **7d,** respectively, indicating that the conjugated fluorophores are attached to the antibody. Due to the low equivalents used to attach to the antibodies, the SDS analysis not reveals significant changes in the conjugates mass. In contrast using a more accurate technique to determine the mass of the conjugates (ESI) it was possible to determine that at least two molecules of compounds **7c** and **7d** was attached to the antibody (**Figure 15**). Furthermore, as **Figure 15C** depicted, the use of more equivalents of the activated carboxylic acid **7d** yield a mixture of conjugates which contains two and three molecules of compound **5h** respectively.

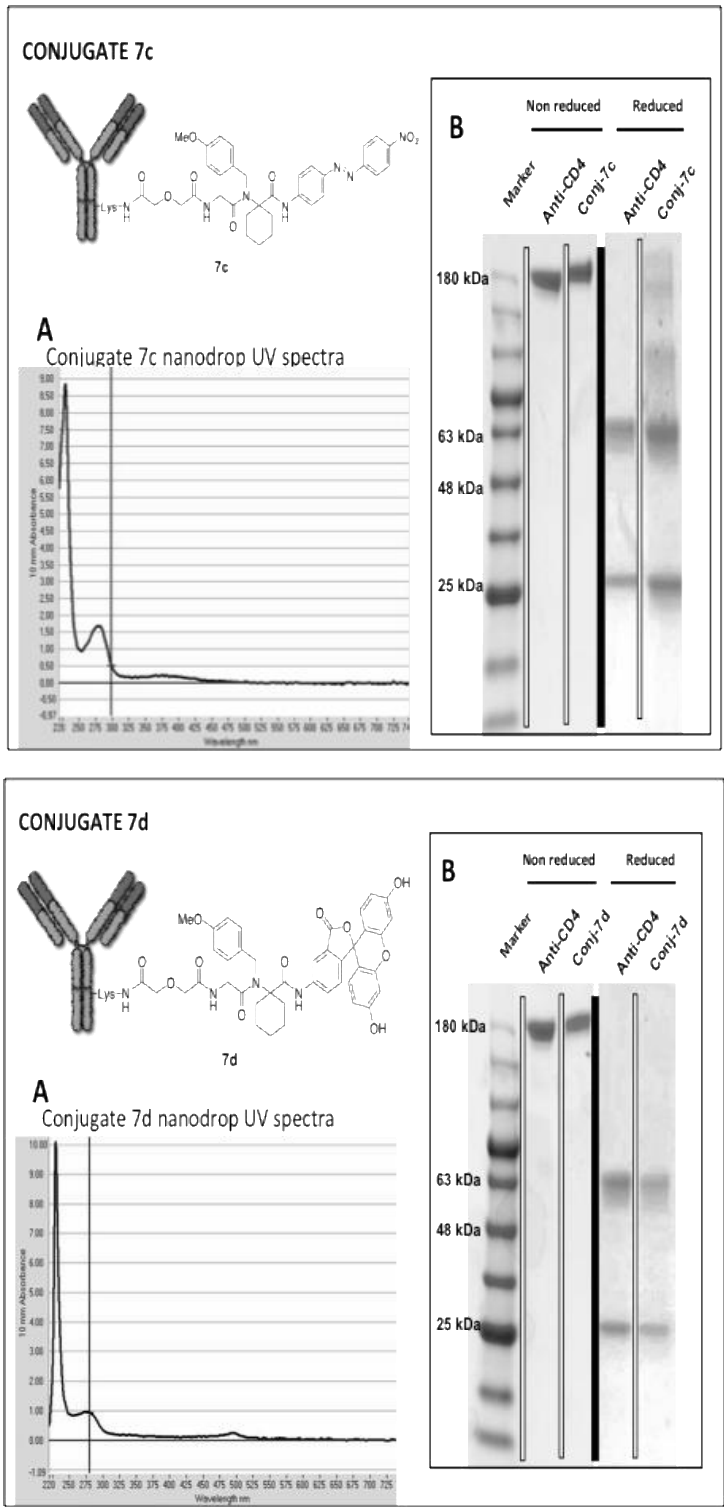


Figure 14. A) UV nanodrop characterization and**; B)** SDS-PAGE electrophoresis using non- and reducing conditions for conjugates **7c (E1) and 7d (E2).**

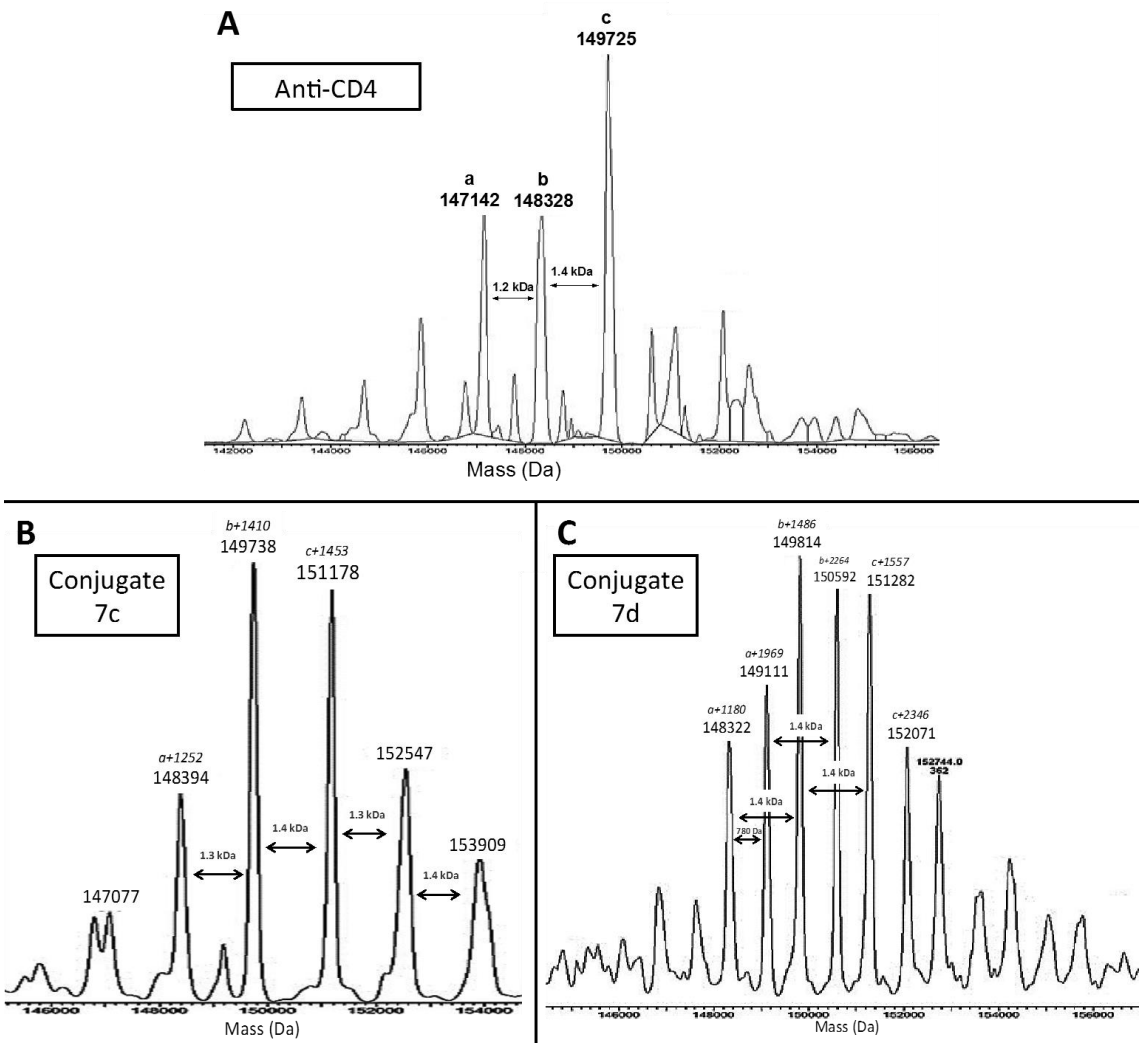


Figure 15. Mass spectra of **A)** deglycosilated anti-CD4**, B**) deglycosilated conjugate **7c** and **C)** deglycosilated conjugate **7d.**

To determine whether the conjugation of the linkers **5g** and **5h** to the anti-CD4 HP2/6 mAbs had any effect on the binding to its epitope, we performed an antibody binding analysis with cell lines that constitutively express CD4 (Jurkat cell line). For this cell line, we used increasing concentrations (0.1-10 µg/ml) of the naked antibody (anti-CD4 HP2/6 mAbs) and its corresponding conjugates (**7c** and **7d**). The naked antibody showed higher mean fluorescence intensity (MFI) and binding affinity compared to different conjugates (**Figure 16, Table 6**).

Accordingly, Conjugates **7c** and **7d** had not similar binding affinity compared to the anti-CD4 mAb (AC_{50} 0.53, 0.57 µg/ml compared to 0.38 µg/ml, respectively), and moreover they reach a lower maximum mean fluorescence intensity value (59 and 58 %, respectively) than that of the anti-CD4 mAb. This result suggests that conjugates **7c** and **7d** would coat more

than 50% of all available CD4 molecules on the cell surface. That binding affinity reduction may be due to the lysine conjugation that could affect to the antigen-binding site or even to the constant region of the antibody, which the secondary antibody recognizes. To exactly determine the part of the antibody where the molecules are attached, digestions experiments should be considered.

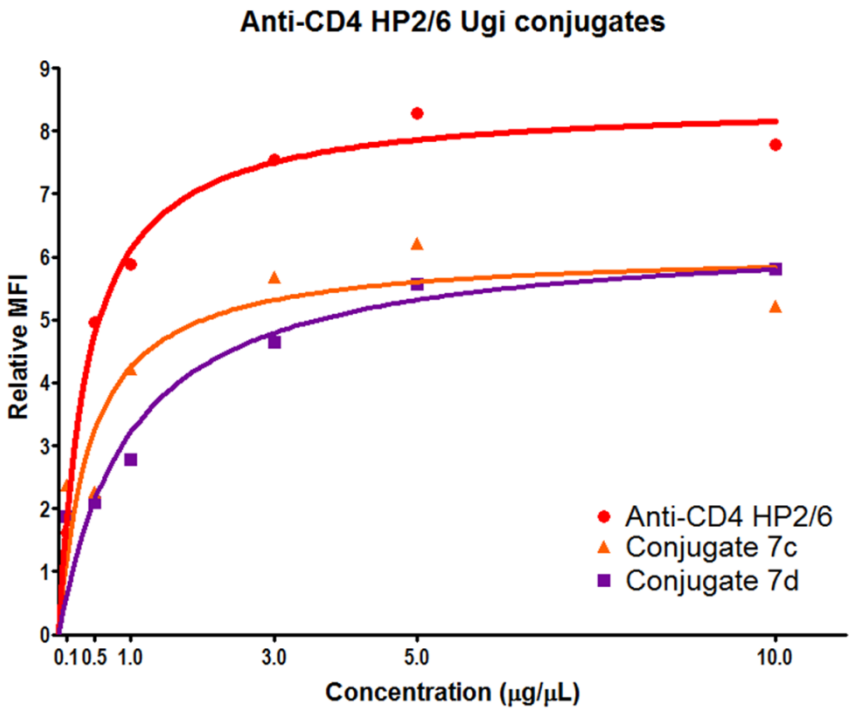


Figure 16. Antibody-Conjugates cell binding analysis by flow cytometry for anti-CD4 and conjugates 7d-c to Jurkat cells. The cell line was incubated with the indicated concentrations of mAb or the conjugates 7d-c, followed by incubation with FITC-labelled goat anti-mouse IgG antibodies, as described in Materials and Methods. Cytometry analysis was performed and the mean fluorescence intensity (MFI) ratio was calculated using as reference the MFI value or an irrelevant mAb (basal MFI value).

Table 6. AC_{50} and Max MFI (%) for mAb anti-CD4 its conjugates (7c-d). AC_{50}, calculated as the antibody concentration (AC) needed to achieve 50% of the highest MFI value for each conjugate**.**

	AC_{50} (µg/mL)	Max MFI (%)
Anti-CD4	0.38	100
Conjutate 7c	0.53	59
Conjugate 7d	0.57	58

As previously mentioned, the biopharmaceutical company PharmaMar (Dr. J. M. Dominguez) has prepared some conjugates with their own cytotoxic compound using some of the described herein linkers decorated with the maleimide functionality. The maleimido-

linker-cytotoxic payload complex was conjugated to the antibody through thiol groups obtained after mild disulfide bond reduction of the mAb. The IC_{50} determination of the prepared conjugates, were assessed by the company using cell viability assays in different human cell lines. The antibody used recognizes specifically the antigen human epidermal growth factor receptor 2 (HER2), which has been described to be overexpressed in a certain aggressive types of breast cancer.[36] Accordingly, two anti-HER2 ADCs were tested using overexpressed HER2 receptor cell lines (HCC1954 and SK-BR3) and to negative HER2 receptor cell lines (MCF7 and MDA-MB-231). As the IC_{50} values are shown in the following table, both ADCs studied present higher efficacy in front $HER2^+$ than $HER2^-$ cell lines. Therefore, the IC_{50} values reveal that the type of conjugation carried out did not affect the antibody-binding site. The results depicted that depending on the linker used for the bioconjugation, the IC_{50} values are directly affected reducing its cytotoxic activity (ADC A *vs* ADC B).

Table 7. IC_{50} values for cell viability assays in front of $HER2^+$/$HER2^-$ cell lines.

HER2 expression	Cell line	IC_{50} (nM)	
		ADC A	ADC B
+	HCC1954	20,3	173,3
	SK-BR3	15,3	133,3
-	MCF7	80	333
	MDA-MB-231	120	333

2.4. Conclusion

In order to find a suitable cleavable linker for bioconjugation a dipeptidic-base 1,4-dicabonilic adducts were studied. Correspondingly, in the present chapter section, a small library of fifteen *N*-alkylated α,α-dialkylglycines were synthetized and conveniently characterized from moderate to high yield by Ugi multicomponent reaction. Accordingly different electron donating/withdrawing and alkyl groups were used in order to improve the acidolytic properties of the mentioned system.

To determine *C*-terminal amide cleavage rates, an acidolysis study with the synthetized compounds 1a-o were carried out. Of the repertoire of molecules studied, compounds decorated with a cyclic dialkylglycine presented higher acidolysis rate. We found that factors such as structure rigidity and the *N*-alkyl group electronic behaviour, affect directly the acidolysis rate. With compounds **1i** and **1m** we observed faster amide cleavage than the other compounds ($t_{1/2}$ = 0.1 h) in 1% TFA in MeCN. For other compounds (**1d**, **1h** and **1l**) the *C*-terminal amide was cleaved fast enough but however, they tend to decompose using the mentioned acidolytic conditions. Although **1k** C-terminal amide was cleaved using 1% TFA in MeCN, unfortunately it amide remains stable under MES 0.1 M buffer at pH = 4.8.

The C-terminal modification of our linkers was optimized for the incorporation of aliphatic and aromatic amines into some of the hydrolysed compounds (**2c**, **2g** and **2i**). The amide formation was successfully performed using standard coupling conditions (EDC·HCl and Oxyma Pure®). Furthermore, the N-termini modification was performed by the simultaneous protecting group elimination and acylation with a symmetric anhydride in one pot to yield a free carboxylic acid. The reaction conditions were optimized to finally obtain elevated reaction yields and high crude purity.

The corresponding carboxylic acids obtained (**5b, 5d**, **5f-h**) were activated with DCC in AcOEt:dioxane (1:1) as *N*-hydroxysuccinimide ester for lysine conjugation to different complex biomolecules. As lysine precursors, a pentapeptide, BSA protein and anti-CD4 mAb were used. The corresponding conjugates (**7a-d**) were conveniently characterized using different techniques.

Additionally, to determine if the synthetized antibody conjugates are biologically affected, antibody/conjugate-antigen binding affinity experiments were carried out. It is noteworthy that during the conjugation process the reaction conditions could affect directly to its properties as immunoglobulin misplacing in fact it binding affinity. However, the synthetized conjugates **7c-d** maintain their ability of binding to CD4, as the naked antibodies showed, but with a slight reduction of it affinity. This fact indicates that this kind of conjugation strategy did not disrupt enough the antibody activity, being the Ugi adducts suitable linkers for amine bioconjugation.

The biotechnological company PharmaMar prepared in a collaborative project, cytotoxic anti-HER2 ADC's using some of the described herein Ugi linkers. The cell viability studies with the mentioned ADC's have shown high specificity in front of $HER2^+$ cell lines and also low IC_{50} values. Although no acidolysis was observed experimentally using biological-like conditions, the synthetized antibody drug conjugates has shown high cell-killing activity and moreover good cell specificity. The versatility of Ugi adducts to fine-tuning it C-terminal cleavage rate, highlighted as really interesting systems for their use as a cleavable linkers.

2.5. References

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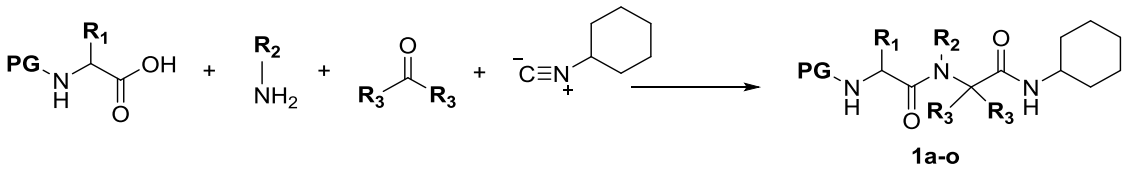
2.6. Experimental section

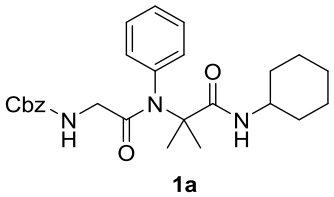
Ugi Reactions

General procedure:



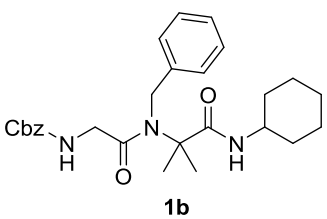
The dialkylglycine derivatives were prepared by the initial imine formation by mixing the corresponding ketone (1 eq. or excess) and the primary amine (1 eq.) during 15 min at room temperature in presence of Na_2SO_4. The reaction solvent is indicated in each particular reaction case. Then *N*-protected amino acid (1 eq.) was added to the mixture and then it was stirred during 10 min. After that time, cyclohexyl isocyanide (1 eq.) was added to the mixture and it was left to react from 1 h to 2-3 weeks depending on the reaction advance at room temperature and in dark to avoid side reactions. Then the suspension was filtered and the solvent was eliminated under vacuum. Then the crude was purified through chromatographic techniques or by recrystallization as indicated below.

1. Cbz-Gly-N-phenyl-α,α-dimethylglycine cyclohexylamide (1a)



Aniline (873 µL, 9.6 mmol), acetone (20 mL, excess), Na_2SO_4 (1.0 g, excess), Cbz-Gly-OH (2.0 g, 9.6 mmol) and cyclohexyl isocyanide (1.2 mL, 9.6 mmol). The reaction was left to react 12 days and then the product was isolated by recrystallization in AcOEt:Hexane (20:60) to yield **1a** as white solid (1.2 g, 28 %). **RP-HPLC**: [linear gradient H_2O/MeCN (95:5) to (0:100) over 8 min] t_R = 7.51 min; **MS-ESI**: *m/z* calculated for $C_{26}H_{33}N_3O_4$ = 451.2, found: 452.1 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.46 – 7.39 (m, 4H), 7.36 – 7.27 (m, 6H), 5.71 (d, *J* = 8.1 Hz, 1H), 5.63 – 5.58 (m, 1H), 5.03 (s, 2H), 3.85 – 3.74 (m, 1H), 3.51 (d, *J* = 4.7 Hz, 2H), 2.01 – 1.93 (m, 2H), 1.77 – 1.67 (m, 2H), 1.66 – 1.58 (m, 1H), 1.35 (m, 8H), 1.24 – 1.13 (m, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 173.5, 168.5, 156.2, 138.1, 136.6, 130.3, 129.8, 129.4, 128.6, 128.1, 128.0, 66.8, 63.1, 48.8, 44.7, 33.1, 25.8, 25.5, 25.1.

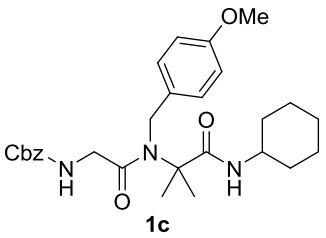
1. Cbz-Gly-N-benzyl-α,α-dimethylglycine cyclohexylamide (1b)



Benzylamine (1.05 mL, 9.6 mmol), acetone (20 mL, excess), Na_2SO_4 (1.0 g, excess), Cbz-Gly-OH (2.0 g, 9.6 mmol) and cyclohexyl isocyanide (1.2 mL, 9.6 mmol). The reaction was left to react 21 days and then the product was isolated by recrystallization in MeOH to yield **1b** as a pale yellow solid (3.4 g, 77 %). **RP-HPLC**: [linear gradient H_2O/MeCN (70:30) to (0:100) over 8 min] t_R = 6.19 min; **MS-ESI**: *m/z* calculated for $C_{27}H_{35}N_3O_4$ = 465.59, found: 466.62 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.46 (d, *J* = 7.6 Hz, 2H), 7.41 – 7.24 (m, 8H), 5.70 (s, 1H), 5.53 (d, *J* = 8.2 Hz, 1H), 5.07 (s, 2H), 4.60 (s, 2H), 4.00 (d, *J* = 4.6

Hz, 2H), 3.83 – 3.70 (m, 1H), 1.99 – 1.88 (m, 2H), 1.75 – 1.57 (m, 3H), 1.44 (s, 6H), 1.40 – 1.30 (m, 2H), 1.26 – 1.08 (m, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 173.6, 169.4, 156.3, 137.6, 136.6, 129.2, 128.6, 128.2, 128.1, 127.7, 126.0, 66.9, 63.1, 48.6, 47.0, 43.7, 33.2, 25.8, 25.1, 24.3.

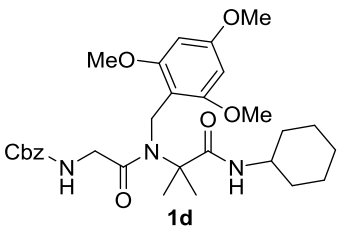
2. Cbz-Gly-N-(4-methoxybenzyl)-α,α-dimethylglycine cyclohexylamide (1c)



1c

4-methoxybenzylamine (1.25 mL, 9.6 mmol), acetone (20 mL, excess), Na_2SO_4 (1.0 g, excess), Cbz-Gly-OH (2.0 g, 9.6 mmol) and cyclohexyl isocyanide (1.2 mL, 9.6 mmol). The reaction was left to react 15 days and then the product was isolated by recrystallization with ethyl acetate to yield **1c** as a white solid (3.3 g, 69 %). **RP-HPLC**: [linear gradient H_2O/MeCN (70:30) to (0:100) over 8 min] t_R = 6.07 min; **MS-ESI**: *m/z* calculated for $C_{28}H_{37}N_3O_5$ = 495.62, found: 496.71 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.39 – 7.28 (m, 7H), 6.91 (d, *J* = 8.5 Hz, 2H), 5.71 – 5.66 (m, 1H), 5.51 (d, *J* = 8.2 Hz, 1H), 5.08 (s, 2H), 4.54 (s, 2H), 4.01 (d, *J* = 4.5 Hz, 2H), 3.80 (s, 3H), 3.78 – 3.74 (m, 1H), 1.97 – 1.89 (m, 2H), 1.74 – 1.66 (m, 2H), 1.58 (s, 3H), 1.43 (m, 4H), 1.39 – 1.30 (m, 2H), 1.20 – 1.08 (m, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 173.5, 169.1, 159.0, 156.2, 136.4, 129.3, 128.4, 128.0, 127.9, 127.1, 114.4, 66.8, 62.9, 55.3, 48.4, 46.2, 43.6, 33.0, 25.6, 24.9, 24.2.

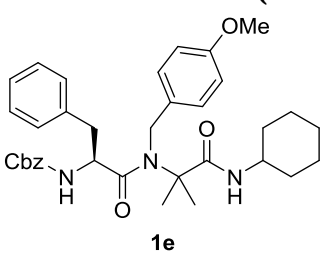
3. Cbz-Gly-*N*-(2,4,6-trimethoxybenzyl)-α,α-dimethylglycine cyclohexylamide (1d)



1d

2,4,6-trimethoxybenzylamine (1.89 mg, 9.6 mmol), acetone (20 mL, excess), Na_2SO_4 (1.0 g, excess), Cbz-Gly-OH (2.0 g, 9.6 mmol) and cyclohexyl isocyanide (1.2 mL, 9.6 mmol). The reaction was left to react 15 days and then the product was isolated was isolated by recrystallization with ethyl acetate to yield **1d** as a white solid (4.1 g, 77 %). **RP-HPLC**: [linear gradient H_2O/MeCN (70:30) to (0:100) over 8 min] t_R = 6.44 min; **MS-ESI**: *m/z* calculated for $C_{30}H_{41}N_3O_7$ = 555.67, found: 556.72 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.38 – 7.28 (m, 5H), 6.14 (s, 2H), 5.84 (s, 1H), 5.69 (d, *J* = 8.3 Hz, 1H), 5.12 (s, 2H), 4.52 (s, 2H), 4.26 (d, *J* = 4.3 Hz, 2H), 3.82 (s, 3H), 3.80 (s, 6H), 3.74 – 3.59 (m, 1H), 1.87 – 1.81 (m, 2H), 1.70 – 1.55 (m, 4H), 1.30 (s, 7H), 1.18 – 1.02 (m, 1H), 0.98 – 0.85 (m, 2H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 174.4, 169.4, 161.1, 159.0, 156.3, 136.6, 128.4, 128.0, 127.9, 106.0, 91.0, 66.6, 63.5, 55.7, 55.3, 47.9, 44.4, 37.4, 33.2, 25.7, 25.0, 23.2.

4. Cbz-Phe-N-(4-methoxybenzyl)-α,α-dimethylglycine cyclohexylamide (1e)

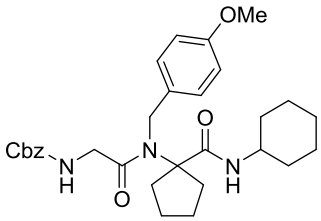


1e

4-methoxybenzylamine (1.25 mL, 9.6 mmol), acetone (20 mL, excess), Na_2SO_4 (1.0 g, excess), Cbz-L-Phe-OH (2.9 g, 9.6 mmol) and cyclohexyl isocyanide (1.2 mL, 9.6 mmol). The reaction was left to react 15 days and then the product was isolated recrystallization with ethyl acetate to yield **1e** as a white solid (4.2 g, 74 %). **RP-HPLC**: [linear gradient H_2O/MeCN (70:30) to (0:100) over 8 min] t_R = 7.40 min; **MS-ESI**: *m/z* calculated for

$C_{35}H_{43}N_3O_5$ = 585.75, found: 586.8 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.39 – 7.24 (m, 7H), 7.23 – 7.19 (m, 3H), 7.00 – 6.93 (m, 2H), 6.87 (d, *J* = 8.4 Hz, 2H), 5.57 (d, *J* = 8.1 Hz, 1H), 5.33 (d, *J* = 7.9 Hz, 1H), 5.12 – 4.96 (m, 2H), 4.69 – 4.53 (m, 2H), 4.38 (d, *J* = 18.1 Hz, 1H), 3.79 (s, 3H), 3.77 – 3.60 (m, 0H), 2.96 (dd, *J* = 13.6, 6.5 Hz, 1H), 2.82 (dd, *J* = 13.6, 7.8 Hz, 1H), 1.93 (dd, *J* = 33.4, 12.6 Hz, 3H), 1.68 (d, *J* = 13.3 Hz, 2H), 1.64 – 1.53 (m, 3H), 1.47 (s, 3H), 1.42 – 1.29 (m, 4H), 1.19 – 1.03 (m, 3H).

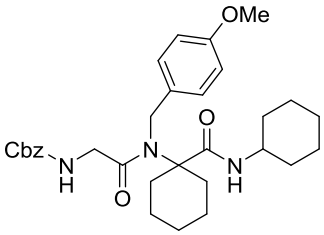
5. Cbz-Gly-N-(4-methoxybenzyl)-cyclopentylglycine cyclohexylamide (1f)



1f

4-methoxybenzylamine (1.25 mL, 9.6 mmol), cyclopentanone (20 mL, excess), Na_2SO_4 (1.0 g, excess), Cbz-Gly-OH (2.0 g, 9.6 mmol) and cyclohexyl isocyanide (1.2 mL, 9.6 mmol). The reaction was left to react 21 days and then the product was isolated by automatic purification on a pre-packed Redisep Rf Gold C18 43 g column by using H_2O/MeCN from 90:10 to 0:100 over 40 min. The collected fractions were lyophilized to yield **1f** as pale yellow solid (3.2 g, 65 %). **RP-HPLC**: [linear gradient H_2O/MeCN (70:30) to (0:100) over 8 min] t_R = 6.84 min; **MS-ESI**: *m/z* calculated for $C_{30}H_{39}N_3O_5$ = 521.3, found: 522.2 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.37 – 7.27 (m, 5H), 7.20 (d, *J* = 8.3 Hz, 2H), 6.90 (d, *J* = 8.5 Hz, 2H), 6.22 (s, 1H), 5.69 – 5.62 (m, 1H), 5.08 (s, 2H), 4.59 (s, 2H), 3.95 (d, *J* = 4.7 Hz, 2H), 3.80 (s, 3H), 3.76 – 3.66 (m, 1H), 2.58 – 2.50 (m, 2H), 1.92 – 1.80 (m, 4H), 1.70 – 1.54 (m, 7H), 1.41 – 1.29 (m, 2H), 1.23 – 1.06 (m, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 172.5, 170.7, 159.0, 156.2, 136.4, 129.5, 128.5, 128.1, 127.9, 126.8, 114.5, 73.9, 66.8, 55.3, 49.0, 48.3, 43.9, 36.0, 32.8, 25.6, 24.7, 23.4.

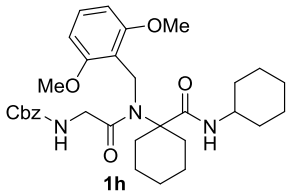
6. Cbz-Gly-N-(4-methoxybenzyl)-cyclohomoleucine cyclohexylamide (1g)



1g

4-methoxybenzylamine (1.25 mL, 9.6 mmol), cyclohexanone (20 mL, excess), Na_2SO_4 (1.0 g, excess), Cbz-Gly-OH (2.0 g, 9.6 mmol) and cyclohexyl isocyanide (1.2 mL, 9.6 mmol). The reaction was left to react 5 days and then the product was isolated by automatic purification on a pre-packed Redisep Rf Gold C18 43 g column by using H_2O/MeCN from 90:10 to 0:100 over 40 min. The collected fractions were lyophilized to yield **1g** as white solid (3.2 g, 61 %). **RP-HPLC**: [linear gradient H_2O/MeCN (70:30) to (0:100) over 8 min] t_R = 7.28 min; **MS-ESI**: *m/z* calculated for $C_{31}H_{41}N_3O_5$ = 535.3, found: 536.2 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.36 – 7.28 (m, 5H), 7.25 (d, *J* = 8.7 Hz, 2H), 6.90 (d, *J* = 8.5 Hz, 2H), 6.04 (d, *J* = 8.0 Hz, 1H), 5.72 – 5.68 (m, 1H), 5.09 (s, 2H), 4.55 (s, 2H), 4.01 (d, *J* = 4.5 Hz, 2H), 3.80 (s, 3H), 3.81 – 3.69 (m, 1H), 2.43 – 2.36 (m, 2H), 1.93 – 1.85 (m, 2H), 1.72 – 1.53 (m, 10H), 1.43 – 1.30 (m, 2H), 1.24 – 1.10 (m, 4H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 172.0, 170.1, 159.1, 156.3, 136.5, 129.6, 128.6, 128.2, 128.1, 127.2, 114.6, 67.0, 66.8, 55.5, 48.5, 46.6, 44.3, 33.0, 32.9, 25.8, 25.5, 24.9, 23.0.

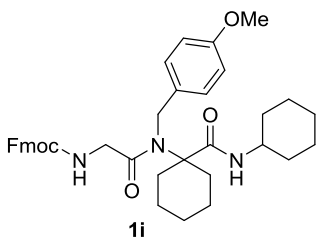
7. Cbz-Gly-N-(2,6-dimethoxybenzyl)-cyclohomoleucine cyclohexylamide (1h)



1h

2,6-dimethoxybenzylamine (802.6 mg, 4.80 mmol), cyclohexanone (1 mL, 9.6 mmol), Na_2SO_4 (1.0 g, excess), Cbz-Gly-OH (1.0 g, 4.80 mmol) and cyclohexyl isocyanide (596.8 µL, 4.80 mmol) in dry MeOH (5 mL). The reaction was left to react 3 days and then the product was isolated by automatic purification on a pre-packed Redisep Rf Gold C18 43 g column by using H_2O/MeCN from 90:10 to 0:100 over 40 min. The collected fractions were lyophilized to yield **1h** (0.70 g, 26 %) as white solid. **RP-HPLC**: [linear gradient H_2O/MeCN (70:30) to (0:100) over 8 min] t_R = 6.85 min; **MS-ESI**: *m/z* calculated for $C_{32}H_{43}N_3O_6$ = 565.3, found: 566.2 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.33 – 7.10 (m, 6H), 6.51 (d, *J* = 8.4 Hz, 2H), 5.89 (d, *J* = 8.2 Hz, 1H), 5.78 (t, *J* = 4.2 Hz, 1H), 5.05 (s, 2H), 4.49 (s, 2H), 4.21 (d, *J* = 4.3 Hz, 2H), 3.76 (s, 6H), 3.60 – 3.42 (m, 1H), 1.99 – 1.90 (m, 2H), 1.74 – 1.45 (m, 10H), 1.41 – 1.31 (m, 2H), 1.29 – 1.16 (m, 2H), 1.15 – 0.94 (m, 2H), 0.87 – 0.75 (m, 2H). **^{13}C NMR** (100 MHz, CDCl3) δ 173.45, 169.99, 158.29, 156.26, 136.61, 129.44, 128.44, 127.97, 127.86, 113.92, 104.46, 66.62, 66.46, 55.88, 47.65, 44.78, 37.51, 33.21, 31.86, 25.76, 25.59, 24.99, 22.63.

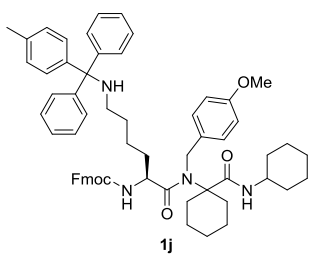
8. Fmoc-Gly-N-(4-methoxybenzyl)-cyclohomoleucyne cyclohexylamide (1i)



1i

4-methoxybenzylamine (1.25 mL, 9.6 mmol), cyclohexanone (20 mL, excess), Na_2SO_4 (1.0 g, excess), Fmoc-Gly-OH (2.85 g, 9.6 mmol) and cyclohexyl isocyanide (1.2 mL, 9.6 mmol). The reaction was left to react 6 days and then the product was isolated by automatic purification on a pre-packed Redisep Rf Gold C18 43 g column by using H_2O/MeCN from 90:10 to 0:100 over 40 min. The collected fractions were lyophilized to yield **1i** as white solid (2.9 g, 49 %). **RP-HPLC**: [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 7.02 min; **MS-ESI**: *m/z* calculated for $C_{38}H_{45}N_3O_5$ = 623.3, found: 624.3 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, CDCl3): δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.4 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.34 – 7.28 (m, 2H), 7.29 – 7.22 (m, 2H), 6.98 – 6.81 (m, 2H), 6.04 (d, *J* = 7.9 Hz, 1H), 5.75 (d, *J* = 4.4 Hz, 1H), 4.57 (s, 2H), 4.34 (d, *J* = 7.3 Hz, 2H), 4.21 (t, *J* = 7.0 Hz, 1H), 4.04 (d, *J* = 4.5 Hz, 2H), 3.86 – 3.72 (m, 3H), 2.44 – 2.36 (m, 2H), 2.02 – 1.87 (m, 4H), 1.74 – 1.51 (m, 6H), 1.46 – 1.30 (m, 4H), 1.27 – 1.10 (m, 4H). **^{13}C NMR** (100 MHz, CDCl3): δ 172.1, 170.1, 159.1, 156.4, 144.0, 141.4, 129.5, 127.8, 127.3, 127.2, 125.3, 120.1, 114.6, 67.3, 66.9, 55.5, 48.5, 47.3, 46.6, 44.3, 33.1, 32.9, 25.8, 25.5, 24.9, 23.1.

9. Fmoc-Lys(Mtt)-N-(4-methoxybenzyl)-cyclohomoleucine cyclohexylamide (1j)



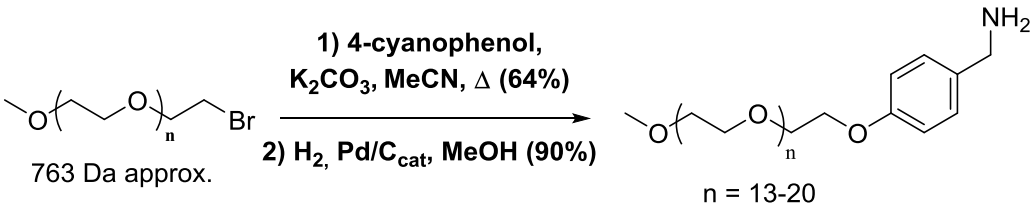
1j

4-methoxybenzylamine (209 µL, 1.6 mmol), cyclohexanone (166 µL, 1.6 mmol), Na_2SO_4 (0.25 g, excess), Fmoc-Lys(Mtt)-OH (1.0 g, 1.6 mmol) and cyclohexyl isocyanide (199 µL, 1.6 mmol) in dry MeOH (5 mL). The reaction was left to react 2 days and then the product was isolated by automatic purification on a pre-packed Redisep Rf Gold C18 43 g column by using H_2O/MeCN from 90:10 to 0:100 over 40 min.

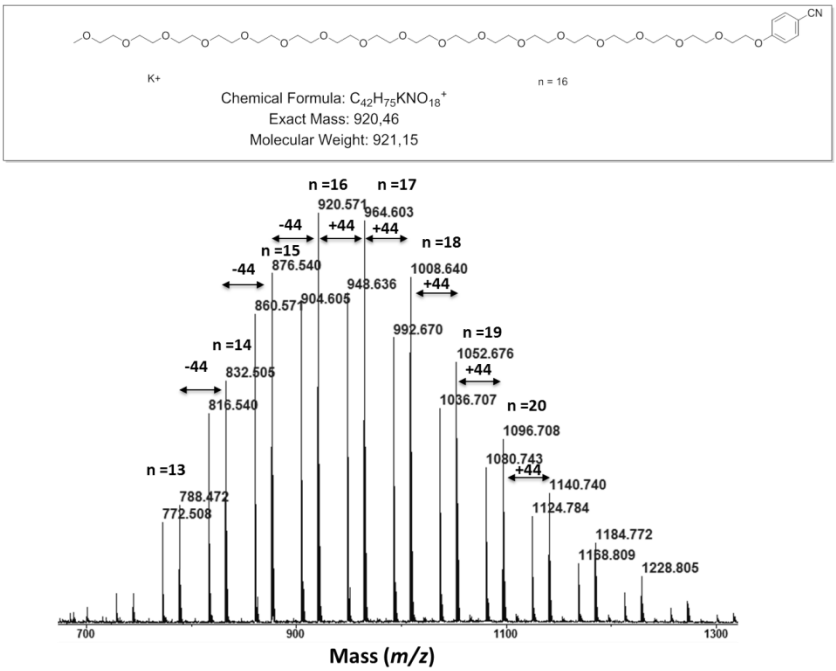
The collected fractions were lyophilized to yield **1j** as white solid (294 mg, 20 %). **RP-HPLC**: [linear gradient H_2O/MeCN (60:40) to (0:100) over 8 min] t_R = 7.89 min; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.75 (dd, *J* = 7.2, 2.8 Hz, 2H), 7.56 (t, *J* = 6.7 Hz, 2H), 7.45 – 7.35 (m, 6H), 7.33 – 7.27 (m, 10H), 7.20 (d, *J* = 8.3 Hz, 2H), 7.11 (d, *J* = 8.2 Hz, 2H), 6.87 (d, *J* = 8.2 Hz, 2H), 5.88 (d, *J* = 7.8 Hz, 1H), 5.82 – 5.75 (m, 1H), 4.67 – 4.48 (m, 3H), 4.34 – 4.25 (m, 1H), 4.21 – 4.12 (m, 2H), 3.76 (s, 4H), 3.65 – 3.54 (m, 1H), 3.05 – 2.92 (m, 1H), 2.87 – 2.76 (m, 1H), 2.42 – 2.22 (m, 5H), 1.92 – 1.80 (m, 1H), 1.75 – 1.38 (m, 15H), 1.38 – 0.96 (m, 8H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 172.38, 172.36, 159.2, 147.1, 144.1, 143.9, 141.4, 139.3, 138.9, 136.0, 129.6, 128.9, 128.80, 128.77, 128.75, 128.03, 127.99, 127.86, 127.82, 127.31, 127.21, 127.16, 125.3, 120.1, 114.6, 76.5, 67.4, 66.8, 55.4, 52.0, 48.4, 47.16, 47.13, 46.1, 32.9, 25.7, 25.5, 24.9, 24.8, 23.2, 22.8, 21.1.

10. Fmoc-Gly-N-(4-methoxyPEG9-benzyl)-cyclohomoleucine cyclohexylamide (1k).

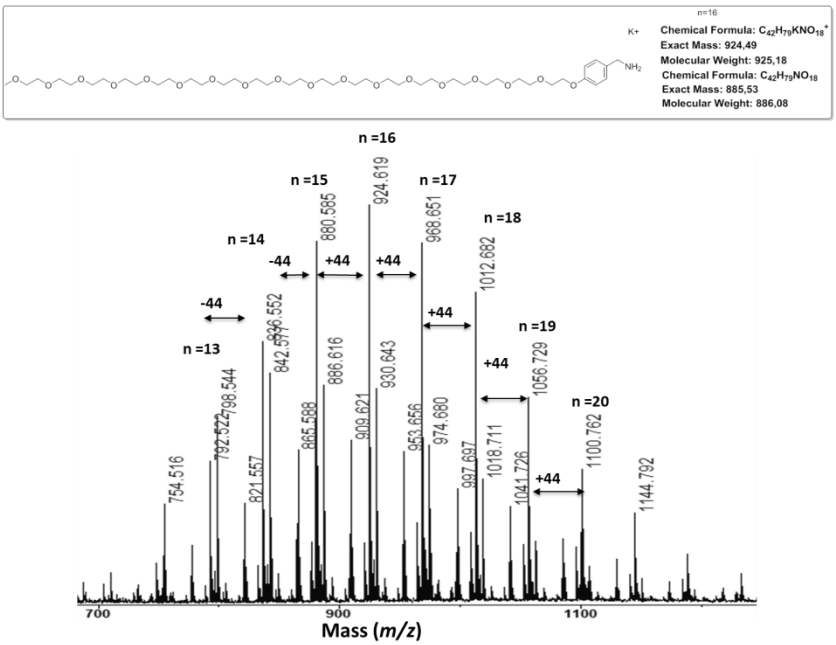
a. 4-Methoxy-PEG-benzylamine preparation:



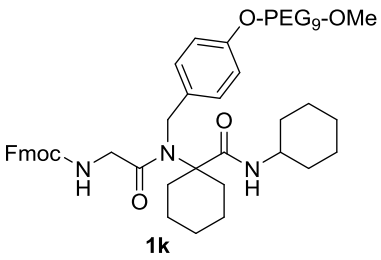
4-methoxyPEG$_{7\text{-}17}$ benzylamine was previously prepared in two steps. Firstly, a mixture of MeO-PEG-Br (1.0 g, 1.31 mmol), 4-cyanophenol (187.3 mg, 1.57 mmol) and K_2CO_3 (363.0 mg, 2.6 mmol) in MeCN (30 mL) was refluxed (110 ºC) under N_2 during 17 h. After that time, the mixtures was cooled to room temperature, and filtered. Then the solvent was removed under reduced pressure and the solid was dissolved in Et_2O, filtered, and then the solvent was removed first under reduced pressure and then it was lyophilized, obtaining the pegylated cyanophenol as a white solid (681.0 mg, 64 %). **RP-HPLC**: [linear gradient H_2O/MeCN (95:5) to (0:100) over 8 min] t_R = 5.26 min; **MALDI***: m/z* calculated for $C_{42}H_{75}NO_{18}$ = 882.1, found: from 788.5 (n = 13) to 1184.8 (n = 21) with average of 920.6 (n = 16) corresponding to $[M_{n=16}+Na]^+$.



Secondly, the pegylated cyanophenol (677.0 mg, 0.77 mmol) in MeOH (25 mL) was treated with 10% Pd/C (77.0 mg). The reaction mixture was treated successively with hydrogen and vacuum and finally stirred under hydrogen atmosphere for 24 h when the HPLC analysis revealed that cyanide had been consumed. Then the catalyst was removed by filtration over Celite and the resulting solution was evaporated under reduced pressure to furnish the PEGylated amine (615 mg, 90 %), which was identified by ESI-MS and used in the Ugi-4CR conjugation procedure without further purification. **RP-HPLC**: [linear gradient H_2O/MeCN (95:5) to (0:100) over 8 min] t_R = 3.92 min; **MALDI***: m/z* calculated for $C_{42}H_{79}NO_{18}$ = 886.08, found: from 754.5 (n = 12) to 1144.8 (n = 21) with average of 924.6 (n = 16) corresponding to $[M_{n=16}+Na]^+$.

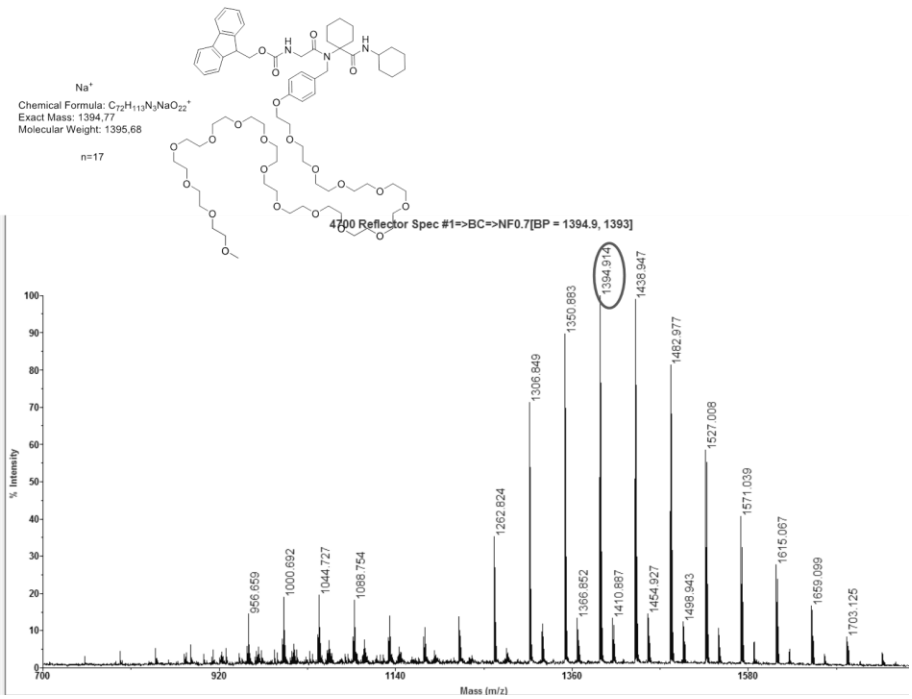


b. Fmoc-Gly-N-(4-methoxyPEG9-benzyl)-cyclohomoleucine cyclohexylamide (1k).

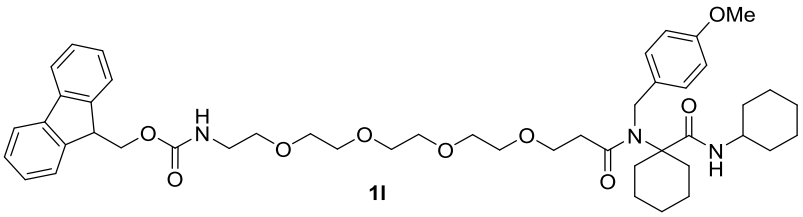


4-methoxyPEG$_9$ benzylamine (614.7 mg, 0.69 mmol), cyclohexanone (15 mL, excess), Na_2SO_4 (0.4 g, excess), Fmoc-Gly-OH (206 mg, 0.69 mmol) and cyclohexyl isocyanide (86.3 µL, 0.69 mmol). The reaction was left to react 3 days and then the product was isolated by automatic purification on a pre-packed Redisep Rf Gold C18 26 g column by using H_2O/MeCN from 90:10 to 0:100 over 30 min. The collected fractions were lyophilized to yield **1k** as pale green oily solid (515.4 mg, 54 %). **RP-HPLC**: [linear gradient H_2O/MeCN (50:50) to (20:80) over 8 min] t_R = 4.81 min; **MALDI***: m/z* calculated for $C_{72}H_{113}N_3O_{22}$ = 1371.8, found: from 1262.8 (n = 14) to 1659.1 (n = 23) with average of 1394.9 (n = 17) corresponding to $[M+Na]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.7 Hz, 2H), 7.59 (d, *J* = 7.6 Hz, 2H), 7.38 (t, *J* = 7.5 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 2H), 7.23 (d, *J* = 8.3 Hz, 2H), 6.95 – 6.84 (m, 2H), 6.07 (d, *J* = 8.0 Hz, 1H), 5.79 (t, *J* = 4.5 Hz, 1H), 4.56 (s, 2H), 4.33 (d, *J* = 7.3 Hz, 2H), 4.20 (t, *J* = 7.3 Hz, 1H), 4.10 (t, *J* = 4.9 Hz, 2H), 4.04 (d, *J* = 4.4 Hz, 2H), 3.84 (t, *J* = 5.7, 4.0 Hz, 2H), 3.73 – 3.69 (m, 2H), 3.67 – 3.59 (m, 62H), 3.56 – 3.52 (m, 2H), 3.37 (s, 3H), 2.42 – 2.35 (m, 2H), 1.94 – 1.86 (m, 2H), 1.72 – 1.52 (m, 9H), 1.40 – 1.09 (m, 6H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 172.3, 170.3, 158.3, 156.4, 144.0, 141.4, 129.6, 127.8, 127.2, 127.2, 125.3, 120.1, 115.3, 72.0, 70.9, 70.67, 70.65, 70.61, 70.59, 70.57, 70.52, 69.8, 67.6, 67.3, 66.8, 59.1, 48.6, 47.2, 46.6, 44.3, 33.0, 32.8, 31.1, 25.7, 25.4, 24.9, 24.6, 23.0.

MALDI



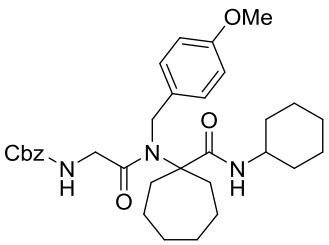
11. Fmoc-d4PEG-N-(4-methoxybenzyl)-cyclohomoleucine cyclohexylamide (1l)



1l

4-methoxybenzylamine (128 µL, 0.97 mmol), cyclohexanone (101 µL, 0.97 mmol), Na_2SO_4 (0.25 g, excess), Fmoc-NH-dPEG(4)-COOH (500 mg, 1.02 mmol) and cyclohexyl isocyanide (121 µL, 0.97 mmol) in dry MeOH (5 mL). The reaction was left to react 18 h and then the product was isolated by automatic purification on a pre-packed Redisep Rf Gold C18 43 g column by using H_2O/MeCN from 90:10 to 0:100 over 40 min. The collected fractions were lyophilized to yield **1l** as a pale yellow oily solid (501.0 mg, 64 %). **RP-HPLC**: [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 6.56 min; **MS-ESI**: *m/z* calculated for $C_{47}H_{63}N_3O_9$ = 813.5, found: 836.4 corresponding to $[M+Na]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.75 (d, J = 7.6 Hz, 2H), 7.60 (d, J = 7.4 Hz, 2H), 7.43 – 7.34 (m, 2H), 7.30 (td, J = 7.5, 1.2 Hz, 2H), 7.20 (d, J = 8.6 Hz, 2H), 6.90 – 6.83 (m, 2H), 6.27 – 6.20 (m, 1H), 5.56 – 5.44 (m, 1H), 4.60 (s, 2H), 4.38 (d, J = 7.0 Hz, 2H), 4.21 (t, J = 7.0 Hz, 1H), 3.79 (s, 3H), 3.73 (t, J = 6.5 Hz, 3H), 3.62 (s, 11H), 3.58 – 3.54 (m, 2H), 3.37 (q, J = 5.1 Hz, 2H), 2.60 (t, J = 6.6 Hz, 2H), 2.45 – 2.33 (m, 4H), 1.92 – 1.83 (m, 2H), 1.70 – 1.62 (m, 2H), 1.61 – 1.51 (m, 7H), 1.42 – 1.28 (m, 2H), 1.24 – 1.07 (m, 4H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 173.4, 172.4, 158.7, 144.0, 141.3, 130.6, 130.5, 127.6, 127.2, 127.0, 125.1, 119.9, 114.2, 70.5, 70.4, 70.4, 70.3, 70.1, 67.8, 66.6, 66.2, 55.3, 48.1, 47.3, 47.2, 40.9, 35.9, 33.1, 32.9, 25.7, 25.4, 24.8, 22.9.

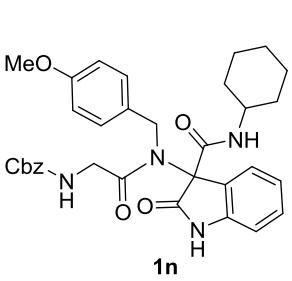
12. Cbz-Gly-N-(4-methoxybenzyl)-cyclopentylglycine cyclohexylamide (1m)

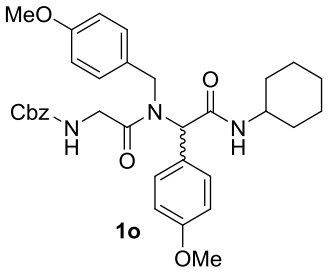


1m

4-methoxybenzylamine (625 µL, 4.78 mmol), cycloheptanone (563.8 µL, 4.78 mmol), Na_2SO_4 (0.6 g, excess), Cbz-Gly-OH (1.0 g, 4.78 mmol) and cyclohexyl isocyanide (594.3 µL, 4.78 mmol) in dry MeOH (5 mL). The reaction was left to react 21 days and then the product was isolated by automatic purification on a pre-packed Redisep Rf Gold C18 43 g column by using H_2O/MeCN from 90:10 to 0:100 over 40 min. The collected fractions were lyophilized to yield **1m** as white solid (0.8 g, 30 %). **RP-HPLC**: [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 5.78 min; **MS-ESI**: *m/z* calculated for $C_{32}H_{43}N_3O_5$ = 549.3, found: 550.3 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.37 – 7.28 (m, 7H), 6.91 (d, *J* = 8.6 Hz, 2H), 5.71 – 5.64 (m, 1H), 5.57 (d, *J* = 8.1 Hz, 1H), 5.07 (s, 2H), 4.58 (s, 2H), 3.96 (d, *J* = 4.6 Hz, 2H), 3.80 (s, 3H), 3.78 – 3.70 (m, 1H), 2.45 – 2.17 (m, 2H), 1.95 – 1.80 (m, 4H), 1.74 – 1.01 (m, 16H). **^{13}C NMR** (100 MHz, $CDCl_3$): δ 173.6, 169.7, 159.1, 156.4, 136.6, 129.8, 128.6, 128.2, 128.1, 127.3, 114.7, 70.2, 66.9, 55.5, 48.5, 46.7, 44.1, 35.2, 33.1, 30.2, 25.8, 25.0, 23.8.

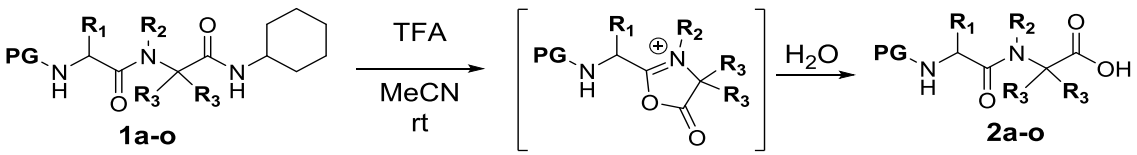
13. Cbz-Gly-N-(4-methoxybenzyl)-isatineglycine cyclohexylamide (1n and 1o)





4-methoxy-benzylamine (625 µL, 4.78 mmol), isatine (703.3 mg, 4.78 mmol), Na_2SO_4 (0.5 g, excess), Cbz-Gly-OH (1.0 g, 4.78 mmol) and cyclohexyl isocyanide (594 µL, 4.78 mmol) in dry MeOH (5 mL). The reaction was left to react 16 days and then the product was isolated by automatic purification on a pre-packed Redisep Rf Gold C18 43 g column by using H_2O/MeCN from 90:10 to 0:100 over 40 min. The collected fractions were lyophilized to yield **1n** as white solid (0.36 g, 13 %) and **1o** as pale yellow solid (0.38 g, 14 %), respectively. **Compound 1n) RP-HPLC**: [linear gradient H_2O/MeCN (95:5) to (0:100) over 8 min] t_R = 7.35 min; **MS-ESI**: *m/z* calculated for $C_{33}H_{36}N_4O_6$ = 584.2, found: 585.2 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.26 (s, 1H), 7.40 (d, *J* = 7.5 Hz, 1H), 7.33 – 7.14 (m, 7H), 7.21 (td, *J* = 7.7, 1.2 Hz, 1H), 7.05 (t, *J* = 7.6 Hz, 1H), 6.86 (d, *J* = 8.3 Hz, 2H), 6.83 – 6.70 (m, 2H), 5.59 (t, *J* = 4.5 Hz, 1H), 5.01 (s, 2H), 4.97 (d, *J* = 18.2 Hz, 1H), 4.73 (d, *J* = 18.0 Hz, 1H), 4.13 (dd, *J* = 17.5, 4.9 Hz, 1H), 3.97 (dd, *J* = 17.3, 4.5 Hz, 1H), 3.77 (s, 3H), 3.49 – 3.37 (m, 1H), 1.70 – 1.62 (m, 1H), 1.56 – 1.39 (m, 3H), 1.27 – 0.90 (m, 5H), 0.69 – 0.54 (m, 1H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 175.0, 170.0, 161.6, 159.2, 156.1, 140.8, 136.3, 129.6, 128.4, 128.0, 128.0, 127.8, 127.3, 126.5, 126.2, 123.2, 114.3, 110.1, 77.3, 77.0, 76.7, 71.4, 66.8, 55.4, 49.2, 48.5, 43.6, 32.1, 31.2, 25.3, 24.4. **Compound 1o) RP-HPLC**: [linear gradient H_2O/MeCN (95:5) to (0:100) over 8 min] t_R = 5.75 min; **MS-ESI**: *m/z* calculated for $C_{33}H_{39}N_3O_6$ = 573.6, found: 574.3 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.36 – 7.28 (m, 5H), 7.28 – 7.21 (m, 2H), 6.94 (d, *J* = 8.2 Hz, 2H), 6.80 (d, *J* = 8.7 Hz, 2H), 6.73 (d, *J* = 8.1 Hz, 2H), 5.73 – 5.66 (m, 2H), 5.54 (d, *J* = 8.8 Hz, 1H), 5.08 (s, 2H), 4.56 (d, *J* = 17.5 Hz, 1H), 4.41 (d, *J* = 17.4 Hz, 1H), 4.08 (dd, *J* = 17.3, 4.5 Hz, 1H), 3.86 (dd, *J* = 17.2, 4.6 Hz, 1H), 3.77 (s, 3H), 3.74 (s, 3H), 1.93 – 1.80 (m, 2H), 1.72 – 1.52 (m, 3H), 1.40 – 1.21 (m, 3H), 1.15 – 0.97 (m, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 170.1, 168.4, 159.9, 158.8, 156.2, 136.4, 131.1, 128.45, 128.3, 128.0, 127.9, 127.5, 126.4, 114.3, 114.0, 77.3, 77.0, 76.7, 66.8, 63.4, 55.3, 55.2, 49.0, 48.7, 43.5, 32.8, 25.4, 24.8, 24.7.

Acidolysis study



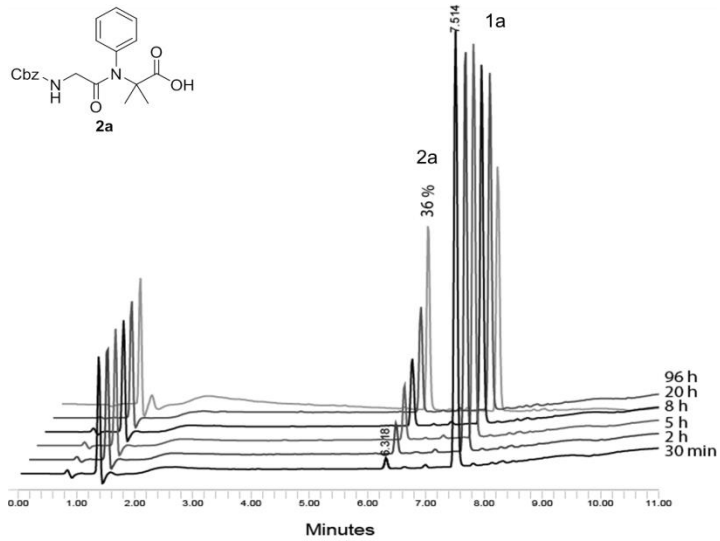
General procedure

The acidolysis study was carried out treating the protected amides **1a-o** at 1 mg/mL in MeCN with 1% of trifluoroacetic acid (TFA). When is indicated, the acidolysis was carried out in H_2O/MeCN (1:1). The reaction was performed in sealed HPLC-glass vials of 2 mL, analyzing the mixture from 2 min to 24 h and the kinetic Overlay HPLC chromatograms are

showed below. Only compounds **2c, 2e, 2g, 2i-l** were isolated by the indicated technique for further studies.

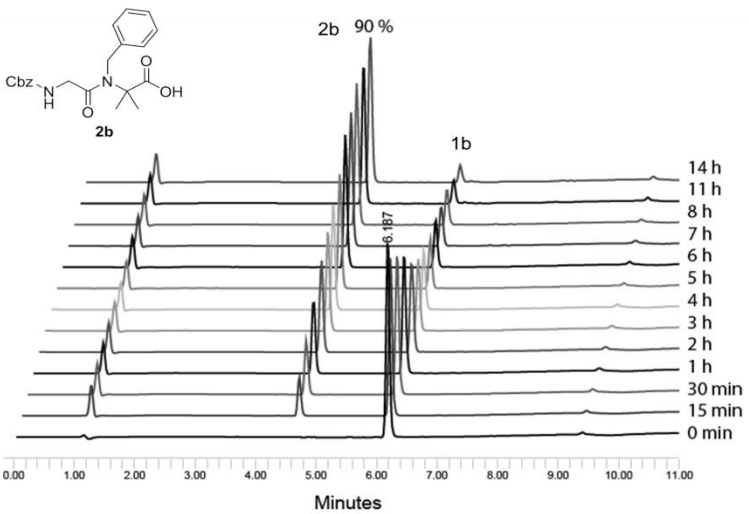
1. **Acidolysis of compound 1a.**

RP-HPLC linear gradients of H_2O/MeCN (95:5) to (0:100) over 8 min.



2. **Acidolysis of compound 1b.**

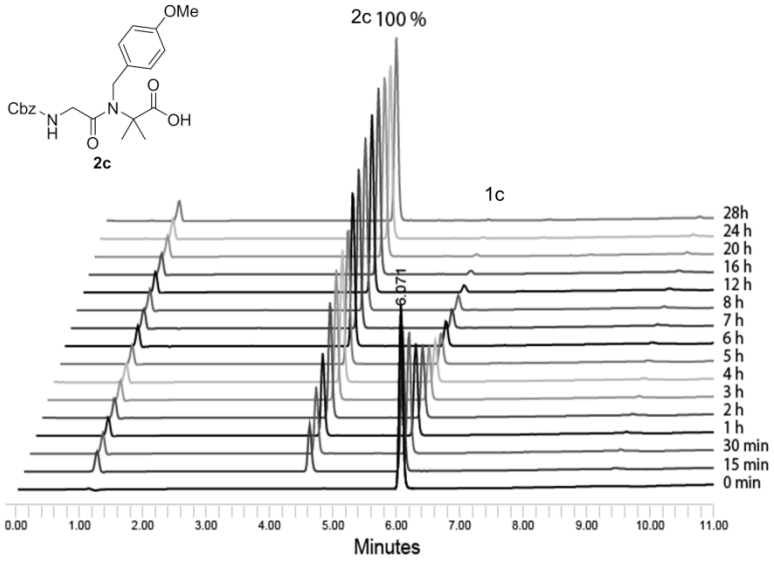
RP-HPLC linear gradients of H_2O/MeCN (70:30) to (0:100) over 8 min.



3. **Acidolysis of compound 1c.**

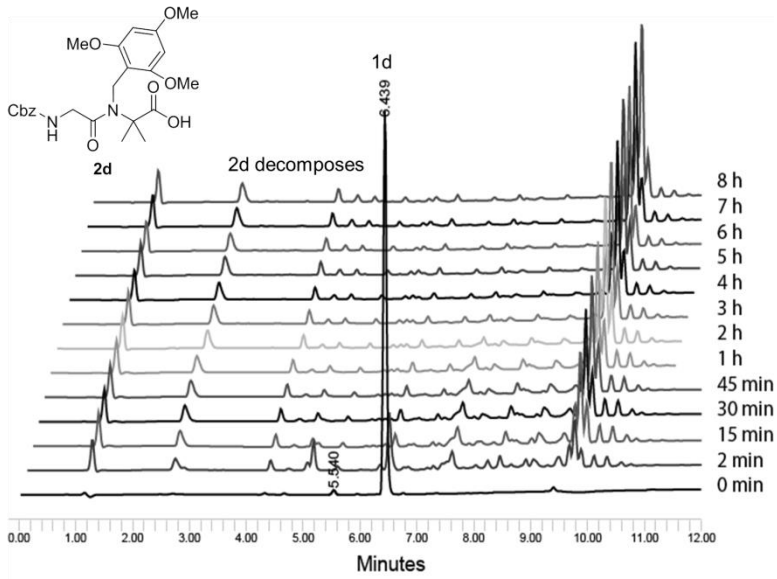
Compound **1c** (297.8 mg, 0.6 mmol) was treated with a solution of 1% TFA in MeCN (40 mL) during 28 h. The solvent was removed and the solid was solved in DCM (40 mL) and washed with HCl 0.1 N (3 × 30 mL) and water (3 × 30 mL). Then the organic layer was dried over $MgSO_4$, filtered and eliminated under reduced pressure to yield compound 2e as a white solid (223.8 mg, 90% yield). **RP-HPLC** linear gradients of H_2O/MeCN (70:30) to (0:100)

over 8 min. **MS-ESI**: *m/z* calculated for $C_{22}H_{26}N_2O_6$ = 414.5, found: 415.4 corresponding to $[M+H]^+$.



4. Acidolysis of compound 1d.

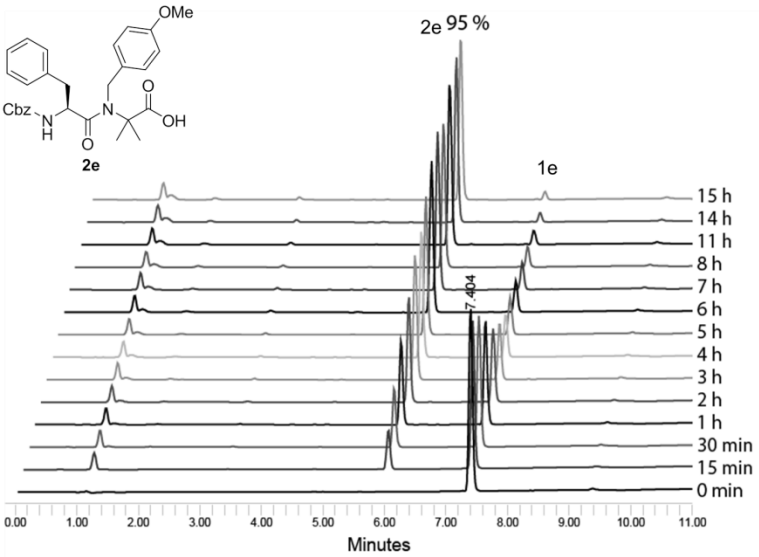
RP-HPLC linear gradients of H_2O/MeCN (70:30) to (0:100) over 8 min. Complete decomposition of **1d** was observed, compound **2d** was not detected.



5. Acidolysis of compound 1e.

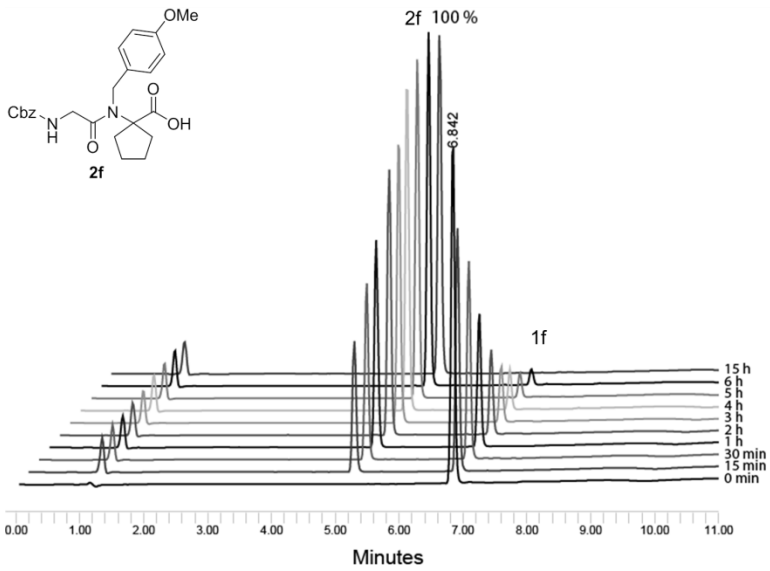
Compound **1e** (101.9 mg, 0.17 mmol) was treated with a solution of 1% TFA in MeCN (40 mL) during 15 h. The solvent was removed and the solid was solved in DCM (40 mL) and washed with HCl 0.1 N (3 × 30 mL) and water (3 × 30 mL). Then the organic layer was dried over $MgSO_4$, filtered and eliminated under reduced pressure to yield compound 2e as a white solid (31.8 mg, 36% yield). **RP-HPLC** linear gradients of H_2O/MeCN (70:30) to (0:100) over

8 min. **MS-ESI**: *m/z* calculated for $C_{29}H_{32}N_2O_6$ = 504.6, found: 505.2 corresponding to $[M+H]^+$.



6. Acidolysis of compound 1f.

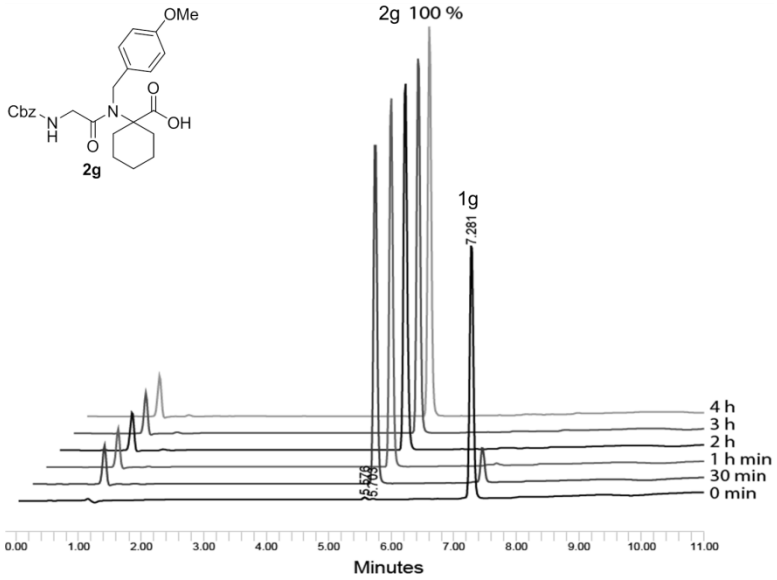
RP-HPLC linear gradients of H_2O/MeCN (70:30) to (0:100) over 8 min. **MS-ESI**: *m/z* calculated for $C_{24}H_{28}N_2O_6$ = 440.2, found: 441.1 corresponding to $[M+H]^+$.



7. Acidolysis of compound 1g.

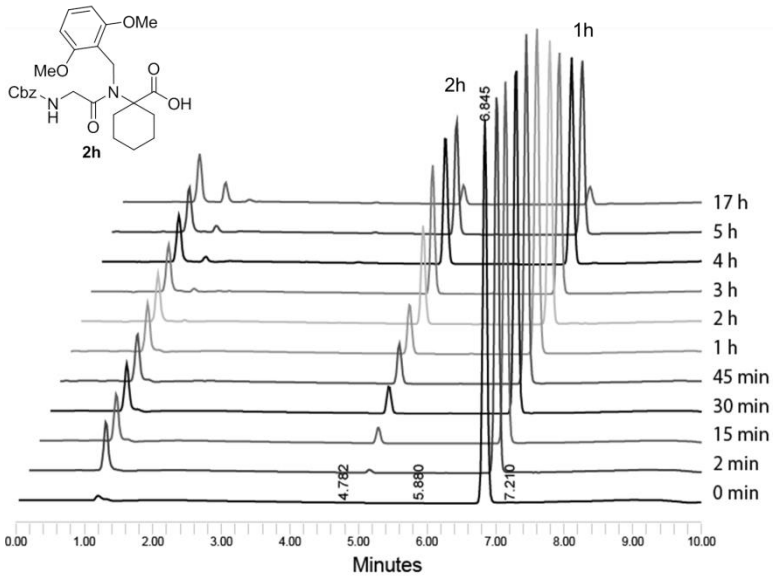
Compound **1g** (400.7 mg, 0.75 mmol) was treated with a solution of 1% TFA in MeCN (40 mL) during 17 h. The solvent was removed and the solid was solved in DCM (40 mL) and washed with HCl 0.1 N (3 × 30 mL) and water (3 × 30 mL). Then the organic layer was dried over $MgSO_4$, filtered and eliminated under reduced pressure to yield compound 2g as a white solid (287.7 mg, 85% yield). **RP-HPLC** linear gradients of H_2O/MeCN (70:30) to (0:100)

over 8 min. **MS-ESI**: *m/z* calculated for $C_{25}H_{30}N_2O_6$ = 454.2, found: 455.1 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.33 (d, *J* = 8.6 Hz, 2H), 7.28 – 7.21 (m, 5H), 6.95 (d, *J* = 8.7 Hz, 2H), 6.65 (t, *J* = 5.5 Hz, 1H), 5.06 (s, 2H), 4.55 (s, 2H), 4.11 (d, *J* = 5.5 Hz, 2H), 3.82 (s, 3H), 2.26 – 2.19 (m, 2H), 1.74 – 1.32 (m, 7H), 1.08 – 0.94 (m, 1H). **^{13}C NMR** (100 MHz, $CDCL_3$) δ 175.8, 171.8, 159.1, 157.2, 136.7, 128.7, 128.5, 128.0, 127.9, 127.2, 114.6, 77.5, 77.2, 76.8, 66.9, 65.8, 55.5, 46.2, 43.7, 31.9, 25.3, 22.4.



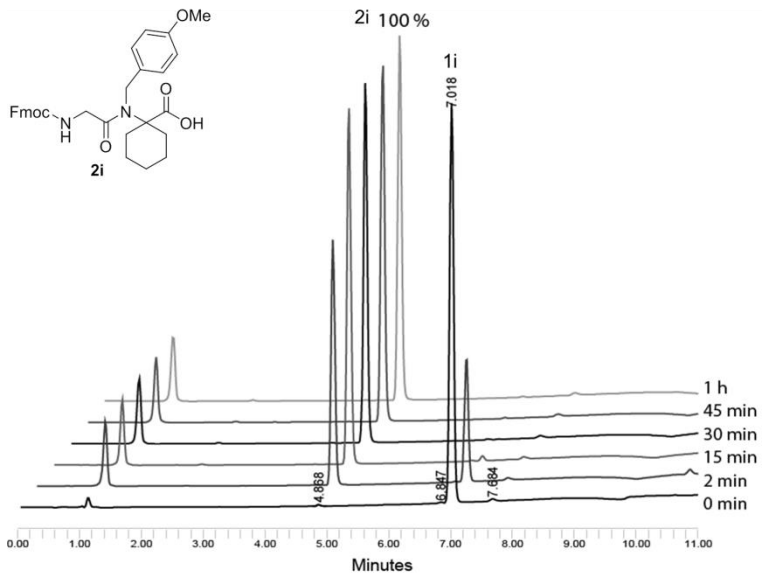
8. Acidolysis of compound 1h.

RP-HPLC linear gradients of H_2O/MeCN (60:40) to (0:100) over 8 min. Complete decomposition of **1h** was observed (TFA 1% in MeCN), compound **2h** was detected by acidolysis treating 1h with 1% TFA in MeCN/H_2O (1:1), but suffer decomposition after 17 h of treatment. **MS-ESI**: *m/z* calculated for $C_{26}H_{32}N_2O_7$ = 484.5, found: 485.2 corresponding to $[M+H]^+$.



9. Acidolysis of compound 1i.

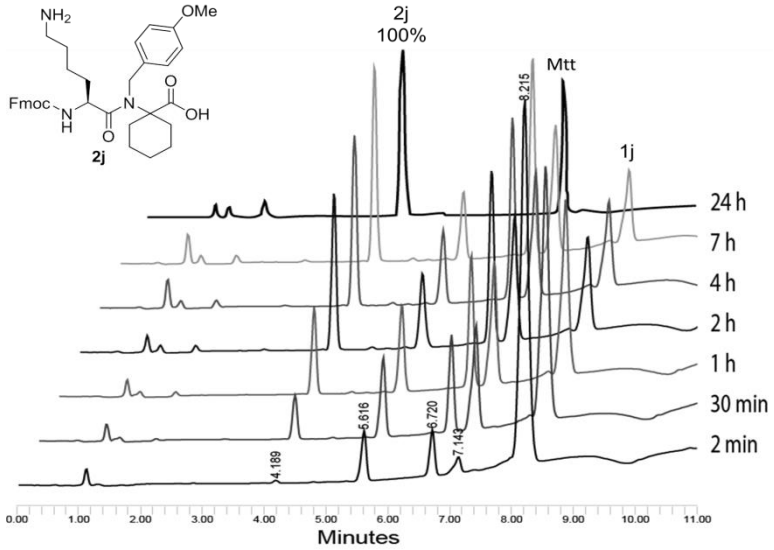
Compound **1i** (476.6 mg, 0.76 mmol) was treated with a solution of 1% TFA in MeCN (25 mL) during 48 h. The solvent was removed and the solid was solved in DCM (40 mL) and washed with HCl 0.1 N (3 × 30 mL) and water (3 × 30 mL). Then the organic layer was dried over $MgSO_4$, filtered and eliminated under reduced pressure to yield compound 2e as a white solid (348.0 mg, 84% yield). **RP-HPLC** linear gradients of H_2O/MeCN (50:50) to (0:100) over 8 min. **Melting point**: 225-226 ºC. **MS-ESI**: *m/z* calculated for $C_{32}H_{34}N_2O_6$ = 542.1, found: 543.2 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, DMSO-d_6) δ 12.01 (s, 1H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.72 (d, *J* = 7.4 Hz, 2H), 7.44 – 7.30 (m, 7H), 6.92 (d, *J* = 8.3 Hz, 2H), 4.60 (s, 2H), 4.26 – 4.18 (m, 3H), 3.80 (d, *J* = 6.0 Hz, 2H), 3.74 (s, 3H), 2.10 – 2.04 (m, 2H), 1.61 – 1.39 (m, 7H), 1.04 (d, *J* = 12.6 Hz, 1H). **^{13}C NMR** (100 MHz, DMSO-d_6) δ 173.7, 169.3, 158.1, 156.4, 143.8, 140.7, 130.6, 127.6, 127.1, 127.1, 125.3, 120.1, 113.8, 65.7, 64.1, 55.0, 46.6, 44.9, 42.9, 31.3, 24.7, 22.0.



10. Acidolysis of compound 1j.

Compound **1j** (100.0 mg, 0.11 mmol) was treated with a solution of 1% TFA in MeCN (25 mL) during 24 h. The reaction was quenched with Na_2CO_3 to pH = 7 and then the solvent was removed under reduced pressure. The obtained solid was automatically purified on a pre-packed Redisep Rf Gold C18 43 g column by using H_2O/MeCN from 90:10 to 0:100 over 40 min. The fractions were collected and lyophilized obtaining compound 2j as a white powder (44.9 mg, 70%). **RP-HPLC** linear gradients of H_2O/MeCN (60:40) to (0:100) over 8 min. **MS-ESI**: *m/z* calculated for $C_{36}H_{43}N_3O_6$ = 613.3, found: 614.2 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.81 – 7.69 (m, 4H), 7.63 – 7.55 (m, 2H), 7.34 (q, *J* = 7.6 Hz, 4H), 7.30 – 7.22 (m, 2H), 6.86 (d, *J* = 8.2 Hz, 2H), 6.52 (d, *J* = 8.6 Hz, 1H), 4.83 (d, *J* = 18.2 Hz, 1H), 4.59 (d, *J* = 18.2 Hz, 1H), 4.54 – 4.47 (m, 1H), 4.37 – 4.22 (m, 2H), 4.16 (t, *J* = 7.6 Hz, 1H), 3.75 (s, 3H), 3.08 (s, 2H), 2.52 – 2.43 (m, 1H), 2.17 – 1.92 (m, 2H), 1.84 – 1.16 (m, 13H), 1.09 – 0.93 (m, 1H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 176.3, 167.1, 158.9, 156.1, 143.9,

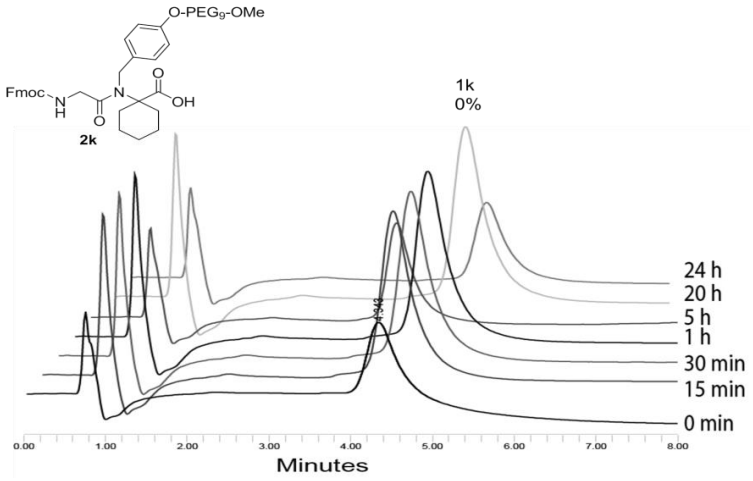
143.8, 141.2, 141.2, 129.3, 127.7, 127.4, 127.0, 125.2, 119.9, 114.2, 67.2, 65.8, 55.2, 51.9, 47.0, 46.5, 39.3, 32.6, 31.1, 31.0, 26.3, 25.2, 22.5, 22.4, 22.2.



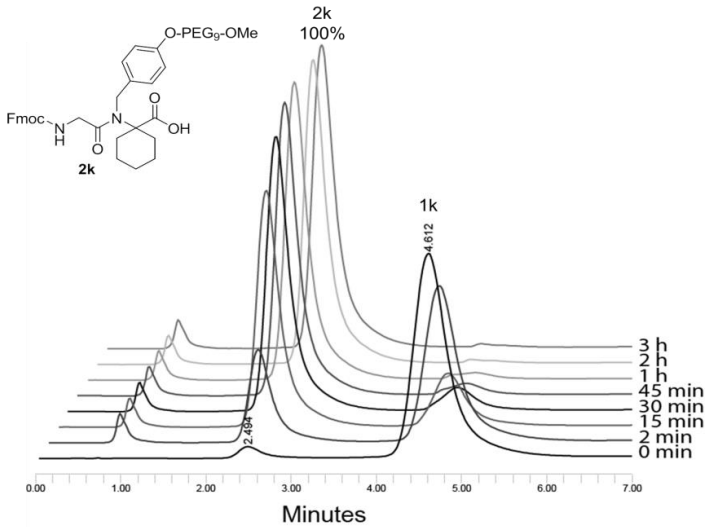
11. Acidolysis of compound 1k.

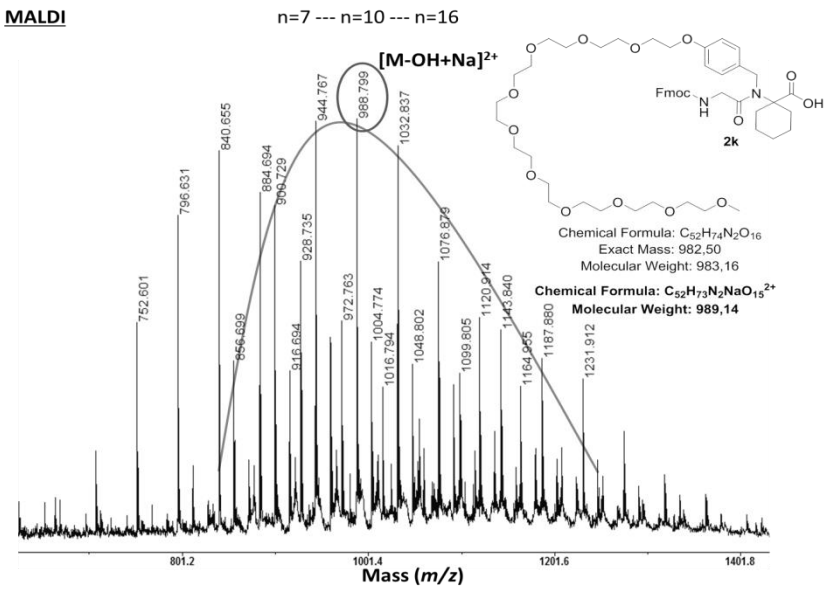
Compound **1k** (96.2 mg, 0.07 mmol) was treated with a solution of 1% TFA in MeCN (25 mL) during 24 h. The reaction was quenched with Na_2CO_3 to pH = 7 and then the solvent was removed under reduced pressure. The obtained solid was automatically purified on a pre-packed Redisep Rf Gold C18 43 g column by using H_2O/MeCN from 90:10 to 0:100 over 40 min. The fractions were collected and lyophilized obtaining compound **2k** as a white oily solid (74.5 mg, 86%). **RP-HPLC** linear gradients of H_2O/MeCN (80:20) to (0:100) over 8 min. **MALDI***: m/z* calculated for $C_{52}H_{74}N_2O_{16}$ = 983.2, found: from 856.7 (n = 6) to 1231.9 (n = 15) with average of 988.8 (n = 9) corresponding to $[M\text{-}OH+Na]^{2+}$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.69 (d, *J* = 7.5 Hz, 2H), 7.54 (d, *J* = 7.5 Hz, 2H), 7.31 (t, *J* = 8.0 Hz, 4H), 7.21 (t, *J* = 7.3 Hz, 2H), 6.90 (d, *J* = 8.3 Hz, 2H), 6.69 (t, *J* = 5.0 Hz, 1H), 4.55 (s, 2H), 4.25 (d, *J* = 7.5 Hz, 2H), 4.16 – 4.10 (m, 2H), 4.10 – 4.05 (m, 1H), 3.84 – 3.79 (m, 2H), 3.71 – 3.66 (m, 2H), 3.60 (s, 65H), 3.54 – 3.48 (m, 2H), 3.34 (s, 3H), 2.28 (d, *J* = 12.0 Hz, 2H), 1.81 – 1.68 (m, 2H), 1.54 – 1.34 (m, 3H), 1.06 – 0.93 (m, 1H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 175.6, 171.3, 158.2, 156.8, 143.9, 141.1, 128.8, 127.6, 127.1, 127.0, 125.2, 119.8, 115.1, 71.9, 70.78, 70.59, 70.56, 70.52, 70.46, 70.38, 69.7, 67.5, 67.3, 65.6, 59.0, 47.0, 46.1, 43.5, 31.8, 25.2, 22.4.

- **MES (0.1 M), NaCl (137 mM) and KCl (2.7 mM) at pH=4.8 and 37 ºC:**



- **1% TFA in MeCN:**

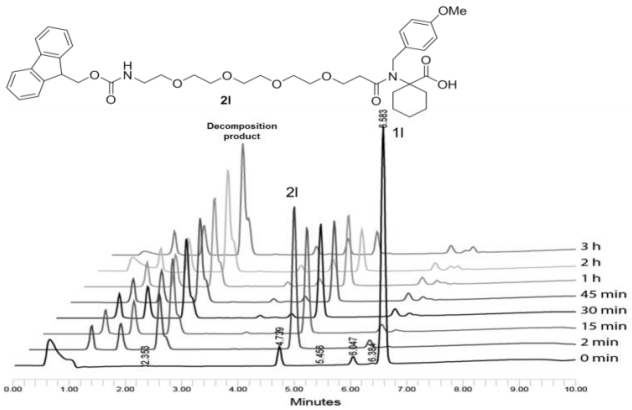




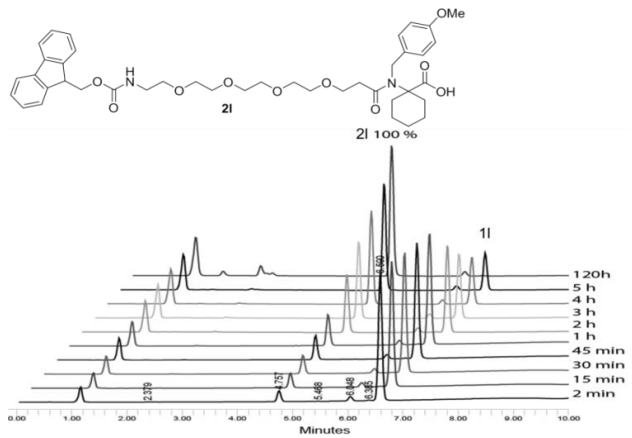
12. Acidolysis of compound 1l.

Compound **1l** (500.0 mg, 0.12 mmol) was treated with a solution of 1% TFA in H_2O/MeCN (1:1) (50 mL) during 24 h. The reaction was quenched with $NaHCO_3$ to pH = 7 and then the MeCN was removed under reduced pressure. Extra water was added (40 mL) to the aqueous phase and then it was extracted with DCM (5 × 50 mL) and the organic layer was dried over $MgSO_4$ filtered and evaporated under reduced pressure. The obtained solid was automatically purified on a pre-packed Redisep Rf Gold C18 43 g column by using H_2O/MeCN from 90:10 to 0:100 over 40 min. The fractions were collected and lyophilized obtaining compound **2l** as a white powder (316.3 mg, 70%). **RP-HPLC** linear gradients of H_2O/MeCN (50:50) to (0:100) over 8 min. **MS-ESI**: *m/z* calculated for $C_{41}H_{52}N_2O_{10}$ = 732.4, found: 733.3 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.64 – 7.56 (m, 2H), 7.39 (t, *J* = 7.5 Hz, 2H), 7.34 – 7.27 (m, 4H), 6.88 (d, *J* = 8.7 Hz, 2H), 6.55 (s, 2H), 4.60 (s, 2H), 4.39 (d, *J* = 7.0 Hz, 2H), 4.25 – 4.17 (m, 1H), 3.79 (s, 3H), 3.76 – 3.72 (m, 2H), 3.69 – 3.50 (m, 14H), 3.44 – 3.37 (m, 2H), 2.57 (t, *J* = 5.8 Hz, 2H), 2.28 (d, *J* = 12.8 Hz, 2H), 1.83 – 1.66 (m, 2H), 1.64 – 1.49 (m, 3H), 1.42 (td, *J* = 13.0, 4.2 Hz, 2H), 1.15 – 0.99 (m, 1H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 173.3, 158.9, 144.6, 144.1, 141.5, 141.4, 130.0, 127.8, 127.2, 127.2, 125.3, 120.1, 114.3, 70.8, 70.7, 70.5, 70.4, 67.2, 66.8, 64.9, 55.4, 47.4, 46.8, 41.0, 34.9, 32.2, 25.4, 22.6.

- **1% TFA in MeCN**

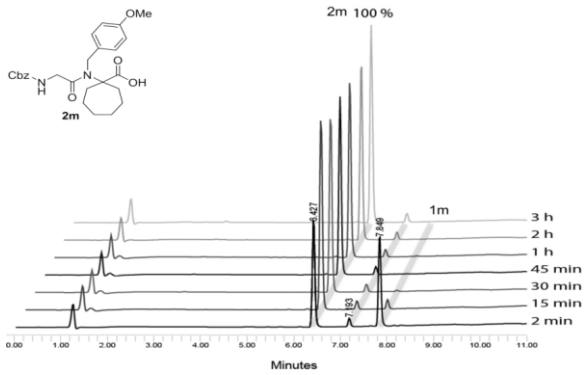


- **1% TFA in MeCN/H_2O (1:1)**



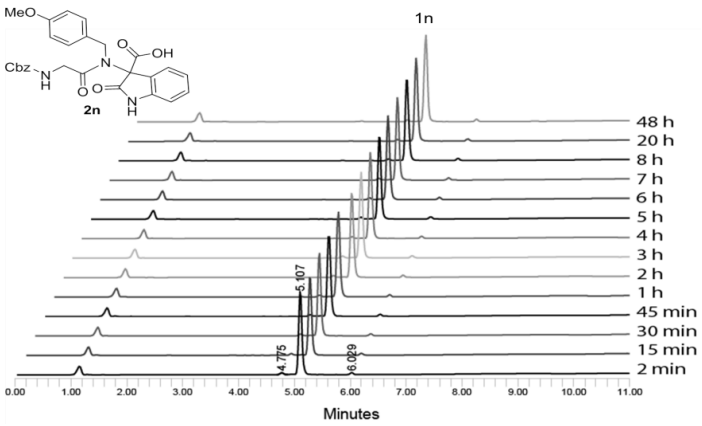
13. Acidolysis of compound 1m.

RP-HPLC linear gradients of H_2O/MeCN (80:10) to (0:100) over 8 min. **MS-ESI**: *m/z* calculated for $C_{26}H_{32}N_2O_6$ = 468.5, found: 469.2 corresponding to $[M+H]^+$.



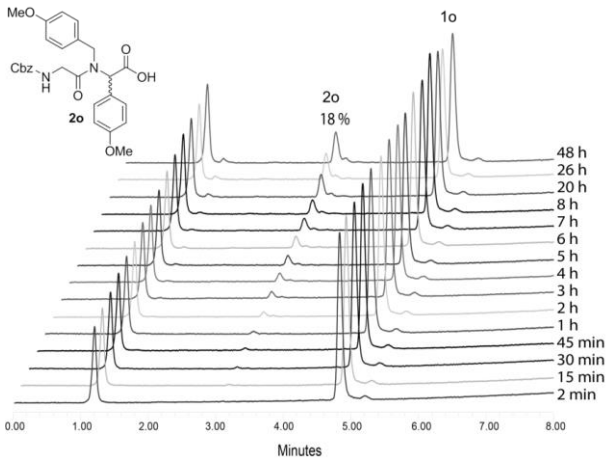
14. Acidolysis of compound 1n.

RP-HPLC linear gradients of H_2O/MeCN (60:40) to (0:100) over 8 min. Compound **1n** was stable into 1% TFA in MeCN.

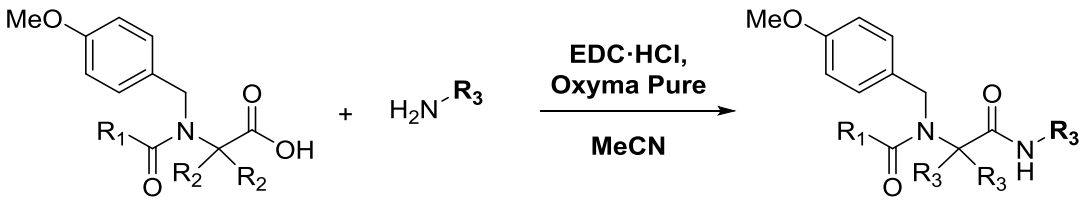


15. Acidolysis of compound 1o.

RP-HPLC linear gradients of H_2O/MeCN (50:50) to (0:100) over 8 min. **MS-ESI**: *m/z* calculated for $C_{27}H_{28}N_2O_7$ = 492.5, found: 493.2 corresponding to $[M+H]^+$.



***C*-terminal functionalization**



2c: R_1=Z-Gly-, R_2=Me
2g: R_1=Z-Gly-, R_2=*c*C_5H_{10}
2i: R_1=Fmoc-Gly-, R_2=*c*C_5H_{10}

3a-e

Entry	Carboxylic acid	H_2N-R_3	Product	Yield (%)
1	2c	H_2N-Ala-Val-NH_2	3a	37
2	2g	H_2N-Ala-Val-NH_2	3b	15
3	2g	H_2N-Phe	3c	22
4	2i	H_2N-Disperse Orange 3	3d	44
5	2i	H_2N-Fluorescein	3e	78

General procedure: The amide bond formation was carried out by treating the free *C*-terminal carboxylic acid dipeptide (1 eq.) with the corresponding amine (1 to 5 eq.) using EDC·HCl (1 eq to excess) as coupling agent and Oxyma Pure (1 to 9 eq.) as an additive in MeCN. Then the mixture was left to react until the starting carboxylic acid was consumed, the reaction was monitored by HPLC. When it was needed an extra amount of EDC·HCl was added to the reaction mixture. Once the starting materials was consumed, the solvent was removed under reduced pressure, and the solid was solved in AcOEt (25 mL), then the organic layer was washed with water (3×20 mL), Na_2CO_3 sat (3×20 mL), and brine (3×20 mL). The organic layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure, when is needed the final product was isolated by automatic purification on a pre-packed Redisep Rf Gold C18 13 g column by using H_2O/MeCN from 90:10 to 0:100 over 20 min. Then the collected fractions were lyophilized to yield 3a-d.

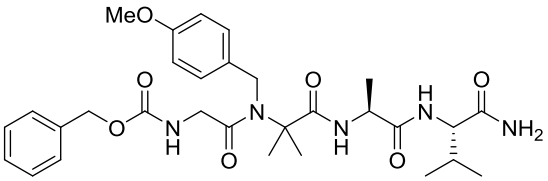
1. Ala-Val-NH_2 *C*-terminal functionalization of compound 2c.

a) Z-Ala-Val-NH_2 preparation

Z-Ala-OH (1.0 g, 4.48 mmol), H-Val-NH_2·HCl (686.9 mg, 4.49 mmol), EDC·HCl (864.4 mg, 4.50 mmol), Oxyma Pure (636.7 mg, 4.48 mmol) and DIEA (1.0 mL, 5.74 mmol) in MeCN (25 mL), 3h. The solvent was removed under reduced pressure and the solid was solved in DCM (50 mL). Then the organic layer was washed with water (3 × 50 mL), HCl 0.1N (3 × 50 mL), Na_2CO_3 sat. (3 × 50 mL) and brine (3 × 50 mL), then the organic layer was dried over $NaSO_4$ and filtered. Then the solvent was removed under reduced pressure and the obtained solid was precipitated with Et_2O. Then the solid was dried on vacuum to yield the protected dipeptide Z-Ala-Val-NH_2 as a white solid (1.25 g, 87% yield, 99% purity). **RP-HPLC** linear gradients of H_2O/MeCN (95:5) to (0:100) over 8 min, t_R = 4.98 min. **MS-ESI**: *m/z* calculated for $C_{16}H_{23}N_3O_4$ = 321.4, found: 322.0 corresponding to $[M+H]^+$.

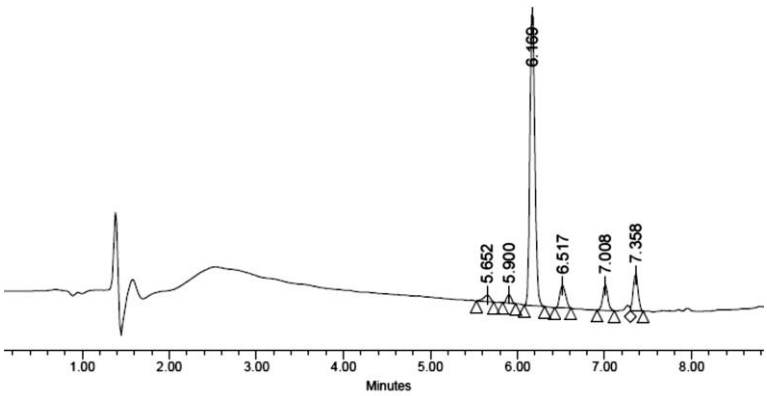
b) Z-Ala-Val-NH_2 hydrogenation

Z-Ala-Val-NH_2 (302.7 mg, 0.94 mmol), Pd/C 10% (30 mg) in MeOH (20 mL) under H_2. 17h. A white solid was obtained (175.0 mg, 99%). **RP-HPLC**: [linear gradient H_2O/MeCN (95:5) to (0:100) over 8 min] t_R = 1.38 min, purity 91%.

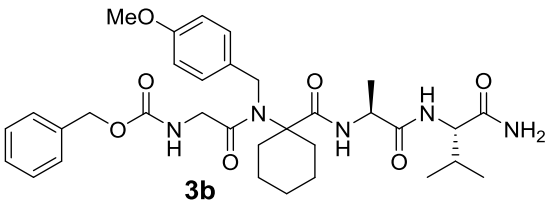


3a

Entry 1: Compound 2c (110.7 mg, 0.27 mmol). H-Ala-Val-NH_2 (50 mg, 0.27 mmol), EDC·HCl (51.2 mg, 0.27 mmol), Oxyma pure (37.9 mg, 0.27 mmol) was stirred in MeCN (25 mL) until no starting acid was detected by HPLC analysis. After purification, compound 3a was obtained as a pale yellow solid (57.2 mg, 37 %). **RP-HPLC** linear gradients of H_2O/MeCN (95:5) to (0:100) over 8 min, purity 74 %. **MS-ESI**: *m/z* calculated for $C_{30}H_{41}N_5O_7$ = 583.3, found: 584.2 corresponding to $[M+H]^+$.

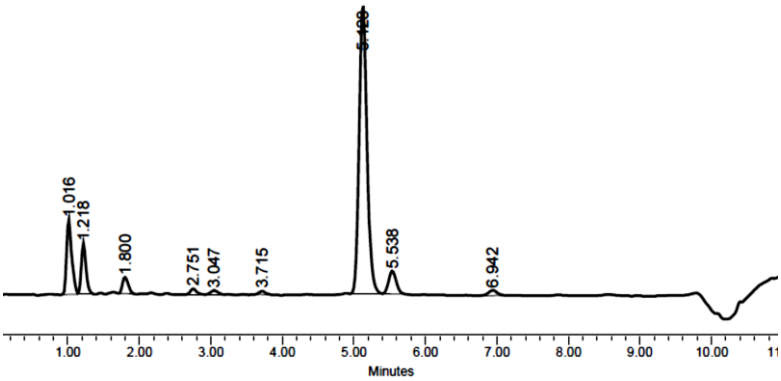


2. Ala-Val-NH2 C-terminal functionalization of compound 2g.



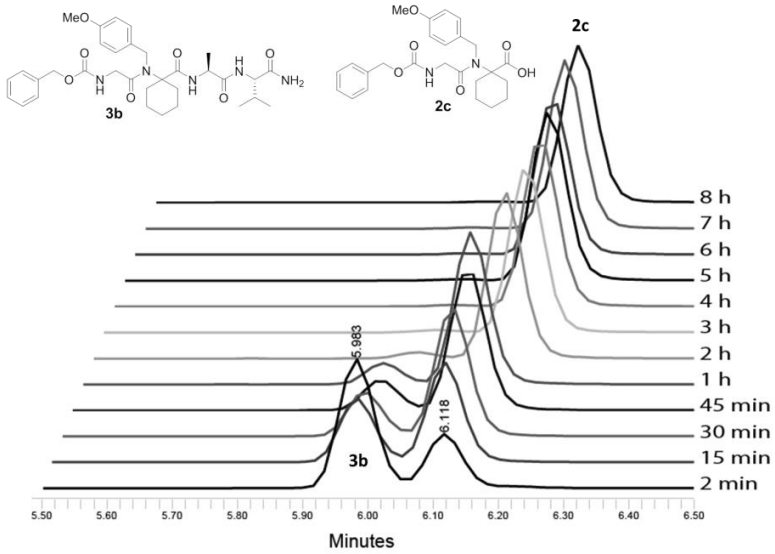
3b

Entry 2: Compound **2g** (100.0 mg, 0.22 mmol). H-Ala-Val-NH_2 (41.2 mg, 0.21 mmol), EDC·HCl (119.5 mg, 0.62 mmol), Oxyma pure (73.8 mg, 0.52 mmol) was stirred in MeCN (10 mL) until no starting acid was detected by HPLC analysis. After purification, compound 3b was obtained as a white (20.4 mg, 15 %). **RP-HPLC**: [linear gradient H_2O/MeCN (55:45) to (50:50) over 8 min] t_R = 5.13 min; purity 69%. **MS-ESI**: *m/z* calculated for $C_{33}H_{45}N_5O_7$ = 623.3, found: 624.3 corresponding to $[M+H]^+$.

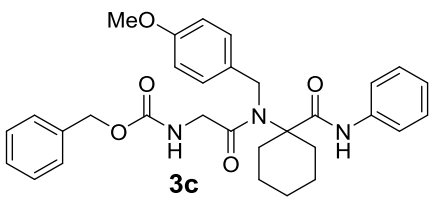


- Acidolysis 1 % TFA MeCN

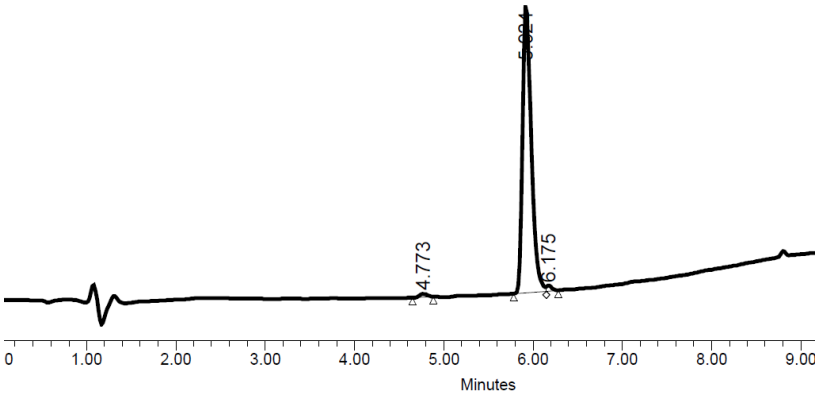
RP-HPLC: linear gradient H_2O/MeCN (80:20) to (0:100) over 8 min.



3. Aniline C-terminal functionalization of compound 2c.

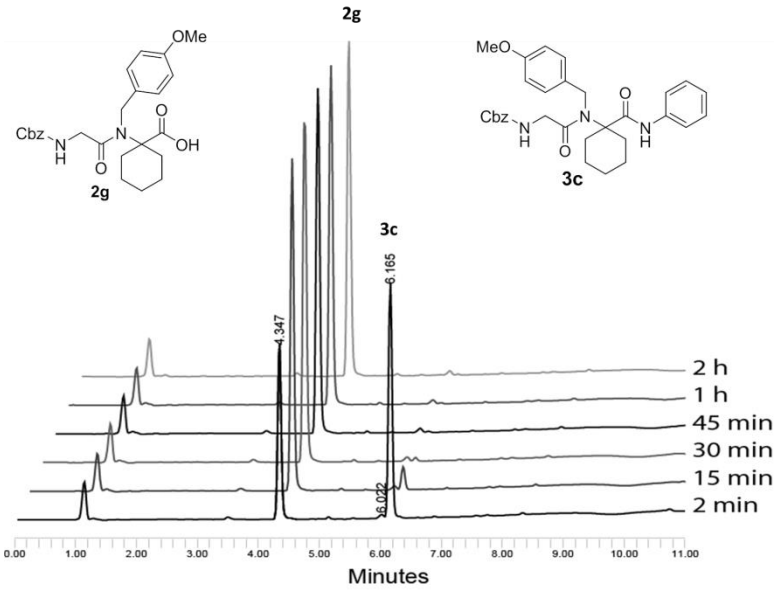


Entry 3: Compound 2g (121.6 mg, 0.27 mmol), aniline (43 µL, 0.47 mmol), EDC·HCl (90.0 mg, 0.47 mmol), Oxyma pure (67.2 mg, 0.47 mmol) and DIEA (100 µL, 0.57 mmol) were stirred in MeCN (15 mL) until no starting acid was detected by HPLC analysis. After purification, a pale yellow powder was obtained (121.6 mg, 86 %). **RP-HPLC**: [linear gradient H_2O/MeCN (60:40) to (0:100) over 8 min] t_R = 5.92 min, 98% purity; **MS-ESI**: *m/z* calculated for $C_{31}H_{35}N_3O_5$ = 529.3, found: 552.3 corresponding to $[M+Na]^+$. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.32 (s, 1H), 7.48 – 7.44 (m, 2H), 7.34 – 7.27 (m, 7H), 7.23 (d, *J* = 8.4 Hz, 2H), 7.11 – 7.06 (m, 1H), 6.88 (d, *J* = 8.5 Hz, 2H), 5.75 – 5.63 (m, 1H), 5.07 (s, 2H), 4.58 (s, 2H), 4.08 (d, *J* = 4.7 Hz, 2H), 3.79 (s, 3H), 2.48 (d, *J* = 13.4 Hz, 2H), 1.86 – 1.75 (m, 2H), 1.68 – 1.57 (m, 5H), 1.27 (d, *J* = 9.5 Hz, 1H).

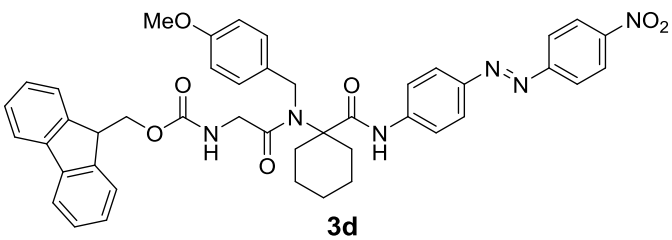


- Acidolysis 1 % TFA MeCN

RP-HPLC: linear gradient H_2O/MeCN (60:40) to (0:100) over 8 min.

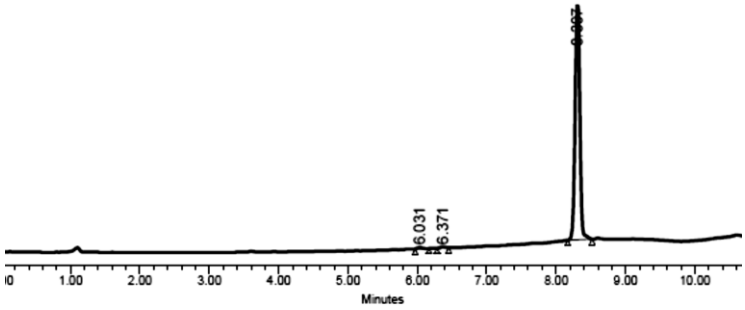


4. Disperse Orange 3 C-terminal functionalization of compound 2i.

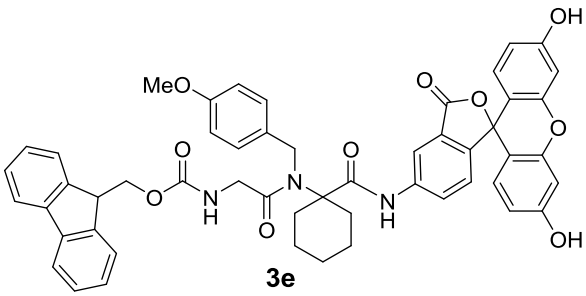


Entry 4: Compound 2i (100.6 mg, 0.19 mmol), disperse orange 3 (224.0 mg, 0.93 mmol), EDC·HCl (746.0 mg, 3.89 mmol) and Oxyma pure (263.0 mg, 1.85 mmol) were stirred in DMF (5 mL) until no starting acid was detected by HPLC analysis (7 h). After purification, a brown-orange powder was obtained (63.0 mg, 44 %). **RP-HPLC**: [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 8.31 min, purity 98.9%; **MS-ESI**: *m/z* calculated for $C_{44}H_{42}N_6O_7$ = 766.3, found: 767.4 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, DMSO-d_6) δ 9.69 (s, 1H), 8.41 (d, *J* = 8.6 Hz, 2H), 8.03 (d, *J* = 8.6 Hz, 2H), 7.93 (d, *J* = 8.8 Hz, 2H), 7.88 (d, *J* = 8.8 Hz, 2H), 7.84 (d, *J* = 7.5 Hz, 2H), 7.67 (d, *J* = 7.5 Hz, 2H), 7.58 (s, 1H), 7.40 – 7.33 (m, 4H), 7.26 (t, *J* = 7.4 Hz, 2H), 6.94 (d, *J* = 8.2 Hz, 2H), 4.73 (s, 2H), 4.20 (d, *J* = 7.1 Hz, 2H), 4.17 – 4.11 (m, 1H), 3.99 (d, *J* = 6.0 Hz, 2H),

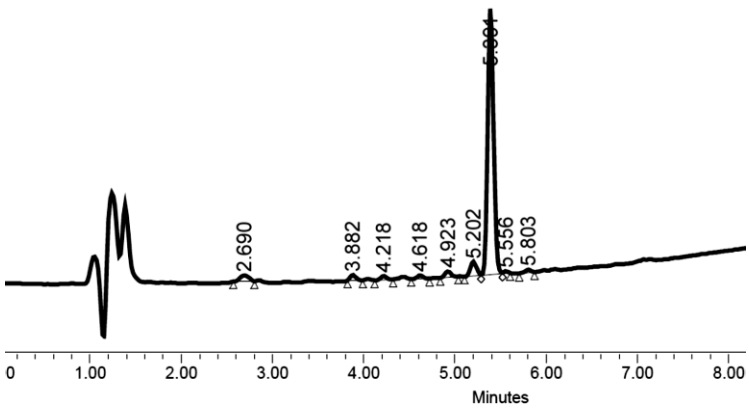
3.74 (s, 3H), 2.38 (d, *J* = 11.9 Hz, 2H), 1.70 – 1.55 (m, 2H), 1.52 – 1.31 (m, 5H), 0.87 – 0.73 (m, 1H).



5. Fluoresceinamine C-terminal functionalization of compound 2i.



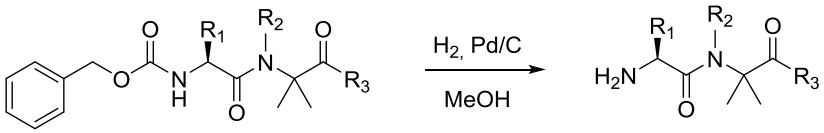
Entry 5: Compound 2i (100.3 mg, 0.19 mmol), fluoresceinamine (323.0 mg, 0.93 mmol), EDC·HCl (428.0 mg, 2.23 mmol) and Oxyma pure (263.0 mg, 1.85 mmol) were stirred in DMF (5 mL) until no starting acid was detected by HPLC analysis (4 h). After purification, an orange powder was obtained (125.4 mg, 78 %). **RP-HPLC**: [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 5.39 min, purity 85.4%; **MS-ESI**: *m/z* calculated for $C_{52}H_{45}N_3O_{10}$ = 871.3, found: 872.4 corresponding to $[M+H]^+$. **^{1}H NMR** (400 MHz, DMSO-d_6) δ 9.51 (s, 1H), 8.28 (s, 1H), 8.02 – 7.81 (m, 3H), 7.71 (d, *J* = 7.5 Hz, 2H), 7.56 (t, *J* = 6.0 Hz, 1H), 7.45 – 7.27 (m, 9H), 7.14 (d, *J* = 8.3 Hz, 2H), 6.96 (d, *J* = 8.4 Hz, 2H), 6.66 – 6.59 (m, 2H), 6.55 – 6.47 (m, 2H), 4.72 (s, 2H), 4.28 (d, *J* = 7.1 Hz, 2H), 4.24 – 4.16 (m, 1H), 4.02 – 3.97 (m, 2H), 3.76 (s, 3H), 2.39 (d, *J* = 11.6 Hz, 2H), 1.67 (d, *J* = 6.9 Hz, 2H), 1.54 – 1.36 (m, 5H), 0.89 – 0.76 (m, 1H).



N-terminal functionalization and activation

Cbz protecting group removal:

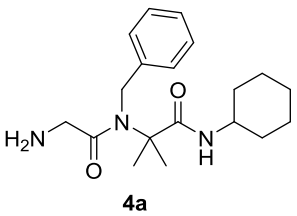
General procedure



1b: R_1 = H, R_2 = Bzl, R_3 = NH-Chx
1c: R_1 = H, R_2 = 4-MeO-Bzl, R_3 = NH-Chx
1d: R_1 = H, R_2 = 2,4,6-triMeO-Bzl, R_3 = NH-Chx
1e: R_1 = Bzl, R_2 = 4-MeO-Bzl, R_3 = NH-Chx
2e: R_1 = Bzl, R_2 = 4-MeO-Bzl, R_3 = OH

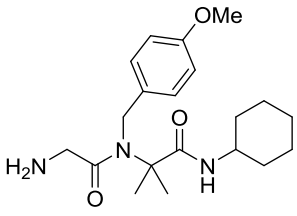
4a: R_1 = H, R_2 = Bzl, R_3 = NH-Chx
4b: R_1 = H, R_2 = 4-MeO-Bzl, R_3 = NH-Chx
4c: R_1 = H, R_2 = 2,4,6-triMeO-Bzl, R_3 = NH-Chx
4d: R_1 = Bzl, R_2 = 4-MeO-Bzl, R_3 = OH
4e: R_1 = Bzl, R_2 = 4-MeO-Bzl, R_3 = cyclic DKP

The indicated quantity of the corresponding Cbz protected dipeptide was solved in the indicated quantity of MeOH. Then the indicated catalytic amount of Pd/C (10% weight) was added to the solution and the system was sealed with a septum, and the balloon was purged first with N_2 and then with H_2. Then the reaction was stirred under H_2 atmosphere at room temperature until the complete Cbz elimination. The reaction was monitored by HPLC. The solution was filtered over Celite® and the solvent was removed under reduced pressure to yield the free-amine dipeptide.



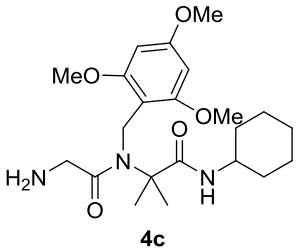
4a

1b (198.1 mg, 0.43 mmol), Pd/C 10% (36.1 mg) in MeOH (25 mL). 17h. **4a** was obtained as a white solid (137.0 mg, 97%). **RP-HPLC**: [linear gradient H_2O/MeCN (60:40) to (50:50) over 8 min] t_R = 1.65 min; **MS-ESI**: *m/z* calculated for $C_{20}H_{31}N_3O_3$ = 331.5, found: 332.1 corresponding to $[M+H]^+$.



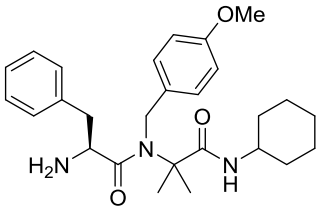
4b

1c (100 mg, 0.20 mmol), Pd/C 10% (10 mg) in MeOH (20 mL). 7h. **4b** was obtained as a white solid (72.9 mg, 72%). **RP-HPLC**: [linear gradient H_2O/MeCN (60:40) to (50:50) over 8 min] t_R = 1.65 min; **MS-ESI**: *m/z* calculated for $C_{20}H_{31}N_3O_3$ = 361.2, found: 362.2 corresponding to $[M+H]^+$.



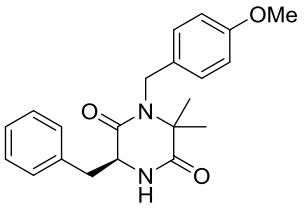
4c

1d (110.8 mg, 0.20 mmol), Pd/C 10% (10 mg) in MeOH (20 mL), 7h. **4c** was obtained as a white solid (81.6 mg, 97%). **RP-HPLC**: [linear gradient H_2O/MeCN (60:40) to (0:100) over 8 min] t_R = 1.90 min; **MS-ESI**: *m/z* calculated for $C_{22}H_{35}N_3O_5$ = 421.5, found: 422.2 corresponding to $[M+H]^+$.



4d

1e (100 mg, 0.17 mmol), Pd/C 5% (16 mg) in MeOH (15 mL). 9h. **4d** was obtained as a white solid (77.6 mg, 99 %). **RP-HPLC**: [linear gradient H_2O/MeCN (60:40) to (50:50) over 8 min] t_R = 3.75 min, purity 97%; **MS-ESI**: *m/z* calculated for $C_{27}H_{37}N_3O_3$ = 451.6, found: 452.7 corresponding to $[M+H]^+$.

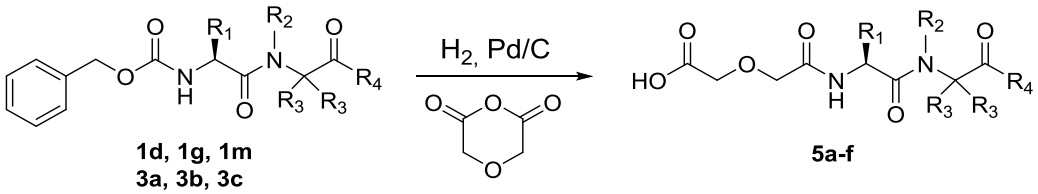


4e

2e (31.8 mg, 0.06 mmol), Pd/C 5% (10.6 mg) in MeOH (10 mL). 7h. A white solid was obtained (20.1 mg, 86%) which correspond to the DKP formation. **RP-HPLC**: [linear gradient H_2O/MeCN (60:40) to (50:50) over 8 min] t_R = 3.75 min, purity 97%; **MS-ESI**: *m/z* calculated for $C_{21}H_{27}N_2O_4$ = 370.5, found: 353.06 corresponding to DKP. **^{1}H NMR 4e** (400 MHz, $CDCl_3$) δ 7.35 – 7.22 (m, 6H), 7.13 (d, *J* = 8.7 Hz, 2H), 6.84 – 6.80 (m, 2H), 4.60 (q, *J* = 15.4 Hz, 2H), 4.39 – 4.33 (m, 1H), 3.79 (s, 3H), 3.40 (dd, *J* = 13.9, 3.7 Hz, 1H), 3.12 – 3.02 (m, 1H), 1.39 (s, 3H), 1.17 (s, 3H). **^{13}C NMR 4e** (100 MHz, $CDCl_3$) δ 166.3, 158.7, 135.6, 130.1, 129.8, 129.0, 128.7, 128.5, 127.9, 127.4, 113.9, 61.7, 55.9, 55.2, 45.3, 40.2, 26.2, 25.7, 24.2.

One pot Cbz protecting group removal and amine acylation:

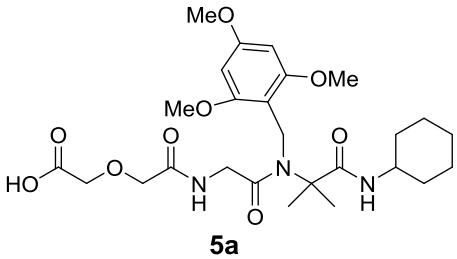
General procedure



1d, 1g, 1m
3a, 3b, 3c

5a-f

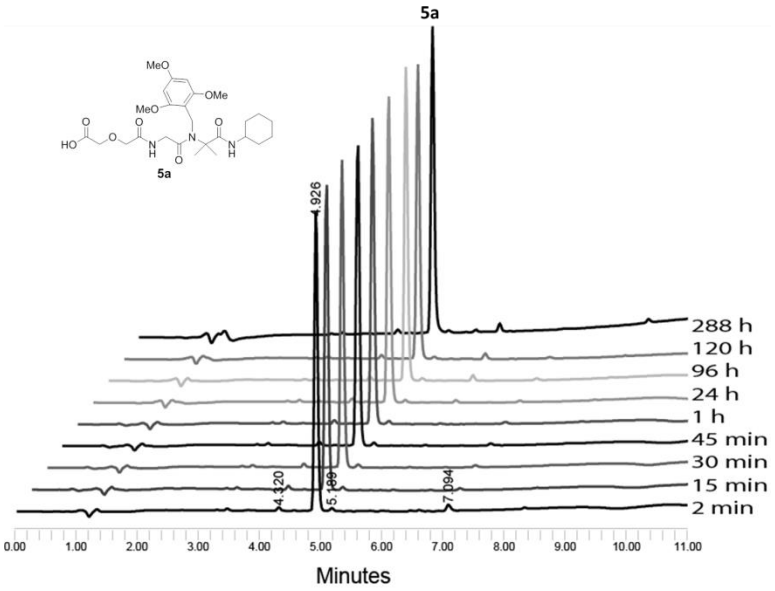
The indicated quantity of the corresponding Cbz protected dipeptide was solved in the indicated quantity of dry THF. Then the indicated quantity of diglycolic anhydride (1 eq.) and a catalytic amount of Pd/C (10% weight) was added to the solution and then the system was sealed with a septum and purged with N_2 and H_2. Then the reaction was stirred under H_2 atmosphere at room temperature until the complete Cbz elimination. The reaction was monitored by HPLC. The solution was filtered over Celite® and the solvent was removed under reduced pressure to yield the *N*-terminal acylated dipeptide.

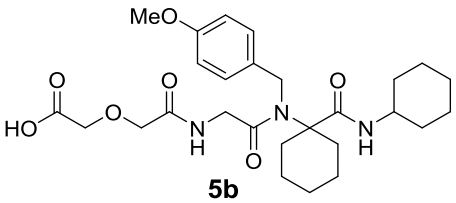


5a

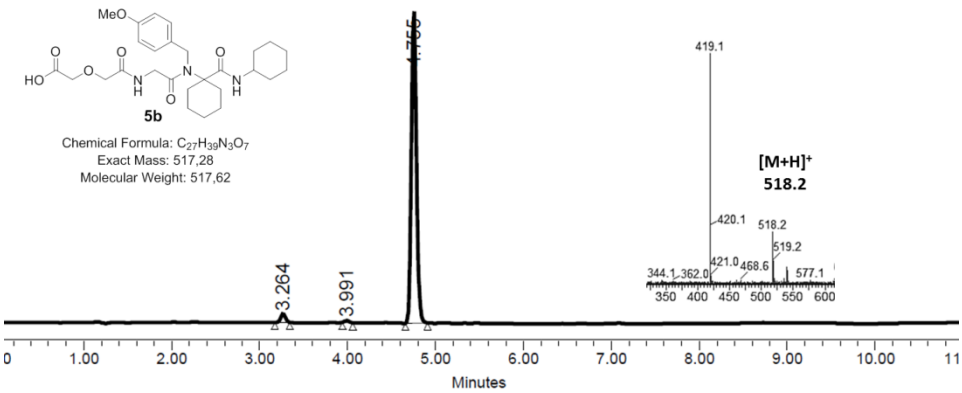
Entry 1: Compound **1d** (101.2 mg, 0.18 mmol), diglycolic anhydride (20.9 mg, 0.18 mmol), and Pd/C 10% (10.0 mg), THF (15 mL) was stirred at room temperature under H_2 for 3 h. After isolation, compound **5a** was obtained as a white solid (94.9 mg, 97% yield, 96% HPLC purity). **RP-HPLC**: [linear gradient H_2O/MeCN (60:40) to (0:100) over 8 min] t_R = 2.79 min.

- Compound **5a** acidolysis with MES (0.1 M) at pH = 4.8

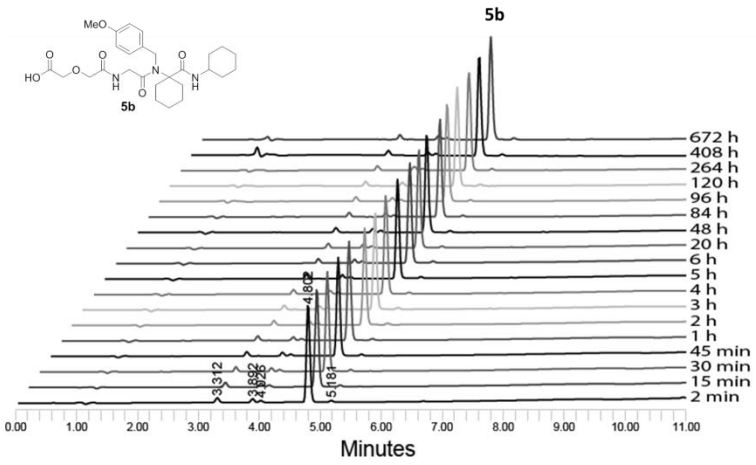




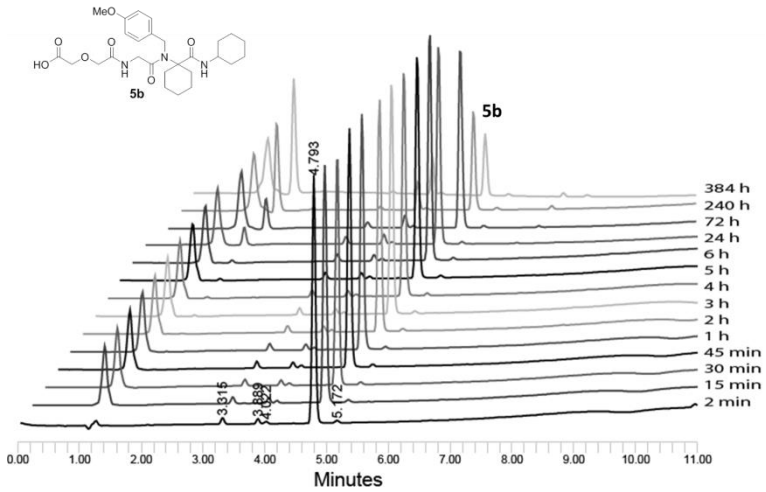
Entry 2: Compound **1g** (50.0 mg, 0.093 mmol), diglycolic anhydride (11.3 mg, 0.097 mmol), and Pd/C 10% (10.0 mg), THF (15 mL) was stirred at room temperature under H_2 for 5 h. After isolation, compound **5b** was obtained as a white solid (47.4 mg, 98% yield, 97% HPLC purity). **RP-HPLC**: [linear gradient H_2O/MeCN (70:30) to (0:100) over 8 min] t_R = 4.76 min; **MS-ESI**: *m/z* calculated for $C_{27}H_{39}N_3O_7$ = 517.6, found: 518.2 corresponding to $[M+H]^+$.



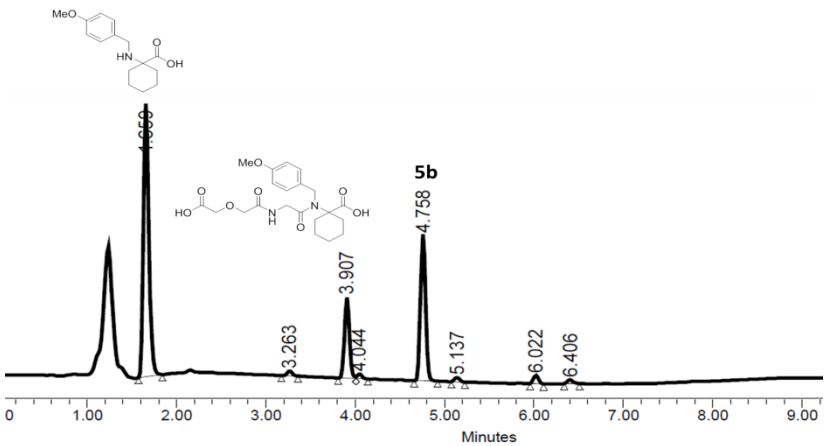
- Compound **5b** acidolysis with MES (0.1 M) at pH = 4.8



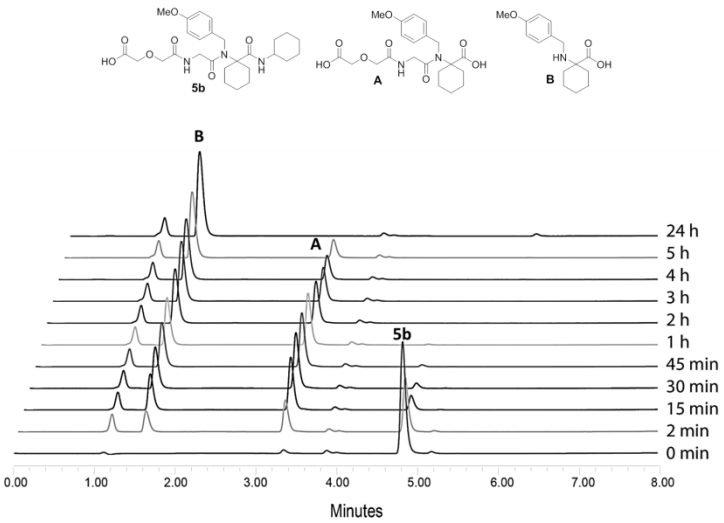
- Compound **5b** acidolysis with 1% TFA in H_2O.



t = 16 days:

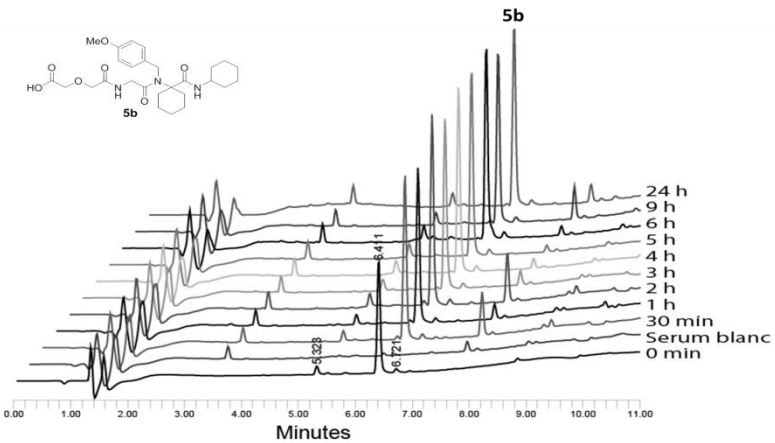


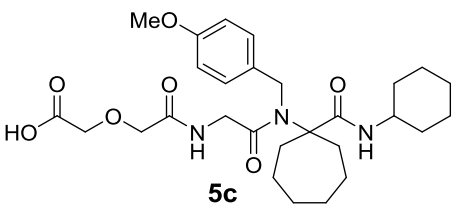
- Compound **5b** acidolysis with 1% TFA in MeCN.



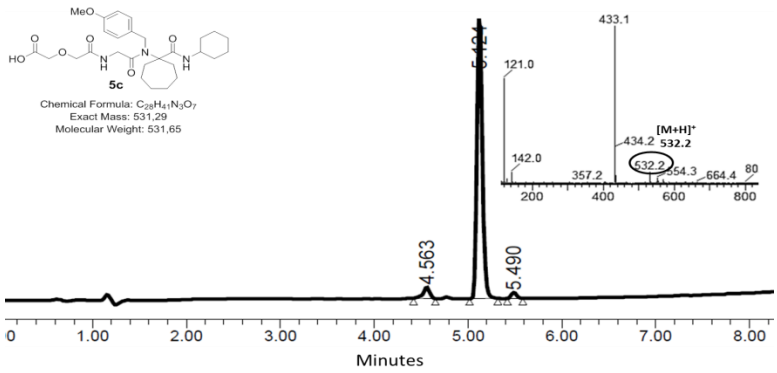
- Compound **5b** human serum stability

Compound **5b** stability was tested using male human serum type AB (Aldrich) and Hank's Balanced Salt solution (HBSS) (Aldrich). By triplicate, compound **5e** was dissolved in a Serum-HBBS solution (9:1) at a concentration of 1 mg/mL and it was incubated at 37 ºC. 50 µL samples were collected at different time points (30 min to 24 h) and poured into 200 µL of cold methanol to precipitate the proteins. Then samples were centrifuged at 15000 rpm for 15 min at 4 ºC. The supernatants were filtered and analyzed by HPLC.

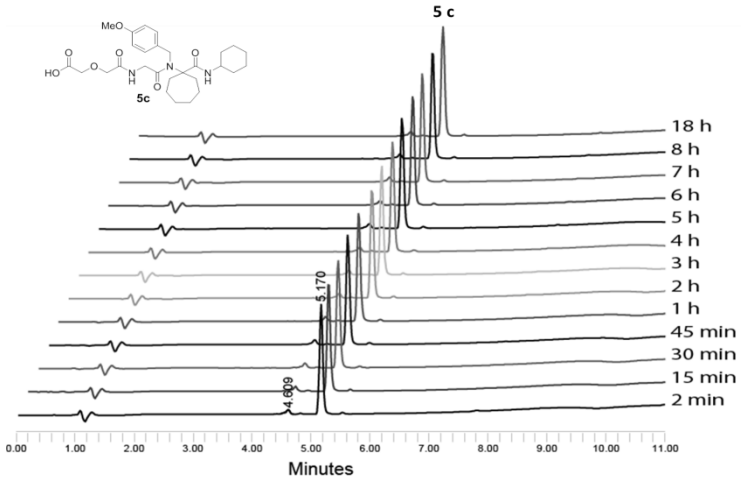




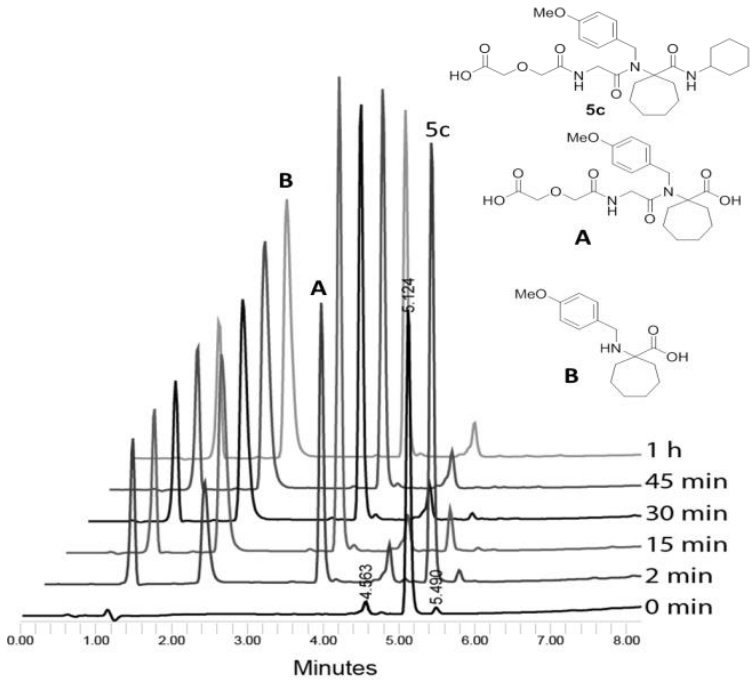
Entry 3: Compound **1m** (50.0 mg, 0.09 mmol), diglycolic anhydride (10.5 mg, 0.09 mmol), and Pd/C 10% (2.5 mg) in THF (20 mL) was stirred at room temperature under H_2 for 1 h. After isolation, compound **5c** was obtained as a white solid (43.6 mg, 90% yield, 94% purity). **RP-HPLC**: [linear gradient H_2O/MeCN (70:30) to (0:100) over 8 min] t_R = 5.12 min; **MS-ESI**: *m/z* calculated for $C_{28}H_{41}N_3O_7$ = 531.6, found: 532.2 corresponding to $[M+H]^+$.

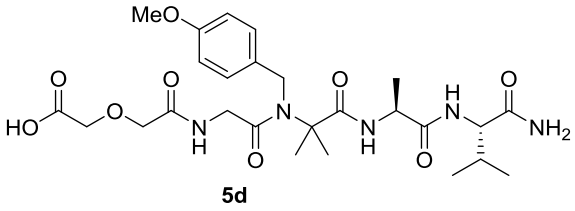


- Compound **5c** acidolysis MES (0.1 M) at pH = 4.8



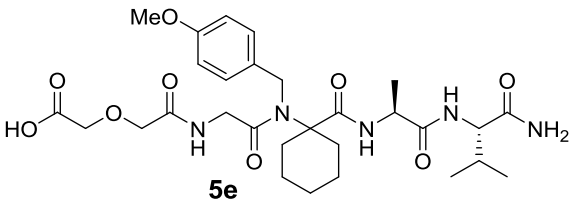
- Compound **5c** acidolysis 1 % TFA MeCN





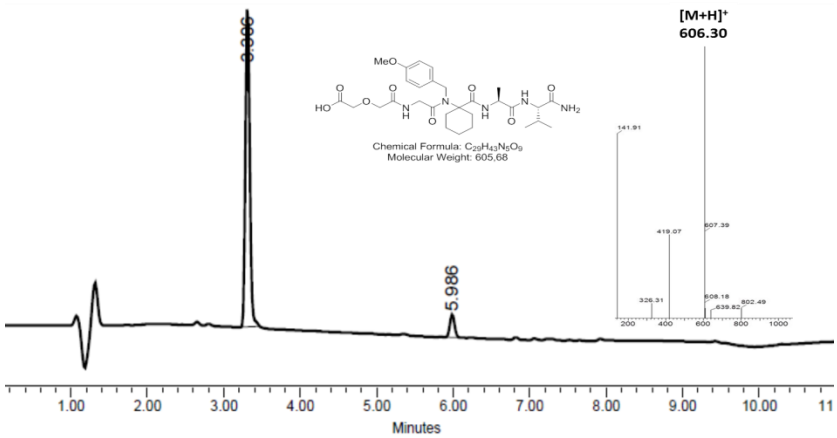
5d

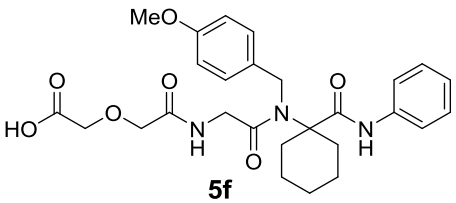
Entry 4: Compound **3a** (50.5 mg, 0.087 mmol), diglycolic anhydride (16.3 mg, 0.14 mmol) and Pd/C 10% (5.0 mg) in THF (10 mL) was stirred at room temperature under H_2 for 17 h. After isolation, compound **5d** was obtained as a white solid (47.7 mg, 97% yield, 83% HPLC purity). **RP-HPLC**: [linear gradient H_2O/MeCN (95:5) to (0:100) over 8 min] t_R = 4.61 min; **MS-ESI**: *m/z* calculated for $C_{26}H_{39}N_5O_9$ = 565.6, found: 566.2 corresponding to $[M+H]^+$.



5e

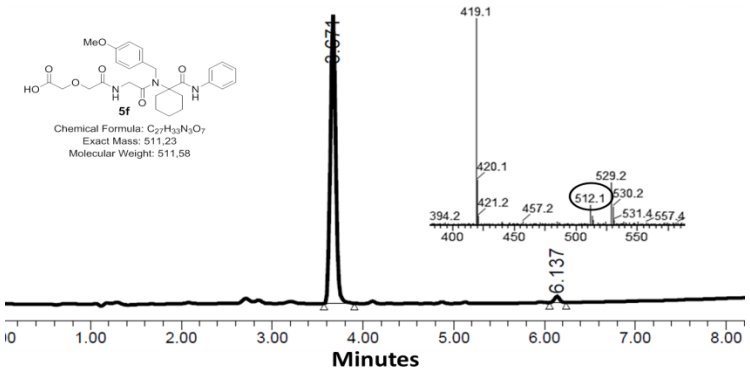
Entry 5: Compound **3b** (20.4 mg, 0.030 mmol), diglycolic anhydride (4.18 mg, 0.033 mmol) and Pd/C 10% (2.0 mg) in THF (10 mL) was stirred at room temperature under H_2 for 1 h. After isolation, compound **5e** was obtained as a white solid (19.6 mg, 99% yield, 93% HPLC purity). **RP-HPLC**: [linear gradient H_2O/MeCN (70:30) to (0:100) over 8 min] t_R = 3.31 min; **MS-ESI**: *m/z* calculated for $C_{29}H_{43}N_5O_9$ = 605.7, found: 606.3 corresponding to $[M+H]^+$.



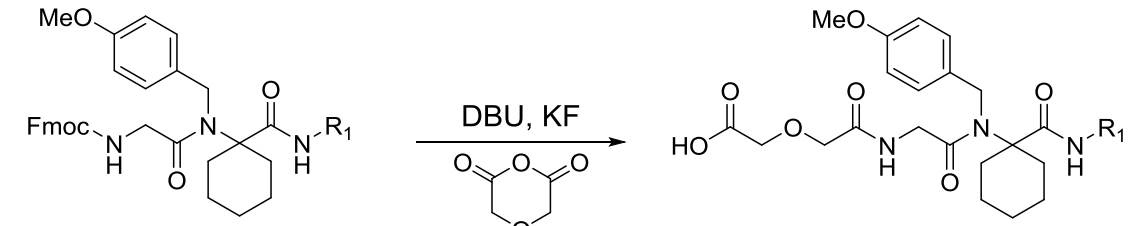


5f

Entry 6: Compound **3c** (100.5 mg, 0.190 mmol), diglycolic anhydride (24.2 mg, 0.208 mmol), and Pd/C 10% (20.0 mg) in THF (10 mL) was stirred at room temperature under H_2 for 17 h. After isolation, compound **5f** was obtained as a white solid (94.3 mg, 97% yield, 98% HPLC purity). **RP-HPLC**: [linear gradient H_2O/MeCN (60:40) to (0:100) over 8 min] t_R = 4.76 min; **MS-ESI**: *m/z* calculated for $C_{27}H_{33}N_3O_7$ = 511.2, found: 512.1 and 529.2 corresponding to $[M+H]^+$ and $[M+Na]^+$ respectively.



One pot Fmoc protecting group removal and amine acylation:

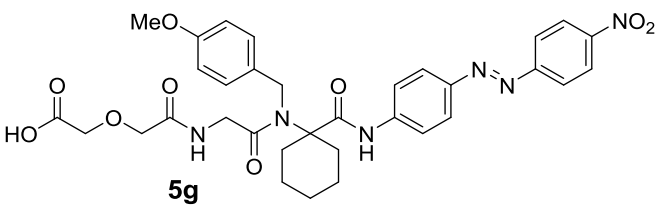


3d: R_1 = Disperse orange 3
3e: R_1 = Fluorescein amine

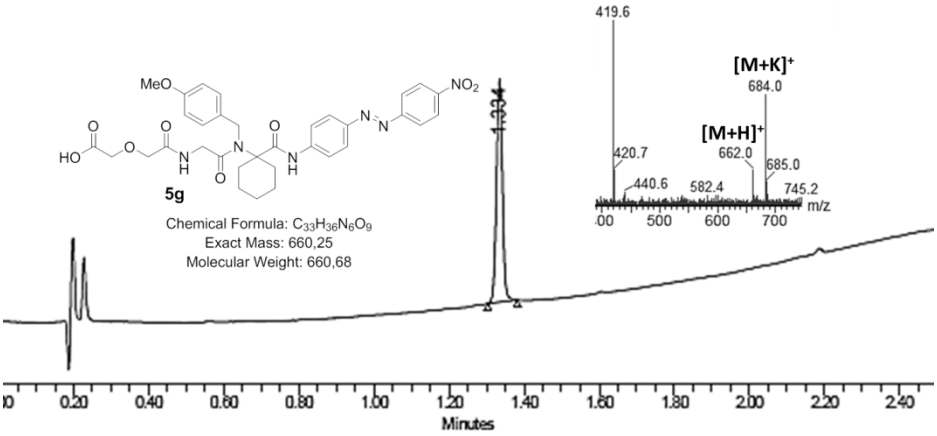
5g: R_1 = Disperse orange 3
5h: R_1 = Fluorescein amine

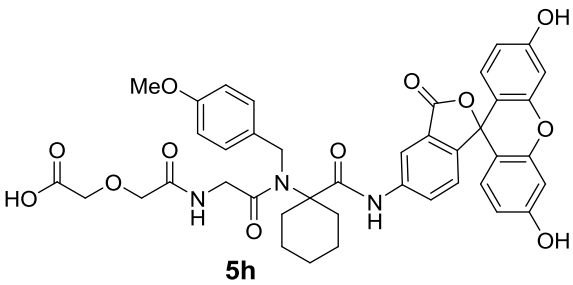
General procedure:

To a solution of the Fmoc protected compounds **3d** or **3e** (1 eq.) in the indicated solvent. Diglycolic anhydride (1 eq.), KF supported on Al_2O_3 (3 eq.) and DBU (1.2 eq) was added and the mixture was stirred under N_2 until the Fmoc was completely removed. Then the mixture was filtered to remove the KF/Al_2O_3 excess and the solvent was removed under reduced pressure. The obtained solid was precipitated with cold Et_2O and centrifuged. The obtained solid was automatically purified on a pre-packed Redisep Rf Gold C18 13 g column using H_2O/MeCN from 90:10 to 0:100 over 20 min. The fractions were collected and lyophilized obtaining the *N*-acylated compounds **5g** and **5h.**

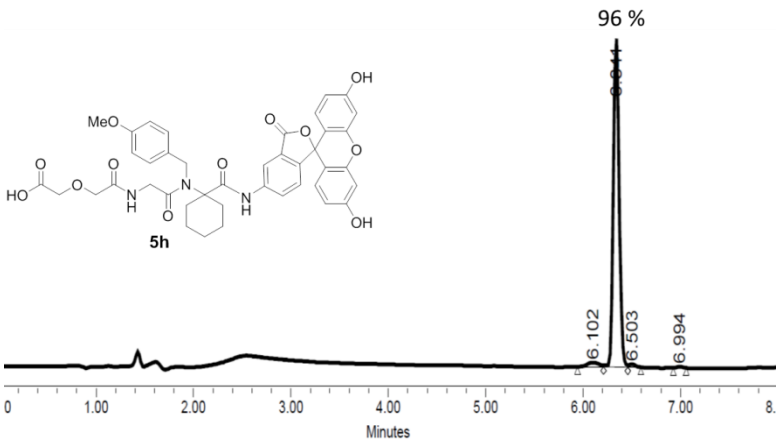


Entry 1: Compound **3d** (30.6 mg, 0.04 mmol), diglycolic anhydride (9.29 mg, 0.08 mmol), KF/Al_2O_3 (25.5 mg, 5.5 mmol/g) and DBU (7.2 µL, 0.047 mmol) was stirred in THF (10 mL) during 3 h. Compound **5g** was obtained as an orange powder (2.3 mg, 9%). **RP-UPLC**: [linear gradient H_2O/MeCN (50:50) to (0:100) over 2 min] t_R = 1.33 min; **MS-ESI**: *m/z* calculated for $C_{33}H_{36}N_6O_9$ = 660.7, found: 661.9 and 684.0 corresponding to $[M+H]^+$ and $[M+K]^+$.

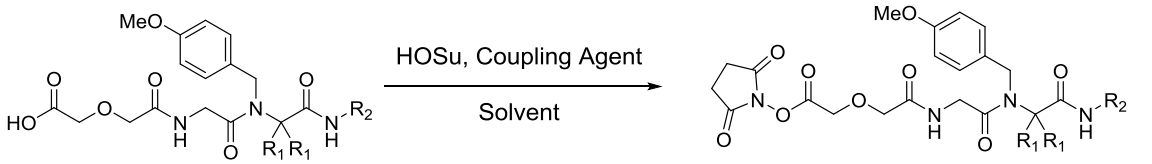




Entry 2: Compound **3e** (50.1 mg, 0.057 mmol), diglycolic anhydride (13.3 mg, 0.11 mmol), KF/Al_2O_3 (46.0 mg, 5.5 mmol/g) and DBU (10.1 µL, 0.067) was stirred in DMF (4 mL) during 17 h. Compound **5h** was obtained as an orange powder (13.1 mg, 30%). **RP-HPLC**: [linear gradient H_2O/MeCN (95:5) to (0:100) over 8 min] t_R = 6.35 min; **^{1}H NMR 5h** (400 MHz, DMSO-d_6) δ 10.10 (s, 2H), 9.48 (s, 1H), 8.27 (d, J = 1.9 Hz, 1H), 7.99 (t, J = 5.3 Hz, 1H), 7.94 (dd, J = 8.4, 1.9 Hz, 1H), 7.41 (d, J = 8.5 Hz, 2H), 7.18 (d, J = 8.3 Hz, 1H), 6.97 (d, J = 8.5 Hz, 2H), 6.67 (d, J = 2.4 Hz, 2H), 6.63 (s, 1H), 6.60 (s, 1H), 6.56 (d, J = 2.3 Hz, 1H), 6.54 (d, J = 2.4 Hz, 1H), 4.74 (s, 2H), 4.14 – 4.09 (m, 4H), 3.99 (s, 2H), 3.77 (s, 3H), 2.43 – 2.37 (m, 2H), 1.71 – 1.60 (m, 2H), 1.53 – 1.40 (m, 5H), 0.84 (s, 1H).



HOSu Activation:

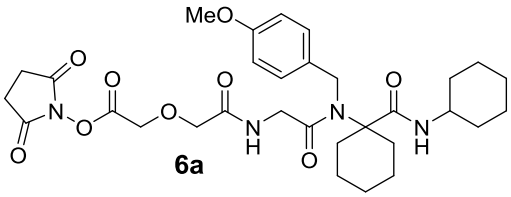


5b: R_1 = *c*-C_6H_{10}, R_2 = Chx
5d: R_1 = CH_3, R_2 = Ala-Val-NH_2
5f: R_1 = *c*-C_6H_{10}, R_2 = Phe
5g: R_1 = *c*-C_6H_{10}, R_2 = Disperse Orange 3
5h: R_1 = *c*-C_6H_{10}, R_2 = Fluorescein

6a: R_1 = *c*-C_6H_{10}, R_2 = Chx
6b: R_1 = CH_3, R_2 = Ala-Val-NH_2
6c: R_1 = *c*-C_6H_{10}, R_2 = Phe
6d: R_1 = *c*-C_6H_{10}, R_2 = Disperse Orange 3
6e: R_1 = *c*-C_6H_{10}, R_2 = Fluorescein

General procedure:

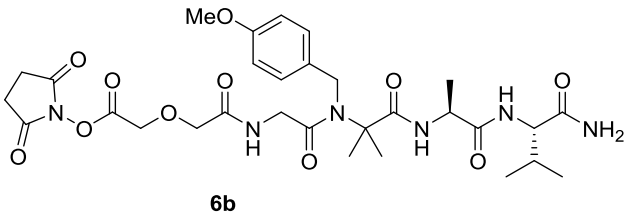
A solution of the indicated carboxylic acid (5b, 5d, 5f-h) in the indicated solvent was treated with *N*-hydroxysuccinimide and the mentioned coupling agent at the indicated quantities. The reaction was monitored by HPLC until the starting carboxylic acid disappeared. Then the solution was filtered over Celite to remove the excess of coupling agent and the solvent was removed under reduced pressure. After observed product decomposition during work-up and isolation, the products were used for conjugation without further purification.



6a

Entry 1: Compound **5b** (20.4 mg, 0.039 mmol), *N*-hydroxysuccinimide (4.37 mg, 0.039 mmol) and EDC·HCl (17.9 mg, 0.094 mmol) were stirred in MeCN (15 mL) for 3 h. The obtained solid was solved in DCM (20 mL) and washed with brine (1×20 mL). The organic layer was dried over $MgSO_4$, filtered and the solvent was removed under vacuum. The product decomposed during the isolation and it was impossible to isolate.

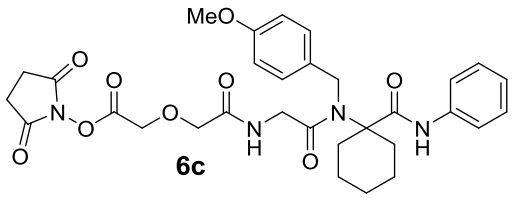
Entry 2: Compound **5b** (26.9 mg, 0.05 mmol), *N*-hydroxysuccinimide (5.98 mg, 0.05 mmol) and DCC (15.5 mg, 0.075 mmol) were stirred in Dioxane/AcOEt (1:1) (10 mL) at room temperature during 5 h. After isolation and solvent elimination, compound **6a** was obtained as a white solid (31.7 mg, 99% yield, 88% HPLC purity). **RP-HPLC**: [linear gradient H_2O/MeCN (70:30) to (0:100) over 8 min] t_R = 5.50 min; **^{1}H NMR 6a** (400 MHz, $CDCl_3$) δ 7.41 (t, J = 4.5 Hz, 1H), 7.24 (d, J = 8.7 Hz, 2H), 6.89 (d, J = 8.7 Hz, 2H), 6.01 (d, J = 8.0 Hz, 1H), 4.56 (s, 2H), 4.54 (s, 2H), 4.16 (s, 2H), 4.12 – 4.08 (m, 2H), 3.79 (s, 3H), 3.49 – 3.40 (m, 1H), 2.86 (s, 4H), 2.40 – 2.35 (m, 1H), 1.90 (d, J = 3.8 Hz, 2H), 1.71 – 1.66 (m, 2H), 1.57 (dd, J = 8.7, 4.1 Hz, 6H), 1.39 – 1.05 (m, 9H).



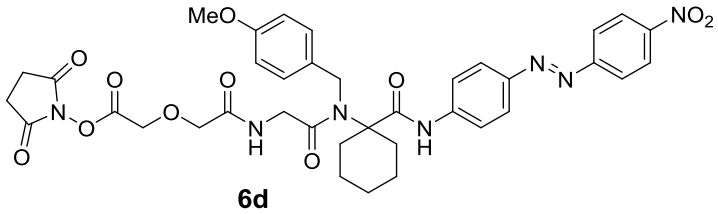
6b

Entry 3: Compound **5d** (47.7 mg, 0.084 mmol), *N*-hydroxysuccinimide (30.2 mg, 0.26 mmol) and DCC (53.6 mg, 0.26 mmol) were stirred in THF (10 mL) at room temperature during 18 h. The desired

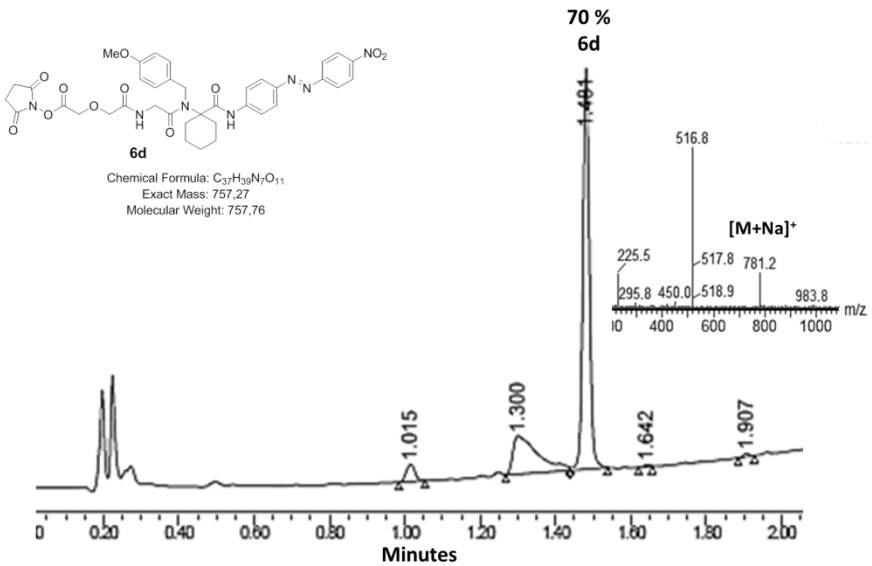
product was observed by MS-ESI but during the purification the compound **6b** was hydrolyzed. The final product was not isolated.

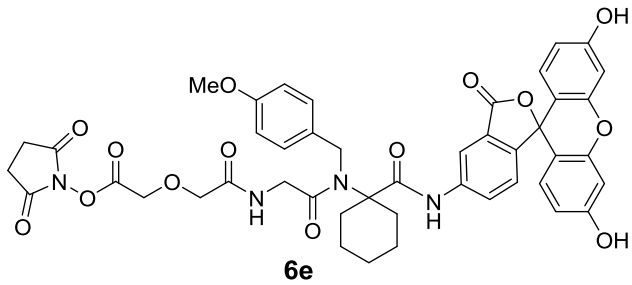


Entry 4: Compound **5f** (82.6 mg, 0.16 mmol), *N*-hydroxysuccinimide (20.4 mg, 0.17 mmol) and DCC (49.9 mg, 0.24 mmol) were stirred in Dioxane/AcOEt (1:1) (10 mL) at room temperature during 1h. After filtration and solvent elimination, compound **6c** was obtained as a white solid. (129.0 mg, >99% yield, starting materials was found in the mixture, 95% HPLC purity). **RP-HPLC**: [linear gradient H_2O/MeCN (60:40) to (0:100) over 8 min] t_R = 4.49 min; **^{1}H NMR 6c** (400 MHz, $CDCl_3$) δ 8.29 (s, 1H), 7.46 (dd, J = 8.6, 1.2 Hz, 2H), 7.43 – 7.40 (m, 1H), 7.34 – 7.28 (m, 2H), 7.23 (d, J = 8.7 Hz, 2H), 7.11 – 7.06 (m, 1H), 6.91 – 6.86 (m, 2H), 4.61 (s, 2H), 4.52 (s, 2H), 4.18 (d, J = 4.7 Hz, 2H), 4.16 (s, 2H), 3.79 (s, 3H), 2.82 (s, 4H), 2.53 – 2.45 (m, 2H), 1.96 – 1.88 (m, 2H), 1.83 – 1.56 (m, 3H), 1.39 – 1.03 (m, 3H). The NMR spectra contain solvent and dicyclohexylurea impurities.



Entry 5: Compound **5g** (2.3 mg, 3.5 µmol), *N*-hydroxysuccinimide (0.9 mg, 7.8 µmol) and DCC (2.0 mg, 9.7 µmol) were stirred in Dioxane/AcOEt (1:1) (2 mL) at room temperature during 1h. After filtration and solvent elimination, compound **6d** was obtained as a brown solid which was not purified (3.9 mg, >99% yield, starting materials was found in the mixture). **MS-ESI**: *m/z* calculated for $C_{37}H_{39}N_7O_{11}$ = 757.76, found: 781.2 corresponding to $[M+Na]^+$.





6e

Entry 6: Compound **5h** (3.8 mg, 4.9 µmol), *N*-hydroxysuccinimide (0.63 mg, 5.5 µmol) and DCC (3.6 mg, 17.4 µmol) were stirred in Dioxane/AcOEt (1:1) (2 mL) at room temperature during 1h. After filtration and solvent elimination, compound **6e** was obtained as an orange solid which was not purified (5.3 mg, >99% yield, starting materials was found in the mixture).

Bioconjugation

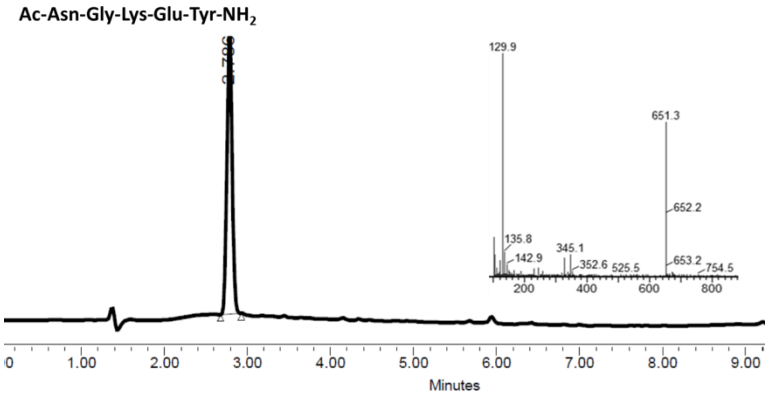
Peptides

Synthesis of Lys-containing pentapeptide :

Peptide P1 was synthetized manually by conventional solid-phase peptide synthesis procedures. 500 mg Fmoc-Rink amide resin (f = 0.70 mmol/g), Coupling agents: Oxyma/DIC (3 eq) DMF 1h. Final amine acetylation: Ac2O/DIEA (10 eq) (2×15 min). Cleavage TFA/TIS/H_2O (95:2.5:2.5) 1 h

Ac-Asn-Gly-Lys-Glu-Tyr-NH_2

RP-HPLC: [linear gradient H_2O/MeCN (95:5) to (0:100) over 8 min] t_R = 2.79 min, purity 97%; **MS-ESI**: *m/z* calculated for $C_{28}H_{42}N_8O_{10}$ = 650.7, found: 651.2 corresponding to $[M+H]^+$.



Peptide Conjugation:

To a solution of pentapeptide **P1** (2.0 mg, 3.07 µmol) in PBS (2 mL) at pH = 7.4, the compound **6a (**2.1 mg, 3.4 µmol) in DMF (100 µL) was added dropwise. The mixture was left to react during 17 h at room temperature. The final product was not isolated, but the reaction crude was analyzed by HPLC and MS-ESI detecting the compound **7a.**

RP-HPLC: [linear gradient H_2O/MeCN (95:5) to (0:100) over 8 min] t_R = 5.51 min; **MS-ESI**: *m/z* calculated for $C_{55}H_{79}N_{11}O_{16}$ = 1150.3, found: 1172.8 corresponding to $[M+Na]^+$.

Protein conjugation:

BSA conjugation

To a solution of bovine serum albumin (BSA) (22.4 mg, 0.35 µmol) in PBS (2 mL) at pH = 7.4, 50 µL of a solution of compound **6c (**12.4 mg, 20.4 µmol) in DMSO (100 µL) was added dropwise. The mixture was left to react during 24 h at 4 ºC and the purified on a PD10 desalting column over water as eluent. Then, the protein-containing fractions were collected and lyophilized obtaining the conjugate 7b as a pale yellow powder (18.4 mg, 65%). **RP-HPLC**: [linear gradient H_2O/MeCN (100:0) to (0:100) over 30 min on BioSuite pC18, 500, 7µm RPC 4.6 × 150 mm column] t_R = 18.03 min; **MALDI**: BSA *m/z* found = 67003.6±55.8 Da. Conjugate *m/z* found = 74814.1±258.2Da.

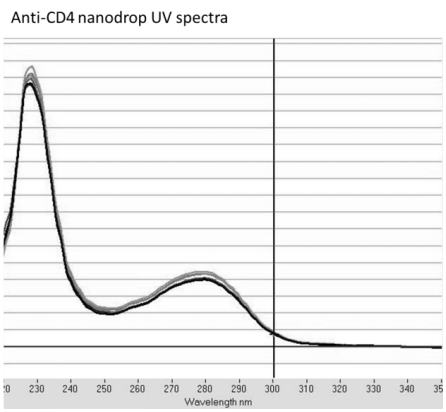
Antibody conjugation:

Conjugation to anti-CD4

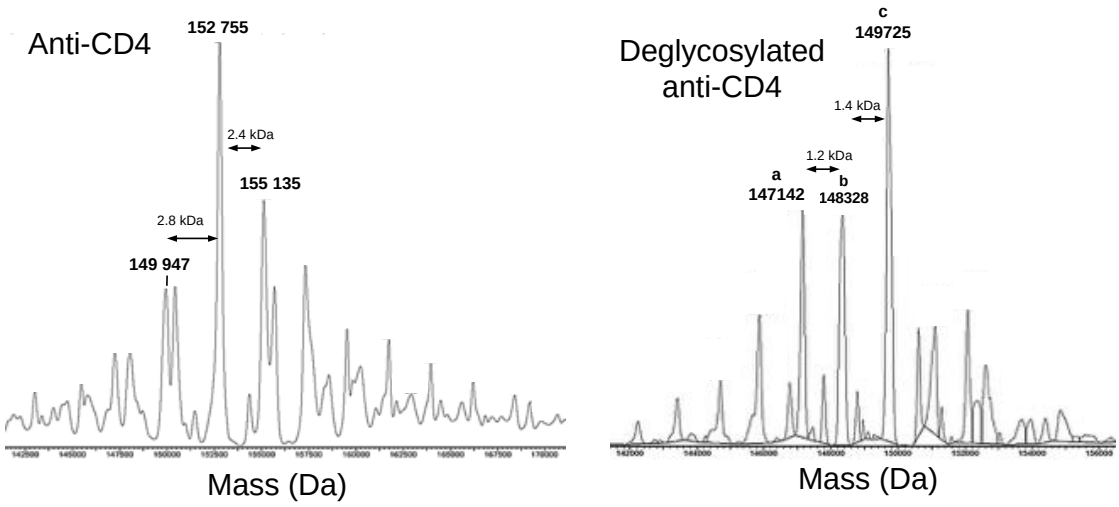
General procedure

The Lys conjugation was performed by treating a solution of anti-CD4 (approximately 1 mg/mL) PBS at pH = 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) with a solution of the HOSu-activated carboxylic acid dissolved in DMSO (5-10%) and it was left to react during 17 h at 4 ºC. Then the crude was purified using PD Minitrap G-25 column and water as eluent for the antibody analysis. The collected samples (approximately 200 µL) were analysed by UV nanodrop in order to determine their concentration as well as by SDS in reducing and non-reducing conditions, and mass spectrometry. For mass analysis, an aliquot of the antibody solution in PBS (18 µL) was treated with PNGase F 500 000 U/mL (1 µL) at 37 ºC during 17 h in order to perform the conjugate deglycosilation to improve the mass analysis spectra.

Anti-CD4 analysis



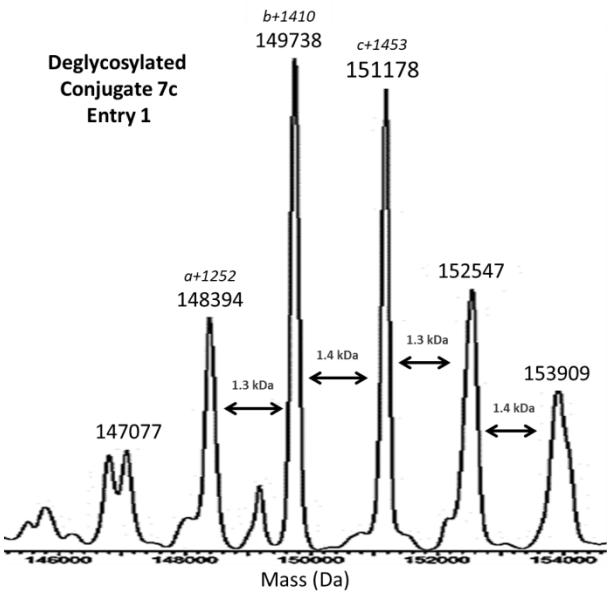
MS-ESI analysis



Conjugate 7c: Compound 6d conjugated to anti-CD4.

0.5 mL of Anti-CD4 (1.5 mg/mL, 10 µM, 5 nmol) in PBS was treated with a solution (10.7 µL) of compound **6d** (2.6 mg, 3.5 µmol) in DMSO (2 mL). After purification, two samples of 0.94 and 0.83 mg/mL (150 µL) were collected.

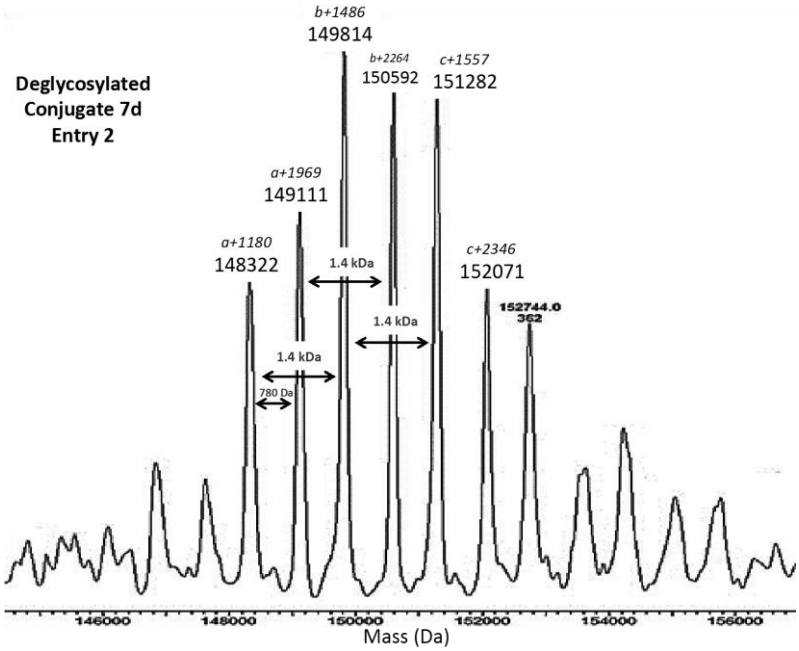
Mass analysis:



Conjugate 7d: Compound 6e conjugated to anti-CD4.

0.5 mL of Anti-CD4 (1.0 mg/mL, 6.7 µM, 3.3 nmol) in PBS was treated with a solution (50 µL) of compound **6e** (1.2 mg, 1.4 µmol) in DMSO (5 mL). After purification, three samples of approximately 150 µL compressed between 0.1 and 0.7 mg/mL were collected.

Mass analysis:



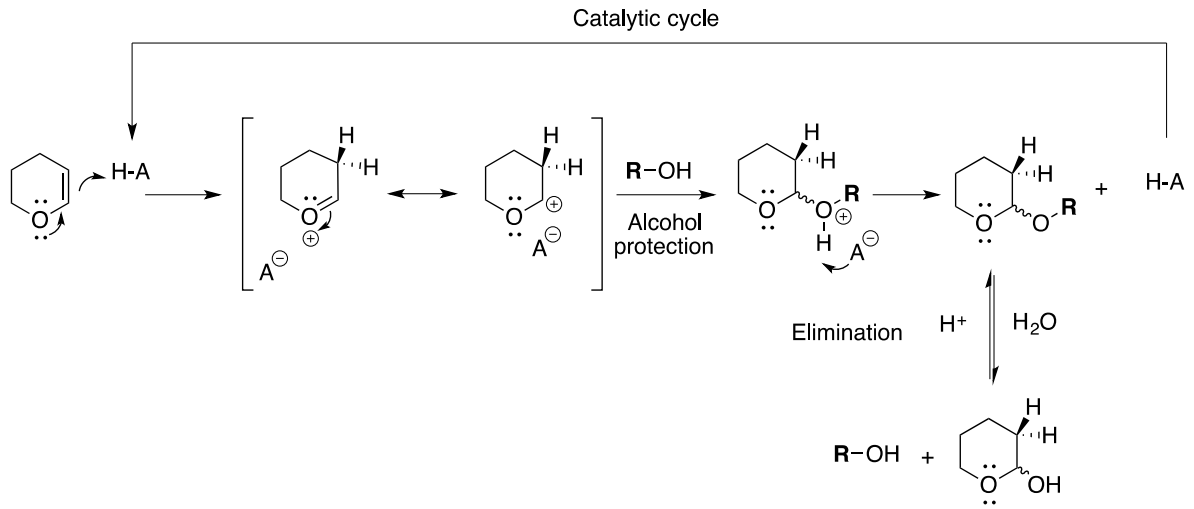
Chapter I. Cleavable linkers

2. Tetrahydropyran (Thp) linker

2.1. Introduction

Tetrahydropyranyl (Thp) group has been recognized as the useful and representative method for the protection of alcohols in organic synthesis due to its low cost, ease installation, general stability to most nonacidic reagents, and the ease with which it can be removed.[1] The extensive contribution of R. Paul to the chemistry of pyran and furan rings attributes to him the discovery of Thp as hydroxyl protecting group. Accordingly, in 1934[2] appeared the first evidence of a introduction of an alcohol (MeOH) into a 2,3-dihydropyran ring and moreover, the acetal hydrolysis by the action of diluted acids. Shortly after, Woods and Kramer[3] studied more in deep the addition different alcohols to the dihydropyran ring and also they postulated the stability of this tetrahypyran acetals under basic conditions. But the Thp description of Thp as alcohol protecting group was not until 1948 by Parham and Anderson,[4] where they highlighted the conversion of hydroxyl compounds to acetals and their readily cleavage in aqueous mild conditions with the regeneration of the hydroxyl group.

Nowadays, several possibilities for tetrahydropyran ether formation are described but the hydroxyl addition to dihydropyran (DHP) mediated by acidic-catalysis continues being one of the most used and productive ways to protect hydroxyls groups[1] (**Scheme 10**).



Scheme 10. Tetrahydropyran (Thp) alcohol protection and it elimination mechanisms.

In this sense, *p*-toluensulfonic acid is considered one of the most commonly used catalyst, and its action it found to be superior to other acidic catalysts[5] such as hydrochloric acid, phosphoryl chloride, or boron trifluoride etherate.[1,6] Owing to the strong acidity of the mentioned acids, the tetrahydropyranylation of highly acid-sensitive molecules still undesirable. Accordingly, Miyashita and collaborators[7] developed milder acidic catalyst for

THP ether formation, pyridinium *p*-toluensulfonate, which are compatible with acid-sensitive compounds.

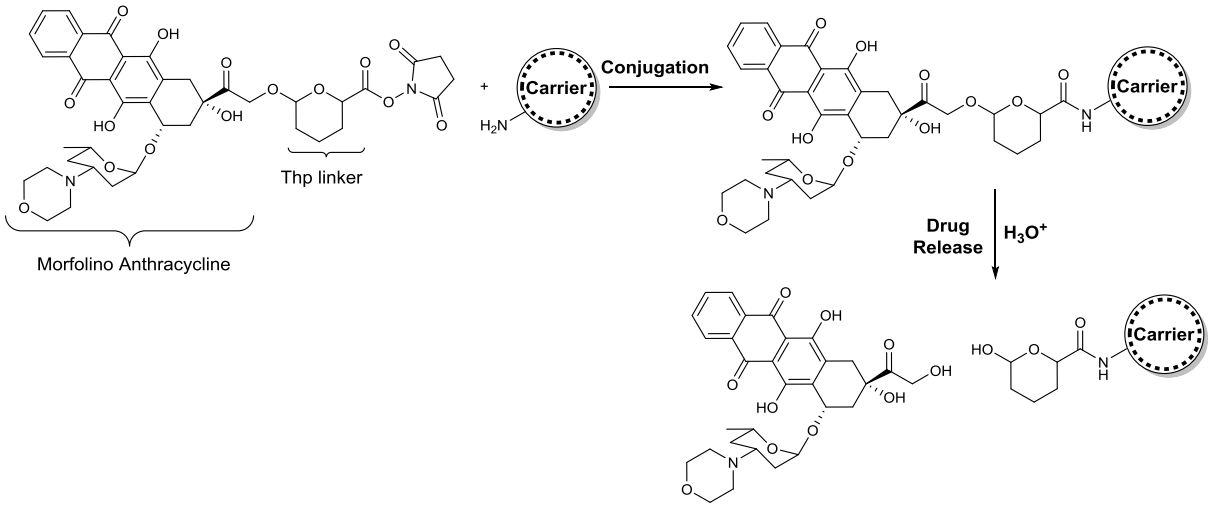
Apart of being broadly used as alcohol protecting group, Thp have found also efficient and stable for thiols groups.[8] Parham and DeLaitsch[4,9] studied and compared the pyranylation of hydroxyl and thiols groups, observing higher reactivity for hydroxyls groups pyranylation and less stability for the resulting tetrahydropyran acetal than for thioacetal. In 1958 Holland and Cohen[10] investigated the use of dihydropyran for the sulfhydryl group into cysteine for peptide synthesis.

Regarding the Thp protecting group elimination, several methodologies have been studied and nowadays chemist have on his hands a wide range of possibilities to easily and selectively eliminate this protecting group. The use of strong protics (i.e. H_2SO_4) or Lewis acids may affect to other functional groups present in the Thp-protected molecule. Accordingly, milder acidic conditions have been developed for this special protecting group. Aqueous acetic acid,[11,12] Selectfluor™[13] or PPTS[7] in alcoholic solvents are able to remove Thp group in high yields. On contrary Thp-thioacetal removal, needs higher acid concentrations or heat due to its higher stability.[9]

Besides the above-mentioned uses as hydroxyl and thiol protecting group, the tetrahydropyran moiety was also studied as cleavable linker for solid-phase peptide synthesis. Thompson and Ellman[14] introduced the use of dihydropyran-functionalized support which provides a general and straightforward method for primary and secondary alcohols attachment through the base-stable tetrahydropyranyl ether linkage. Dihydropyran-decorated supports have found convenient for the synthesis of a wide range of hydroxyl-containing organic compounds[15–18] as well as for peptides synthesis anchoring trifunctional aminoacids such as threonine (Thr)[19] or serine (Ser)[20] through its side chains.

Accordingly, Thp group was also utilized for peptides, proteins and antibodies conjugation, but their studies was only depicted in a 1998's US patent property of Pharmacia and Upjohn.[21,22] In this regard, they invented some anthracycline conjugates characterized by the presence of an acid sensitive acetalic bond, result of the linkage between a dyhydropyran and hydroxyl group. They used the Thp linker to attached hydroxyl-containing anthracycline derivatives to different carriers through an amine group (**Scheme 11**). The resulting

conjugates are found to target certain mammalian tumours as well as improve the drug potency, therapeutic efficacy and moreover, reduce the toxic effects.

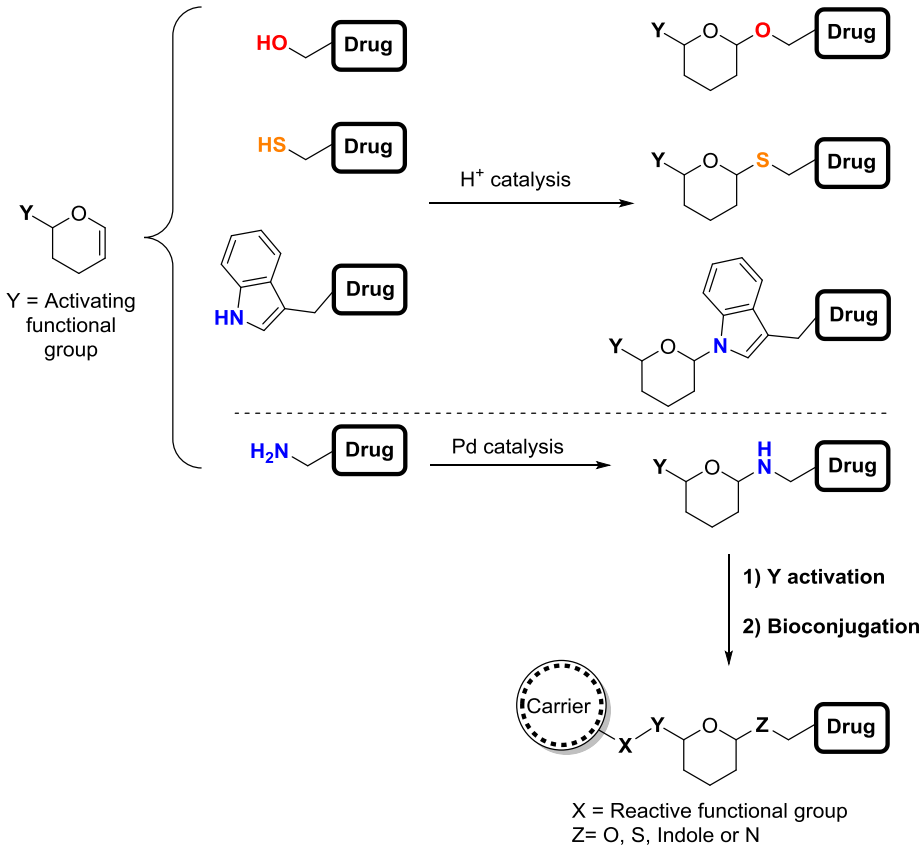


Scheme 11. Morfolino anthracycline tetrahydropyranyl-mediated conjugation and it acetalic bond acidolysis.

However, the solid support dihydropyranylation of nitrogen-based functional groups has not been extensively studied. The reversible union of purines[23,24] and benzimidazoles[25] through one of the imidazole nitrogens has been reported as well as the indolic nitrogen for the synthesis of 2,3-disubstituted indoles.[26] More recently Nicolás group[27] use the dihydropyranyl linker to anchoring the indolic nitrogen from the amino acid tryptophan to a solid support for the synthesis of tryptophan containing diketopiperazines. On the one hand they yield the hemiaminal linkage by heating the mixture of a tryptophan derivative and the dihydropyran ring in presence of acidic mild conditions provided by pyridine *p*-toluenesulfonate (PPTS) all dissolved in 1,2-dichloroethane. On the other hand the Thp removal is achieved using strong acid concentration (TFA) in presence of a carbocation scavenger.

Using a fashionable palladium mediated methodology for the carbon-nitrogen bond formation, Cheng and Hii[28] achieved the incorporation of different amino groups into a saturated dihydropyran ring yielding the corresponding hemiaminal linkage, which considering the above mentioned studies we assume that it could be acid-labile, and concretely interesting for used as cleavable linker for bioconjugation. Therefore, studies with glucosylamines demonstrated the lability of the *N*-glycosidic bond in front of low acid concentration.[29,30]

In this sense Thp protecting group may be removed from the masked hydroxyl, sulfhydryl or amine groups under very mildly acidic conditions, fact that is important to highlight for our purpose to develop new cleavable linkers in acidic conditions (**Scheme 12**).



Scheme 12. Versatility of the Dhp ring functionalization for it further bioconjugation.

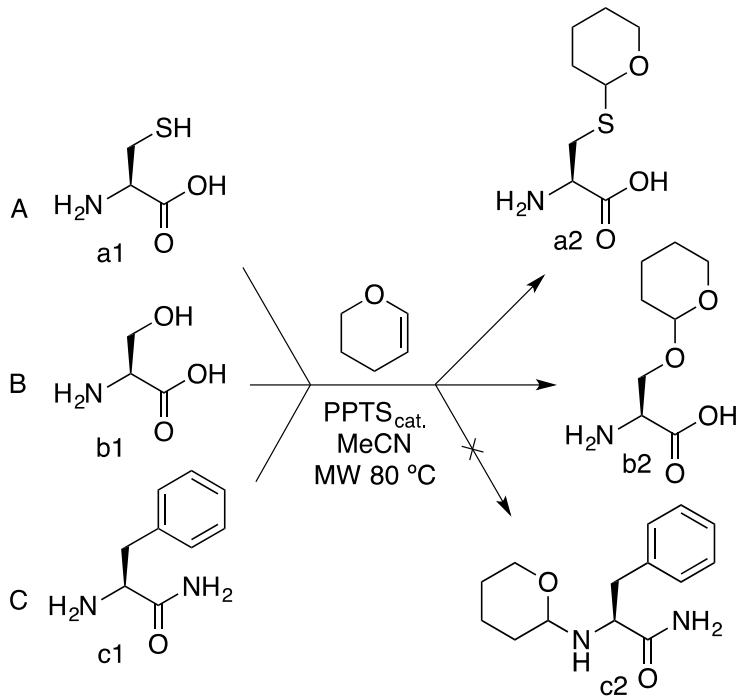
Due to its ability to introduce different functional groups, the versatility of the pyran ring and the possibility to further functionalize it with potentials reactive groups to be attached to a biomolecule, Thp group attract our attention in order to use it as a promising cleavable linker for bioconjugation. As the Thp linker was studied for hydroxyl groups, in the present thesis we focus on developing the methodology for it use as cleavable linker for bioconjugation through other functional groups such as amines or sulfhydryl.

2.2. Objectives

- Study and optimize thiol and amine tetrahydropyranilation for their further bioconjugation.
- Study Thp stability and it elimination conditions in order to use as cleavable linker.

2.3. Results and discussion

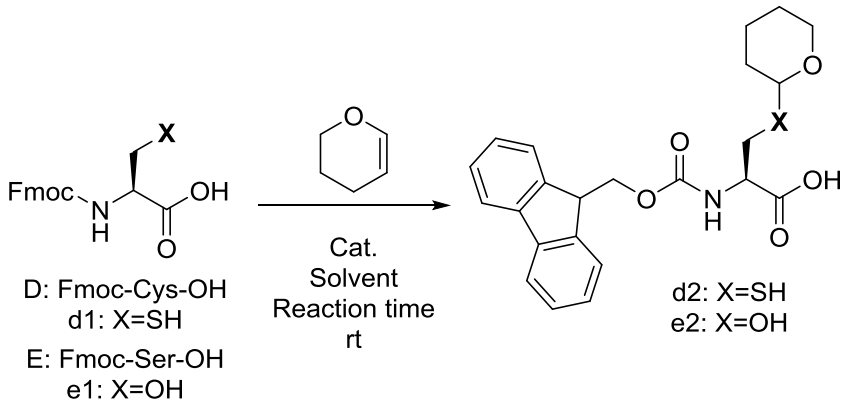
In order to test the ability of the pyran ring as a potential linker, as a first approach we test the ability to introduce into thiols, alcohols and amines the pyran ring obtaining the corresponding thioacetal (A), acetal (B) or hemiaminal (C) respectively (**Scheme 13**). The protection and elimination of the mentioned functional groups with the tetrahydropyran group will allow crucial information about their behaviour as cleavable linker or not. For that we choose the tetrahydropyran moiety as the simples model in order to determine the optimal introduction and elimination conditions with cheapest starting materials.



Scheme 13. First approach of **A**) Cysteine, **B**) serine and **C**) tetrahydropyranylation, under microwave conditions in MeCN and using PPTS as catalyst.

The first approach that we tackled resulted fruitful as we expected for Cys and Ser (A and B respectively), the mass spectra of the analysed reaction crudes reveals that the pyranylation took place. However no evidence of the Phe amine pyranylation (C) was found. For A and B, the zwitterionic character of the amino acids did not affect to the acidic catalyst deprotonation, meanwhile for the H-Phe-NH_2, the catalyst proton could be sequestered by it primary amine disabling the catalyst action which is crucial for the Dhp activation. Furthermore, A and B reactions demonstrated that the pyranylation took place in presence of other functional groups, fact which increase the potential of the Thp as a possible cleavable linker due to the chemoselectivity in front of hydroxyl or thiols groups.

Due to the absence of UV chromophores of the obtained products a2 and b2, their detection through the available chromatographic techniques found complicated. For that reason, we though to study the introduction of the Thp group and it elimination using the 9-fluorenylmethoxycarbonyl (Fmoc) amine protecting group which is stable to acids. Accordingly, we study the tetrahydropyranylation of the Fmoc-Aaa-OH (being Aaa: Cys and Ser respectively) using milder conditions than the previously described yielding the Thp protected amino acids through it side chain (**Scheme 14**).



Scheme 14. Fmoc-Cys-OH and Fmoc-Ser-OH Thp protection.

Entry	Reaction	Catalyst	Solvent	Reaction time	Yield (%)
1	D	PPTS (0.1 eq)	DCM	5 days	72
2	D	*p*-TsOH (0.1 eq)	DCM	1 h	87
3	E	*p*-TsOH (0.1 eq)	DCM	17 h	89

The tested experiments demonstrated that with p-toluensulfonic acid (*p*-TsOH) the tetrahydropyranylation took place faster than with a milder acid catalyst (PPTS). Herein is important to highlight the high reaction yields as well as the easy and economic incorporation of the tetrahydropyran into thiols and hydroxyls groups. It is considered that the main drawback of the Thp group is the formation of a new stereocenter that leads to diastereomeric mixtures. Nevertheless, as with other protecting groups or linkers, their use is temporary and in concordance, the formation of the stereocenter is not a limitation.

With the protected amino acids in hand, we tested different Thp elimination conditions in acidic media in order to determine if the Thp linker is found as an efficient cleavable linker for bioconjugation **(Table 8).**

Table 8. Fmoc-Cys(Thp)-OH and Fmoc-Ser(Thp)-OH protecting group elimination study.

Entry	Compound	Cocktail Composition	Reaction time	Deprotected Cys, yield (%)
1	d2	MES[a] (100 mM) pH = 4.8	48 h	0
2	e2		40 h	50
3	d2	MES/NaCl/KCl (100:137:2.7 mM) pH = 4.8	48 h	0
4	e2		20 h	25
5	d2	MES/TIS (0.1 M:1.5%) pH = 4.8	48 h	0
6	d2	PBS PO_4^{3-}/KCl/NaCl (10:2.7:137 mM) pH = 7.4	120 h	0
7	e2			0
8	d2	TFA/H_2O/DCM (1:1:98) pH ≈ 1	48 h	29
9	d2	TFA/H_2O/DCM (10:1:89) pH ≤ 1	48 h	94

[a] 2-(N-morpholino)ethanesulfonic acid

During the Thp elimination studies we observed that the thioacetal bond (d2) is more stable into mild acidic conditions than the acetal bond (e2). As it was expected, the Thp group was eliminated of the compound e2 easily with mild acids. Accordingly a hydrolysis study at pH = 4.8 during 7 days was carried out by HPLC (**Figure 17A**). In contrast, Fmoc-Cys(Thp)-OH needs strong acidic conditions for it elimination being more suitable for synthetic purposes as we expose in the following section than for use it as a cleavable linkers for bioconjugation. Also an acidolytic study for Cys-Thp elimination was carried out during 48 h using strong acid conditions (**Figure 17B**).

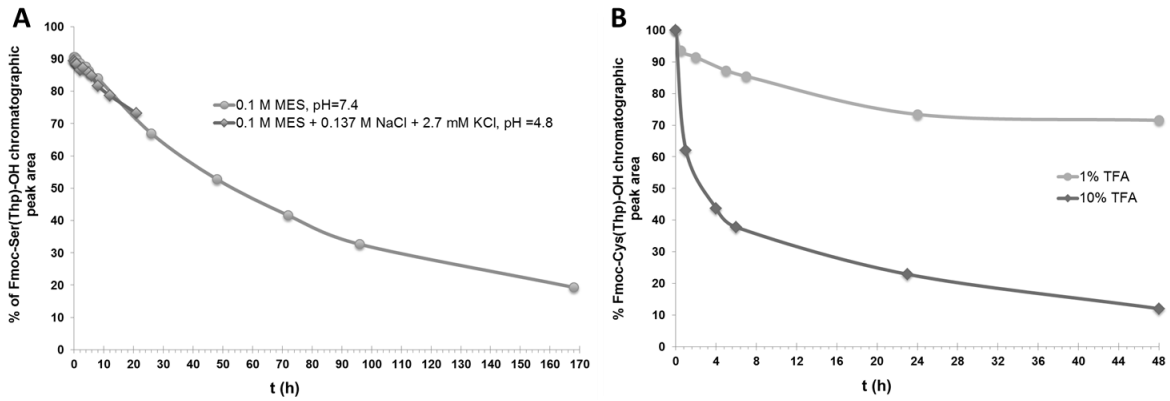
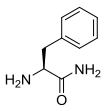
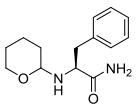
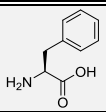
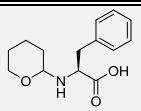
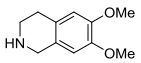
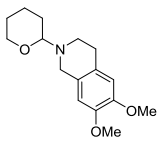
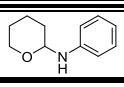


Figure 17. A) Fmoc-Ser(Thp)-OH and **B)** Fmoc-Cys(Thp)-OH acydolisis kinetic study.

Is important to remember that for bioconjugation the linkage between both entities must be stable at physiological pH, and moreover be hydrolysable in the proximities or inside the tumour cells where the pH decrease in comparison to the systemic circulation. In this sense, the *S*-Thp linkage found stable into physiological-like conditions (PBS) but however into the lysosomal-like hydrolytic conditions (pH = 4.8).

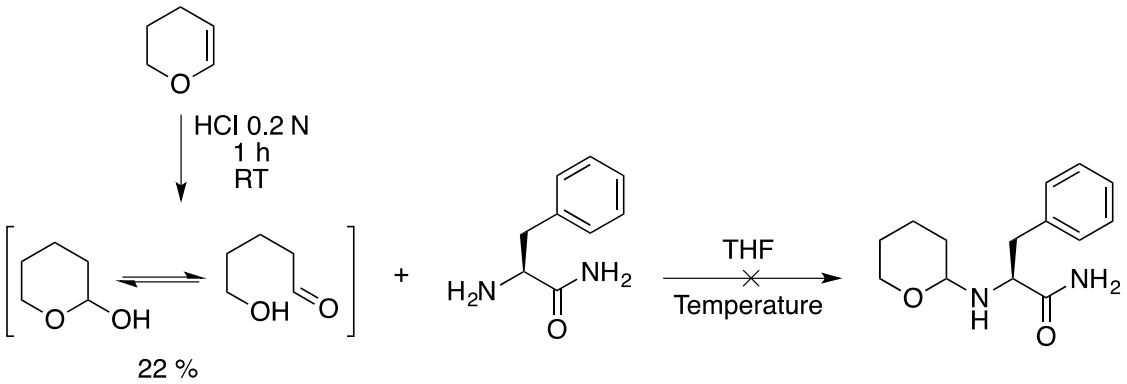
Accordingly, the tetrahydropyranilation of amines was tackled using the methodology developed by Cheng and Hii[28] which is based on Pd-catalysis. With this purpose in mind, we used different commercially available Pd catalysts and also we prepared the catalyst which the mentioned authors postulated as the most efficient for the *N*-Thp linkage, $K_2Pd(SCN)_4$. The following table comprise the amines, catalysts, reaction times and solvents which were tested (Table 9).

Table 9. Reaction conditions tested for Pd-mediated amine tetrahydropyranylation.

Entry	Amine	Solvent	Catalyst	Temp (ºC)	Reaction time (h)	Expected product
1		DMF	$Pd(AcO)_2$	rt	72	
2				80 **MW**	1	
3				80 **MW**	1.5	
4		DMF	$Pd(AcO)_2$	rt	72	
5				80 **MW**	1	
6		DMF	$Pd(AcO)_2$	rt	19	
7				80 **MW**	1	
8				100 **MW**	0.5	
9				80 **MW**	1	
10				100 **MW**	1	
11				100 **MW**	7	
12				100 **Δ**	19	
13			$Pd(ACN)_2Cl_2$	rt	19	
14				80 **MW**	1	
15				100 **MW**	1	
16			$Pd(PPh_3)_2Cl_2$	rt	19	
17				80 **MW**	1	
18				100 **MW**	1	
19			$Pd(PEt_3)_2Cl_2$	rt	19	
20				80 **MW**	1	
21				100 **MW**	1	
22			$Pd(CF_3CO_2)_2$	rt	19	
23				80 **MW**	1	
24			$K_2Pd(SCN)_4$	100 **MW**	1.5	
25			$Pd(AcO)_2$	80 **MW**	1	

Unfortunately, all the reaction conditions tested, even the performed using the above-mentioned manuscript conditions did not work at all for the Pd-mediated hydroamination of 2,3-dihydropyran. Accordingly, we decided to drop out the related studies using Pd catalysis for the *N*-Thp protection.

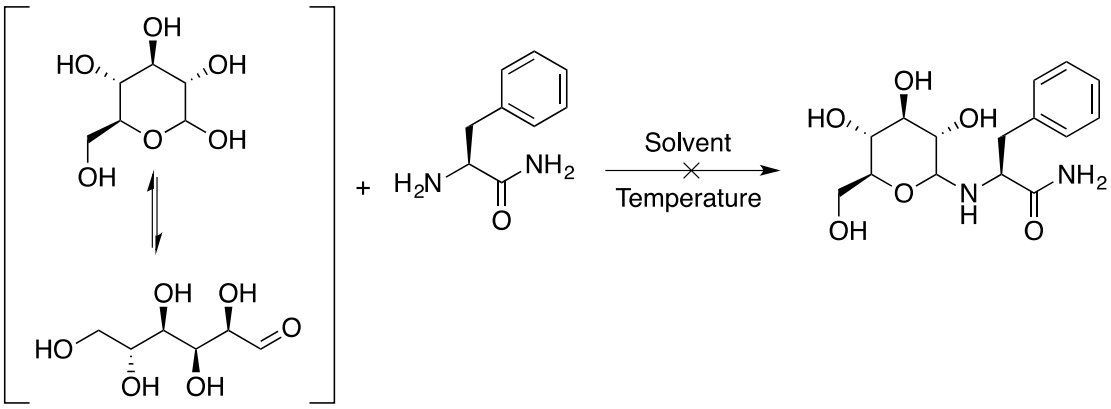
Therefore we decided to try as a final attempt the formation of an hemiaminal using the same procedures for the described synthesis of *N*-glucosamides which also contains an hemiaminal bond.[29,31] For that, first of all we hydrolyse the double bond of the 1,3-dyhydropyran ring in order to obtain the reactive aldol which afterwards we used to try the formation of an hemiaminal bond (**Scheme 15**). Again, we didn't observe the final product with the conditions tested.



Scheme 15. Attempt to phenylalanine amine tetrahydropyranylation.

Entry	Temperature	Time (h)	Yield (%)
1	Reflux 70 ºC	24	0
2	70 ºC ^{MW}	1	0
3	rt	24	0

Finally, as the final attempt to achieve the amine pyranylation, we decided to test the *N*-glycosidic bond with the commercially available glucose, which naturally we found the aldehyde on is aldol form, and as Capon and Connet postulated it is possible to attach an amine to the glucose core. Therefore, mimicking the described conditions, we try to obtain that much desired *N*-glycosidic bond (**Scheme 16**).



Scheme 16. Attempt to *N*-glycosidic bond formation using glucose and phenylalanine.

Entry	Additive	Solvent	Temperature	Time (h)	Yield (%)
1	$MgSO_4$	THF	Reflux, 80 ºC	24	0
2	-	MeOH	Reflux, 80 ºC	24	0

The results for the *N*-glycosidic bond formation using comercial glucose were found unsatisfactory. Again mimicking the reaction conditions described in the literature, we didn't observe the desired products. Due to the failed consecutives attempts to achieve the *N*-Thp protection, we decided to give up this strategy and focus on the development of the use *S*-Thp as protecting group as is explained in the following point of this chapter.

2.4. Conclusions

Alcohol, thiol and amine tetrahydropyranylation were tackled in order to find the optimal system, which could work as cleavable linker for bioconjugation. Alcohol and thiol pyranylation was successfully achieved, however the *N*-Thp protection was not reached even using the described conditions for that purpose.

Furthermore, the Thp group elimination was studied in order to find out which systems suffer the hydrolysis under similar to lysosomal degradation conditions. Considering that the thioacetal bond remains stable at the most similar lysosomal conditions studied in terms of pH (pH = 4.8), the *S*-Thp linkage lack of utility as a cleavable linker for bioconjugation. In contrast, as it publications demonstrated, the lability of the *O*-Thp at pH = 4.8 and it stability into physiological-like conditions (PBS) highlighted as outstanding cleavable linker but due to it use was previously described it lack of research utility for our purpose. Although *S*-Thp was not hydrolysed in mild acidic conditions, it acidolysis using higher acid concentration could be interesting for it use in SPPS as we deeply study in the following section.

2.5. References

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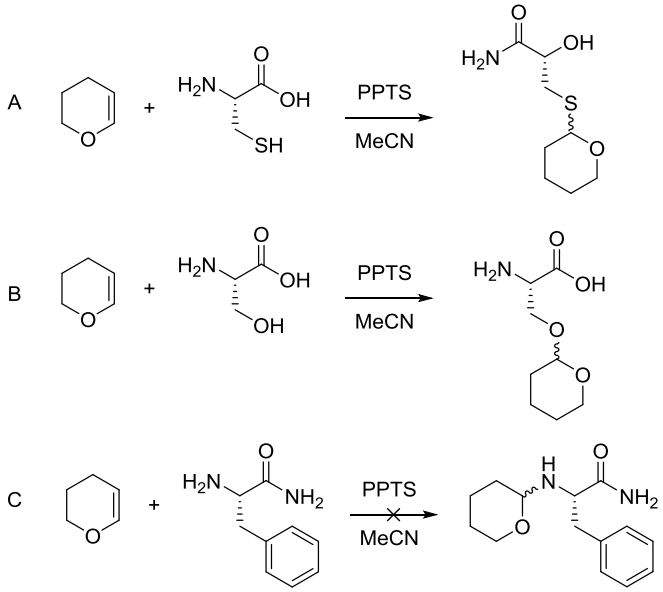
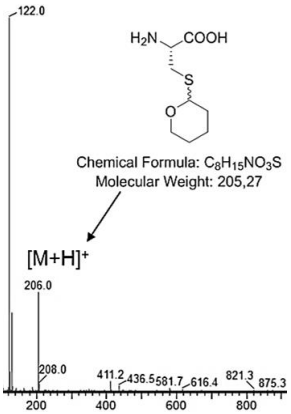
2.6. Experimental section

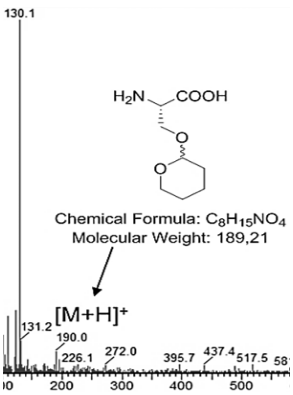
Thiol, alcohol and Amine Thp protection:



A) Cysteine pyranylation: DHP (9.4 µL, 0.1 mmol), H-Cys-OH (12.5 mg, 0.1 mmol), PPTS (2.6 mg, 0.01 mmol), MeCN (5 mL). The reaction was left to react during 1 h at rt and not appreciable change was observed by the analysis by HPLC and MS-HPLC. Then the mixture was heated during 30 min at 80 ºC using microwave. The desired product was observed by mass analysis.



B) Serine pyranylation: DHP (8.8 µL, 0.1 mmol), H-Ser-OH (10.1 mg, 0.1 mmol), PPTS (2.4 mg, 0.01 mmol), MeCN (5 mL). The reaction was left to react during 1 h at rt and not appreciable change was observed by the analysis by HPLC and MS-HPLC. Then the mixture was heated during 30 min at 80 ºC using microwave. The desired product was observed by mass analysis.



C) Phenylalanine amide pyranylation: DHP (6.0 µL, 0.07 mmol), H-Phe-NH_2 (10.8 mg, 0.07 mmol), PPTS (1.65 mg, 0.007 mmol), MeCN (5 mL). The reaction was left to react

during 1 h at rt and no appreciable change was observed by the analysis by HPLC and MS-HPLC. Then the mixture was heated during 30 min at 80 ºC using microwave. The desired product was not observed by mass analysis.

Preparation of Fmoc-Cys-OH (d1):

To a mixture of Fmoc-Cys(Trt)-OH (2.0 g, 3.4 mmol) in DCM (45 mL), TIS (0.75 mL) and TFA (5 mL) was added. The reaction mixture was stirred at room temperature for 1h. Then the solvent was removed under reduced pressure and the crude was washed and precipitated with hexane (6×40 mL). Afterwards the solid was lyophilized obtaining 1.1 g of pure Fmoc-Cys-OH (95% yield) as a white powder. **HPLC** (H_2O/MeCN from 95:5 to 0:100 over 8 min) t_R : 6.8 min). *m/z* calculated for $C_{18}H_{17}NO_4S$ = 343.4; found = 344.1 $[M+H]^+$, being M the MW of Fmoc-Cys-OH (1). **^{1}H NMR (**400 MHz, DMSO-*d*6) δ 12.86 (s, 1H), 7.90 (dt, *J* = 7.6, 1.0 Hz, 2H), 7.74 (d, *J* = 7.3 Hz, 1H), 7.69 (d, *J* = 8.2 Hz, 1H), 7.42 (t, *J* = 7.2 Hz, 1H), 7.34 (td, *J* = 7.5, 1.2 Hz, 2H), 4.34 – 4.30 (m, 2H), 4.24 (t, *J* = 7.0 Hz, 1H), 4.13 (td, *J* = 8.4, 4.4 Hz, 1H), 2.94 – 2.85 (m, 1H), 2.79 – 2.69 (m, 1H), 2.54 – 2.47 (m, 1H). **^{13}C NMR** (100 MHz, DMSO-*d*6): δ 171.8, 156.0, 143.8, 143.7, 140.7, 127.6, 127.1, 125.3, 120.1, 65.7, 56.6, 46.6, 25.4.

D) Fmoc-Cys-OH pyranylation:

Entry 1: Preparation of Fmoc-Cys(Thp)-OH (d2):

To a suspension of Fmoc-Cys-OH (101.8 mg, 0.296 mmol) and 3,4-dihydro-2*H*-pyran (DHP) (32.5 µL, 0.356 mmol) in DCM (10 mL), PPTS (7.4 mg, 0.0296 mmol) was added. Then the mixture was left to react at room temperature for 5 days under N_2. Afterwards the organic layer was washed with $NaHCO_3$ (3×20 mL), brine (3×20 mL) and water (3×20 mL) then it was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure, furnishing Fmoc-Cys(Thp)-OH as white solid (90.8 mg, 72% yield, 89% HPLC purity). **HPLC** (H_2O/MeCN from 95:5 to 0:100 over 8 min): t_R: 7.6 min. *m/z* calculated for $C_{23}H_{25}NO_5S$ = 427.1; found = 428.0 $[M+H]^+$, being M the MW of Fmoc-Cys(Thp)-OH. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.78 – 7.73 (m, 2H), 7.63 – 7.59 (m, 2H), 7.43 – 7.36 (m, 2H), 7.34 – 7.28 (m, 2H), 6.97 (d, *J* = 8.3 Hz, 1H), 6.40 (d, *J* = 6.8 Hz, 0.6H), 5.44 (s, 1.7H), 4.77 – 4.64 (m, 1.6H), 4.54 – 4.39 (m, 2H), 4.28 – 4.18 (m, 1H), 4.04 – 3.89 (m, 1H), 3.46 – 3.38 (m, 1H), 3.20 (dd, J = 14.6, 5.1 Hz, 0.4H), 3.06 (dd, J = 14.5, 6.8 Hz, 0.4H), 2.89 (dd, *J* = 14.7, 3.9 Hz, 1H), 1.99 – 1.86 (m, 1H), 1.85 – 1.77 (m, 1H), 1.74 – 1.65 (m, 1H), 1.62 – 1.50 (m, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 174.3, 156.8, 144.1, 143.9, 143.9, 141.5, 141.5, 141.4, 127.9, 127.8, 127.2, 127.2, 125.2, 125.2, 120.1, 120.0, 84.7, 83.6, 67.3, 67.0, 66.5, 65.9, 54.2, 47.3, 47.3, 35.2, 32.9, 31.6, 31.3, 25.3, 25.2, 22.6, 22.3, 15.3.

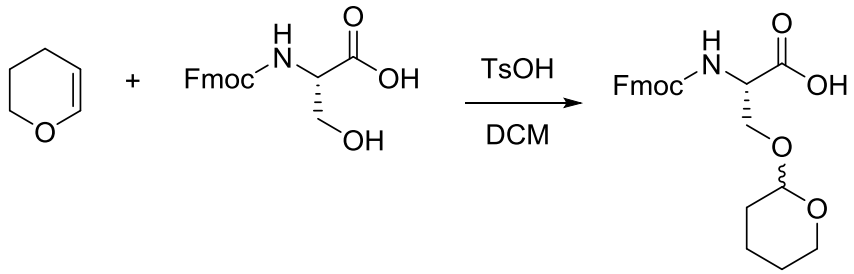
Entry 2: Preparation of Fmoc-Cys(Thp)-OH (d2):

To a suspension of Fmoc-Cys-OH (1.8 g, 5.2 mmol) and 3,4-dihydro-2*H*-pyran (DHP) (575 µL, 6.2 mmol) in DCM (40 mL), *p*-toluensulfonic acid (99.7 mg, 0.52 mmol) was added.

Then the mixture was left to react at room temperature for 1h under N_2. Afterwards the organic layer was washed with brine (3×50 mL) and water (3×50 mL) then it was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure, furnishing a withe solid (2.2 g) which was automatically purified on a pre-packed Redisep Rf Gold C18 43g column by using H_2O/MeCN from 90:10 to 0:100 over 30 min. The collected fractions were lyophilized to obtain Fmoc-Cys(Thp)-OH as a withe powder (1.95 g, 87% of yield). **HPLC** (H_2O/MeCN from 95:5 to 0:100 over 8 min): t_R: 7.6 min. *m/z* calculated for $C_{23}H_{25}NO_5S$ = 427.1; found = 428.0 $[M+H]^+$, being M the MW of Fmoc-Cys(Thp)-OH. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.78 – 7.73 (m, 2H), 7.63 – 7.59 (m, 2H), 7.43 – 7.36 (m, 2H), 7.34 – 7.28 (m, 2H), 6.97 (d, *J* = 8.3 Hz, 1H), 6.40 (d, *J* = 6.8 Hz, 0.6H), 5.44 (s, 1.7H), 4.77 – 4.64 (m, 1.6H), 4.54 – 4.39 (m, 2H), 4.28 – 4.18 (m, 1H), 4.04 – 3.89 (m, 1H), 3.46 – 3.38 (m, 1H), 3.20 (dd, J = 14.6, 5.1 Hz, 0.4H), 3.06 (dd, J = 14.5, 6.8 Hz, 0.4H), 2.89 (dd, *J* = 14.7, 3.9 Hz, 1H), 1.99 – 1.86 (m, 1H), 1.85 – 1.77 (m, 1H), 1.74 – 1.65 (m, 1H), 1.62 – 1.50 (m, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 174.3, 156.8, 144.1, 143.9, 143.9, 141.5, 141.5, 141.4, 127.9, 127.8, 127.2, 127.2, 125.2, 125.2, 120.1, 120.0, 84.7, 83.6, 67.3, 67.0, 66.5, 65.9, 54.2, 47.3, 47.3, 35.2, 32.9, 31.6, 31.3, 25.3, 25.2, 22.6, 22.3, 15.3.

E) Fmoc-Ser-OH pyranylation:

Entry 3. Preparation of Fmoc-Ser(Thp)-OH (e2):



DHP (12.3 µL, 0.13 mmol), Fmoc-Ser-OH·H_2O (40 mg, 0.12 mmol), TsOH (2.3 mg, 0.012 mmol), DCM (20 mL). The reaction was followed by HPLC analysis at 1, 3, 4 and 17 h but we finally considered that the final product decompose in the HPLC acidic conditions (~0.1% TFA). Then DHP (12.3 µL, 0.13 mmol) was added to the mixture and it was left to react during 15 min. Then the organic layer was washed with $NaHCO_3$ sat (3×20 mL) and brine (3×30 mL) and dried with $NaSO_4$. Then the solvent was reduced under reduced pressure obtaining 45 mg of a white solid (89% yield, 85% HPLC purity). The solid was analyzed by MS-HPLC using basic conditions: expected *m/z* for $C_{23}H_{25}NO_6$ = 411.5; observed *m/z* = 429.5 corresponded to $[M+NH_4]^+$.

Lability experiments:

The lability experiments were performed by using the protected Fmoc-Aa(Thp)-OH aminoacid (1 mg/mL) into mixtures which contains different percentage of acids [2-(*N*-morpholino)ethanesulfonic acid (MES) or trifluoroacetic acid (TFA)], scavengers (such as water and TIS) and DCM at room temperature. The table below indicates the acidic proportion of scavenger used in the acidolytic cocktail. Moreover it indicates the % of Fmoc-Aa-OH determined by the HPLC chromatographic peak area. The acidolytic reaction was monitored by RP-HPLC until the complete elimination of the Thp protecting group by using a linear gradient of H_2O/MeCN (from 95:5 to 0:100) over 8 min.

Entry 1: Fmoc-Ser(Thp)-OH acidolysis:

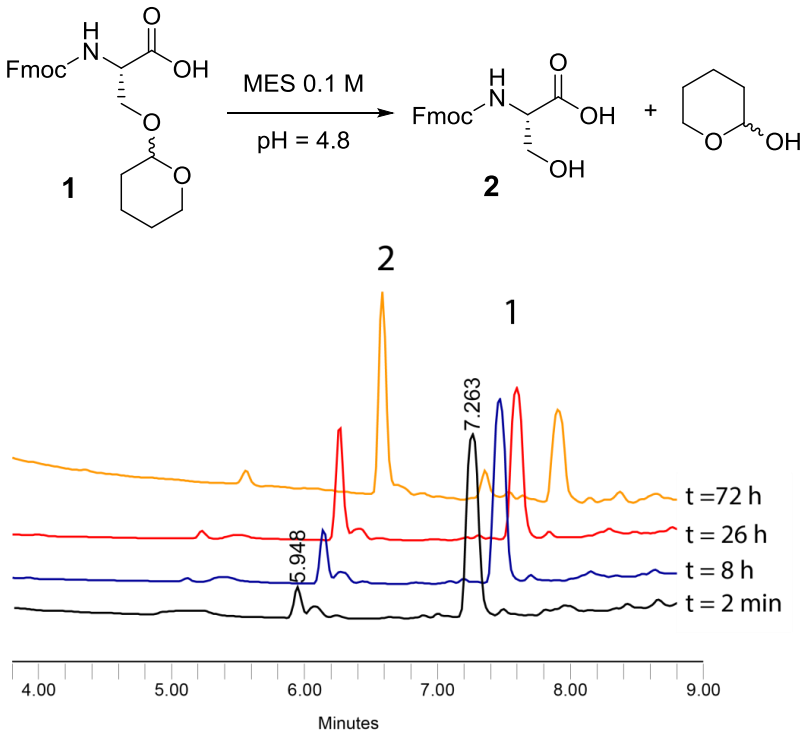


Table 10. Acid lability studies of Fmoc-Ser(Thp)-OH and Fmoc-Cys(Thp)-OH.

Entry	Compound	Cocktail		pH	Reaction time	Deprotected Cys, yield (%)	$t_{1/2}$ (h)[b]
		Composition	Quantity				
1	Fmoc-Ser(Thp)-OH	MES/H_2O	100 mM	4.8	1 h	10	74
					5 h	13	
					8 h	16	
					26 h	33	
					48 h	47	
					72 h	58	
					96 h	67	
2		MES/KCl/NaCl/H_2O	100, 2.7 and 137 mM	4.8	1 h	12	74
					5 h	14	
					8 h	18	
					26 h	27	
3		PBS buffer[a]		7.4	120 h	1	- (Stable)
4	Fmoc-Cys(Thp)-OH	MES/H_2O	100 mM	4.8	48 h	0	- (Stable)
5		MES/KCl/ NaCl/H_2O	0.1 M, 2.7 mM and 0.137 M	4.8	48 h	0	- (Stable)
6		MES/TIS/H_2O	2:1.5:96.5	4.8	48 h	0	- (Stable)
7		TFA/H_2O/DCM	1:1:98	1	1 h	9	>300
					5 h	12	
					8 h	15	
					24 h	26	
					48 h	28	
8		TFA/H_2O/DCM	10:1:89	< 1	1 h	38	3
					5 h	56	
					8 h	62	
					48 h	93.7	
9		TFA/TIS/DCM	10:1.5:88.5	< 1	5 min	>99.0	< 0.1

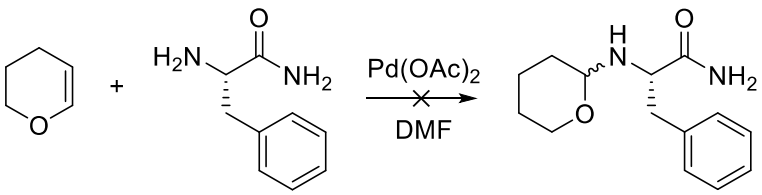
[a] PBS = 0.01 M phosphate buffer, 2.7 mM KCl and 0.137 M NaCl. [b] $t_{1/2}$ is defined as ln 2 / k. The reaction rate constant (k), were determined by the following linear equation: lnA = lnA0 – kt. Where A is the staring material HPLC peak area.

Pd-mediated amine tetrahydropyranylation experiments:

General procedure:

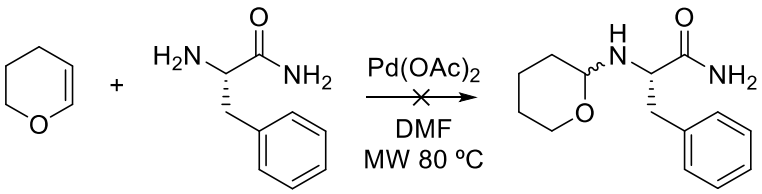
The corresponding amine was treated with 1,3-dihydropyran and the indicated Pd catalyst at the specified quantities and the mixture was left to react in the designated solvent, and temperature conditions during a period of time.

Entry 1:



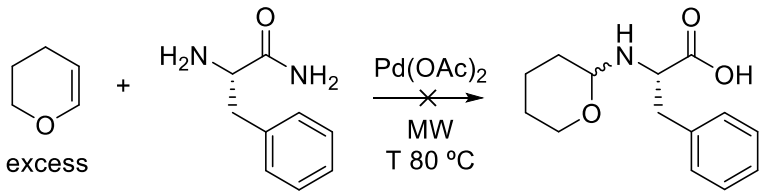
DHP (55 µL, 0.61 mmol), H-Phe-NH_2 (100 mg, 0.61 mmol), $Pd(OAc)_2$ (2.7 mg, 0.012 mmol), DMF (5 mL), room temperature. The reaction was followed by HPLC analysis at 1, 3, 5, 17 and 48 and 72 h but the final product were not observed.

Entry 2:



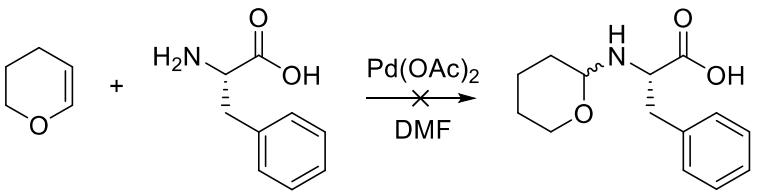
DHP (27.5 µL, 0.30 mmol), H-Phe-NH_2 (50 mg, 0.30 mmol), $Pd(OAc)_2$ (2.3 mg, 0.01 mmol), DMF (5 mL). The reaction was heated at 80 ºC during 20 min and it was analyzed by HPLC. Due to the lack of reactivity, the reaction was heated during 1 h at 80 ºC using microwave and again, the final product was not detected neither by HPLC or MS-HPLC.

Entry 3:



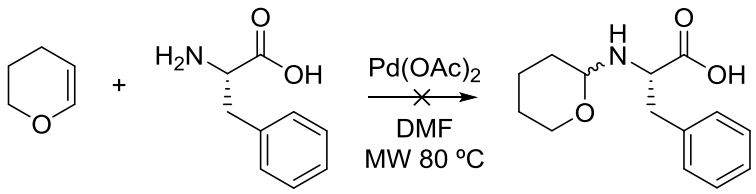
A mixture of DHP (1.0 mL, 10.9 mmol), H-Phe-NH_2 (50 mg, 0.30 mmol) and $Pd(OAc)_2$ (2.0 mg, 0.009 mmol) was heated using MW at 80 ºC during 2 h. The reaction was followed by HPLC and MS-HPLC analysis but the final product was not observed.

Entry 4:



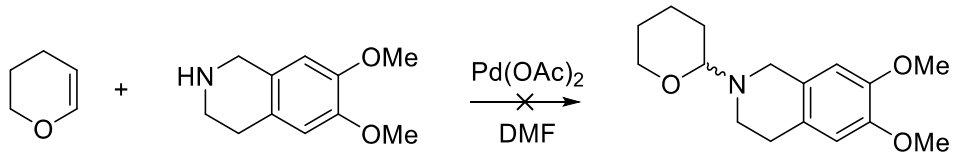
DHP (27.5 µL, 0.30 mmol), H-Phe-OH (50 mg, 0.30 mmol), $Pd(OAc)_2$ (2.1 mg, 0.001 mmol), DMF (5 mL). The reaction was followed by HPLC analysis at 1, 3, 5, 17 and 48 h but the final product was not observed.

Entry 5:



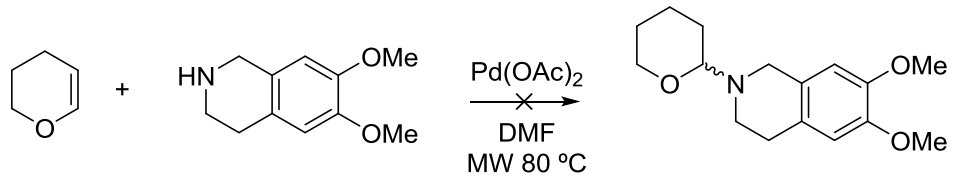
DHP (27.5 µL, 0.30 mmol), H-Phe-NH_2 (50 mg, 0.30 mmol), $Pd(OAc)_2$ (1.4 mg, 0.006 mmol), DMF (5 mL). The reaction was heated at 80 ºC during 20 min and it was analyzed by HPLC. Due to the lack of reactivity, the reaction was heated during 1 h at 80 ºC using microwave and again, the final product was not detected neither by HPLC or MS-HPLC and the starting H-Phe-OH was degraded after the MW treatment.

Entry 6:



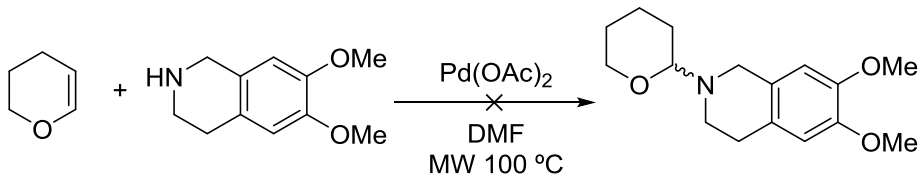
DHP (27.5 µL, 0.30 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (50 mg, 0.26 mmol), $Pd(OAc)_2$ (1.2 mg, 0.005 mmol), DMF (5 mL), room temperature. The reaction was followed by HPLC analysis at 1, 3, 5, 19 h but the final product was not observed.

Entry 7 and 9:



DHP (27.5 µL, 0.30 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (50 mg, 0.26 mmol), $Pd(OAc)_2$ (1.2 mg, 0.005 mmol), DMF (5 mL). The reaction was heated at 80 ºC during 1 h and it was analyzed by HPLC. The starting amine disappeared and a new product appeared. The solution was filtered, and the solvent was eliminated under reduced pressure.

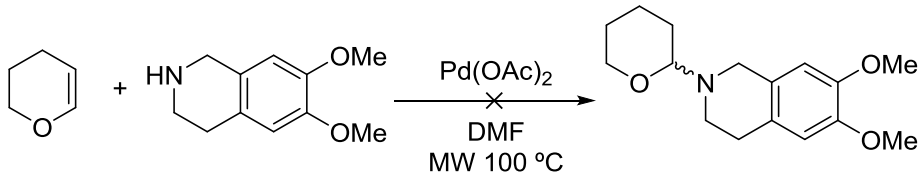
Entry 8:



DHP (47 µL, 0.52 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (100 mg, 0.52 mmol), $Pd(OAc)_2$ (2.3 mg, 0.01 mmol), DMF (2 mL). The reaction was heated at 100 ºC during 1 h and it was analyzed by HPLC at 0.5, 1 and 7 h. The starting amine disappeared and new products correspond to amine decomposition appeared. The solution was filtered, and the solvent was eliminated under reduced pressure. The crude was automatically purified on a pre-packed Redisep Rf Gold C18 13g column by using H_2O/MeCN from 90:10 to 0:100 over 20 min. The collected fractions were lyophilized obtaining 43.9, 9.2 mg of 6,7-dimethoxy-3,4-dihydroisoquinolin-1(2H)-one, 6,7-dimethoxyisoquinolin-1(2H)-one respectively.

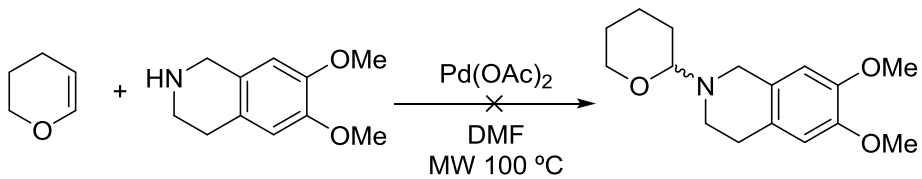
6,7-dimethoxy-3,4-dihydroisoquinolin-1(2H)-one: **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.92 (s, 1H), 7.18 (s, 1H), 6.81 (s, 1H), 3.99 (s, 3H), 3.93 (d, *J* = 8.2 Hz, 1H), 3.89 (s, 3H), 3.80 (m, 1H), 3.09 (t, *J* = 8.2 Hz, 2H). **^{13}C NMR** (100 MHz, $CDCl_3$ δ 164.7, 157.4, 149.1, 133.0, 116.9, 114.8, 111.0, 56.7, 56.4, 41.3, 24.7.
6,7-dimethoxyisoquinolin-1(2H)-one: **^{1}H NMR** (400 MHz, $CDCl_3$) δ 9.43 (s, 1H), 8.38 (d, *J* = 6.1 Hz, 1H), 7.93 (d, *J* = 6.1 Hz, 1H), 7.44 (s, 1H), 7.27 (s, 1H), 4.13 (s, 3H), 4.08 (s, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$ δ 157.8, 153.1, 142.9, 136.6, 131.0, 124.3, 122.4, 106.7, 105.1, 57.0, 56.8.

Entry 10:



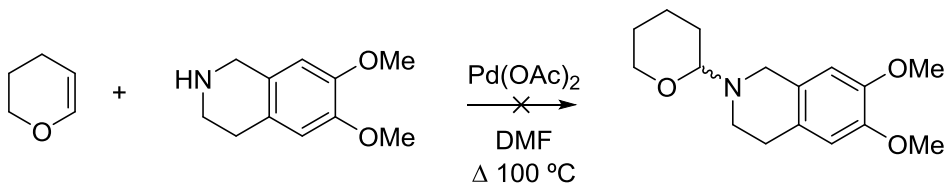
DHP (47 µL, 0.52 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (100 mg, 0.52 mmol), $Pd(OAc)_2$ (2.3 mg, 0.01 mmol), DMF (2 mL). The reaction was heated at 100 ºC during 1 h and it was analyzed by HPLC. The starting amine disappeared and new products correspond to amine decomposition appeared.

Entry 11:



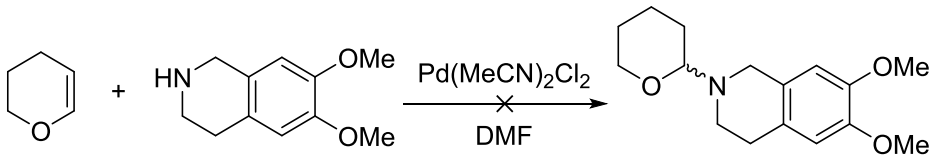
DHP (47 µL, 0.52 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (100 mg, 0.52 mmol), $Pd(OAc)_2$ (3.0 mg, 0.01 mmol), DMF (2 mL). The reaction was heated at 100 ºC during 7 h and it was analyzed by HPLC. The starting amine disappeared and new products correspond to amine decomposition appeared.

Entry 12:



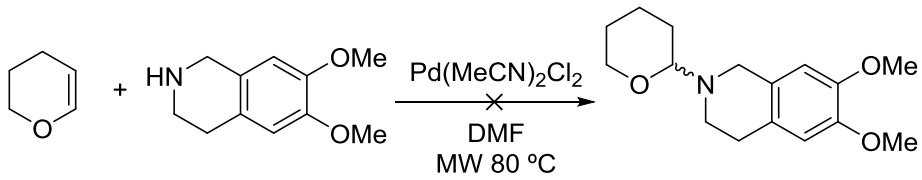
DHP (47 µL, 0.52 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (100 mg, 0.52 mmol), $Pd(OAc)_2$ (2.3 mg, 0.01 mmol), DMF (5 mL). The reaction was heated to reflux at 100 ºC during 19 h and it was analyzed by HPLC but the final product was not observed and not appreciable changes in the starting material were observed.

Entry 13:



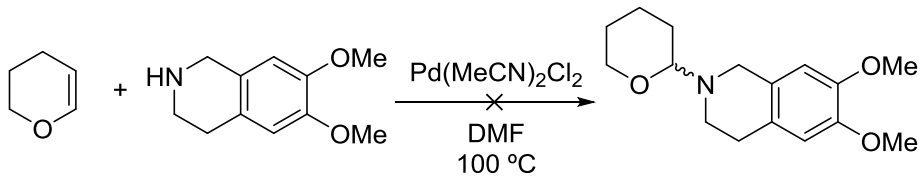
DHP (23.7 µL, 0.26 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (50 mg, 0.26 mmol), $Pd(MeCN)_2Cl_2$ (1.3 mg, 0.005 mmol), DMF (2 mL) at room temperature. The reaction was followed by HPLC analysis at 1, 3, 5, 19 h but the final product was not observed and not appreciable changes in the starting material were observed.

Entry 14:



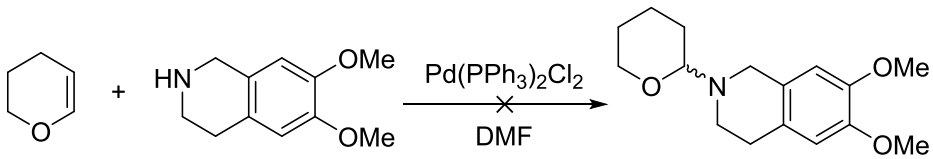
DHP (23.7 µL, 0.26 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (50 mg, 0.26 mmol), $Pd(MeCN)_2Cl_2$ (1.2 mg, 0.005 mmol), DMF (2 mL). The reaction was heated at 80 ºC during 1 h and it was analyzed by HPLC. The starting amine disappeared and new products correspond to the amine decomposition appeared. The solution was filtered, and the solvent was eliminated under reduced pressure.

Entry 15:



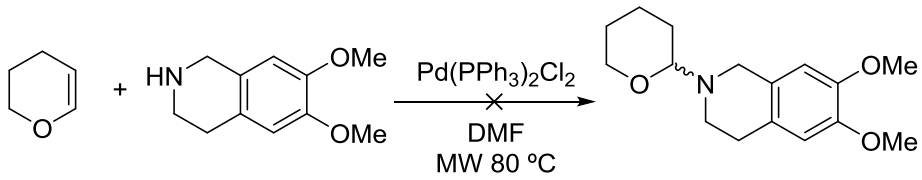
DHP (23.7 µL, 0.26 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (50 mg, 0.26 mmol), $Pd(MeCN)_2Cl_2$ (1.2 mg, 0.005 mmol), DMF (2 mL). The reaction was heated at 100 ºC during 1 h and it was analyzed by HPLC. The starting amine disappeared and new products correspond to the amine decomposition appeared. The solution was filtered, and the solvent was eliminated under reduced pressure.

Entry 16:



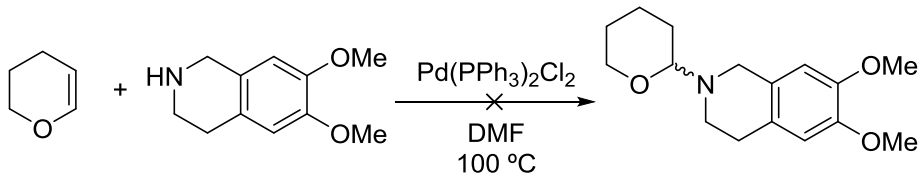
DHP (23.7 µL, 0.26 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (50 mg, 0.26 mmol), $Pd(PPh_3)_2Cl_2$ (3.6 mg, 0.005 mmol), DMF (2 mL) at room temperature. The reaction was followed by HPLC analysis at 1, 3, 5, 19 h but the final product was not observed and not appreciable changes in the starting material were observed.

Entry 17:



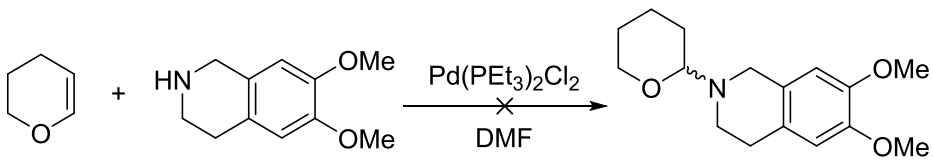
DHP (23.7 µL, 0.26 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (50 mg, 0.26 mmol), $Pd(PPh_3)_2Cl_2$ (3.6 mg, 0.005 mmol), DMF (2 mL). The reaction was heated at 80 ºC during 1 h and it was analysed by HPLC but the final product was not observed and not appreciable changes in the starting material were observed. The solution was filtered, and the solvent was eliminated under reduced pressure.

Entry 18:



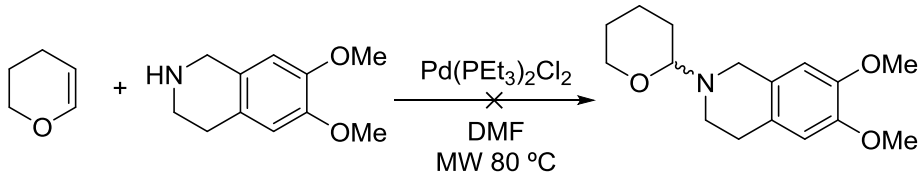
DHP (23.7 µL, 0.26 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (50 mg, 0.26 mmol), $Pd(PPh_3)_2Cl_2$ (3.6 mg, 0.005 mmol), DMF (2 mL). The reaction was heated at 100 ºC during 1 h and it was analysed by HPLC but the final product was not observed and not appreciable changes in the starting material were observed. The solution was filtered, and the solvent was eliminated under reduced pressure.

Entry 19:



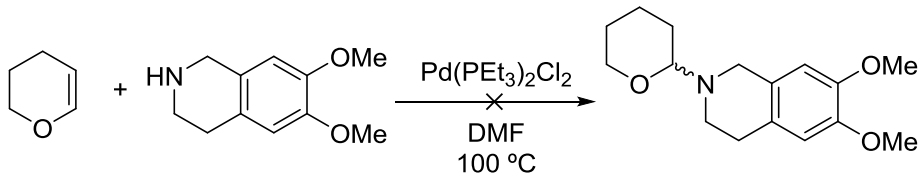
DHP (23.7 µL, 0.26 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (50 mg, 0.26 mmol), $Pd(PEt_3)_2Cl_2$ (2.1 mg, 0.005 mmol), DMF (2 mL). The reaction was followed by HPLC analysis at 1, 3, 5, 19 h but the final product was not observed and not appreciable changes in the starting material were observed.

Entry 20:



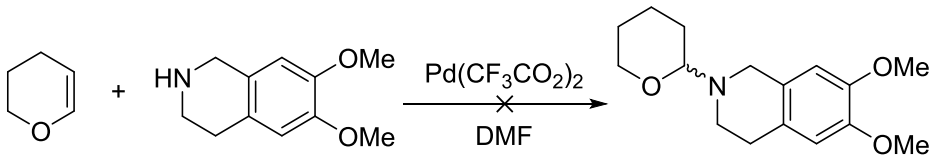
DHP (23.7 µL, 0.26 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (50 mg, 0.26 mmol), $Pd(PEt_3)_2Cl_2$ (2.1 mg, 0.005 mmol), DMF (2 mL). The reaction was heated at 80 ºC during 1 h and it was analyzed by HPLC. The starting amine partially disappeared and new products correspond to amine decomposition appeared. The solution was filtered, and the solvent was eliminated under reduced pressure.

Entry 21:



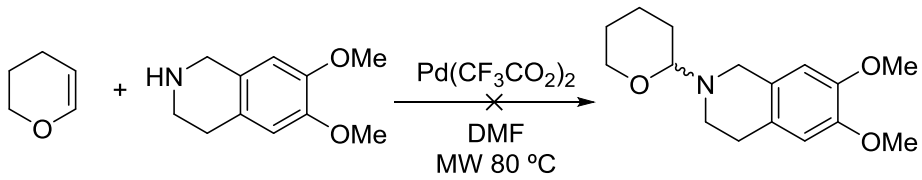
DHP (23.7 µL, 0.26 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (50 mg, 0.26 mmol), $Pd(PEt_3)_2Cl_2$ (2.1 mg, 0.005 mmol), DMF (2 mL). The reaction was heated at 100 ºC during 1 h and it was analyzed by HPLC. The starting amine partially disappeared and new products correspond to amine decomposition appeared. The solution was filtered, and the solvent was eliminated under reduced pressure.

Entry 22:



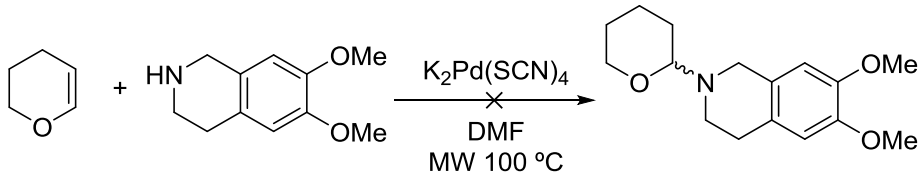
DHP (24 µL, 0.26 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (50 mg, 0.26 mmol), $Pd(CF_3CO_2)_2$ (1.7 mg, 0.005 mmol), DMF (2 mL). The reaction was followed by HPLC analysis at 1, 3, 5, 19 h but the final product was not observed and not appreciable changes in the starting material were observed.

Entry 23:



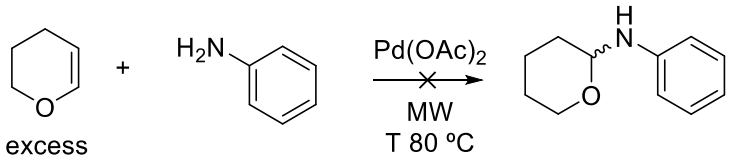
DHP (23.7 µL, 0.26 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (50 mg, 0.26 mmol), $Pd(CF_3CO_2)_2$ (1.7 mg, 0.005 mmol), DMF (2 mL). The reaction was heated at 80 ºC during 1 h and it was analyzed by HPLC. The starting amine disappeared and new products correspond to amine decomposition appeared. The solution was filtered, and the solvent was eliminated under reduced pressure.

Entry 24:



DHP (27 µL, 0.29 mmol), 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (25 mg, 0.13 mmol), $K_2Pd(SCN)_4$[28] (4.0 mg, 0.01 mmol), DMF (2 mL). The reaction was heated at 100 ºC during 1.5 h and it was analyzed by HPLC. The starting amine partially disappeared and new products correspond to amine decomposition, appeared. The solution was filtered, and the solvent was eliminated under reduced pressure.

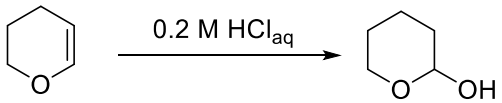
Entry 25:



A mixture of DHP (1.0 mL, 10.9 mmol), aniline (50 µL, 0.55 mmol) and $Pd(OAc)_2$ (1.2 mg, 0.005 mmol) was heated using MW at 80 ºC during 2 h. The reaction was followed by HPLC and MS-HPLC analysis but the final product was not observed.

Attempt to *N*-Glycosidic bond formation:

Dyhydropyran hydrolysis[32]

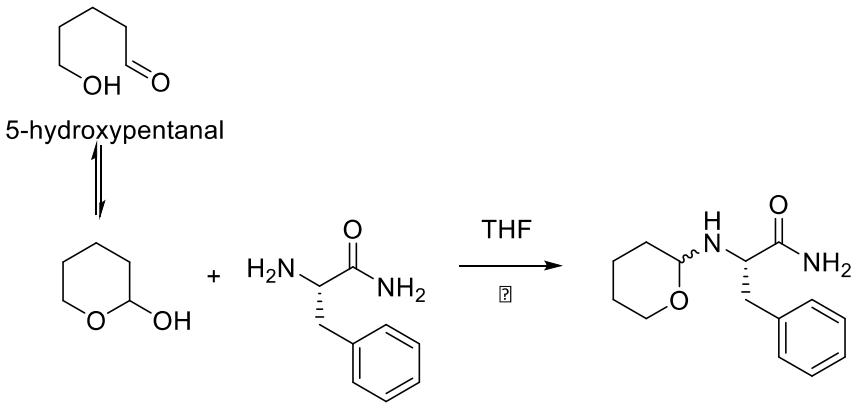


To 2,3-dihydropyran (7.0 g, 83.2 mmol), 0.2 M HCl aq. (15 mL) was added at 0 ºC and it was stirred during 15 min, and afterwards at room temperature during 1 h. The mixture was extracted with DCM and the combined organic layer was washed with $NaHCO_3$ (3×20 mL), and brine (3×20 mL) and dried over $MgSO_4$, filtered and the organic solvent was removed under reduced pressure to yield a colorless oil which was automatically purified on a pre-packed Redisep Gold Silica 13g column by using hexane/AcOEt from 100:0 to 0:100 over 20 min. The solvent from the collected fractions was eliminated under reduced pressure to yield the cyclic hemiacetal (1.86 g, 22 %) and a complex mixture which contains the linear hemiacetal and the dimerization product.

Tetrahydro-2*H*-pyran-2-ol **^{1}H NMR** (400 MHz, $CDCl_3$) δ 4.90 – 4.76 (m, 1H), 4.71 (s, 1H), 3.97 – 3.82 (m, 1H), 3.53 – 3.38 (m, 1H), 1.79 – 1.63 (m, 2H), 1.46 – 1.32 (m, 4H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 94.2, 63.6, 31.8, 25.1, 20.2.

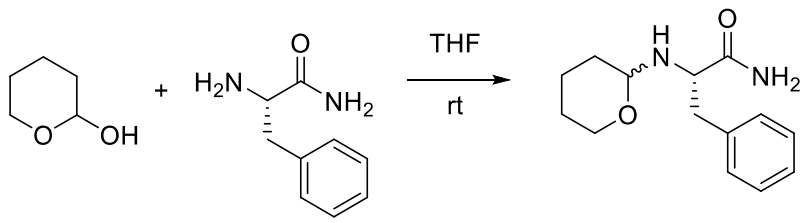
5-hydroxy pentanal and H-Phe-NH_2 condensation:

Entry 1: [31]



THP-OH (29 µL, 0.30 mmol), H-Phe-NH_2 (50 mg, 0.30 mmol), THF (10 mL). The reaction was heated to reflux at 80 ºC during 19 h and it was analyzed by HPLC and MS-HPLC but the final product was not detected.

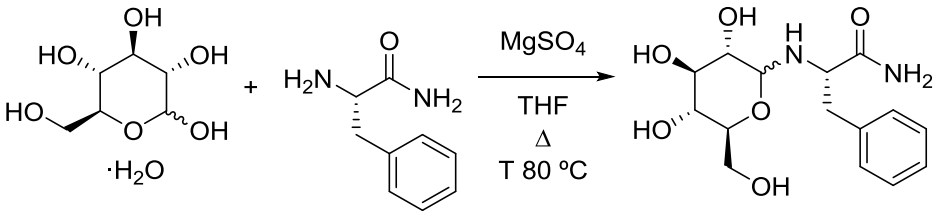
Entry 2:



THP-OH (344.5 mg, 3.4 mmol) and H-Phe-NH_2 (558 mg, 3.4 mmol) in THF (2 mL) was stirred at room temperature during 24 h and it was analyzed by HPLC and MS-HPLC but the final product was not detected.

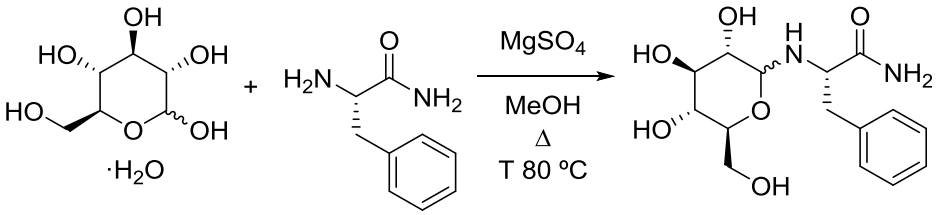
Glucose and H-Phe-NH_2 condensation:

Entry 1:



Glucose·H_2O (77 mg, 0.43 mmol), H-Phe-NH_2 (66 mg, 0.40 mmol), THF (15 mL). The reaction was heated to reflux at 80 ºC during 4 h and it was analyzed by HPLC and MS-HPLC but the final product was not detected.

Entry 2: [29]



Glucose·H_2O (109.7 mg, 0.60 mmol), H-Phe-NH_2 (100 mg, 0.60 mmol), MeOH (20 mL). The reaction was heated to reflux at 80 ºC during 2 h and then the mixture was cooled to room temperature. Then the solvent was removed until 10 mL approximately and Et_2O was added to the mixture and it was crystalized at 0 ºC. A white powder was obtained but no changes were observed neither by HPLC and MS-HPLC. By NMR we observed the glycosidic epimerization of the starting glucose.

Chapter I. Cleavable linkers

3. Tetrahydropyran as a Cys protecting group for Fmoc chemistry.

The following results were published on:

Ramos-Tomillero, I.; Rodríguez, H.; and Albericio, F. Tetrahydropyranyl, a Nonaromatic Acid-Labile Cys Protecting Group for Fmoc Peptide Chemistry. *Org. Lett.* **2015,** *17*, 1680–1683.

3.1. Introduction

Since the discovery of solid-phase peptide synthesis (SPPS) by Merrifield,[1] the peptide field has suffered a dramatic change in a wide set of points such as the development of new solid supports, the discovery of highly efficient coupling reagents and additives, as well as the fashionable design of linkers and protecting groups. Consequently, the SPPS is considered the most efficient way to synthesize a broad number of complex molecules. Into the complete process of this synthetic methodology, a large number of side reactions occur, in consequence during the past decades tremendous efforts have been done to avoid these drawbacks that jeopardize the efficiency of this procedure. Among all known side reactions, amino acid racemization is still an issue in SPPS because of the similarity between the desired product and the concomitant racemic product. In this sense, several factors such as coupling reagents, handles or protecting groups, or even the basic conditions used during the peptide synthesis, could affect directly the integrity of the α-proton, which are able to modulate the amino acid racemization.

Although SPPS is highly efficient, amino acid racemization is still an issue and measures for its minimization are needed. Of the repertoire of natural amino acids, cysteine (Cys) and histidine (His) are the most problematic due to its high tendency to lose the integrity.[2–4]

In nature, Cys is crucial due to its capacity to confer stability to peptides and proteins through the disulfide bonds formation, which also constrain the specific conformation of these biomolecules.[5,6] In addition, its use has modernized the chemical synthesis of large polypeptides and proteins by Native Chemical Ligation.[7] Accordingly, Cys is one of the most problematic amino acid in SPPS, due to the high instability of its α-proton by the proximity of the thiol group, which promotes the racemization of its chiral center. In this regard, it has been shown that the degree of racemization depends on the protection of the β-thiol group, which can modulate the acidity of the α-proton.[4] Furthermore, β-elimination followed by piperidine addition, oxidation and alkylation are other examples of potentials Cys side reactions.[8] All these Cys collateral reactions are responsible of the complex crude products obtained during the synthesis of Cys-rich peptides.[9] Nowadays, extensive possibilities to avoid these Cys promoted side reactions have been studied. In that sense, the development of new coupling reagents and additives for SPPS, assisted the by-products minimization.[10] Additionally, special efforts are focused into Cys side chain protection and as a result, a

myriad of Cys protecting groups stable to a set of chemical conditions have been developed for their use in SPPS.[3]

The stability and lability of protecting groups play an important role into the strategy selection criteria for SPPS. While Boc/Bzl chemistry requires base-labile protecting groups, the Fmoc/*t*Bu strategy involves the use of acid cleavable groups. The conferred stability is related to the kind of binding between the β-thiol group of Cys and the protecting group. As reported in the literature, thioether linkage is the most commonly used thiol protection form, due to the stability under several conditions. Thus, in recent years the development of the most successful protecting groups for Cys are based on this special linkage. In this regard, herein we will focus our attention in acid-labile Cys protecting groups in general, and in the development of a non-aromatic acid-labile Cys protecting group, in particular.

Acid-labile Cys protecting groups

The conscious design of protecting groups had triggered a myriad of groups (**Figure 18**), which are stable under different range of acidic conditions. Therefore, the selective protecting group elimination is possible, depending on the acidic conditions used.

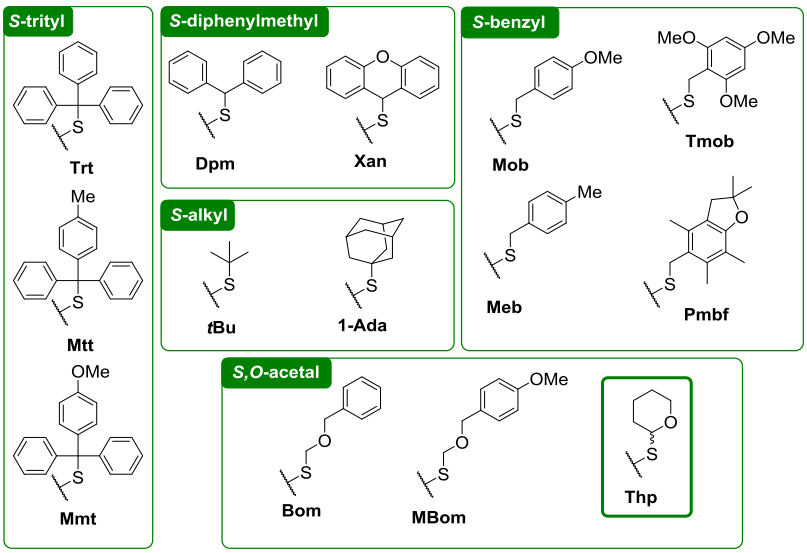


Figure 18. Acid-labile Cys protecting groups.

As a result of years of studies in the field, there are several Cys protecting groups available for both liquid or solid phase peptide synthesis; some of them are labile in low acid concentration such as Mmt,[11] Trt[12–14] or Thp;[15] others are more stable in low concentrations of trifluoroacetic acid (TFA) but cleavable using higher quantity of TFA - Diphenylmethyl

(Dpm),[12,14,16] *t*Bu[17,18] or 4-methoxybenzyloxy-methyl (MBom)[19] and moreover, there are groups which are completely stable to TFA and removable using harsh conditions such as HF, for example Bom,[20] Meb or Mob[21–23] groups.

Due to their chemical properties, most protecting groups and linkers used in SPPS[24] are based on benzyl, diphenylmethyl, and triphenylmethyl structures. The mechanisms of elimination are based on the protonation of the thiol group, and followed by the carbocation formation. Herein, the stability of the liberated carbocation in acidic conditions manages the protecting group lability. Consequently, the more acid labile the more stable carbocation.[25] Some factors contribute to their stability such as steric hindrances or inductive effects, but the most imperative effect correspond to the electronic delocalization, which increase the carbocation stability.[26]

Tetrahydropyranyl (Thp) for Fmoc peptide Chemistry

While the most used thiol protection is in form of thioethers, trityl-, diphenylmethyl-, and benzyl-like scaffolds are the most used for the protection of Cys, the groups of Yajima and Nishiuchi developed *S,O*-acetal protecting groups, such as the benzyloxymethyl (Bom) for Boc chemistry,[20] and more recently MBom for Fmoc chemistry.[19] Bom and MBom, whose syntheses are not straightforward, result in a very low level of racemization;[4] however, their use can hamper the quality of the final product because formaldehyde is formed as a side product during cleavage and accompanied by concomitant hydroxymethylation.

Regarding the molecular structures (**Figure 1**), most of the protecting groups available contain aromatics rings, except *t*Bu, 1-Ada and Thp. In that sense, in the production of protected hydrophobic peptides, the use of bulky protecting groups in SPPS, such as trityl-like scaffolds as well as *t*Bu, 1-Ada, affects their inter/intrachain Interaction during peptide elongation and this fact jeopardize the purity of the final product. Thp is extensively used as hydroxyl protecting group[27–30] as well as a linker,[31–34] due to its chemical properties and its stability in strong bases, and acylating and alkylating agents among others.

With the same idea of exploiting the *S,O*-acetal protecting group concept, in the present thesis we introduced tetrahydropyranyl (Thp) as Cys protecting group[15] for SPPS. Thp has the advantage over benzyl-based protecting groups (Trt, Dmp, Mmt, Bom) that it lacks of aromaticity. In addition to producing more protected hydrophobic peptides, the use of bulky

aromatic protecting groups in SPPS affects to the inter/intra-chain interaction during peptide elongation and therefore jeopardizes the purity of the final product.[3,6] Accordingly, Thp is an excellent candidate for use in SPPS, but it is known that the main drawback should be the formation of a new stereocenter that leads to diastereomeric mixtures. Nevertheless, as with other protecting groups or linkers, their use is temporary. Consequently, the formation of the stereocenter is not a limitation.

3.2. Objectives

- Study and optimize Cys thiotetrahydropyranilation.
- Study and optimize Cys-Thp elimination and compare it stability with other commercially available Cys protecting groups.
- Study Thp stability and racemization ratio during SPPS synthesis for inner, C and N-terminal Cys.
- Use Fmoc-Cys(Thp)-OH for the synthesis of Cys-containing peptides.

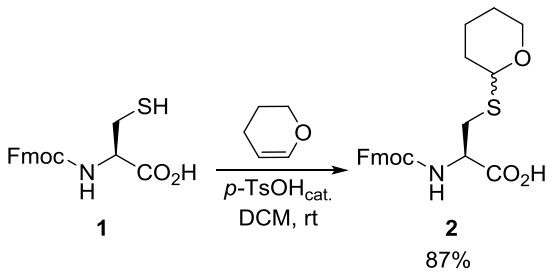
3.3. Results and discussion

In order to amplify the methodological spectrum of peptide synthesis, especially for Cys-containing molecules, and taking to account that *S,O*-acetal is an excellent choice as Cys protecting group, here we present Thp as a non-aromatic Cys protecting group for the Fmoc/*t*Bu strategy in SPPS. Although Thp was introduced by Holland *et al.*[15] as a Cys protecting group half century ago for the synthesis in solution of insulin peptides, to date it has not been used in Fmoc chemistry.

The Thp group has been widely used as hydroxyl protecting group due to the stability of the acetal derivative towards strongly basic conditions, organometallics, hydrides, and acylating and alkylating reagents. Thp elimination is usually performed in acidic media through the hydrolysis or alcoholysis of the acetal bond, but also through various Lewis acids.[29,30]

The main drawback of the use of Thp in organic synthesis, is the formation of a new stereocenter that leads to diastereomeric mixtures; however, when used as protecting group, the chiral center formed is temporary. Consequently, the formation of the stereocenter is not a shortcoming.

Fmoc-Cys(Thp)-OH (**2**) was synthesized in good yield by means of the acid-catalyzed reaction between Fmoc-Cys-OH (**1**) and the versatile vinyl ether dihydropyran (DHP), using *p*-toluene sulfonic acid (*p*-TsOH) as catalyst and DCM as solvent (See **Scheme 17**).



Scheme 17. Synthesis of Fmoc-Cys(Thp)-OH (2).

In order to determine the acid stability of Thp as protecting group, Fmoc-Cys(Thp)-OH (**2**) were studied under a range of acidolytic conditions (**See Table 11**).

Table 11. Acid lability studies of Fmoc-Cys(Thp)-OH (2).

Cocktail			Reaction time	Deprotected Cys, yield (%)
Entry	Composition	Quantity (%)[a]		
1	MES/H_2O	2:98	48 h	0
2	MES/TIS/H_2O	2:1.5:96.5	48 h	0
3	TFE/AcOH/DCM	20:20:60	48 h	4.7
4	TFA/H_2O/DCM	1:1:98	48 h	28.5
5	TFA/H_2O/DCM	10:1:89	48 h	93.7
6	TFA/DCM	1:99	24 h	27.4
7	TFA/DCM	10:90	24 h	89.0
8	TFA/TIS/DCM	1:1.5:97.5	2 h	98.5
9	TFA/TIS/DCM	10:1.5:88.5	5 min	>99.0
10	HCl/dioxane	12:88	2 h	>99.0
11	HCl/TIS/dioxane	12:1.5:86.5	2 h	>99.0

a % *w/v* for MES (Entries 1 and 2) and % *v/v* for the others (Entries 3-11).

The acid lability of the Thp group was strongly increased by the presence of triisopropylsilane (TIS) (# 4, 6 *vs.* 8 and 5, 7 *vs.* 9) (**Table 11**). Interestingly, HCl in dioxane (4M) removed Thp. Therefore, conventional cleavage conditions such as TFA/TIS/DCM (10:2.5:87.5), TFA/TIS/H_2O (95:2.5:2.5) and 0.1 N HCl/HFIP-TIS (99:1)[35] will ensure complete elimination of Thp in short treatments.

Inner Cys studies

In order to determine a broader compatibility of the Cys(Thp)-containing peptide with continuous piperidine treatments and coupling conditions, Fmoc-Ala-Cys(Thp)-Leu-X (X=NH_2, **3a** and X=OH, **4a**) were prepared by SPPS using Sieber amide (**3a**) and 2-Chlorotrityl chloride (2CTC) (**4a**) resins as solid supports (**Figure 19, A** and **B**). Moreover, the performance of Thp as Cys protecting group was studied by comparison of the synthesis of **3a** and **4a** with several Fmoc-Ala-Cys(PG)-Leu-X synthesized using as Cys protecting groups (PG): Trt (**3b-c,** and **4b-c**), Dpm (**3d**), and Acm (**3e**) (**Figure 19**).[4] For the incorporation of the protected Cys derivatives, a 5-min pre-activation procedure using Fmoc-Cys(PG)-OH/DIPCDI/Oxyme Pure (2 eq. of each compound) in DMF for 90 min at room temperature was used, which is described to minimize the racemization.[36]

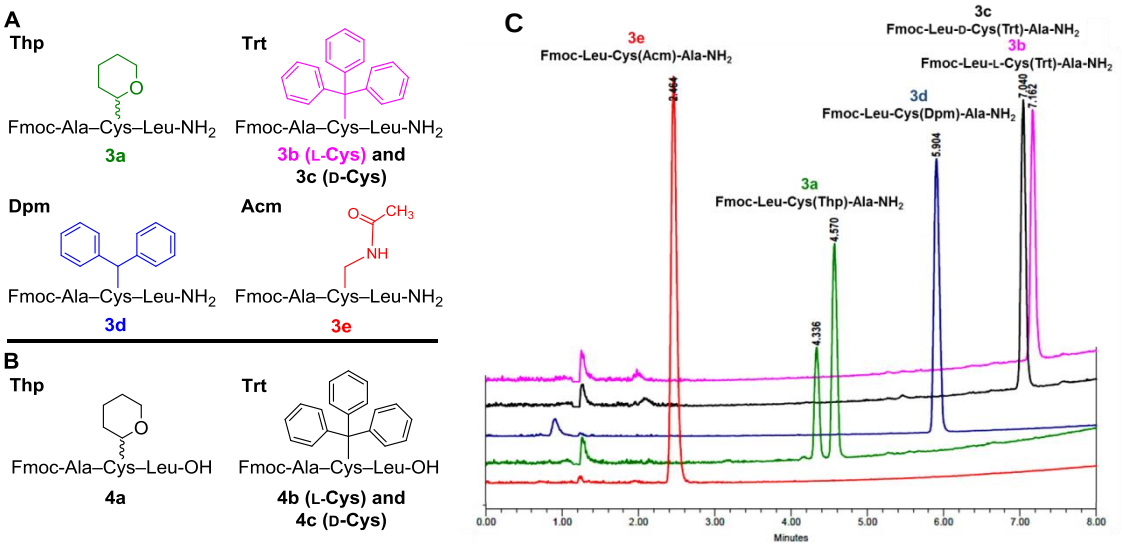


Figure 19. Fully protected synthesized Cys-containing tripeptides on A: Sieber amide resin and B: 2-Chlorotrityl chloride resin. And RP-HPLC profiles of the synthesized C-terminal amide tripeptides 3a-e. Linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min.

All synthesized tripeptides (**3a-e**, **4a-c**) were cleaved from the resin using low TFA concentrations [TFA/DCM (1:99)] and analyzed by RP-HPLC. The chromatograms for the C-terminal amides (**3a-e**) (**Figure 19**, **C**) revealed that the Thp group was stable during the tripeptide elongation and also during cleavage from the resin. Similar behaviour was observed for C-terminal acid tripeptides (**4a-c**) (See SI). The RP-HPLC profiles also corroborate that the use of Thp as thiol-protecting group (**3a**) rendered a more soluble tripeptide, in comparison that achieved with the commonly used Trt (**3b-c**) and Dpm (**3d**) groups. This parameter is relevant during the synthesis of Cys-containing protected peptides.

Furthermore, as expected given the particularity of Thp, a mixture of diastereoisomers was observed in the isolated tripeptide cleaved under low TFA concentrations (**Figure 20A**). Nevertheless, using higher concentrations of TFA in the presence of scavenger [TFA/TIS/DCM (95:2.5:2.5)] as cleavage conditions, only a single pure product (**3**) was obtained (**Figure 20B**).

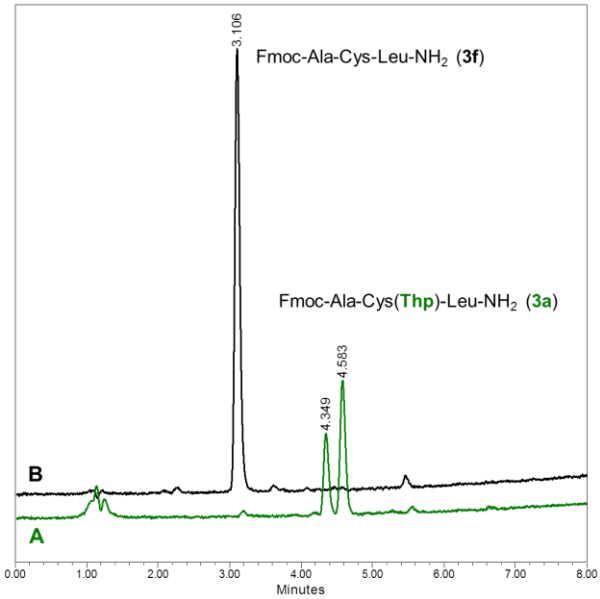


Figure 20. RP-HPLC profiles of A: Fmoc-Ala-Cys(Thp)-Leu-NH_2 (3a), and B: Fmoc-Ala-Cys-Leu-NH_2 (3), using TFA/DCM (1:99), and TFA/TIS/DCM (95:2.5:2.5) as cleavage conditions, respectively, Linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min.

Fmoc removal followed by peptide cleavage with TFA/TIS/DCM (95:1.5: 1.5) yielded H-Ala-Cys-Leu-X tripeptides (X=NH_2, **3'** and X=OH, **4'**). When Thp was used (**3a**), an inappreciable level of racemization ([D-peptide/L-peptide]×100 = 0.74) compared with Trt (**3b**, D/L = 3.3) and Dpm groups (**3d**, D/L = 6.8) was observed (**Table 12**). The model tripeptides were also synthesized under the same coupling conditions, by using Rink amide and 2-chlorotrityl chloride resins, which yielded similar crude products and also low racemization levels as **Table 12** illustrates.

Table 12. Cys racemization studies for Ala-Cys-Leu tripeptide.

Resin	Cys PG	Compound	% Racemization[a]
Sieber Amide	Thp	3a	0.7
	Trt	3b	3.3
	Dpm	3d	6.8
Rink Amide	Thp	3a	0.5
	Trt	3b	2.3
	Dpm	3d	1.6
2-Chlorotrityl chloride	Thp	4a	0.2
	Trt	4b	0.7

a Calculated as (D-peptide peak area/L-peptide peak area)x100

C-terminal Cys studies

As mentioned above, the synthesis of *C*-terminal Cys-containing acid peptides showed increased difficulty due to racemization and formation of 3-(1-piperinidyl)alanine derivatives in response to the repetitive piperidine treatments for Fmoc elimination.[37]

In order to determine the racemization level of the C-terminal cysteine, Boc-Ala-Leu-Cys(PG)-O-Resin (PG = Thp or Trt) were synthesized, both peptides immobilized in 2CTC and Wang resins. Peptidyl-resins were exposed to piperidine-DMF (1:4) (standard basic conditions for Fmoc removal in SPPS), and then a small amount was collected after 2, 4, 6 and 24 h. Each sample was cleaved with TFA/TIS/H_2O (95:2.5:2.5), and the tripeptide crude products were then analyzed by RP-HPLC to determine racemization. The racemization level was determined by the analysis of the area under the curve (AUC) corresponding to H-Ala-Leu-*L*-Cys-OH (**5a**) and H-Ala-Leu-*D*-Cys-OH (**5b**) present in the tripeptides synthesized in the two resins (**Figure 21**).

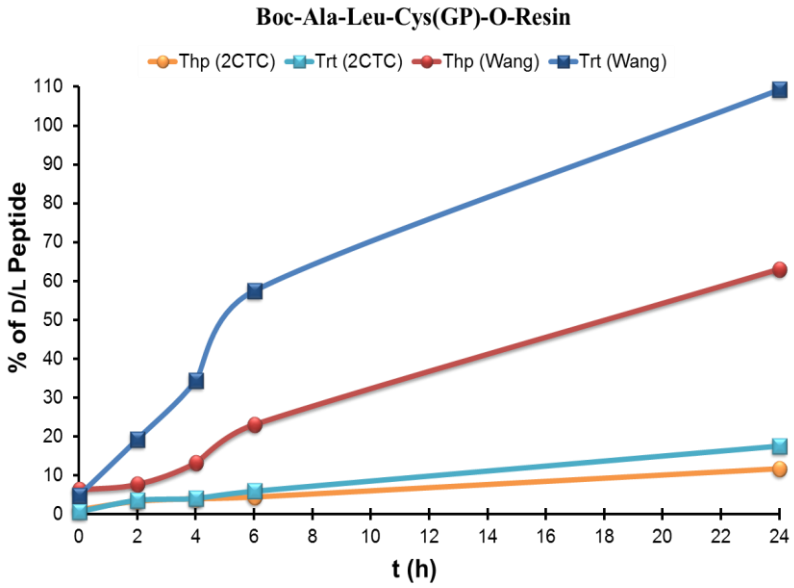


Figure 21. C-terminal cysteine tripeptide racemization studies for Boc-Ala-Leu-Cys(PG)-O-Resin (2CTC and Wang resins), using piperidine/ DMF (1:4) for 24 h. Racemization ratio was defined as (AUC to 5b / AUC to 5a)×100

As reported in similar studies,[37] the racemization ratio was higher for Wang than 2CTC resin. Moreover, for both resins, a greater racemization level was obtained when the Trt group was used to protect C-terminal Cys, and therefore, the racemization of the Cys-

containing Thp group in the C-terminal was considerably lower than the same Trt-protected tripeptide.

Furthermore, the formation of the Ala-Leu-[3-(1-piperidinyl)alanine]-OH (**5d**) was also detected, and its extension was monitored for the SPPS of C-terminal Cys tripeptides **5a** and **5c** in both Wang and 2CTC resins (**Figure 22**).[8] In addition, the alkylation of the thiol group with a *p*-hydroxybenzyl (**5e-f**) $[M+107]^+$ was detected in the SPPS conducted on Wang resin under standard cleavage conditions [TFA/TIS/H_2O (95:2.5:2.5)]. This side-product resulting from the undesired cleavage of the Wang linker.[9,38–40]

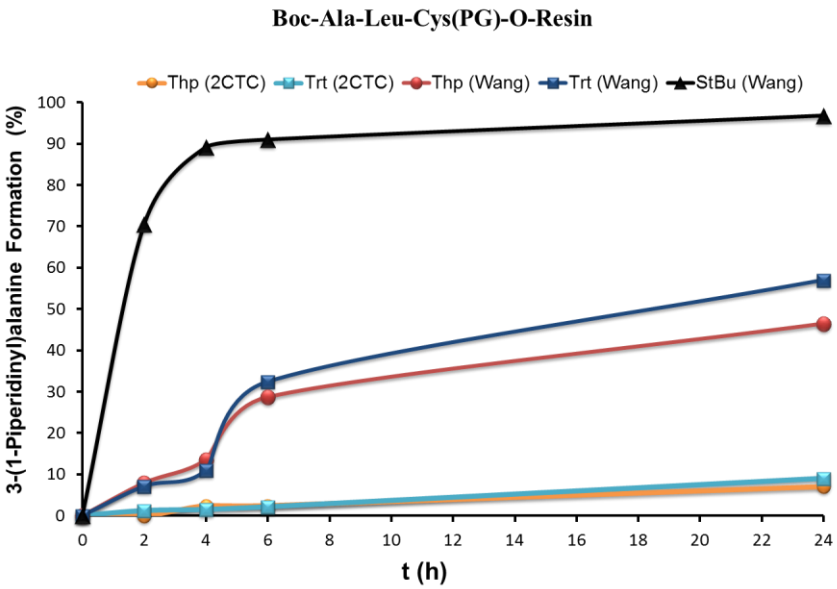
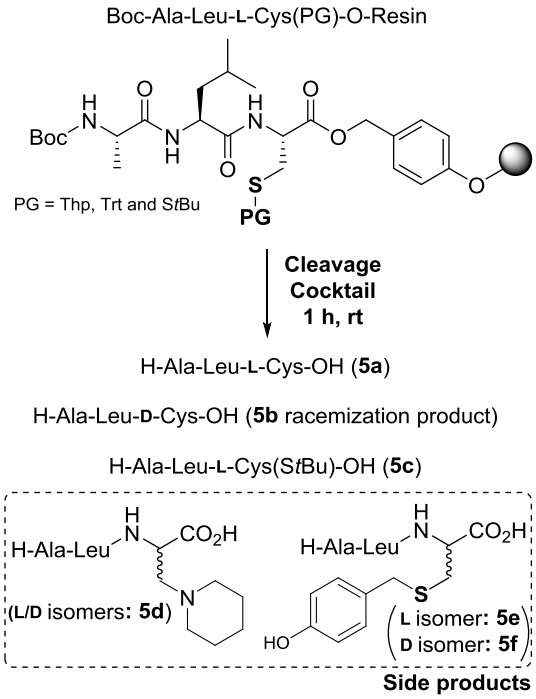


Figure 22. Formation of Ala-Leu-[3-(1-piperidi-nyl)alanine]-OH (5d) during the piperidine/DMF treatment of Boc-Ala-Leu-Cys(PG)-O-Resin. Percentage of 5d was calculated considering the area under the curve (AUC) of 5d, and D/L-tripeptides in the crude product analyzed 5a-b.

The formation of this side-product was reduced using [TFA/TIS/DMB (92.5:2.5:5)] as cleavage cocktail (**Scheme 18**, products from the cleavage from Wang resin).



Scheme 18. Obtained C-terminal Cys tripeptides from Wang resin **5a-c**, and their side-products **5d-f**.

As expected, when *t*Bu was used as a Cys protecting group, the product from the cleavage reaction was the thiol (S*t*Bu)-protected *C*-terminal Cys tripeptide (**5c**).[41]

Comparing the results between Boc-Ala-Leu-Cys(PG)-OH, the protecting groups of which were Thp, Trt and S*t*Bu, we conclude that Thp presented lower *C*-terminal Cys racemization than the tripeptide in which Trt was used as protecting group (Figure 21). Furthermore, less formation of the 3-(1-piperinidyl)alanine adducts **5d** was detected when Thp was used as *C*-terminal Cys protecting group (**Figure 22**).

N-terminal Cys studies

While the MBom presents a straightforward preparation of the protected amino acid unit, Thp is easily introduced into the Cys thiol group by the using a catalytic amount of acid in presence of dihydropyran as above mentioned. Regarding the final protecting group elimination (**Figure 23**), in both cases an aldehyde is generated, formaldehyde and 5-hydroxypentanal for MBom and Thp, respectively. Accordingly, the formaldehyde formation supposes an important drawback because of the hydroxymethylation. An undesirable side reaction is the formation of the thiazolidine (Thz) ring in *N*-terminal Cys.

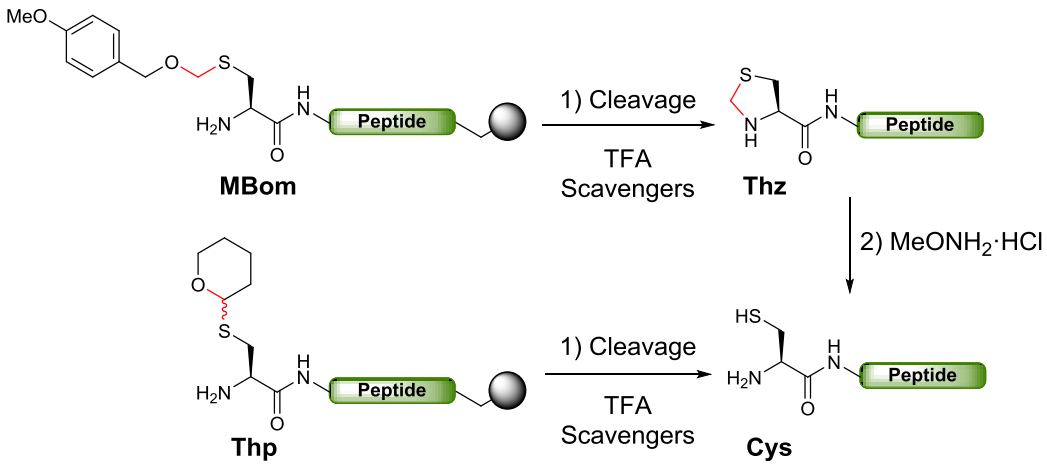
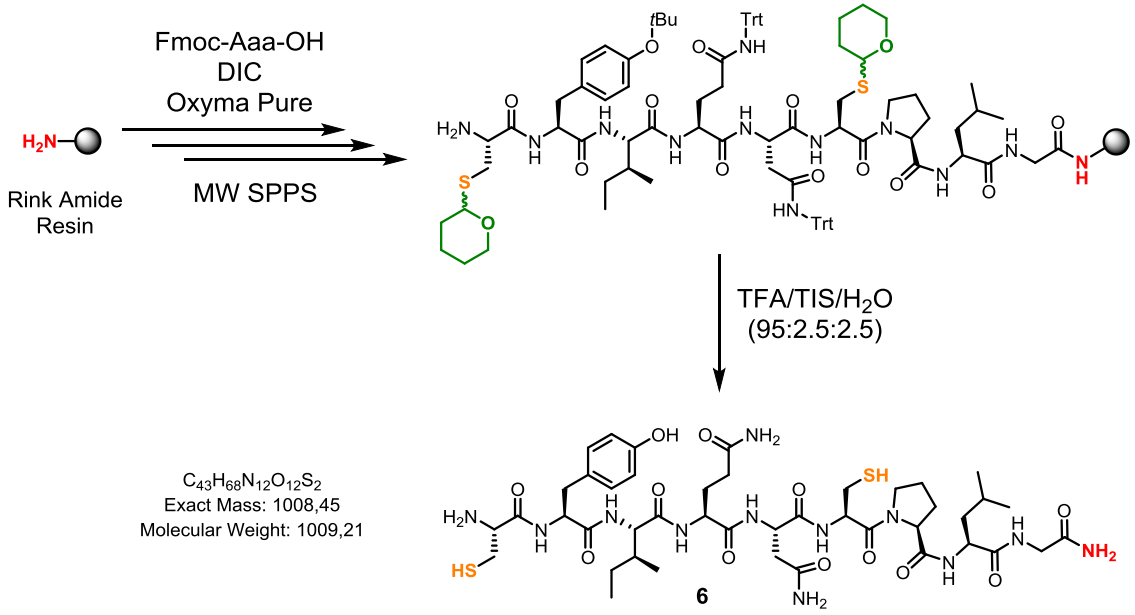


Figure 23. MBom and Thp *N*-terminal Cys protecting group elimination**.**

In that sense, we studied if the above mentioned side reactions occurs using Thp as *N*-terminal Cys protecting group. For that, we automatically synthetized by using rink amide resin and standard microwave conditions the reduced form of Oxytocin (**6**), a natural peptide-like hormone which contain a *N*-terminal Cys in their structure (**Scheme 19).** After the peptide scission from the resin, the reduced oxytocin was yield in 80% of purity as UPLC analysis depicted (Figure 24). We demonstrated that Thp is suitable protecting group for *N*-terminal Cys containing peptides and moreover, that the formation of 5-hydroxypentanal not suppose a problem due to that the hydroxyl group liberated in the Thp molecule, suffer an intramolecular hydroxymethylation, trapping the reactive electrophilic carbon atom and avoiding the undesired Thz-like ring formation.



Scheme 19. Reduced Oxytocin SPPS preparation.

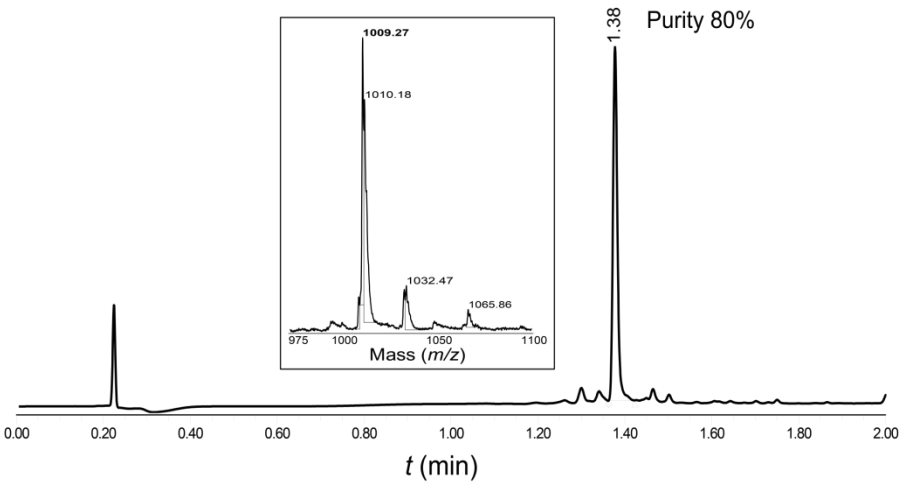


Figure 24. UPLC and MALDI analysis. Linear gradient H_2O/ MeCN (90:10) to (30:70) over 2 min.

3.4. Conclusions

The methodological spectrum available for the synthesis of peptides in solid supports, has suffered during several years an impressive transformation. Always seeking to avoid the side reactions, which jeopardize the synthesis of this kind of special molecules. In that sense, all the scientific contributions in the field make the SPPS an outstanding and rigorous way to obtain the myriad of interesting small molecules, peptides or proteins. In summary, our recent contribution to the field -the Cys protecting group Thp-, demonstrated that now with a lot of work done in the field, Thp allowed lower racemization levels than the conventional protecting groups for Cys (Trt, Dpm, and S*t*Bu), both when using Thp in a peptide sequence (**3a** and **4a,** D/L<1%) or as C-terminal Cys-protecting group (**5a,** D/L<6%). In addition, fewer side products, such as the 3-(1-piperinidyl)alanine adducts, were detected during the synthesis of C-terminal Cys carboxylic acid peptides. Furthermore, the solubility of Thp-protected peptides was improved with respect to strategies involving the commonly used hindered aromatic protecting groups. Additionally, Thp is suitable for *N*-terminal Cys protecting group and in comparison to other protecting groups, Thp avoid formylation in the final deprotection step. Our results reveal Thp as a useful protecting group for Cys when applied to the Fmoc/*t*Bu strategy in SPPS.Due to the scientific ambition, the field is tending to reach the synthetic perfection. The question is: will it be possible?

3.5. References

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3.6. Experimental section

Preparation of Fmoc-Cys-OH (1):

To a mixture of Fmoc-Cys(Trt)-OH (2.0 g, 3.4 mmol) in DCM (45 mL), TIS (0.75 mL) and TFA (5 mL) was added. The reaction mixture was stirred at room temperature for 1h. Then the solvent was removed under reduced pressure and the crude was washed and precipitated with hexane (6×40 mL). Afterwards the solid was lyophilized obtaining 1.1 g of pure Fmoc-Cys-OH (95% yield) as a white powder. **HPLC** (H_2O/MeCN from 95:5 to 0:100 over 8 min) t_R : 6.8 min). *m/z* calculated for $C_{18}H_{17}NO_4S$ = 343.4; found = 344.1 $[M+H]^+$, being M the MW of Fmoc-Cys-OH (1). **^{1}H NMR (**400 MHz, DMSO-*d*6) δ 12.86 (s, 1H), 7.90 (dt, *J* = 7.6, 1.0 Hz, 2H), 7.74 (d, *J* = 7.3 Hz, 1H), 7.69 (d, *J* = 8.2 Hz, 1H), 7.42 (t, *J* = 7.2 Hz, 1H), 7.34 (td, *J* = 7.5, 1.2 Hz, 2H), 4.34 – 4.30 (m, 2H), 4.24 (t, *J* = 7.0 Hz, 1H), 4.13 (td, *J* = 8.4, 4.4 Hz, 1H), 2.94 – 2.85 (m, 1H), 2.79 – 2.69 (m, 1H), 2.54 – 2.47 (m, 1H). **^{13}C NMR** (100 MHz, DMSO-*d*6): δ 171.8, 156.0, 143.8, 143.7, 140.7, 127.6, 127.1, 125.3, 120.1, 65.7, 56.6, 46.6, 25.4.

Preparation of Fmoc-Cys(Thp)-OH (2):

To a suspension of Fmoc-Cys-OH (1.8 g, 5.2 mmol) and 3,4-dihydro-2*H*-pyran (DHP) (575 µL, 6.2 mmol) in DCM (40 mL), *p*-toluensulfonic acid (99.7 mg, 0.52 mmol) was added. Then the mixture was left to react at room temperature for 1h under N_2. Afterwards the organic layer was washed with brine (3×50 mL) and water (3×50 mL) then it was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure, furnishing a withe solid (2.2 g) which was automatically purified on a pre-packed Redisep Rf Gold C18 43g column by using H_2O/MeCN from 90:10 to 0:100 over 30 min. The collected fractions were lyophilized to obtain Fmoc-Cys(Thp)-OH as a withe powder (1.95 g, 87% of yield). **HPLC** (H_2O/MeCN from 95:5 to 0:100 over 8 min): t_R: 7.6 min. *m/z* calculated for $C_{23}H_{25}NO_5S$ = 427.1; found = 428.0 $[M+H]^+$, being M the MW of Fmoc-Cys(Thp)-OH. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.78 – 7.73 (m, 2H), 7.63 – 7.59 (m, 2H), 7.43 – 7.36 (m, 2H), 7.34 – 7.28 (m, 2H), 6.97 (d, *J* = 8.3 Hz, 1H), 6.40 (d, *J* = 6.8 Hz, 0.6H), 5.44 (s, 1.7H), 4.77 – 4.64 (m, 1.6H), 4.54 – 4.39 (m, 2H), 4.28 – 4.18 (m, 1H), 4.04 – 3.89 (m, 1H), 3.46 – 3.38 (m, 1H), 3.20 (dd, J = 14.6, 5.1 Hz, 0.4H), 3.06 (dd, J = 14.5, 6.8 Hz, 0.4H), 2.89 (dd, *J* = 14.7, 3.9 Hz, 1H), 1.99 – 1.86 (m, 1H), 1.85 – 1.77 (m, 1H), 1.74 – 1.65 (m, 1H), 1.62 – 1.50 (m, 3H). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 174.3, 156.8, 144.1, 143.9, 143.9, 141.5, 141.5, 141.4, 127.9, 127.8, 127.2, 127.2, 125.2, 125.2, 120.1, 120.0, 84.7, 83.6, 67.3, 67.0, 66.5, 65.9, 54.2, 47.3, 47.3, 35.2, 32.9, 31.6, 31.3, 25.3, 25.2, 22.6, 22.3, 15.3.

Lability experiments:

The lability experiments were performed by using the protected Fmoc-Cys(Thp)-OH aminoacid (1 mg/mL) into mixtures which contains different percentage of acids [2-(*N*-morpholino)ethanesulfonic acid (MES), trifluoroacetic acid (TFA) and HCl], scavengers (such as water and TIS) and DCM at room temperature. The table below indicates the acidic proportion of scavenger used in the acidolytic cocktail. Moreover it indicates the % of Fmoc-Cys-OH determined by the HPLC chromatographic peak area. The acidolytic reaction was

monitored by RP-HPLC until the complete elimination of the Thp protecting group by using a linear gradient of H_2O/MeCN (from 95:5 to 0:100) over 8 min. t_R = 6.87 min; *m/z* calculated for $C_{18}H_{17}NO_4S$, 343.1; found, 344.0 $[M+H]^+$.

C-terminal amide: Ala-Cys-Leu-NH_2 tripeptides synthesis:

The resins used for the manual preparation of C-Terminal amide tripeptide were Fmoc-Sieber-amide AM-polystyrene resin (f = 0.69 mmol/g) and Fmoc-Rink-amide AM-polystyrene resin (f = 0.70 mmol/g). Initially, both resins were washed with DCM (4×5 mL) and DMF (4×5 mL) and the Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 2×5 mL×5 min), and washed with DCM (4×5 mL) and DMF (4×5 mL). For both resins, Fmoc-Leu-OH (3 eq.) was incorporated by using DIPCDI (3 eq.) and Oxyme Pure (3 eq.) in DMF, after pre-activation of 5 min. The mixtures were left to react for 1 h and 30 min. Afterwards, the resins were washed with DMF (4×5 mL) and DCM (4×5 mL).

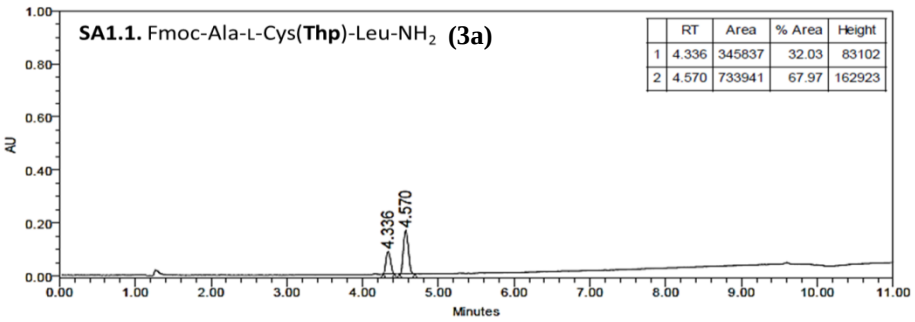
A.1-Sieber amide resin (SA):

The Fmoc-Leu-Sieber amide resin was divided into five fractions (SA1-SA5), which were solvated with DCM (3×5 mL) and DMF (3×5 mL). The following Cys protected aminoacid (2 eq.) –Fmoc-L-Cys(Thp)-OH (SA1), Fmoc-L-Cys(Trt)-OH (SA2), Fmoc-D-Cys(Trt)-OH (SA3), Fmoc-L-Cys(Dpm)-OH (SA4), Fmoc-L-Cys(Acm)-OH (SA5) – were coupled using DICPDI (2 eq.) and Oxyma Pure (2 eq.) in DMF with a 5 min pre-activation and 1 h and 30 min of coupling at 25 ºC. Re-coupling of Cys(Trt) (A2 and A3) and Cys(Dpm) (A4) were needed as the Kaiser test determined after 1 h and 30 min of reaction. For the re-coupling the same conditions as the coupling was used for SA2-SA4 (5 min of pre-activation + 1 h of coupling at 25 ºC). The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 2×5 mL× 5 min). The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL) and then Alanine was incorporated to the peptide chain using Fmoc-Ala-OH
(3 eq.), DIPCDI (3 eq.) and Oxyme Pure (3 eq.): 5 min pre-activation + 1 h 30 min coupling at 25 ºC. The resins were washed with DMF (4 × 5 mL) and DCM (4 × 5 mL).

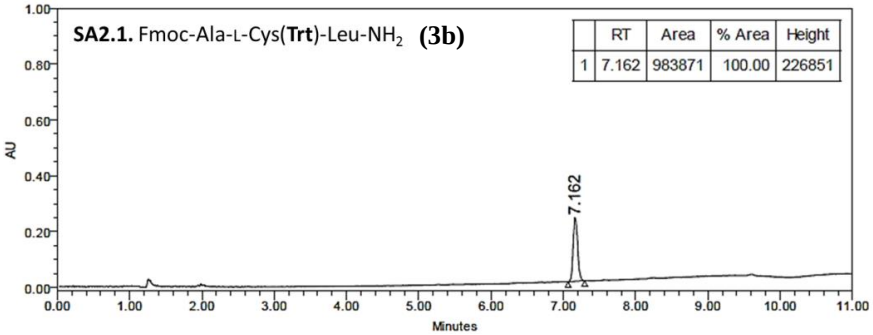
Cleavage: For each cleavage studies, 10 mg of the Fmoc-Ala-Cys(PG)-Leu-NH-Resin were used.

- Fmoc-Ala-Cys(**PG**)-Leu-NH_2 tripeptides: Tripeptides-containing resins were treated with TFA/DCM (1:99) (3×0.5 mL×1 min) and washed with DCM (2×1 mL). Then the filtered washes were evaporated and precipitated with Et_2O. The resulting peptides were dissolved into 2 mL H_2O/MeCN (1:1) for their analysis by RP-HPLC.

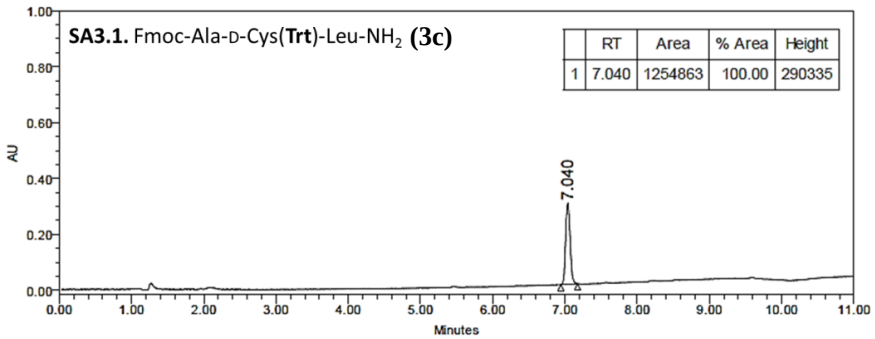
SA1.1. *Fmoc-Ala-L-Cys(**Thp**)-Leu-NH_2 (**3a**):* RP-HPLC [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 4.34 + 4.57 min; Purity = 32 % and 68 % respectively (diastereoisomers). *m/z* calculated for $C_{32}H_{42}N_4O_6S$ = 610.3; found = 611.2 $[M+H]^+$, being M the MW of Fmoc-Ala-L-Cys(**Thp**)-Leu-NH_2.



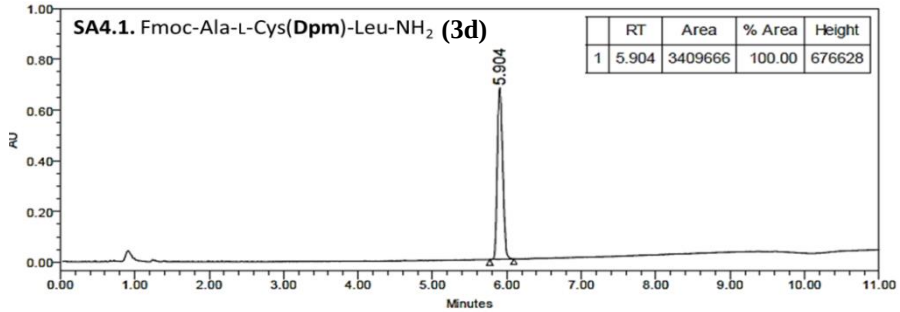
SA2.1. *Fmoc-Ala-L-Cys(**Trt**)-Leu-NH_2 (**3b**):* RP-HPLC [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 7.16 min; Purity = >99 %. *m/z* calculated for $C_{46}H_{48}N_4O_5S$ = 768.3; found = 769.3 $[M+H]^+$, being M the MW of Fmoc-Ala-L-Cys(**Trt**)-Leu-NH_2.



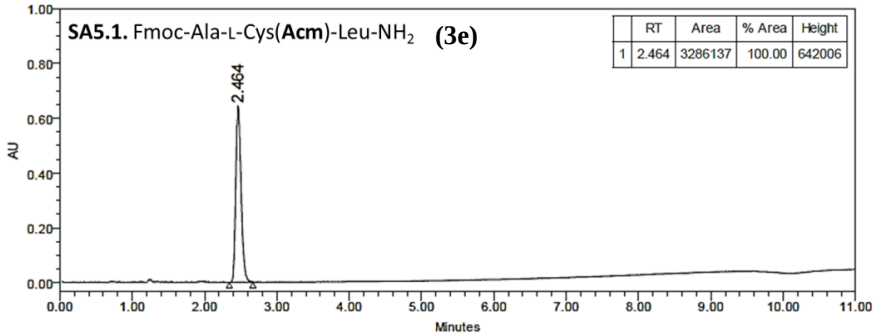
SA3.1. *Fmoc-Ala-D-Cys(**Trt**)-Leu-NH_2 (**3c**):* RP-HPLC [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 7.04 min; Purity = >99 %. *m/z* calculated for $C_{46}H_{48}N_4O_5S$ = 768.3; found = 769.3 $[M+H]^+$, being M the MW of Fmoc-Ala-D-Cys(**Trt**)-Leu-NH_2.



SA4.1. *Fmoc-Ala-L-Cys(**Dpm**)-Leu-NH_2 (**3d**):* RP-HPLC [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 5.90 min; Purity = >99 %. *m/z* calculated for $C_{40}H_{44}N_4O_5S$ = 692.3; found = 693.2 $[M+H]^+$, being M the MW of Fmoc-Ala-L-Cys(**Dpm**)-Leu-NH_2.

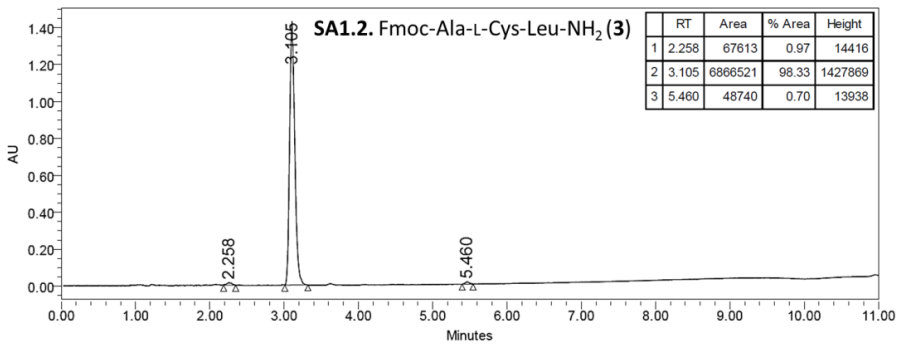


SA5.1. *Fmoc-Ala-L-Cys(**Acm**)-Leu-NH_2 (**3e**):* RP-HPLC [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 2.46 min; Purity = >99 %. *m/z* calculated for $C_{30}H_{39}N_5O_6S$ = 597.3; found = 598.2 $[M+H]^+$, being M the MW of Fmoc-Ala-L-Cys(**Acm**)-Leu-NH_2.

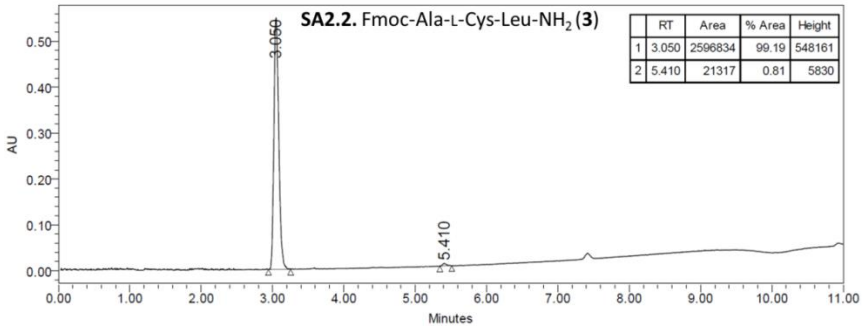


- Fmoc-Ala-L-Cys-Leu-NH_2 tripeptide (**3**): The fully-protected tripeptides resins were treated with TFA/TIS/DCM (95:2.5:2.5) (1×2 mL×30 min) and washed with DCM (2×1 mL). Then the filtered washes were evaporated and precipitated with Et_2O. The resulting peptides were dissolved into 2 mL H_2O/MeCN (1:1) for their analysis by RP-HPLC.

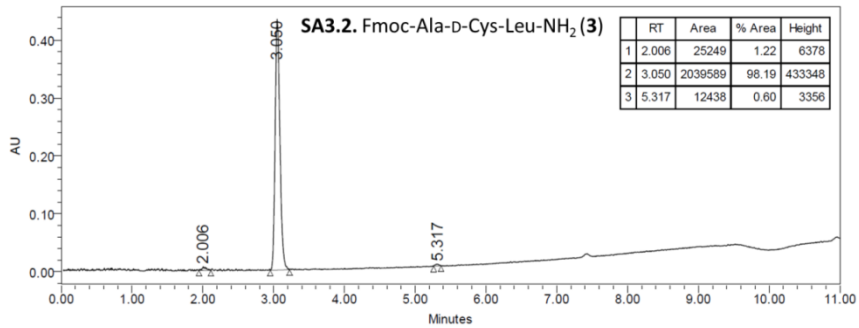
SA1.2. *Fmoc-Ala-L-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 3.10 min; Purity = 98 %.*



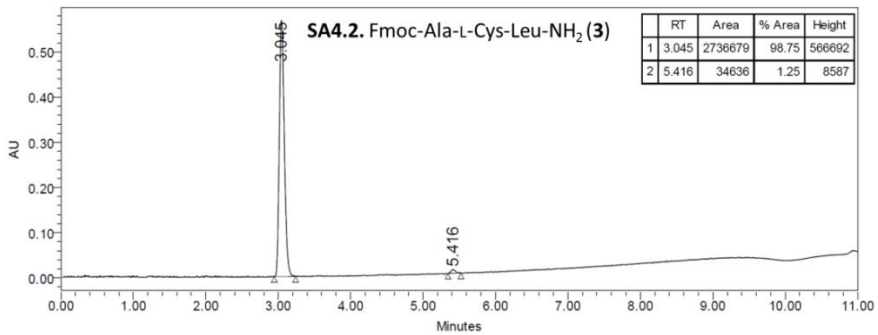
SA2.2. Fmoc-Ala-L-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 3.05 min; Purity = 99 %.



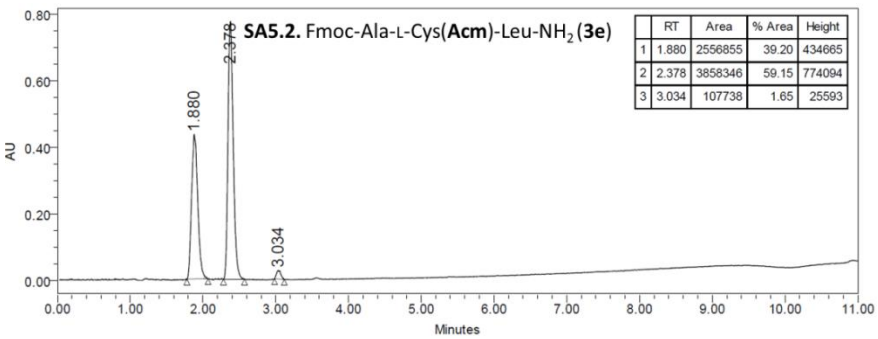
SA3.2. Fmoc-Ala-D-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 3.05 min; Purity = 98 %.



SA4.2. Fmoc-Ala-L-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 3.05 min; Purity = 99 %.

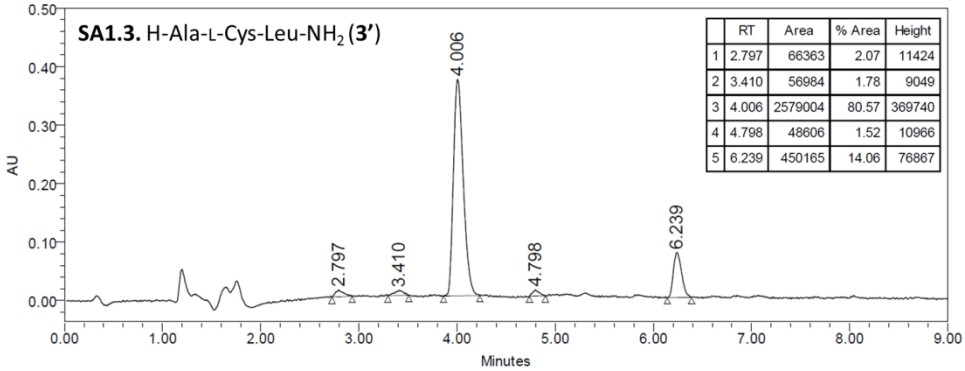


*SA5.2. Fmoc-Ala-L-Cys(**Acm**)-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 2.38 min; Purity = 60 %.*



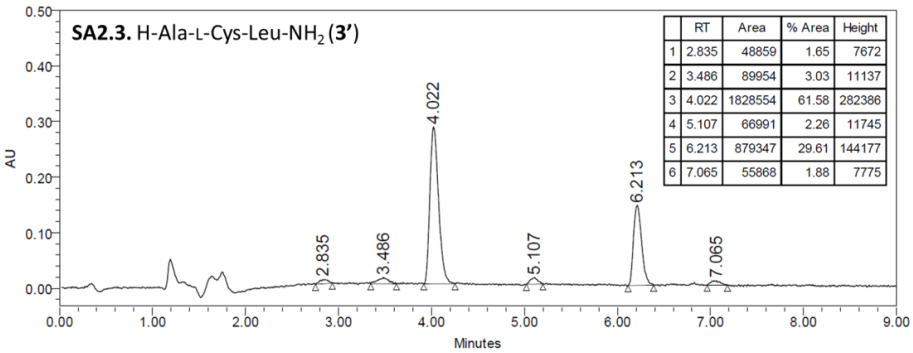
- H-Ala-Cys-Leu-NH_2 tripeptide (**3'**): The resins were solved with DCM (3×2 mL×5 min) and DMF (3×2 mL×5 min). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×2 mL×1 min + 2×2 mL×5 min). Then the resins were washed with DCM (4×5 mL). The complete cleavage from the resins were performed with TFA/TIS/DCM (95:2.5:2.5) (1×2 mL×30 min) then the resins were washed with DCM (2×1 mL). Then the filtered washes were evaporated and precipitated with Et_2O. The resulting peptides were dissolved into 2 mL of TCEP 2 mM in H_2O/MeCN (1:1) for their analysis by RP-HPLC.

SA1.3. H-Ala-L-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 4.01 min; Purity = 81 %.



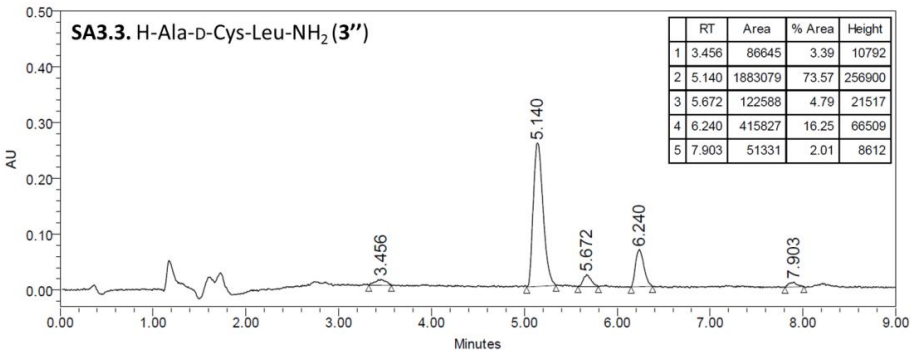
	RT	Area	% Area	Height
1	2.797	66363	2.07	11424
2	3.410	56984	1.78	9049
3	4.006	2579004	80.57	369740
4	4.798	48606	1.52	10966
5	6.239	450165	14.06	76867

SA2.3. H-Ala-L-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 4.02 min; Purity = 63 %.



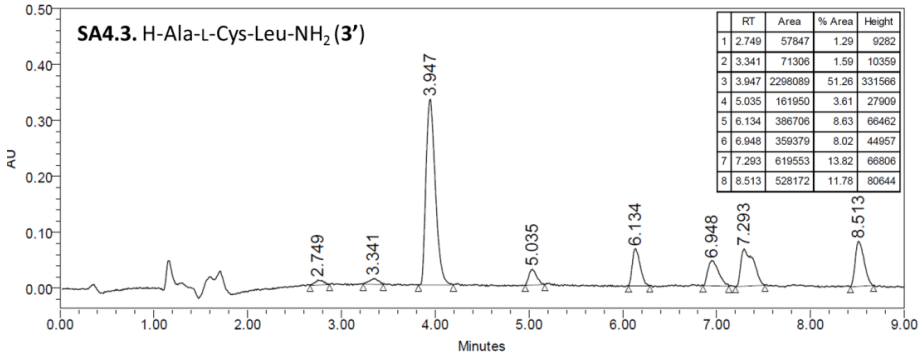
	RT	Area	% Area	Height
1	2.835	48859	1.65	7672
2	3.486	89954	3.03	11137
3	4.022	1828554	61.58	282386
4	5.107	66991	2.26	11745
5	6.213	879347	29.61	144177
6	7.065	55868	1.88	7775

SA3.3. H-Ala-D-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 5.18 min; Purity = 75 %.



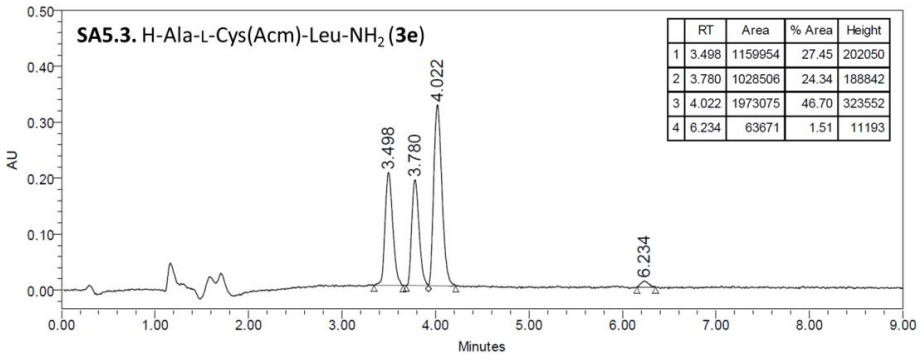
	RT	Area	% Area	Height
1	3.456	86645	3.39	10792
2	5.140	1883079	73.57	256900
3	5.672	122588	4.79	21517
4	6.240	415827	16.25	66509
5	7.903	51331	2.01	8612

SA4.3. H-Ala-L-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 3.95 min; Purity = 52 %.



	RT	Area	% Area	Height
1	2.749	57847	1.29	9282
2	3.341	71306	1.59	10359
3	3.947	2298089	51.26	331566
4	5.035	161950	3.61	27909
5	6.134	386706	8.63	66462
6	6.948	359379	8.02	44957
7	7.293	619553	13.82	66806
8	8.513	528172	11.78	80644

*SA5.3. H-Ala-L-Cys(**Acm**)-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 3.50 + 3.78 + 4.02 [H-Ala-L-Cys-Leu-NH_2] min; Purity = 27 % + 24 % + 47 % respectively.*



	RT	Area	% Area	Height
1	3.498	1159954	27.45	202050
2	3.780	1028506	24.34	188842
3	4.022	1973075	46.70	323552
4	6.234	63671	1.51	11193

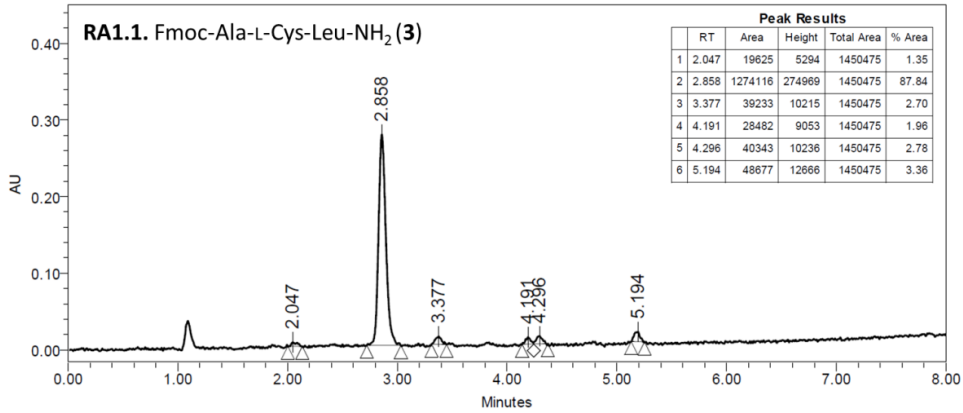
A.2- Rink amide Resin (RA):

The Fmoc-Leu-Rink amide resin was divided into three fractions (RA1-RA3), which were solvated with DCM (3×5 mL) and DMF (3×5 mL). The following Cys protected aminoacid (2 eq.) –Fmoc-L-Cys(Thp)-OH (RA1), Fmoc-L-Cys(Trt)-OH (RA2), Fmoc-L-Cys(Dpm)-OH (RA3)– were coupled using DICPDI (2 eq.) and Oxyma Pure (2 eq.) in DMF with a 5 min pre-activation and 1 h and 30 min of coupling at 25 ºC. The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 2×5 mL×5 min). The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL) and then Alanine was incorporated to the peptide chain using Fmoc-Ala-OH (3 eq.), DIPCDI (3 eq.) and Oxyme Pure (3 eq.): 5 min pre-activation + 1 h 30 min coupling at 25 ºC. The resins were washed with DMF (4×5 mL) and DCM (4×5 mL).

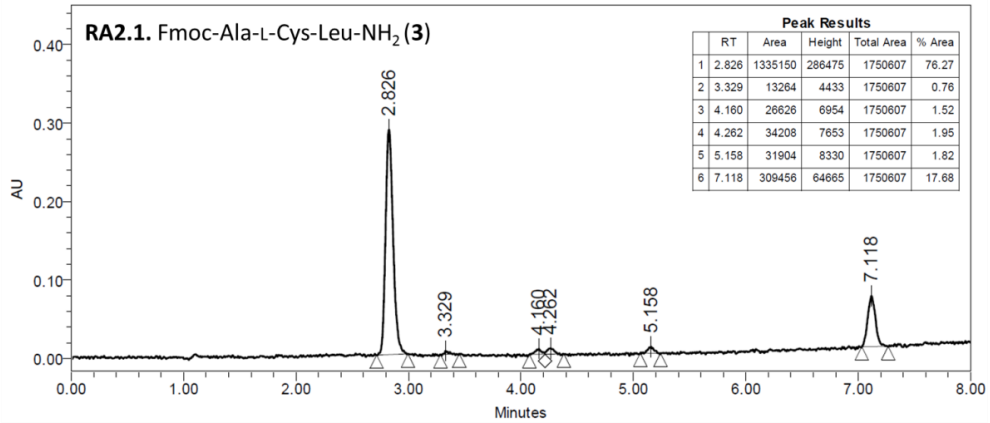
Cleavage: For each cleavage studies, 10 mg of the Fmoc-Ala-Cys(PG)-Leu-NH-Resin were used.

- Fmoc-Ala-Cys-Leu-NH_2 tripeptide (**3**): Tripeptides-containing resins were treated with TFA/TIS/H_2O (95:2.5:2.5) (1×2 mL×1 h) and washed with DCM (2×1 mL). Then the filtered washes were evaporated and precipitated with Et_2O. The resulting peptides were dissolved into 2 mL H_2O/MeCN (1:1) for their analysis by RP-HPLC.

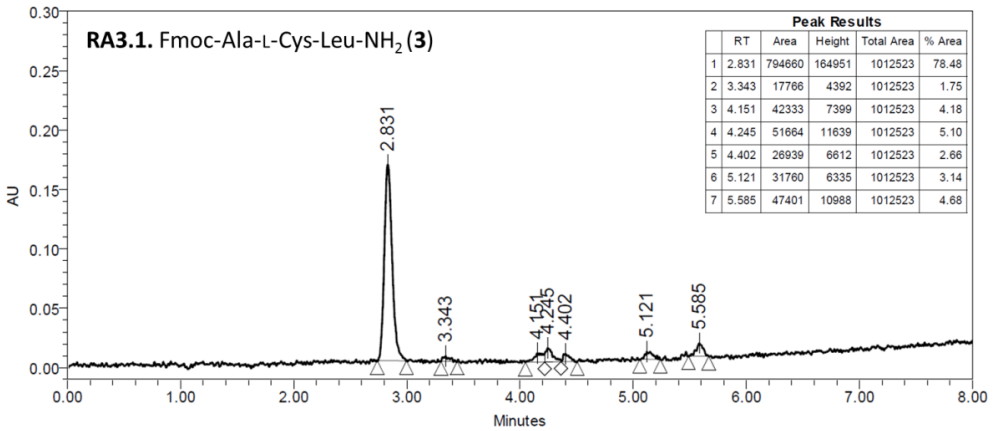
RA1.1. Fmoc-Ala-L-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 2.86 min; Purity = 88 %.



RA2.1. Fmoc-Ala-L-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 2.83 min; Purity = 76 %.



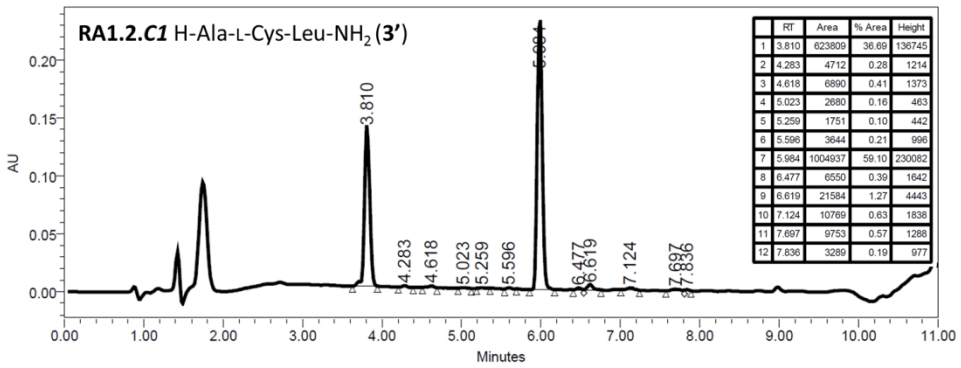
RA3.1. Fmoc-Ala-L-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 2.83 min; Purity = 78 %.



- H-Ala-L-Cys-Leu-NH_2 tripeptide (**3'**): The resins were solved with DCM (3×2 mL×5 min) and DMF (3×2 mL×5 min). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×2 mL×1 min + 2×2 mL×5 min). Then the resins were washed with DCM (4×5 mL). The complete cleavage from the resins were performed with **TFA/TIS/H_2O (95:2.5:2.5)** (1×2 mL×1 h) then the resins were washed with DCM (2×1 mL). Then the

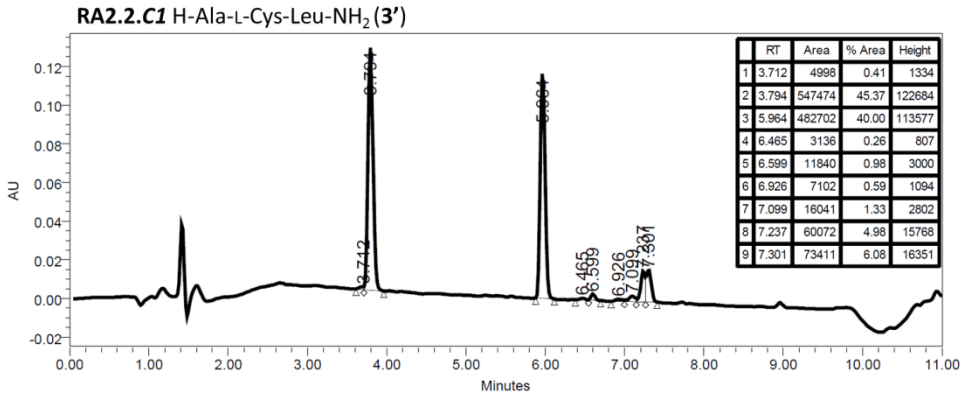
filtered washes were evaporated and precipitated with Et_2O. The resulting peptides were dissolved into 2 mL of TCEP 2 mM in H_2O/MeCN (1:1) for their analysis by RP-HPLC.

RA1.2.C1. *H-Ala-L-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 3.81 min; Purity = 37%.*



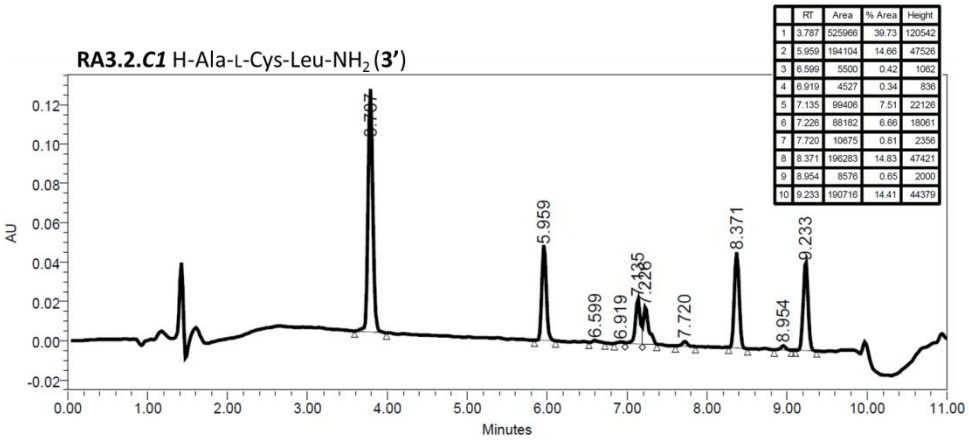
	RT	Area	% Area	Height
1	3.810	623809	36.69	136745
2	4.283	4712	0.28	1214
3	4.618	6890	0.41	1373
4	5.023	2680	0.16	463
5	5.259	1751	0.10	442
6	5.596	3644	0.21	996
7	5.984	1004937	59.10	230082
8	6.477	6550	0.39	1642
9	6.619	21584	1.27	4443
10	7.124	10769	0.63	1838
11	7.697	9753	0.57	1288
12	7.836	3289	0.19	977

RA2.2.C1. *H-Ala-L-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 3.79 min; Purity = 45 %.*



	RT	Area	% Area	Height
1	3.712	4998	0.41	1334
2	3.794	547474	45.37	122684
3	5.964	482702	40.00	113577
4	6.465	3136	0.26	807
5	6.599	11840	0.98	3000
6	6.926	7102	0.59	1094
7	7.099	16041	1.33	2802
8	7.237	60072	4.98	15768
9	7.301	73411	6.08	16351

RA3.2.C1. *H-Ala-L-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 3.79 min; Purity = 40 %.*

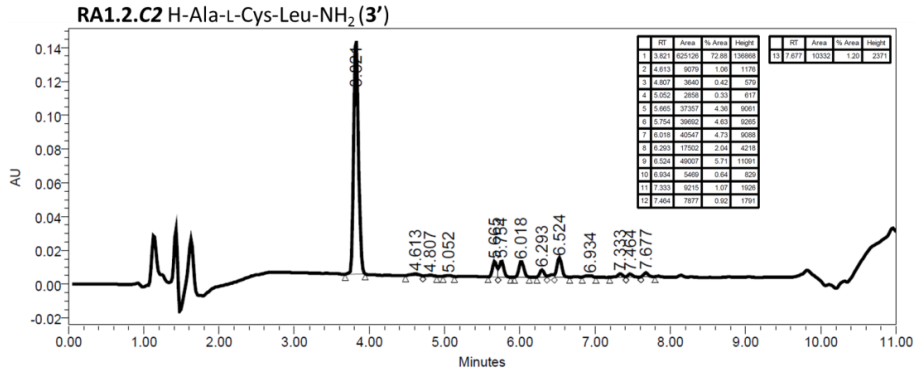


	RT	Area	% Area	Height
1	3.787	525966	39.73	120542
2	5.959	194104	14.66	47526
3	6.599	5500	0.42	1062
4	6.919	4527	0.34	836
5	7.135	99406	7.51	22126
6	7.226	88182	6.66	18061
7	7.720	10675	0.81	2356
8	8.371	196283	14.83	47421
9	8.954	8576	0.65	2000
10	9.233	190716	14.41	44379

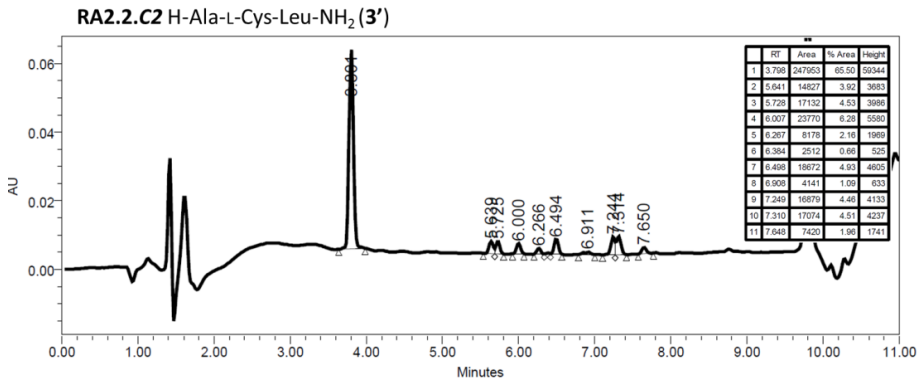
- H-Ala-L-Cys-Leu-NH_2 tripeptide (**3'**): The resins were solved with DCM (3×2 mL×5 min) and DMF (3×2 mL×5 min). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×2 mL×1 min + 2×2 mL×5 min). Then the resins were washed with DCM (4×5 mL). The complete cleavage from the resins were performed with **TFA/TIS/DMB**

(92.5:2.5:5) (1×2 mL×1 h) then the resins were washed with DCM (2×1 mL). Then the filtered washes were evaporated and precipitated with Et_2O. The resulting peptides were dissolved into 2 mL of TCEP 2 mM in H_2O/MeCN (1:1) for their analysis by RP-HPLC.

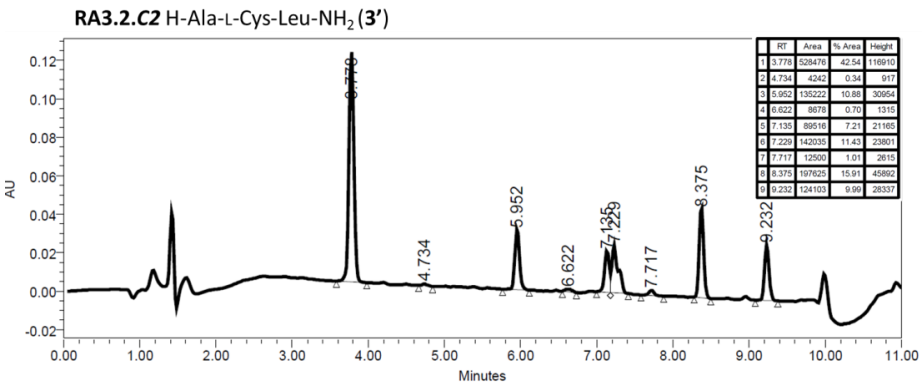
RA1.2.C2. *H-Ala-L-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 3.76 min; Purity = 73 %.*



RA2.2.C2. *H-Ala-L-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 3.80 min; Purity = 67 %.*



RA3.2.C2. *H-Ala-L-Cys-Leu-NH_2: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 3.78 min; Purity = 43 %.*



C-terminal carboxylic acid: Ala-Cys-Leu-OH tripeptides synthesis:

B. 2-Chlorotrityl chloride resin (2-CTC):

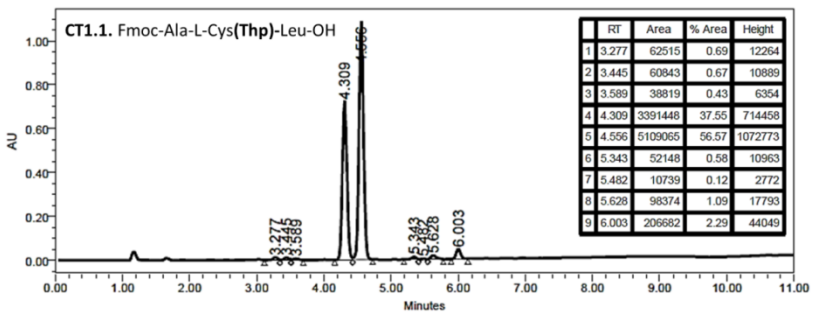
The resin used for the manual preparation of C-Terminal carboxylic acid tripeptide was 2-Chlorotrityl chloride resin (f = 1.60 mmol/g). Initially, the resin was washed with DCM (4×5 mL) and DMF (10×5 mL) and DCM (4×5 mL) again. The attachment of the first amino acid was carried out by the treatment of the resin with Fmoc-Leu-OH (1 eq.) and DIEA (3 eq.) in DCM during 10 min at 25 ºC. Then, more DIEA (7 eq.) was added to the mixture, which was left to react for 40 min at 25 ºC. Thereafter, the resin was capped by adding MeOH (10 eq.) to the mixture, which was left for 10 min at 25ºC. Then the resin was washed with DCM (4×5 mL) and DMF (5×5 mL) and the Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×2 mL×1 min + 2×2 mL×5 min). Being, 0.97 mmol/g the loading for the H-Leu-2ClTrityl as the UV quantification Fmoc group determined. The resins were washed with DMF (4×5 mL), DCM (4×5 mL).

The H-Leu-O-2-CTC Resin was divided into three fractions (CT1-CT3), which were solvated with DCM (3×5 mL) and DMF (3×5 mL). The following Cys protected aminoacid (2 eq.) –Fmoc-L-Cys(Thp)-OH (CT1), Fmoc-L-Cys(Trt)-OH (CT2), Fmoc-D-Cys(Trt)-OH (CT3)– were coupled using DICPDI (2 eq.) and Oxyma Pure (2 eq.) in DMF with a 5 min pre-activation and 1 h and 30 min of coupling at 25 ºC. The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 1×5 mL×3 min). The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL) and then Alanine was incorporated to the peptide chain using Fmoc-Ala-OH (3 eq.), DIPCDI (3 eq.) and Oxyme Pure (3 eq.): 5 min pre-activation + 1 h 30 min coupling at 25 ºC. The resins were washed with DMF (4×5 mL) and DCM (4×5 mL).

Cleavage: For each cleavage studies, 10 mg of the Fmoc-Ala-Cys(PG)-Leu-O-2CTC Resin were used.

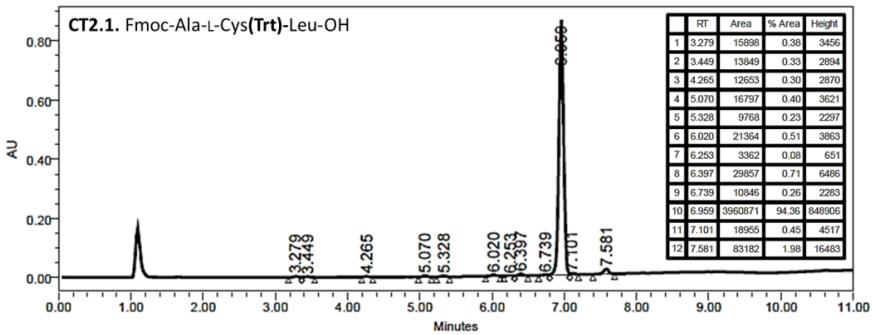
- Fmoc-Ala-Cys(**PG**)-Leu-OH tripeptides (**4a-b**): Tripeptides-containing resins were treated with TFE/AcOH/DCM (20:20:60) (1×2 mL×1 h 30 min) and washed with the cleavage cocktail (2×2 mL) and with DCM (2×2 mL). Then the filtered washes were evaporated and precipitated with Et_2O. The resulting peptides were dissolved into 2 mL H_2O/MeCN (1:1) for their analysis by RP-HPLC.

*2-CTC1.1. Fmoc-Ala-L-Cys(**Thp**)-Leu-OH (**4a**): RP-HPLC [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 4.31 + 4.56 min; Purity = 38 % and 57 % respectively (diastereoisomers).*



	RT	Area	% Area	Height
1	3.277	62515	0.69	12264
2	3.445	60843	0.67	10889
3	3.589	38819	0.43	6354
4	4.309	3391448	37.55	714458
5	4.556	5109065	56.57	1072773
6	5.343	52148	0.58	10963
7	5.482	10739	0.12	2772
8	5.628	98374	1.09	17793
9	6.003	206682	2.29	44049

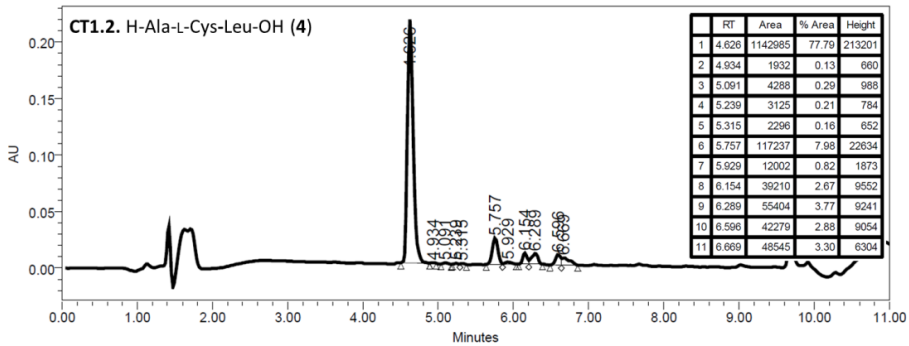
*2-CTC2.1. Fmoc-Ala-L-Cys(**Trt**)-Leu-OH: RP-HPLC [linear gradient H_2O/MeCN (50:50) to (0:100) over 8 min] t_R = 6.96 min; Purity = 94 %.*



	RT	Area	% Area	Height
1	3.279	15898	0.38	3456
2	3.449	13849	0.33	2894
3	4.265	12653	0.30	2870
4	5.070	16797	0.40	3621
5	5.328	9768	0.23	2297
6	6.020	21364	0.51	3863
7	6.253	3362	0.08	651
8	6.397	29857	0.71	6486
9	6.739	10846	0.26	2263
10	6.959	3960871	94.36	848906
11	7.101	18955	0.45	4517
12	7.581	83182	1.98	16483

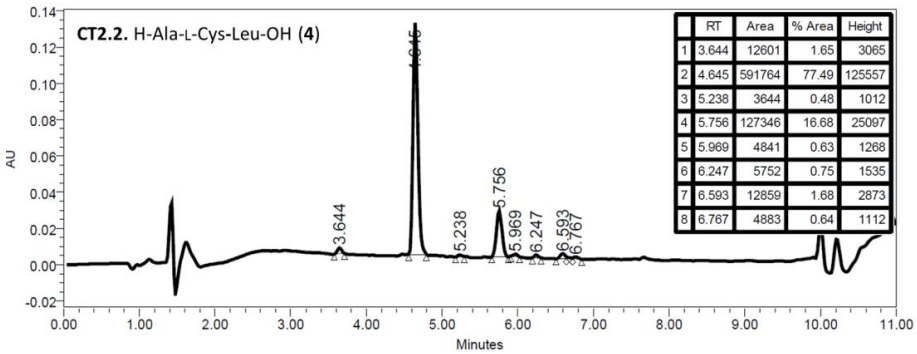
- H-Ala-Cys-Leu-OH tripeptide (**4**): The resins were solved with DCM (3×2 mL×5 min) and DMF (3×2 mL×5 min). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×2 mL×1 min + 2×2 mL×5 min). Then the resins were washed with DCM (4×5 mL). The complete cleavage from the resins were performed with TFA/TIS/DCM (95:2.5:2.5) (1×2 mL×30 min) and washed with DCM (2×1 mL). Then the filtered washes were evaporated and precipitated with Et_2O. The resulting peptides were dissolved into 2 mL of H_2O/MeCN (1:1) for their analysis by RP-HPLC.

2-CTC1.2. H-Ala-L-Cys-Leu-OH: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 4.63 min; Purity = 78 %.



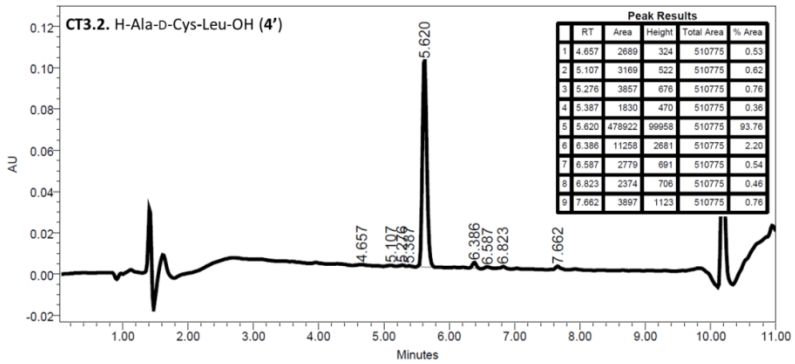
	RT	Area	% Area	Height
1	4.626	1142985	77.79	213201
2	4.934	1932	0.13	660
3	5.091	4288	0.29	988
4	5.239	3125	0.21	784
5	5.315	2296	0.16	652
6	5.757	117237	7.98	22634
7	5.929	12002	0.82	1873
8	6.154	39210	2.67	9552
9	6.289	55404	3.77	9241
10	6.596	42279	2.88	9054
11	6.669	48545	3.30	6304

2-CTC2.2. H-Ala-L-Cys-Leu-OH: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 4.64 min; Purity = 78 %.



	RT	Area	% Area	Height
1	3.644	12601	1.65	3065
2	4.645	591764	77.49	125557
3	5.238	3644	0.48	1012
4	5.756	127346	16.68	25097
5	5.969	4841	0.63	1268
6	6.247	5752	0.75	1535
7	6.593	12859	1.68	2873
8	6.767	4883	0.64	1112

2-CTC3.2. H-Ala-D-Cys-Leu-OH: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 5.82 min; Purity = 94 %.



_C_-terminal carboxylic acid: Ala-Leu-Cys-OH tripeptides synthesis.

2-Chlorotrityl chloride resin

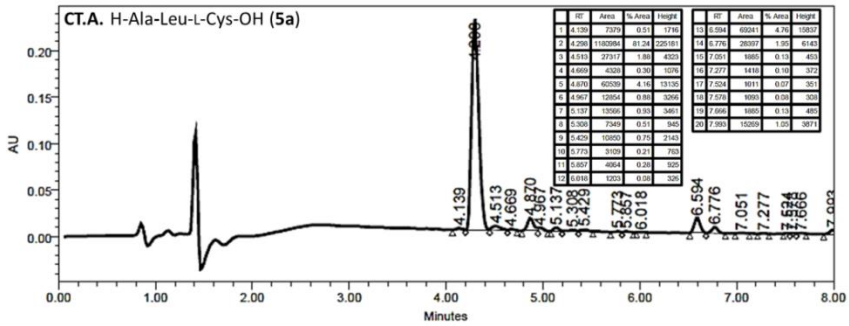
The resin used for the manual preparation of C-Terminal carboxylic acid tripeptide was 2-Chlorotrityl chloride resin (f = 1.60 mmol/g). Initially, the resin was washed with DCM (4×5 mL), DMF (10×5 mL) and DCM (4×5 mL). The attachment of the first amino acid was carried out by the treatment of the resin with the appropriate Fmoc-Cys-(PG)-OH (1 eq.) and DIEA (3 eq.) in DCM during 10 min at 25 ºC. Then, more DIEA (7 eq.) was added to the mixture, and it was left to react for 40 min at 25 ºC. Thereafter, the resin was capped by adding MeOH (10 eq.) to the mixture, which was left for 10 min at 25ºC. Then the resin was washed with DCM (4 × 5 mL) and DMF (5×5 mL) and the Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 2×5 mL×5 min). Being, 0.60, 0.57 and 0.60 mmol/g the loading for the H-L-Cys(Thp)-O-2ClTrityl, H-L-Cys(Trt)-O-2ClTrityl and H-D-Cys(Trt)-O-2ClTrityl resins respectively, as the UV quantification Fmoc group determined. The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (4×5 mL). Then leucine was incorporated into each resin using Fmoc-Leu-OH (3 eq.), DICPDI (3 eq.) and Oxyma Pure (3 eq.) in DMF with a 5 min pre-activation and 1 h and 30 min of coupling at 25 ºC. Then the resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 1×5 mL×3 min). The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL) and then Alanine was incorporated to the peptide chain using Boc-Ala-OH (3 eq.), DIPCDI (3 eq.) and Oxyme Pure (3 eq.): 5 min pre-activation + 1 h 30 min coupling at 25 ºC. The resins were washed with DMF (4×5 mL) and DCM (4×5 mL).

Cleavage: For each cleavage studies, 10 mg of the Boc-Ala-Leu-Cys(PG)-O-2ClTrityl Resins were used.

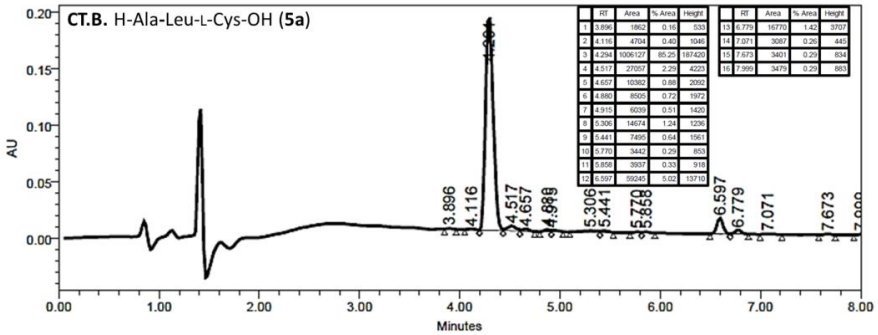
- H-Ala-Leu-Cys-OH tripeptide (**5a**): The resins were solved with DCM (3×2 mL× 5 min) and its complete cleavage were performed with TFA/TIS/DCM (95:2.5:2.5) (1×2 mL×1 h) followed by washes with DCM (3×1 mL). Then the filtered washes were evaporated and

precipitated with Et_2O. The resulting peptides were dissolved into 2 mL of H_2O/MeCN (1:1) for their analysis by RP-HPLC.

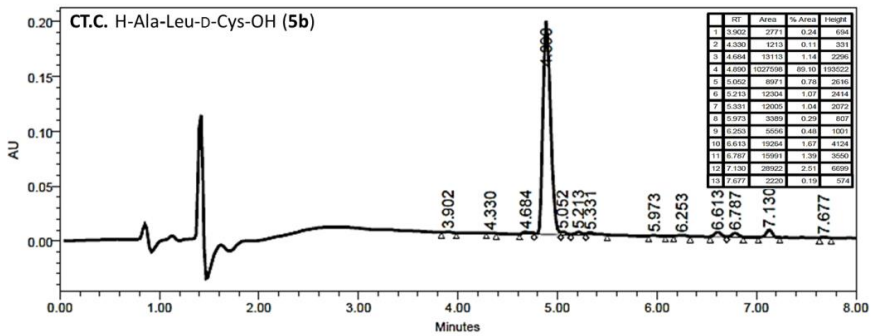
CT.A. H-Ala-Leu-L-Cys-OH: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 4.30 min; Purity = 81 %.



CT.B. H-Ala-Leu-L-Cys-OH: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 4.30 min; Purity = 85 %.



CT.C. H-Ala-Leu-D-Cys-OH: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 4.89 min; Purity = 89 %.



Wang Resin

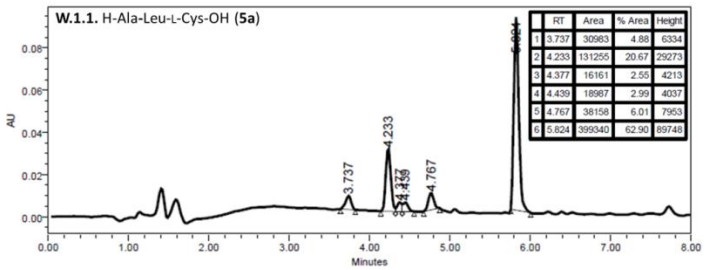
The resin used for the manual preparation of C-Terminal carboxylic acid tripeptides was Wang resin (f = 1.2 mmol/g). Initially, the resins were washed with DCM (4×5 mL), DMF (10×5 mL) and DCM (4×5 mL). The attachment of the first amino acid was carried out by the treatment of the resins with the appropriate Fmoc-Cys-(PG)-OH (5 eq.) derivative, DIPCDI (2.5 eq.), Oxyme Pure (5 eq.) and DMAP (0.1 eq.) in DCM:DMF (9:1) (5 mL) during 3 h at 25 ºC. Then, the resins were washed with DCM (5×5 mL) and it were capped with acetic anhydride (2 eq.) and DIEA (2 eq.) during 30 min at 25 ºC. Thereafter, the resins were

washed with DCM (4×5 mL) and DMF (4×5 mL) and the Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 2×5 mL×5 min). Being, 1.0, 1.1 and 0.9 mmol/g the loading for the H-L-Cys(Thp)-O-Wang, H-L-Cys(Trt)-O-Wang and H-L-Cys(S*t*Bu)-O-Wang resins respectively, as the UV quantification Fmoc group determined. Then the resin was washed with DCM (4×5 mL) and DMF (5×5 mL). Leucine was incorporated into each resin using Fmoc-Leu-OH (3 eq.), DICPDI (3 eq.) and Oxyma Pure (3 eq.) in DMF with a 5 min pre-activation and 1 h and 30 min of coupling at 25 ºC. Then the resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL). Fmoc protecting group was eliminated by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 1×5 mL×3 min). The resins were washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL) and immediately Alanine was incorporated to the peptide chain using Boc-Ala-OH (3 eq.), DIPCDI (3 eq.) and Oxyme Pure (3 eq.): 5 min pre-activation + 1 h 30 min coupling at 25 ºC. The resins were washed with DMF (4×5 mL) and DCM (4×5 mL).

Cleavage: For each cleavage studies, 10 mg of the Boc-Ala-Leu-Cys(PG)-O-Wang Resins were used.

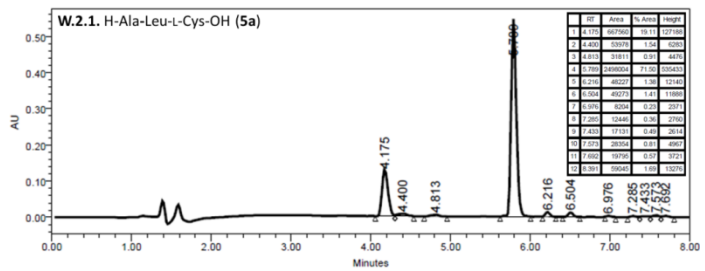
- H-Ala-Leu-Cys-OH tripeptide: The resins were solved with DCM (3×2 mL× 5 min) and its complete cleavage were performed with **TFA/TIS/H_2O (95:2.5:2.5)** (1×2 mL×1 h) followed by washes with DCM (3×1 mL). Then the filtered washes were evaporated and precipitated with Et_2O. The resulting peptides were dissolved into 2 mL of H_2O/MeCN (1:1) for their analysis by RP-HPLC.

W.1.1 H-Ala-Leu-L-Cys-OH: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 4.23 min; Purity = 21 %.

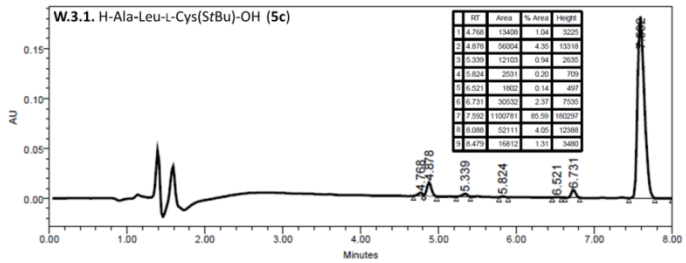


	RT	Area	% Area	Height
1	3.737	30983	4.88	6334
2	4.233	131255	20.67	29273
3	4.377	16161	2.55	4213
4	4.439	18987	2.99	4037
5	4.767	38158	6.01	7953
6	5.824	399340	62.90	89748

W.2.1 H-Ala-Leu-L-Cys-OH: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 4.18 min; Purity = 19 %.

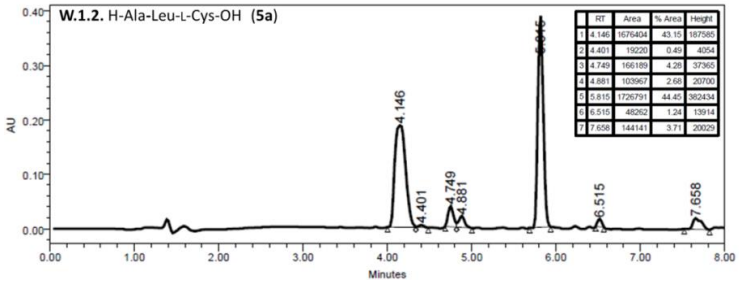


W.3.1 *H-Ala-Leu-L-Cys(StBu)-OH: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 7.59 min; Purity = 86 %.*

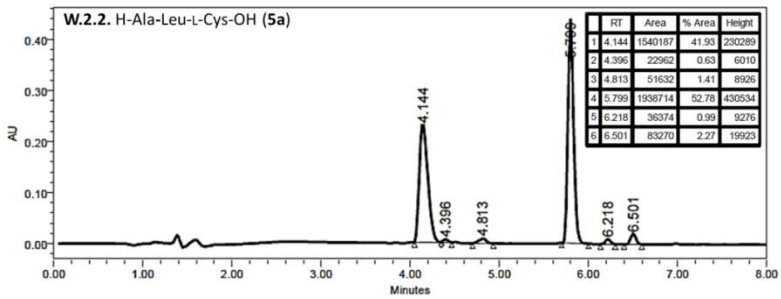


- H-Ala-Leu-Cys-OH tripeptide: The resins were solved with DCM (3×2 mL×5 min) and its complete cleavage were performed with **TFA/TIS/DMB (92.5:2.5:5)** (1×2 mL×1 h) followed by washes with DCM (3×1 mL). Then the filtered washes were evaporated and precipitated with Et_2O. The resulting peptides were dissolved into 2 mL of H_2O/MeCN (1:1) for their analysis by RP-HPLC.

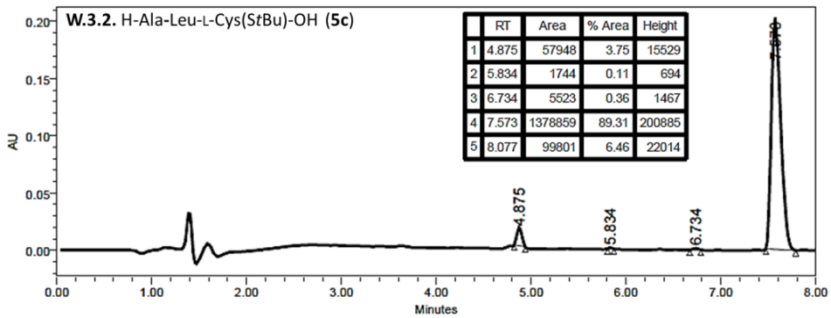
W.1.2 *H-Ala-Leu-L-Cys-OH: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 4.15 min; Purity = 43 %.*



W.2.2 *H-Ala-Leu-L-Cys-OH: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 4.14 min; Purity = 42 %.*



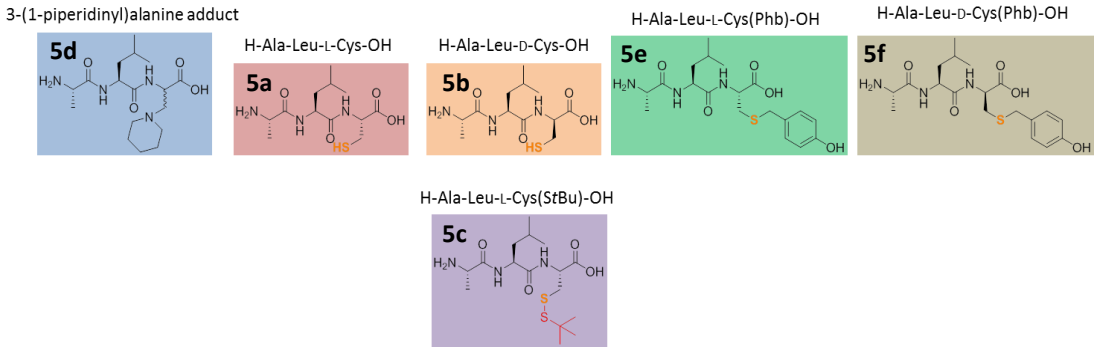
W.3.2 *H-Ala-Leu-L-Cys(StBu)-OH: RP-HPLC [linear gradient H_2O/MeCN (95:5) to (50:50) over 8 min] t_R = 7.57 min; Purity = 89 %.*



C-Terminal Cys Racemization study:

For C-terminal Cysteine racemization studies 100 mg of each Boc-Ala-Leu-Cys(PG)-O-2-CTC and Boc-Ala-Leu-Cys(PG)-O-Wang Resins were used.

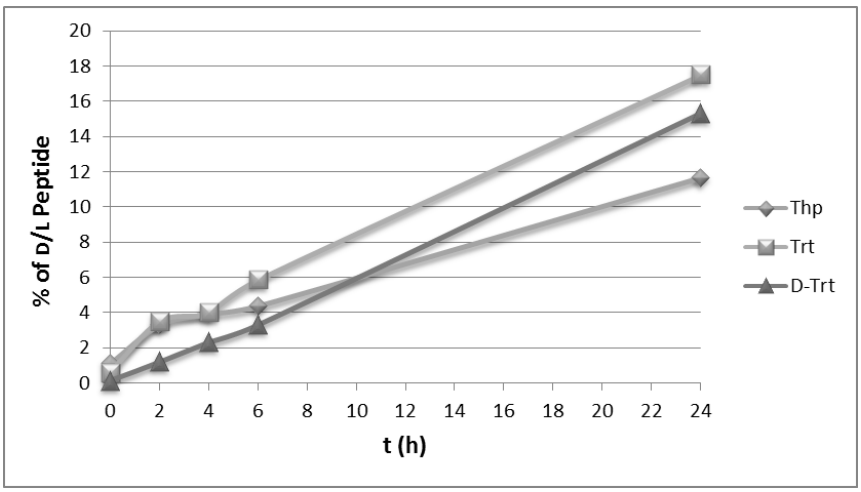
The following products were observed into the analyzed cleavage mixtures.



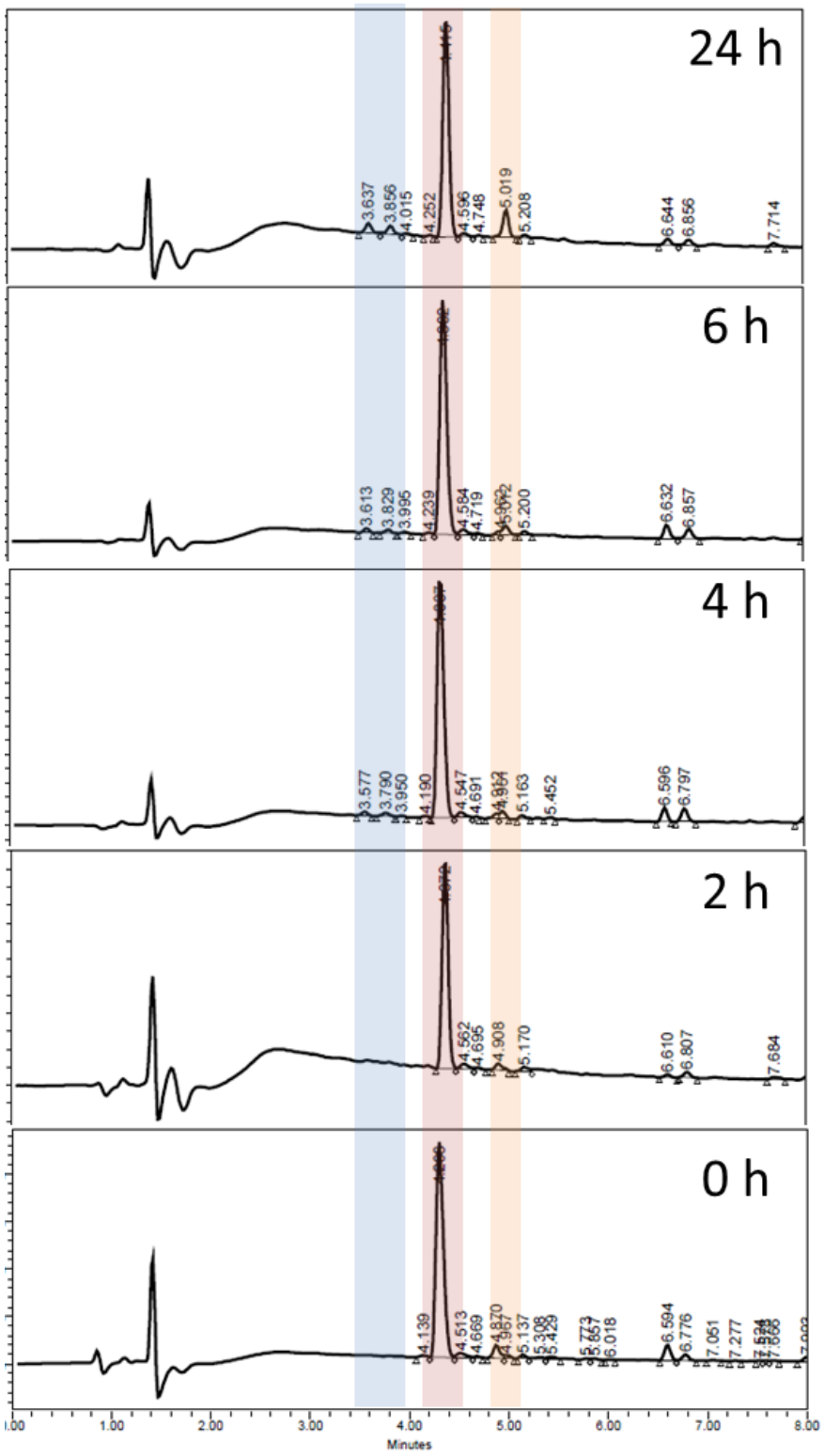
2-CTC resin

The corresponding Boc-Ala-Leu-Cys(PG)-O-2-CTC resins [for PG: Thp, L- and D-Cys(Trt)] were introduced into glass vial and it were treated with DMF/piperidine (4:1) (3 mL) for 24 h at 25 ºC. At 2, 4, 6 and 24 h, a small aliquot of resin were separated and it was introduced into a polypropylene syringe and after washes with DMF (5×2 mL) and DCM (4×2 mL). The peptides were cleaved from the resin using TFA/TIS/H_2O (95:2.5:2.5) (1×2 mL×1 h) followed by washes with DCM (3×1 mL). Then the filtered washes were evaporated and precipitated with Et_2O. The resulting peptides were dissolved into 1 mL of H_2O/MeCN (1:1) for their analysis by RP-HPLC.

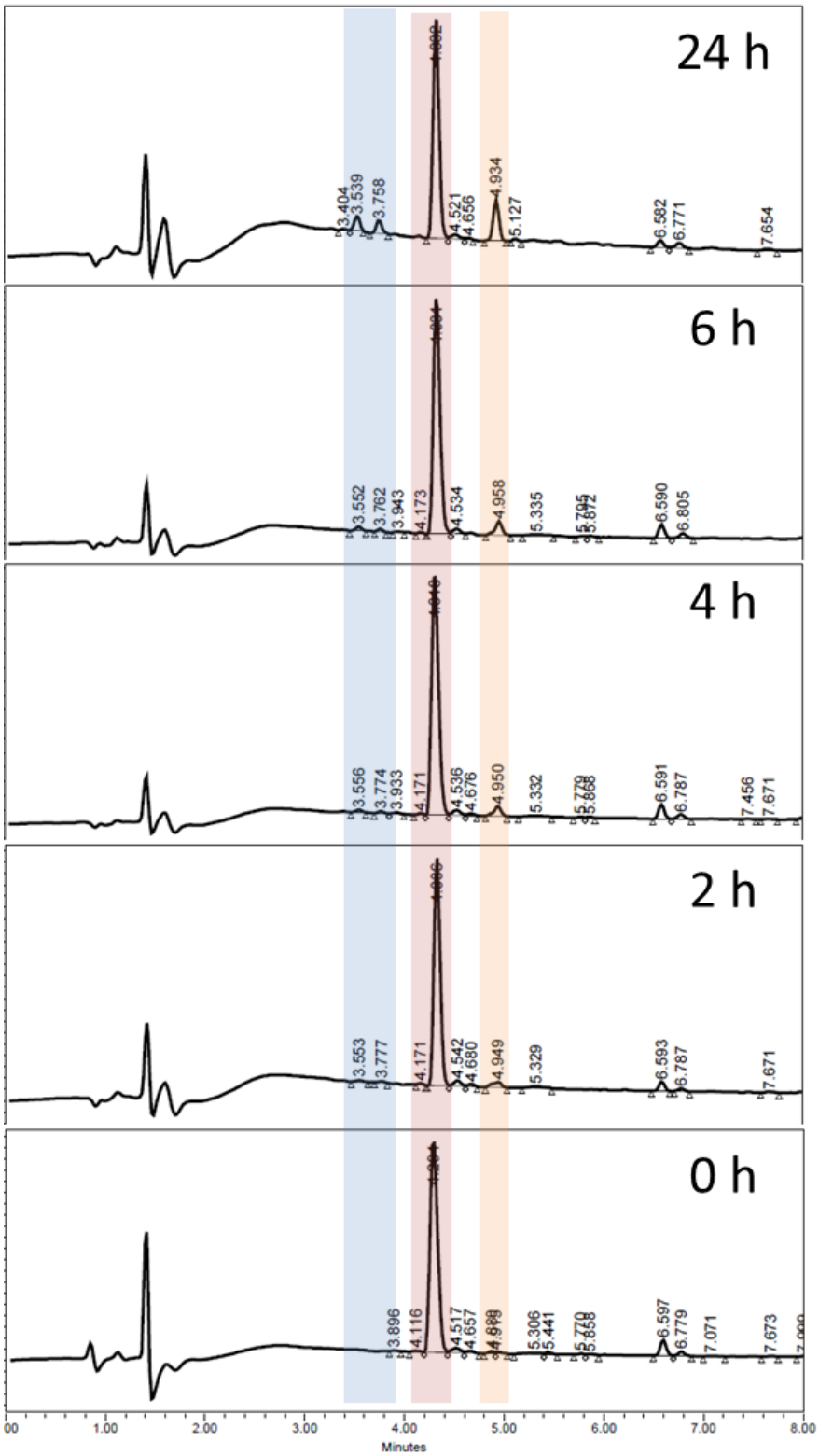
Boc-Ala-Leu-Cys(PG)-O-2-CTC resin



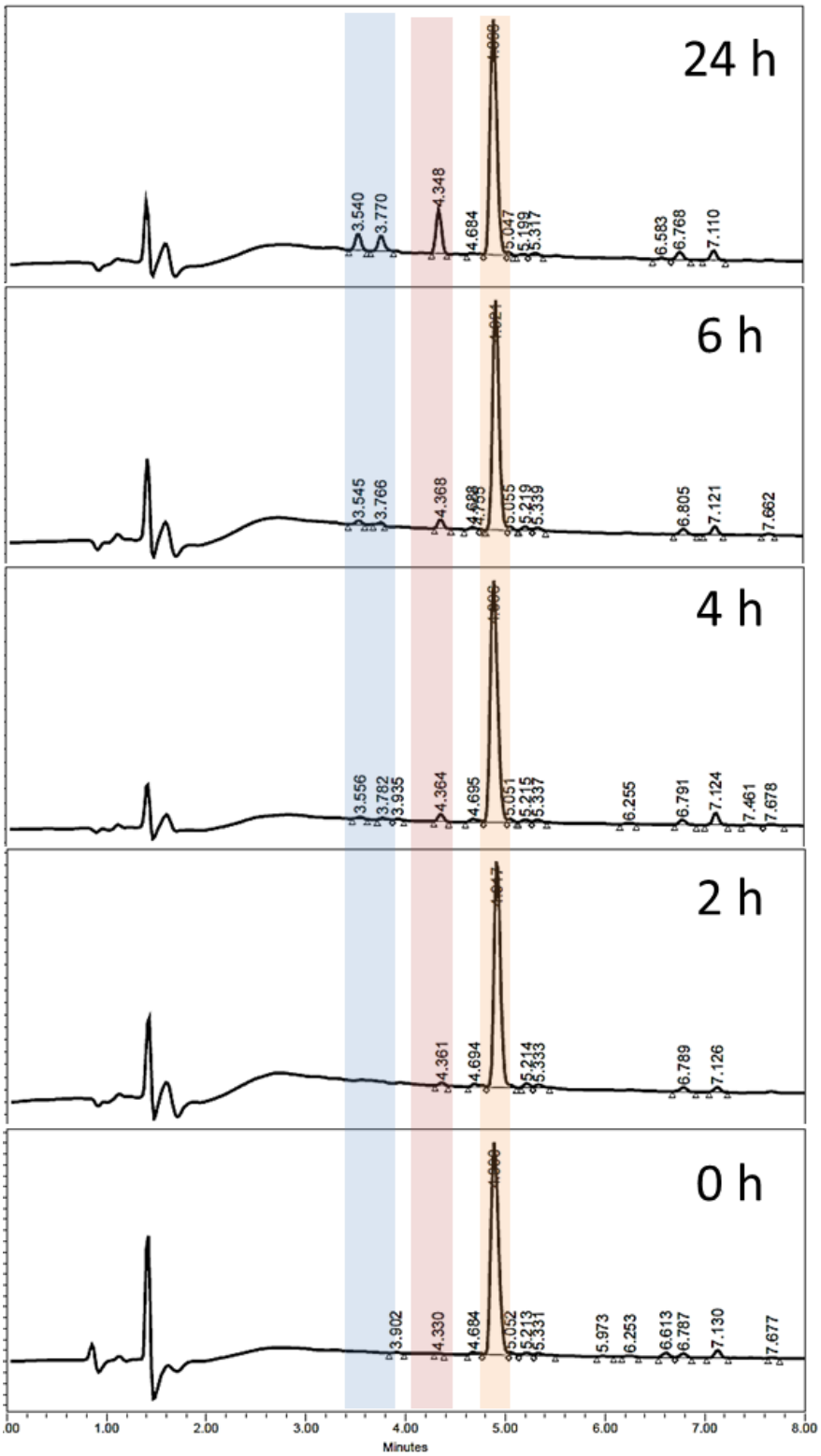
CTA.1. 2-CTC resin with Cys(Thp).



CTB.1. 2-CTC resin with L-Cys(Trt)

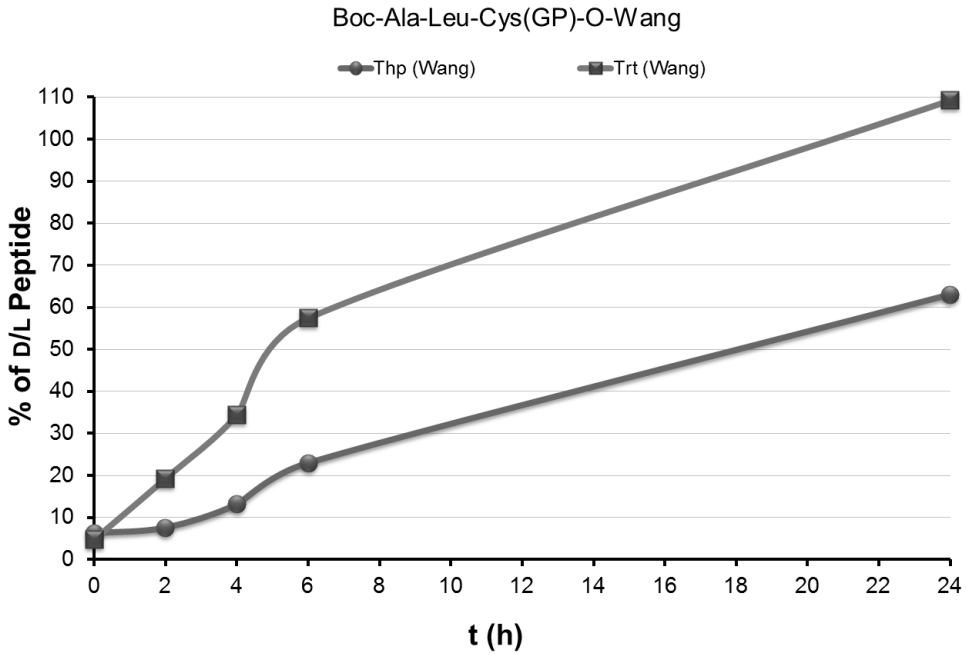


CTC.1. 2-CTC resin with D-Cys(Trt)

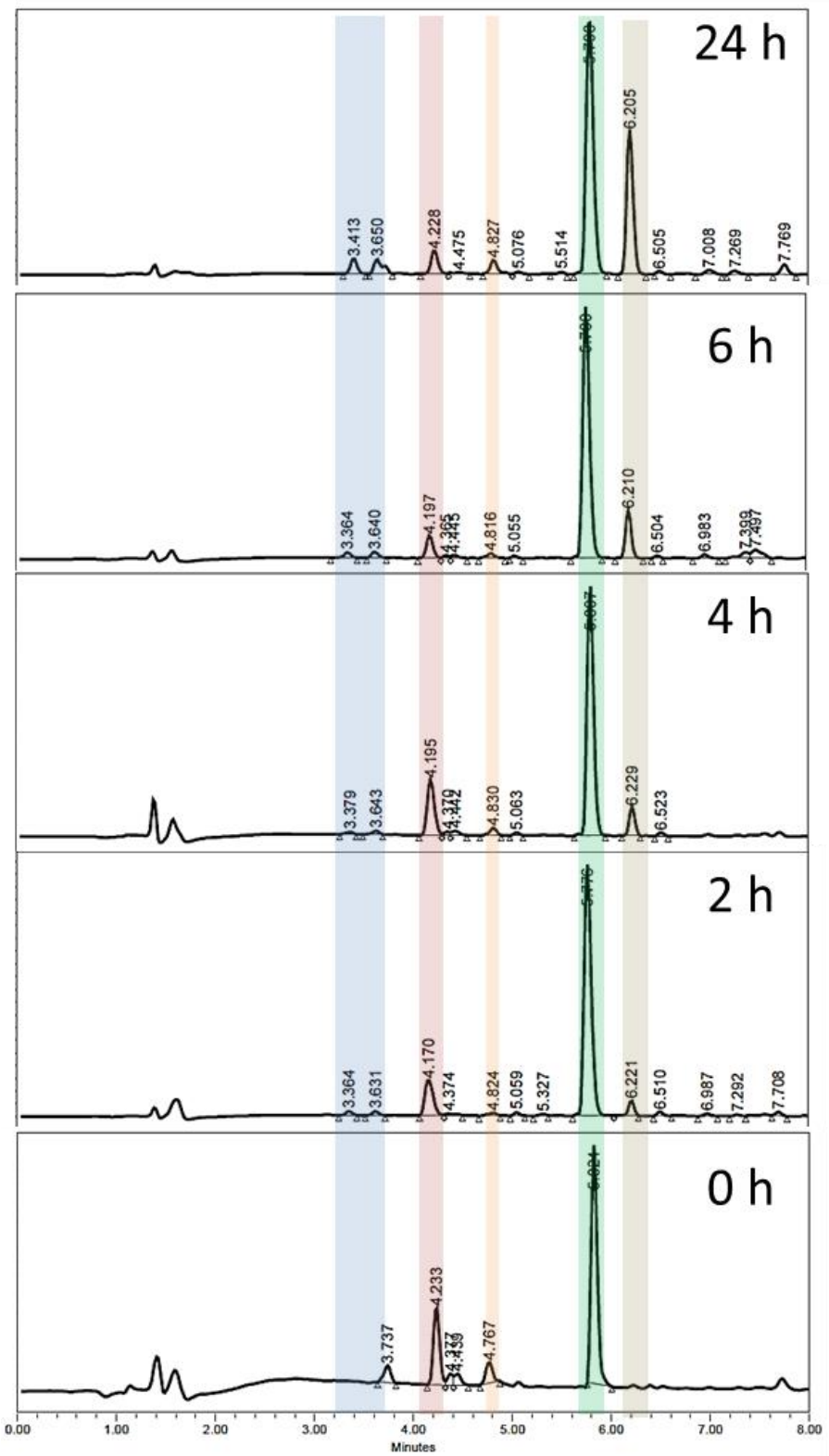


Wang Resin

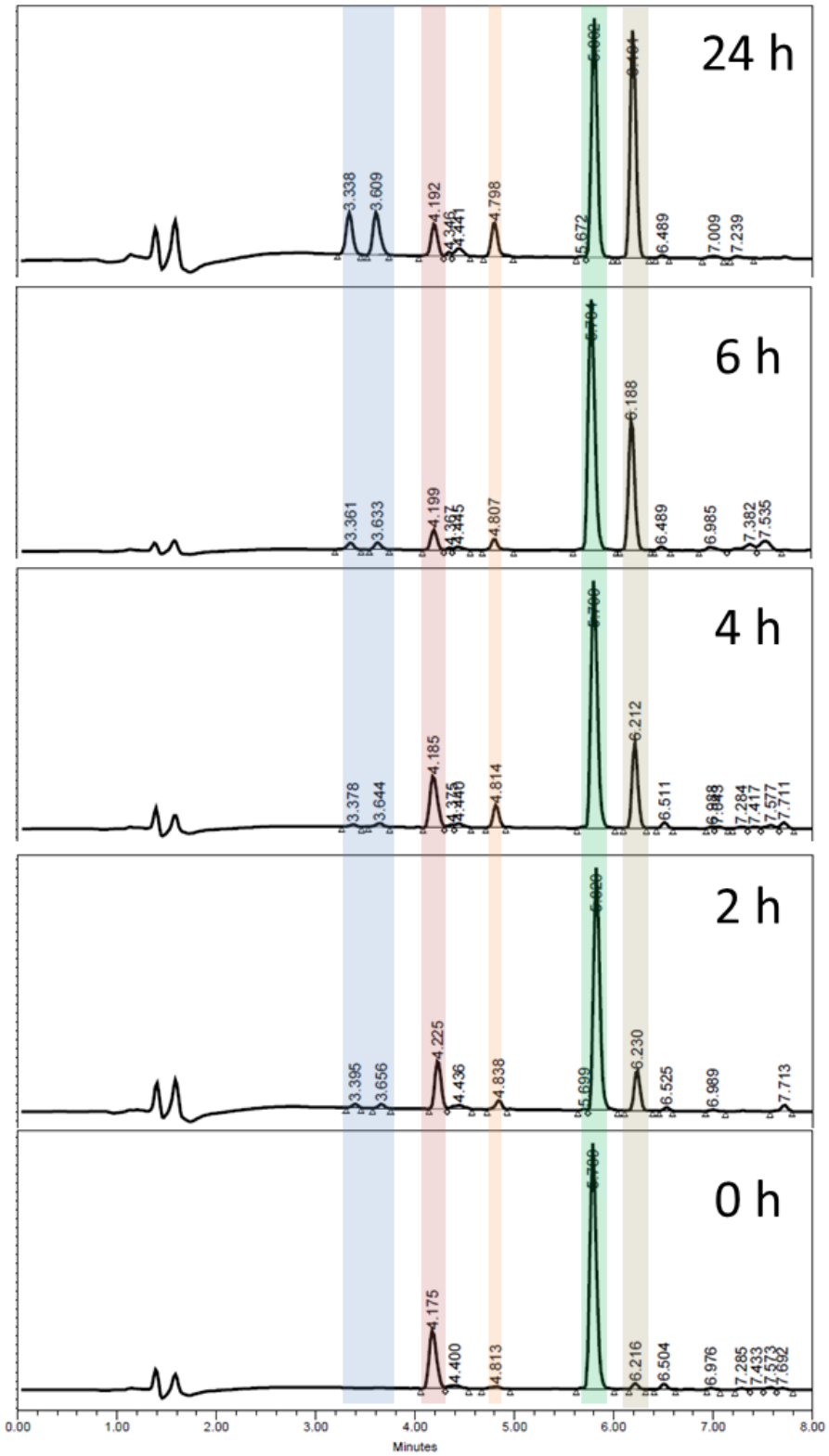
The corresponding Boc-Ala-Leu-Cys(PG)-O-Wang resins [for PG: Thp, L- and D-Cys(Trt)] were introduced into glass vial and it were treated with DMF/piperidine (4:1) (3 mL) for 24 h at 25 ºC. At 2, 4, 6 and 24 h, a small aliquot of resin were separated and it was introduced into a polypropylene syringe and after washes with DMF (5×2 mL) and DCM (4×2 mL). The peptides were cleaved from the resin using **TFA/TIS/H_2O (95:2.5:2.5)** (1×2 mL×1 h) followed by washes with DCM (3×1 mL). Then the filtered washes were evaporated and precipitated with Et_2O. The resulting peptides were dissolved into 1 mL of H_2O/MeCN (1:1) for their analysis by RP-HPLC.



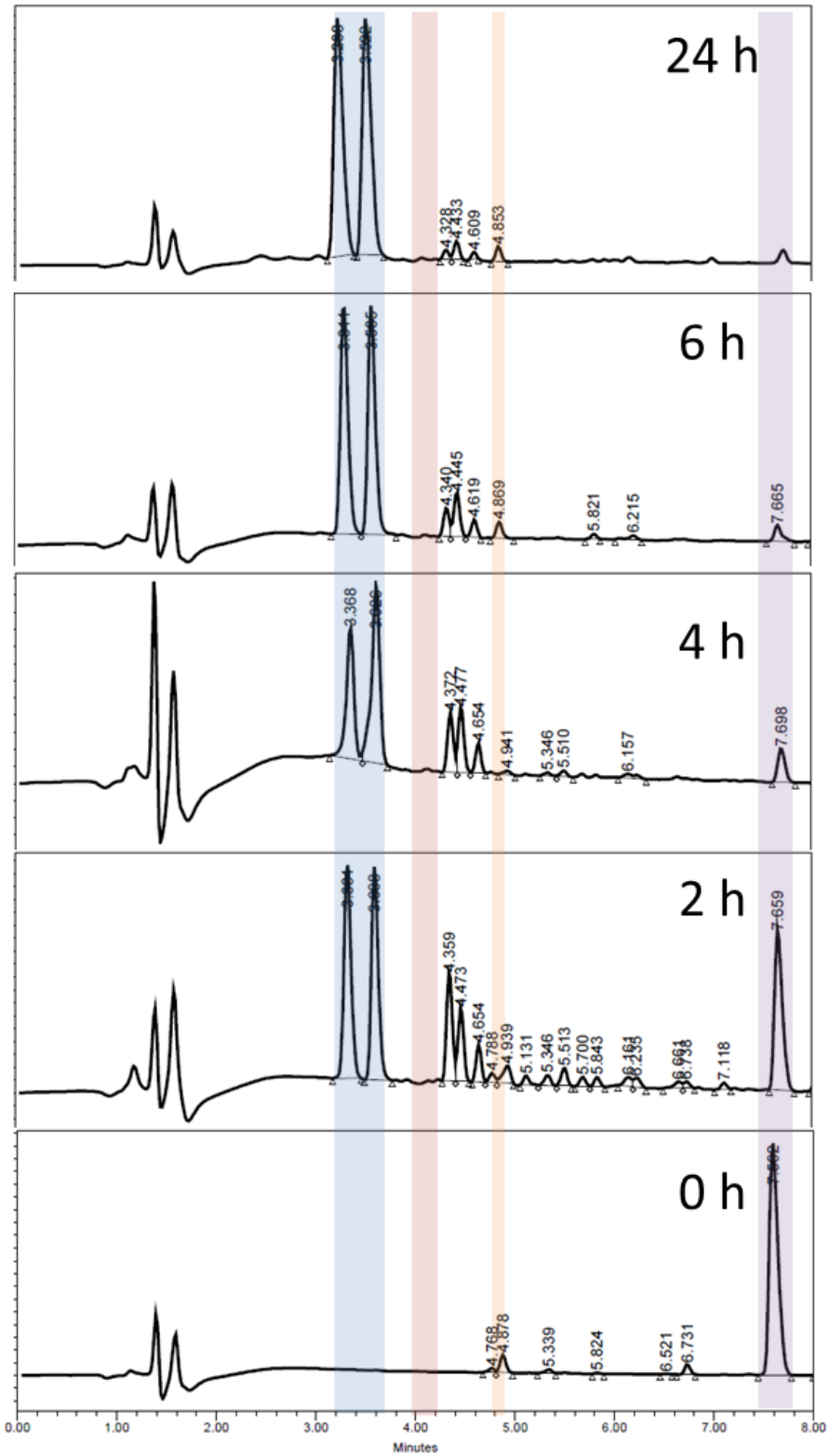
W1.1. Wang resin with Cys(Thp)



W2.1. Wang resin with Cys(Trt)



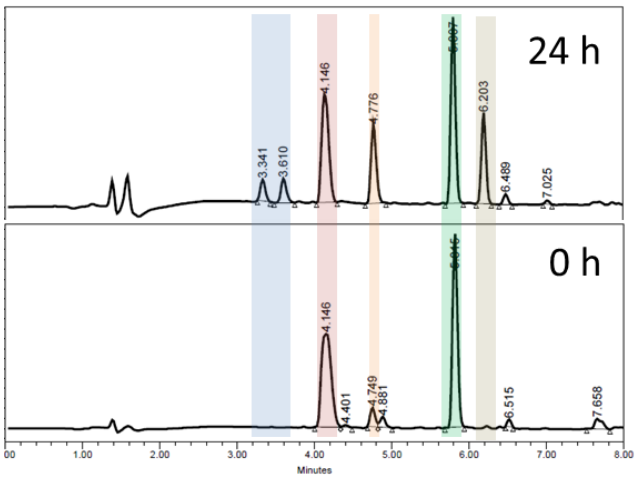
W3.1. Wang resin with Cys(S*t*Bu)



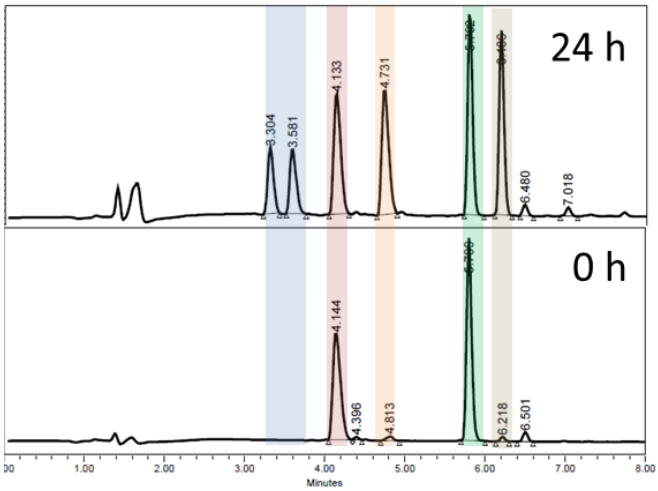
Alternatively, the aliquots after 24 h were cleaved using **TFA/TIS/DMB (92.5:2.5:5)** (1×2 mL×1 h) followed by washes with DCM (3×1 mL). Then the filtered washes were evaporated

and precipitated with Et_2O. The resulting peptides were dissolved into 1 mL of H_2O/MeCN (1:1) for their analysis by RP-HPLC.

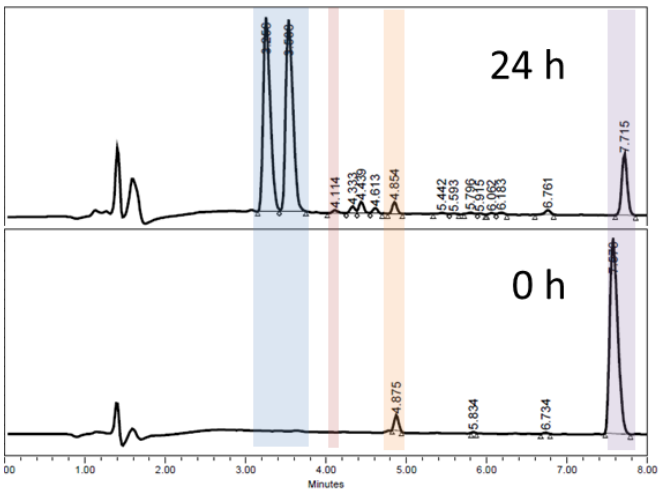
W1.2. Wang resin with Cys(Thp)



W2.2. Wang resin with Cys(Trt)



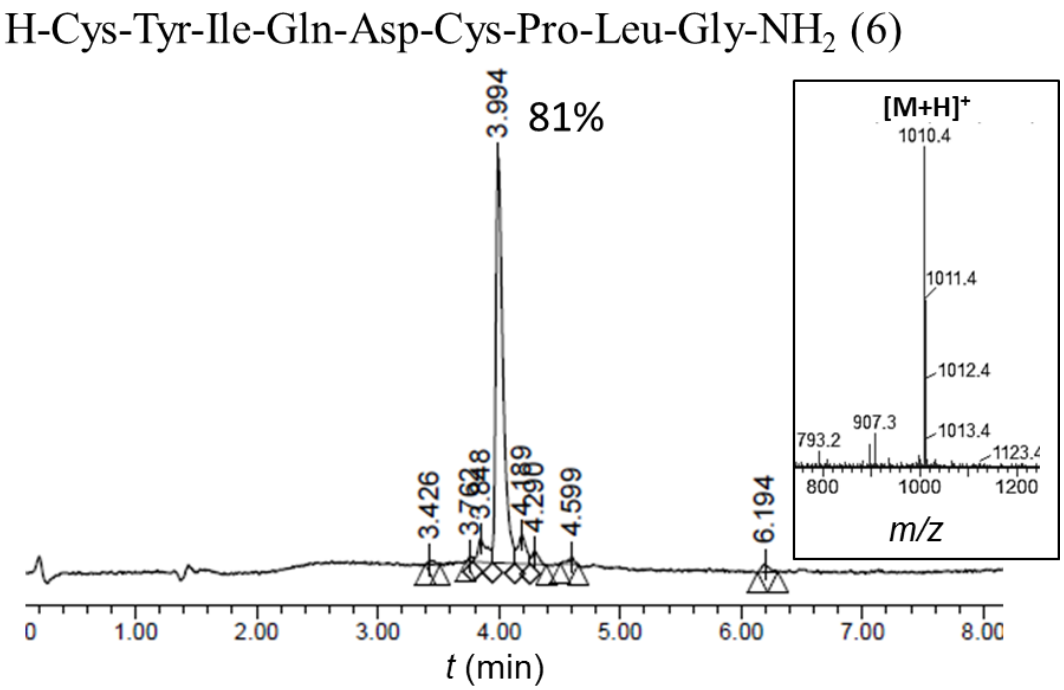
W3.2. Wang resin with Cys(S*t*Bu)



***N*-terminal Cys studies:**

Reduced Oxytocin (H-Cys-Tyr-Ile-Gln-Asp-Cys-Pro-Leu-Gly-NH_2) preparation:

Fmoc-Rink-amide AM-polystyrene resin (f = 0.71 mmol/g) was used for the peptide synthesis. Initially, the resin was washed with DCM (4×5 mL) and DMF (4×5 mL) and the Fmoc protecting group was removed by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 2×5 mL×5 min), and washed with DCM (4×5 mL) and DMF (4×5 mL). Then the first aminoacid was manually incorporated by treating the resin with Fmoc-Gly-OH (3 eq.), DIPCDI (3 eq.) and Oxyme Pure (3 eq.) in DMF, after pre-activation of 5 min. The mixture was left to react for 1 h and 30 min. Afterwards, the resin was washed with DMF (4×5 mL) and DCM (4×5 mL). Then the peptide chain was automatically elongated on the CEM Liberty Blue™ peptide synthesizer using the conditions described above. Fmoc-Cys(Thp)-OH was used for the peptide synthesis. The peptide cleavage was performed treating the peptidyl-resin with TFA/TIS/DMB (92.5:2.5:5) (1×2 mL×1 h) and washing with DCM (2×2 mL). Then the filtered washes were evaporated and precipitated with cold Et_2O. Then, the resulting peptide was directly analyzed without further purification.



Chapter II.

Non-cleavable linkers for Bioconjugation

An extensive part of the results present in this chapter are considered for further publication, the manuscripts are under preparation.

Chapter II. Non-cleavable linkers

1. Mesitylene linker

1.1. Introduction

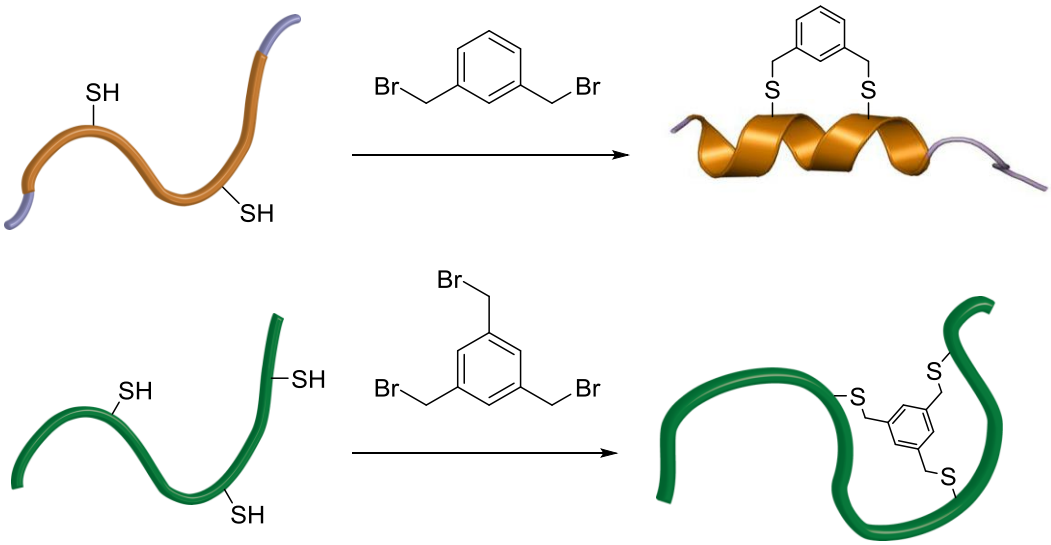
The initial report regarding the use of cysteine (Cys) and 1,3,5-Tris(bromomethyl)benzene (TBMB) was in 1981's, where both compounds were employed in order to understand the conformational behavior of peptides by the synthesis of conformational restricted peptides, which facilitate β-turns into the peptide chain.[1] Making benefit of this studies, the same research group utilized TBMB for the formation of vase-shaped cage compounds, which are able to trap water molecules in their structure.[2] Thus, this mentioned studies provide an important structural information and valuable models for understand peptides interactions with other biomolecules. Shortly after, TBMB was taken place on the preparation of bowl-shaped chiral homotriazacalixarenes, in that sense the mesityl aromatic core improve the synthetic methodology for this architecturally complex macrocycles which is broadly used as receptors for ionic and neutral molecules.[3]

The studies regarding bromobenzyl derivatives which assisted peptide cyclization's was continued by Timmerman group, who carried out the robust formation of single, double and triple loops in Cys containing peptides with di- tri- and tetrabromobenzyl derivatives respectively.[4] In this work it was demonstrated that the cyclization step takes place rapidly in aqueous solvents, allowing diverse polycyclic structures based on peptidic scaffolds.

More recently, Heinis and coworkers[5] exploited TBMB as rigid organic core to build up highly selective Cys-containing bicyclic peptides libraries from phage-display screening, expanding their chemical use. In this regard, Szostak group,[6] used α,α-dibromo-*o*-xylene as cross-linker for Cys residues in order to perform peptide cyclization for the synthesis of macrocyclic target-specific binding peptides libraries, which were tested *in vivo* mRNA-display. These methodologies allows the rapid production and selection of chemically modified peptide libraries, therefore simplifying the isolation of high-affinity binders to interesting pharmacological proteins.[7,8] Moreover, Heinis exploits the use of bis(bromomethyl)benzene in the stabilization of bicyclic peptides with hydrophilic chemical structures at their center to promote noncovalent intramolecular interactions, thereby stabilizing peptide conformation, among peptide-protein interaction. Also, allowing the development of new peptides with high bind affinity capacities, and opening the avenue for the generation of synthetic antibody-mimicking structures.[9] Additionally, using the above-mentioned methodology, Heinis re-

search group tested the ability of small byciclic peptides decorated with the tribenzylic scaffold to penetrate and distribute evenly into solid tumours.[10]

In that sense, the bicyclic peptides have shown more extensive interactions with globular proteins than the linear analogues.[11] In consequence, bicyclic peptides will be more capable to block protein-protein interactions and in comparison with disulfide-bonded bicyclic peptides, covalently-bonded cyclic peptides ensure more stability in front of proteases and reducing environments,[12,13] leading constrained cyclic peptides as interesting potential drugs.[14] Several polybrominated alkyl and benzyl derivatives were studied for peptide cyclization or stapling,[15] and more particularly for α-helix stabilization[16] (**Scheme 20**). In this sense, several peptides stapling or macrocyclization methodologies have been developed to date by modifying peptide main chain either the amino acids side chains.[17] Disulfide bond formation is the natural way to introduce peptide staples,[14,18] on contrary, chemical modifications have been developed for peptide stabilization such as ring closing metathesis[19,20] or "click" chemistry reaction.[21,22]

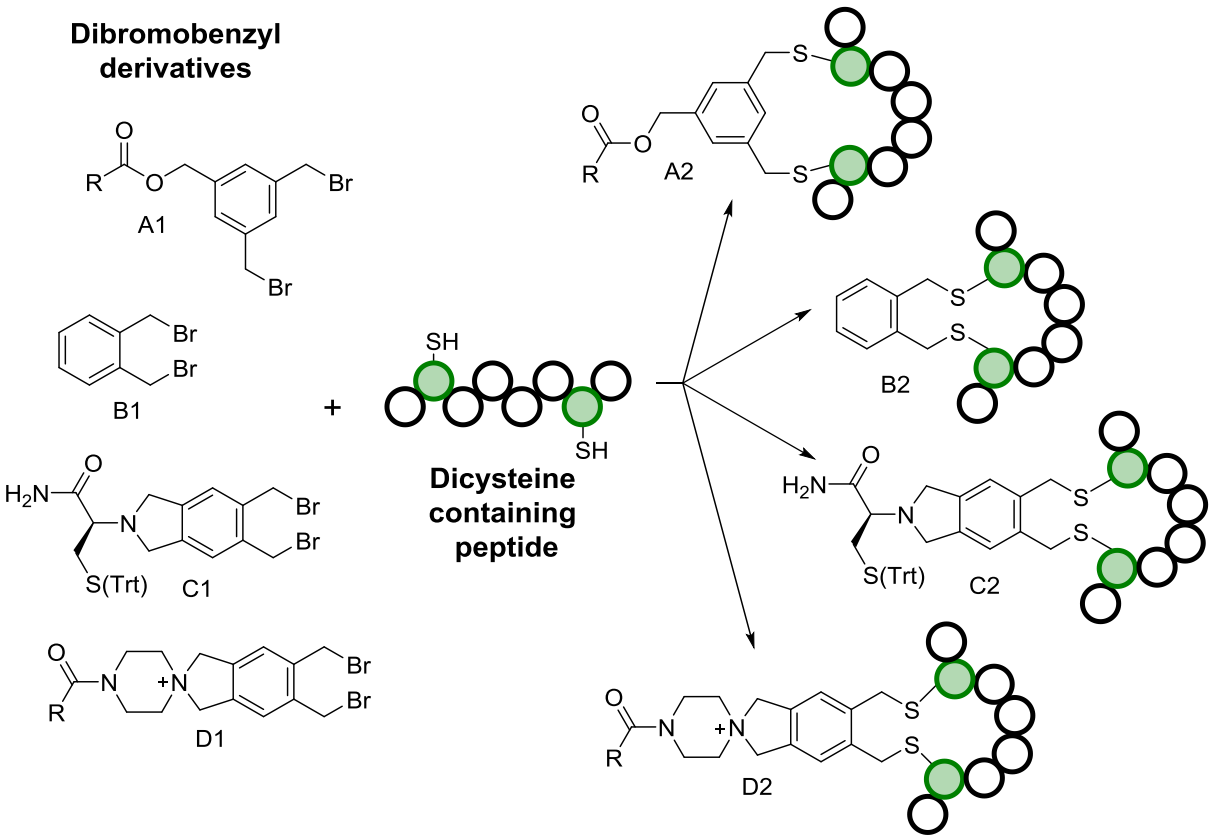


Scheme 20. Bromobenzyl derivatives for peptide stapling and cyclization.

The interesting work carried out by Hartman and collaborators amplify the use of TBMB scaffold in a dual way using it as peptide stapling and moreover as linker for carboxylic acids.[23] Hartman described the chemical modification of TBMB by the monoalkylation with different carboxylic acids in basic conditions yielding a functionalized dibromobenzyl derivatives (**Scheme 21**, A1), which in front of a dicysteine containing peptides, allow the simultaneous cyclization and moreover the peptide labelling (A2). In this sense, Szewczuk *et*

al.[24] introduced the dibromoxylene (B1) for peptide cyclization through Cys-containing peptides in order to restrict the peptide conformation (B2). Furthermore Walker and Johnson,[25] study different bromoalkyl and aryl scaffolds including *m*-dibromoxylene, in order to prepare Cys-decorated cyclic peptidomimetics for its use as a potential serine-proteases inhibitors. All mentioned strategies followed the Mosberg procedure,[26] where the cyclization in dicysteine containing peptides occurs through a linker mediation.

Going a step further and following the CLIPS concept (**C**hemical **L**Inkage of **P**eptides onto **S**caffolds),[27] Timmerman and Maarseveen group[28] improved one of the principal problems present in dibromoxylene products, the water solubility. For that, the polyalkylation of primary and secondary amines with 1,2,4,5-tetrabromodurene yields a tertiary (C1) and a quaternary ammonium ion water-soluble (D1) functionalized *m*-dibromoxylenes, (**Scheme 21**) which are suitable for peptide cyclization in aqueous media.



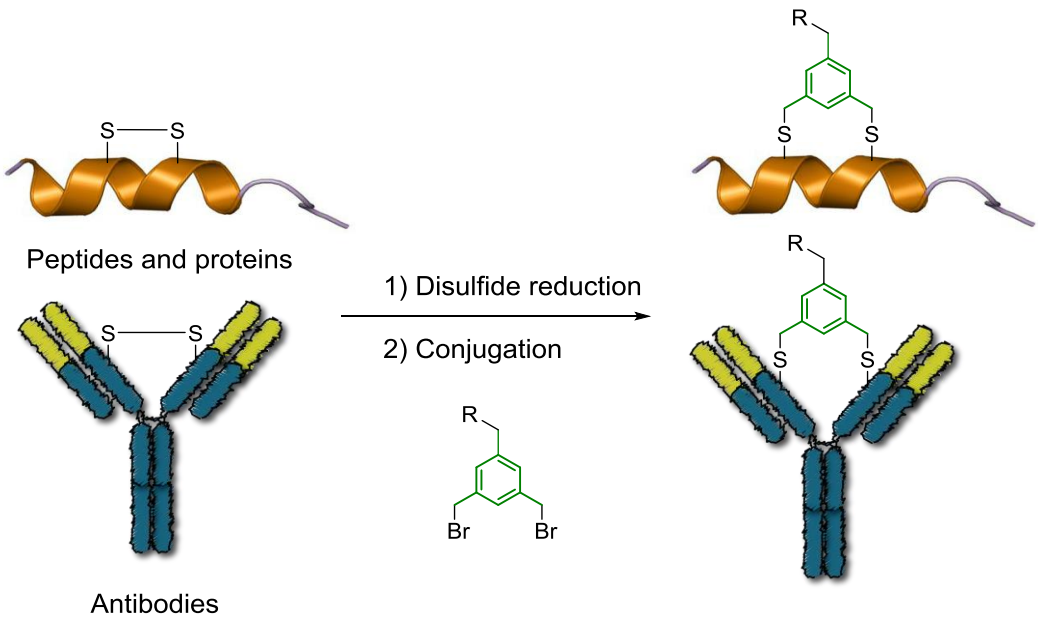
Scheme 21. Dibromobenzyl derivatives as peptides linkers.

The studies done to date regarding dibromobenzyl derivatives as linkers, inspired us for further study of this special system using more complex dicysteine containing compounds as

antibodies are. The chemical stability of the formed thioether into the benzylic scaffold, could be potentially interesting for its use as non-cleavable linkers for bioconjugation in general, and for ADCs in particular.

For that purpose, the bioconjugation strategy to follow comprise the previous functionalization of the 1,3,5-Tris(bromomethyl)benzene (TBMB) as Hartman and co-workers described.[23] The TBMB functionalization with carboxylic acid was discarded, due to the potential instability of the obtained benzylic ester in humans by the enzymatic degradation mediated by esterases, which are widely distributed throughout the body.[29,30] Accordingly, in order to avoid enzymatic cleavage, we decided to study the monoalkylation of TBMB using the thiol group. In this regard, the thiol group is an excellent choice due to their chemical properties and selective reactivity in front of halobenzyl derivatives under physiological conditions.[31] Moreover, the robustness of obtained thioether bond, which is broadly used in bioconjugation, allows a strong binding to protein being stable enough to avoid their degradation into systemic circulation.[32]

The strategy followed also involves a disulfide bond reduction of the peptide, protein or antibody in order to release two thiols groups, which finally react with the functionalized dribromobenzyl derivative yielding the desired tribenzyl-mediated conjugate (**Scheme 22**).



Scheme 22. Bioconjugation strategy.

In this kind of conjugation, the stable dibenzylic dithioether replace the native interchain disulfide bond of the antibody, which are sensitive to reductive agents such as reduced glutathione present into our body,[33] conferring an additional stability to the peptide, protein or antibody. Moreover, the low abundance of Cys, in comparison to other reactive amino acids (i.e. amines from lysines or carboxylic acids from aspartic or glutamic acid) into peptides and proteins structure, transform this methodology even more attractive due to the site-specificity that this fact confers being a potential interesting bioconjugation strategy.

1.2. Objectives

- Study the functionalization via thiol monoalkylation of 1,3,5-tris(bromomethyl)-benzene (TBMB) and its further use as linker in bioconjugation.
- Find optimal bioconjugation conditions for dibromobenzyl derivatives.
- Use dibromobenzyl compounds for antibodies bioconjugation and study if the bioconjugation process affect to the macromolecular entity.

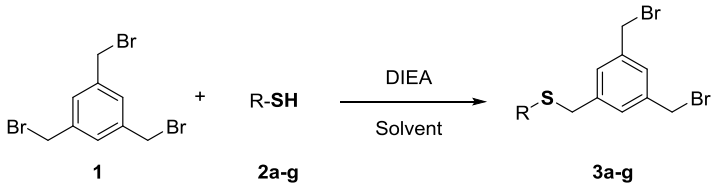
1.3. Results and discussion

Monoalkylation studies and linker-cargo preparation

As starting point, we tested different conditions to monoalkylate the 1,3,5-tris(bromomethyl)benzene (TBMB) with phenolic, benzylic or alkylic thiols in order to prepare dibromobenzyl derivatives (**Scheme 23**, **Table 13**) for its further use in bioconjugation. Some thiol derivatives were commercially available (**2a,b,f**) but some others were previously synthetized for this purpose (**2c-e,g**)(see experimental section).

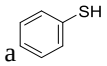
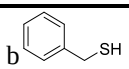
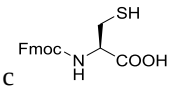
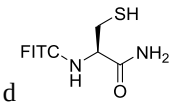
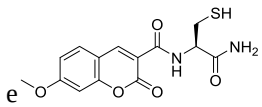
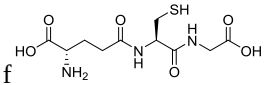
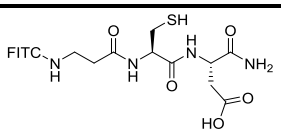
Table 13 illustrates the thiols and the conditions used for testing the monoalkylation reaction, the % of conversion detected by HPLC/UPLC at the indicated time of reaction, temperature, and the isolated product yield. The monoalkylation reaction was performed using the indicated equivalents of TBMB and 1 eq. of the reactive thiol in presence of diisopropyl ethylamine (DIEA) into the indicated solvents.

The results obtained depicted the aliphatic thiols (**2c-g**) as the best choice in terms of HPLC/UPLC reaction conversion, and also by the absence of an awkward smell in comparison with aromatic thiols. Accordingly, monoalkylation of less-polar thiols (entries 1-12) takes place with better results than with polar substrates (entries 13-15). For long reaction times, polialkylation products appear in the reaction mixture hindering the isolation of the desired monoalkylate derivative. Moreover, the average of the reaction conversions corroborated that bimolecular nucleophilic substitution (S_N2) rate are increased in polar solvents (DCM < MeCN < DMF). Although the syntheses of dibromoxylenes derivatives were a challenging task, we obtained the quantity necessary of 3c, 3e and 3g to carry out the bioconjugation experiments. We corroborated that a rigorous control of the reaction conditions such as equivalents, reaction time, temperature and solvents were needed in order to obtain monoaddition products avoiding the undesired polyalkylation, due to the reactivity of the thiol group in front of halobenzylic compounds. Additionally, the product isolation found to be a tricky process where the reaction yields decrease drastically due to the compound decomposition.



Scheme 23. 1,3,5-tris(bromomethyl)benzene (TBMB) thiol monoalkylation.

Table 13. 1,3,5-tris(bromomethyl)benzene (TBMB) thiol monoalkylation.

Entry	R-SH (2)	TBMB (1) (eq.)	DIEA (eq.)	Solvent	Reaction time (h)/ Temp (°C)	HPLC/UPLC conversion	Yield (3a-g)
1	 a	1.0	1.2	DCM	1 h/rt	13%	-[a]
2	 b	1.0	1.2	DCM	1 h/rt	22%	-[a]
3	 c	1.0	1.0	DCM	1 h/rt	21%	×[b]
4		1.5	3.0	DCM	17 h/rt	21%	63%
5		1.0	1.0	DCM	17 h/37°C	46%	×[b]
6				MeCN	17 h/rt	8 %	×[b]
7		1.0	3.0	MeCN	0.5 h/ 0 °C	35%	32%
8		1.0	1.0	DMF	0.5 h /rt	17%	×[b]
9		1.0	1.0	DMF	0.5 h / 0 °C	38%	20%
10	 d	1.0	1.2	MeCN	17 h/rt	20%	×[b]
11		1.0	1.1	DMF	1 h/ 0 °C	40%	×[b]
12	 e	1.0	1.2	DMF	1 h/rt	47%	×[b]
13		1.0	1.2	DMF	1 h/ 0 °C	28%	31%
14	 f	1.0	1.0	DMF	0.5 h / 0 °C	ND[c]	×[b]
15	 g	1.2	1.2	MeCN	1 h/rt	15%	5.7%
16		1.1	2.0	DMF	0.5 h / 0 °C	24%	12%[d]

[a] Not isolated. [b] Product decomposition during Work up (Polyaddition). [c] ND → no determined. [d] The mass detected for the isolated product correspond to thiol monoalkylation but with dibromobenzyl hydrolysis (See experimental section).

Bioconjugation

- **Conjugation with oxytocin**

As a proof of concept, we attempt the bioconjugation into a dithiol containing model. For this purpose, we prepared the reduced form of the natural hormone Oxytocin (**4**) by automatic

microwave assisted solid phase peptide synthesis. For the peptide synthesis, the Cys protecting group recently developed by us (Thp) was used (chapter I.3), which avoid the Cys racemization in SPPS.[34] The conjugation reaction between the dithiol-containing peptide **4** and the dibromobenzyl derivative (**3c**) was performed in basic $NaHCO_3$ buffer at pH = 8 at room temperature (**Figure 25**). Surprisingly the conjugation reaction took place rapidly and as the HPLC chromatogram show (**Figure 25B)**, the chromatographic peak corresponding to the reduced oxytocin (**4**) disappeared after 15 min of reaction obtained the expected cyclic peptide **5**. This result encouraged us to use the dibromobenzyl system for conjugation to antibodies.

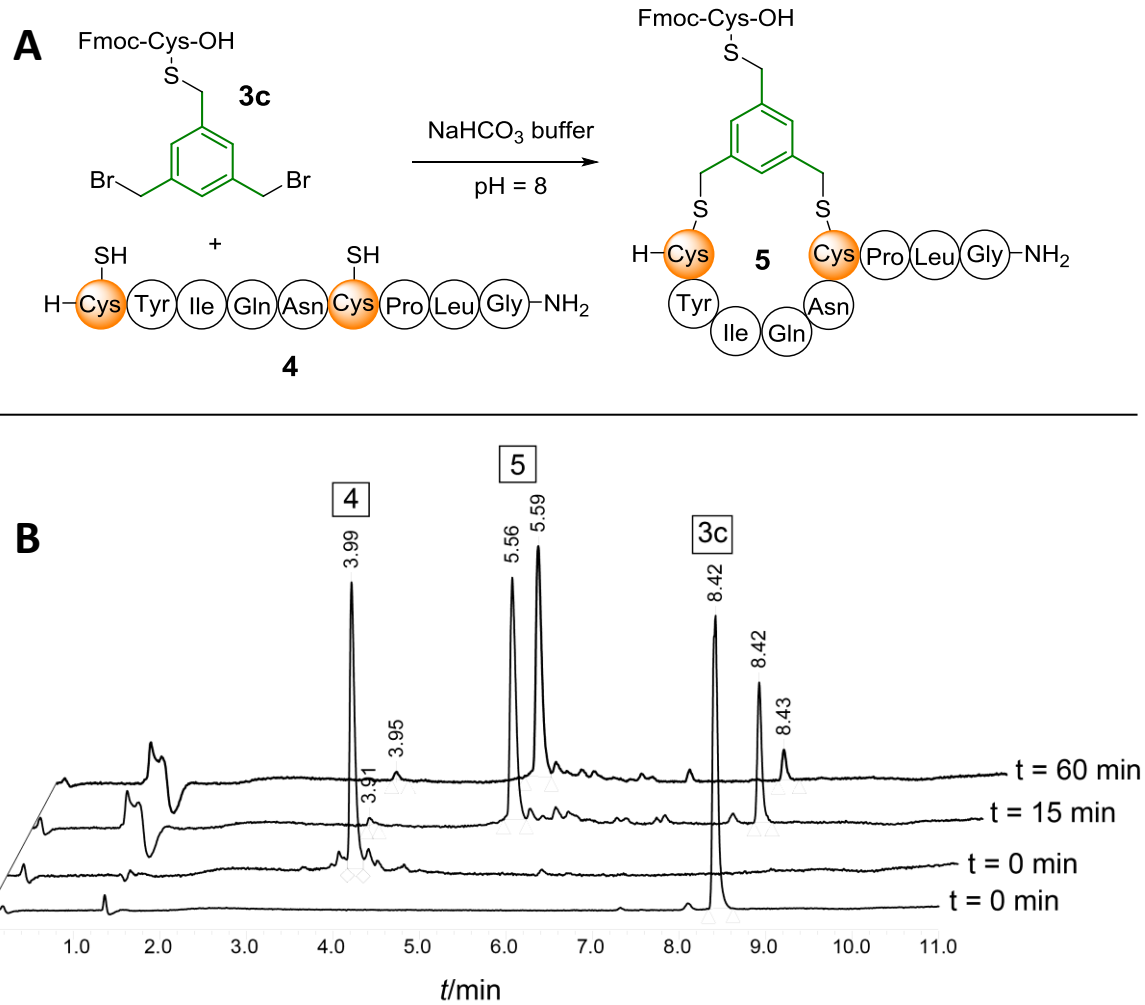


Figure 25. A) Dibromobenzyl derivative **3c** conjugation with reduced oxytocin **4. B)** HPLC chromatograms of the starting materials **3c** and **4**, and the bioconjugation product **5**.

- **Conjugation to Anti-CD4 and Anti-CD13**

In order to carry out the bioconjugation with the dibromoxylene system, a previous disulfide reduction step into the antibody was needed to liberate a reactive thiol groups. In order to study the behavior of the bromobenzyl derivatives in front of two or more reactive

thiols an Anti-CD4 solution (1 mg/mL approximately) was treated with different quantities of TCEP (1, 3 eq. and an excess >100 eq. for conjugates **2 a,b** and **c** respectively) during 1 h at 37 ºC. After disulfide reduction, the dibromobenzyl derivative (**3c**) was added to the antibody solution and it was left to react at 4 ºC (quantities indicated in the experimental section). After 1 h the conjugates (**Conj2a-c**) were purified and conveniently characterized by SDS-Page (**Figure 26A and B**).

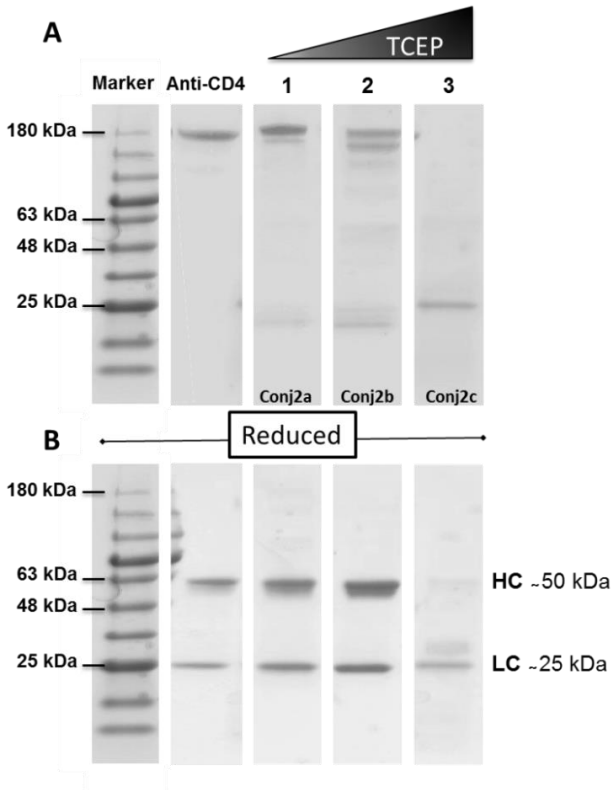
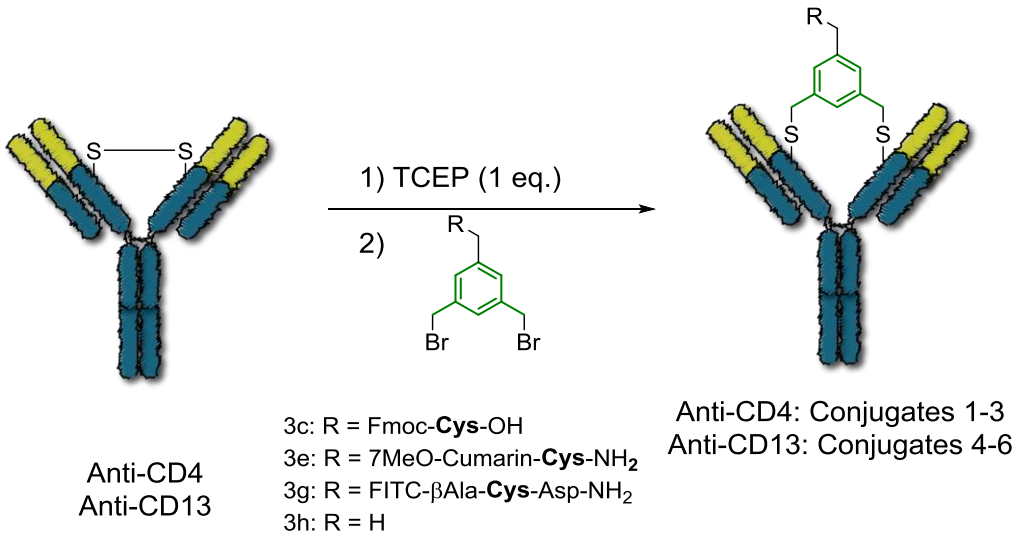


Figure 26. SDS-PAGE analysis of the prepared conjugates in **A**) non-reducing and **B)** reducing conditions. Acrylamide 4-20%. HC and LC correspond to Heavy chain and Light chain, respectively.

The SDS Page under reduced conditions (**Figure 26B**) showed that the reduction step affect the integrity of the conjugate. For the same quantity of the dibromobenzyl **3c,** the more equivalents of TCEP used, the more reduction of the antibody. Using SDS in non-reducing conditions (**Figure 26A**), we expected to observe the entire antibody band (≈150 kDa) and it was observed with the naked antibody and with the conjugate analyzed in the line 1 (**C2a**), where the conjugation was carried out using equimolar quantities of TCEP and the dibromobenzyl **3c**. However, when the quantity of TCEP was higher than **3c** (more than 1 eq.) new bands appeared in the electrophoresis gel. In contrast with reducing SDS analysis, with 1 and 3 eq. of TCEP (lane 1 and 2 from **Figure 26B**) the observed bands corresponds to the light and heavy chains (25 and 50 kDa respectively), meanwhile using excess of TCEP, apart of the light chain band more than one band is observed, indicating that this conditions affects to the

structure of the conjugate. Due to the low molecular weight of the molecule conjugated **3c** {(459.6 g/mol) correspond to $C_{27}H_{25}NO_4S^{2+}$ [**3c**-Br_2]$^{2+}$} in SDS analysis the differences between the conjugate and the naked antibody are minimum and that fact difficult the analysis of results observed by SDS.

Determined the optimal conditions to reduce disulfide bonds without affecting to the integrity of the antibody, the next step will be the preparation of a set of conjugates. For that, selected and previously prepared dibromobenzyl derivatives were conjugated to the antibodies Anti-CD4 and Anti-CD13 after the disulfide reduction with 1 eq. of TCEP followed by the addition of the activated dibromobenzyl derivative and stirred at 4 ºC during 30 min. In order to ensure the conjugation, a slight excess of the dibromobenzyl derivative dissolved in DMSO was used as indicated in the following table (DMSO<10%) (**Table 14**). Additionally to the prepared compounds (**3c**, **3e** and **3g**), we also used the commercially available *m*-dibromoxylene (**3h**) for the bioconjugation (**Scheme 24**).



Scheme 24. Bromobenzyl derivatives antibodies conjugation**.**

Table 14. Mesitylene-antibody conjugation conditions.

Conjugate	Antibody	Conjugated Compound	R	eq. of R
1	Anti-CD4	3h	H	1
2a		3c	Fmoc-**Cys**-OH	2
2b				6
2c				46
3		3g	FITC-βAla-**Cys**-Asp-NH_2	2
4a	Anti-CD13	3h	H	2
4b				4
5a		3c	Fmoc-**Cys**-OH	2
5b				4
6a		3e	Cumarin-**Cys**-NH_2	4
6b				4

The indicated conjugates were fully characterized by UV (**Figure 27**), SDS-PAGE (**Figure 28**), HPLC and mass spectrometry (**Figure 29**) (See experimental section for all the analysis). As the UV analysis of the synthetized conjugates reveals, some changes into the conjugates spectra, in comparison to the naked antibody have found after the conjugates purification by PD-10 columns. Despite the tricky dibromobenzyl compound 3g preparation, one of the most encouraging results was the preparation of the conjugate 3, where the introduction of a labelled tripeptide (3g) into the antibody could ease the identification of the conjugate by their analysis by UV. As **Figure 27A** depicted, the variation into the spectra at approximately 475 nm clearly indicates that the conjugation of a molecule that contains FITC (Conjugate 3) occurs. Unfortunately, the UV analysis for the 7-methoxycumarin conjugates **Figure 27B** (Conjugates **6a**, **6b**) did not reveal a clear conjugation due to the small UV variation at approximately 330 nm, the degradation of the dibromobenzyl compound could decrease the conjugation ratio.

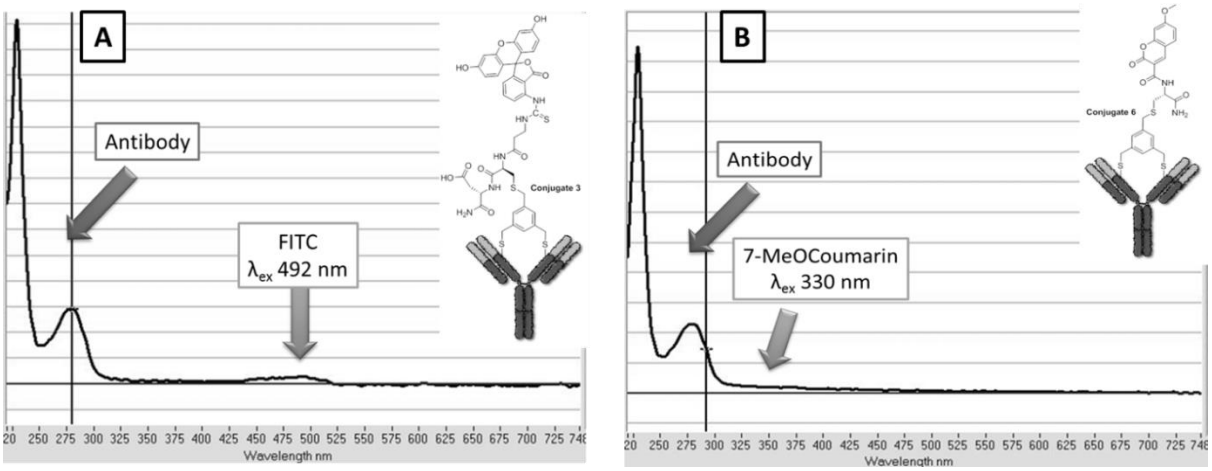
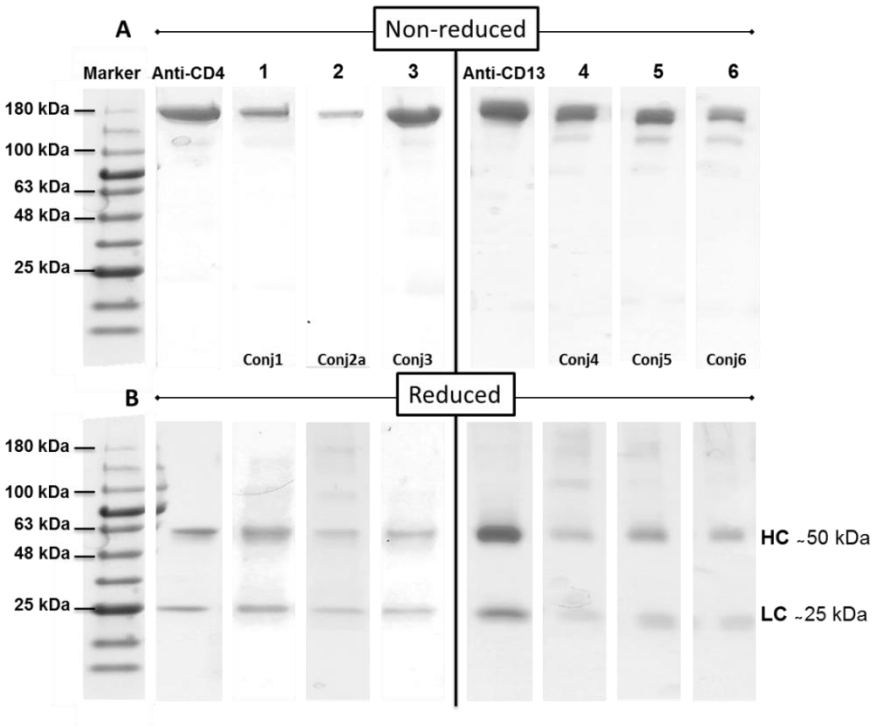


Figure 27. UV Spectra (from 220 to 750 nm) for **A)** Conjugate 3 (Compound **3g** conjugate to Anti-CD4) and **B)** Conjugate 6 (compound 3e conjugated to Anti-CD13).

The analysis by SDS and by mass spectrometry reveals that the integrity of the antibody was maintained during the developed bioconjugation protocol. Their analysis by SDS in non-reducing conditions (**Figure 28A**) reveal that the antibodies were not completely reduced as shown when more than one eq. of TCEP was used (**Figure 28A conj2c**). Moreover, the soft appearance of approximately 100 kDa bands (**Figure 28B**) is a strong indicative that at least some molecules are conjugated to the antibody, linking two high chains (HC) (50+50 kDa) of the mAb. In this sense, the stability of the thioether linkage, maintains linked both chains and for that reason, this bands appeared in the SDS analysis. As not all the HC's are linked each

other, the use more equivalents of dibromobenzyl derivatives are needed for a complete bioconjugation.



184

Figure 28. SDS-PAGE analysis of the antibodies and their conjugates (1-6) in **A)** non-reducing, and **B)** reducing conditions. Acrylamide 4-20%. HC and LC correspond to Heavy chain and Light chain, respectively

Previously to the mass spectrometry analysis, the *N*-glycosids from the antibody were removed by treating the conjugates with PNGase F[35] in PBS during 17 h in order to improve the mass spectra.[36,37] All the conjugates were analyzed for an accurate determination of the number of molecules, which were attached to the antibody using this methodology. As the mass analysis depicted, mostly all the conjugates maintain its integrity after the conjugation step. The analysis of the molecules attached into the antibodies was determined by comparing the naked antibodies mass spectra with the respective conjugate **(Figure 29).**

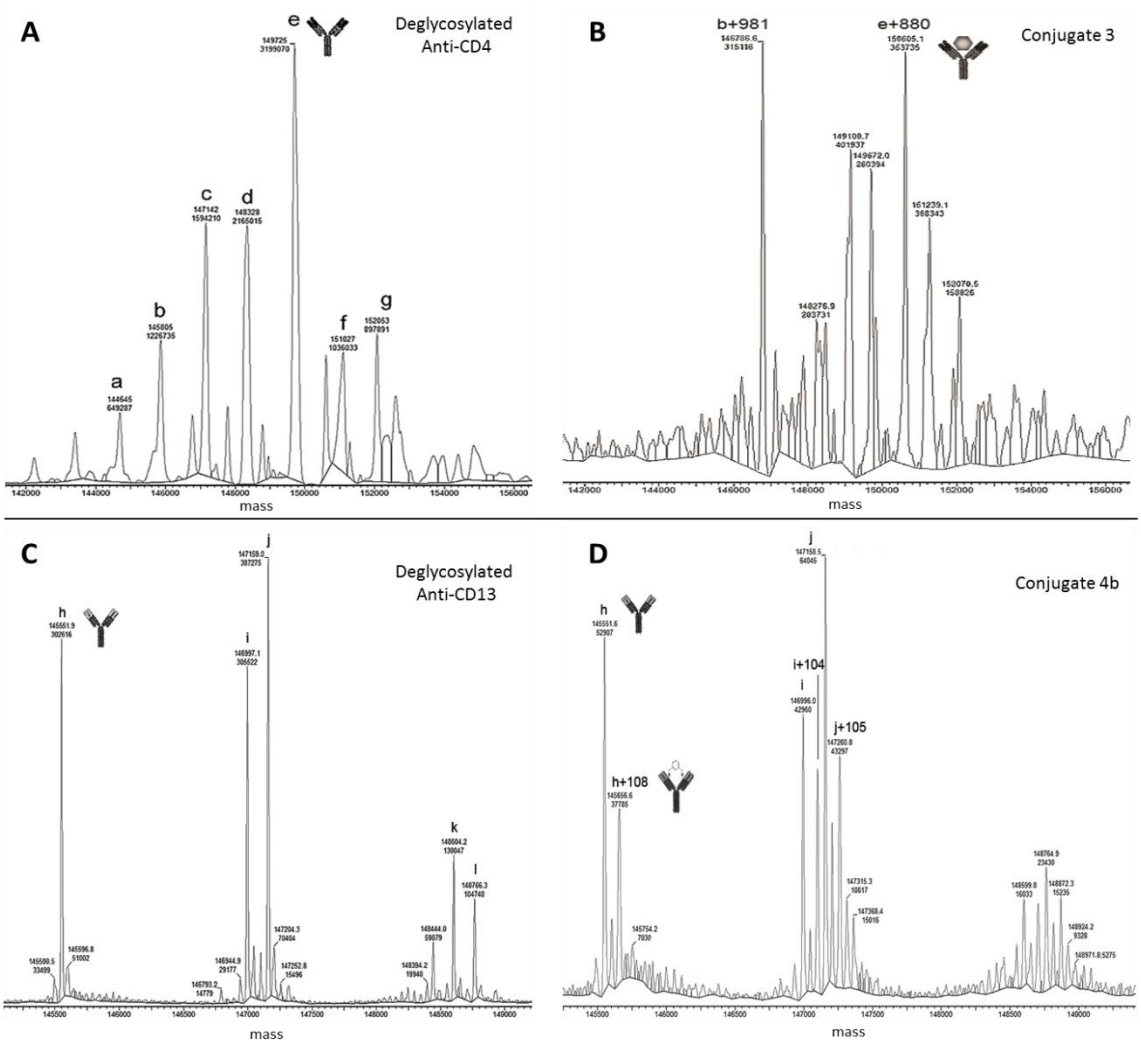


Figure 29. TOF ESI$^+$ for deglycosylated **A)** anti-CD4**, B)** conjugate 3**, C**) anti-CD13 and **D)** conjugate 4b. For conjugates 3 and 4b the molecular weight of the conjugated compounds 3g and 3h are 813.9 and 104.2 Da respectively.

Regarding anti-CD4 mAb and conjugates due to the complexity of the mass spectra, the analysis got unreliable information about the number of molecules covalently attached to the immunoglobulin but a mass increment was observed for the deglycosilated conjugates into its analysis (compare **Figure 29A** and **B**). On contrary, the mass spectra for anti-CD13 present mainly 4 peaks from 144 to 148 kDa approximately (**Figure 29C**), simplifying the conjugates analysis. The clearest evidence of conjugation has shown with conjugates 3 and 4 for anti-CD4 and CD13, respectively (**Figure 29**B and D). Accordingly, the accurate quantification of the molecules conjugated to the antibodies is limited due to the poor sensitivity of compounds with high mass. Thus, completely antibody reduction or enzymatic digestion should improve the analysis sensitivity.

Antibody-Conjugate cell binding analysis.

To determine whether the conjugation of the linkers to the anti-CD4 HP2/6 and anti-CD13 TEA1/8 mAbs had any effect on the binding to their corresponding epitopes, we performed an antibody binding analysis with cell lines that constitutively express CD4 (Jurkat cell line) or CD13 (U937 cell line). For both cell lines, we used increasing concentrations (0.1-10 µg/ml) of the naked antibodies (anti-CD4 HP2/6 and anti-CD13 TEA1/8 mAbs) and their corresponding conjugates (1-3 for anti-CD4 and 4-6 for anti-CD13). As shown in **Figure 30A and B**, the naked antibodies showed a higher mean fluorescence intensity (MFI) and binding affinity compared to those of the different conjugates (**Table 15**).

Comparing how the disulfide reduction affects the conjugation (of anti-CD4), we can observe that a complete reduction of the antibody during the conjugation affects drastically to the immunoglobulin binding properties. In this regard, the conjugate 2c was found to be inefficient due to the poor binding to the antigen CD4. Conjugates 2a and 2b had similar binding affinity compared to the anti-CD4 mAb (AC_{50} 0.3, 0.36 µg/ml compared to 0.38 µg/ml, respectively), although they reach a lower maximum mean fluorescence intensity value (71 and 89 %, respectively) than that of the anti-CD4 mAb. This result suggests that conjugates 2a and 2b would coat mostly all available CD4 molecules on the cell surface. Conjugates 1 and 3 had AC_{50} of 0.49, 0.31 µg/ml, respectively, but it returned low average of the maximum MFI obtained using the naked anti-CD4 mAb. However, for conjugates 1 and 3 the reduction in the maximum MFI obtained might simply reflect a diminished binding of the secondary FITC antibody, as a result of epitope masking caused by the linkers. On contrary, the loss of activity of the conjugate 2c is attributed to the destruction of the antibody due to its complete reduction.

On the other hand, all the analyzed anti-CD13 conjugates showed a reduced binding affinity in comparison to the naked antibody but all of them are suitable to coat the available CD13 molecules present in the cell surface (**Figure 30B**).

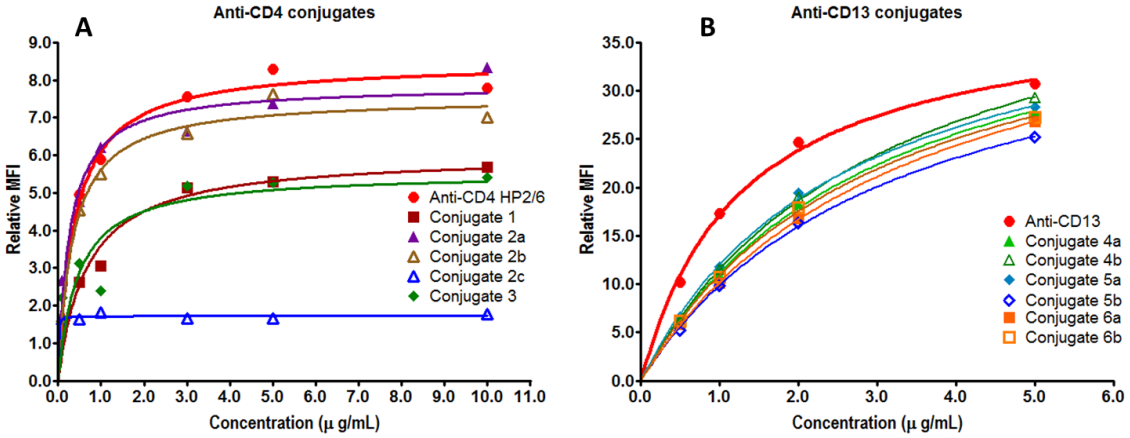


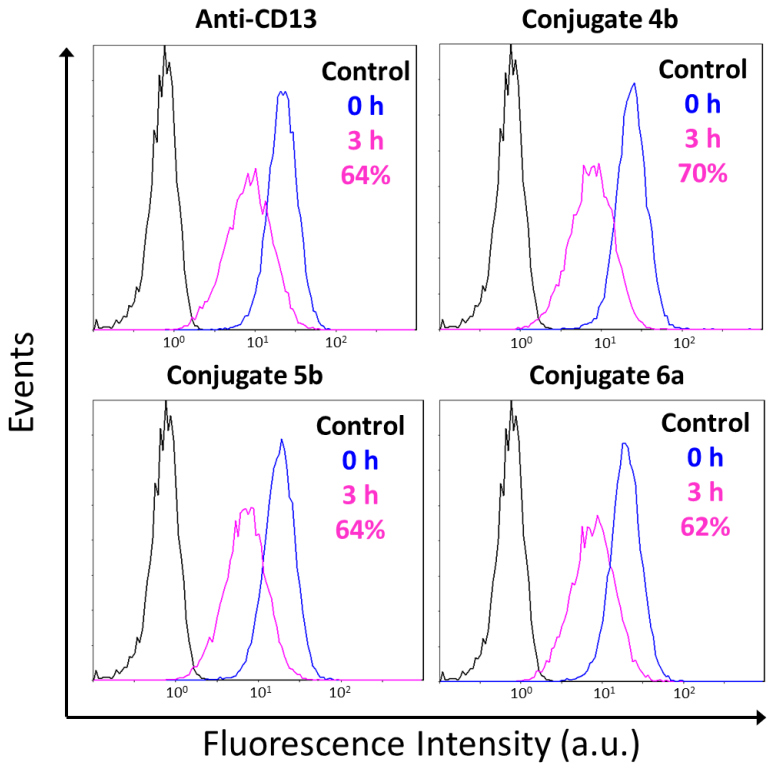
Figure 30. Antibody-Conjugates cell binding analysis by flow cytometry for **A**) anti-CD4 and conjugates 1-3 to Jurkat cells and **B**) anti-CD13 and conjugates 4-6 to U937 cells. Both cell lines were incubated with the indicated concentrations of mAb or the conjugates 1-6, followed by incubation with FITC-labelled goat anti-mouse IgG antibodies, as described in Materials and Methods. Cytometry analysis was performed and the mean fluorescence intensity (MFI) ratio was calculated using as reference the MFI value or an irrelevant mAb (basal MFI value).

Table 15. AC_{50} and Max MFI (%) for mAbs anti-CD4 and anti-CD13 and its conjugates (1-6). AC_{50}, calculated as the antibody concentration (AC) needed to achieve 50% of the highest MFI value for each conjugate

	AC_{50} (µg/mL)	Max MFI (%)		AC_{50} (µg/mL)	Max MFI (%)
Anti-CD4	0.38	100	**Anti-CD13**	0.82	100
Conjugate 1	0.49	57	**Conjugate 4a**	1.23	90
Conjugate 2a	0.30	71	**Conjugate 4b**	1.24	95
Conjugate 2b	0.36	89	**Conjugate 5a**	1.16	92
Conjugate 2c	-	1	**Conjugate 5b**	1.25	82
Conjugate 3	0.31	46	**Conjugate 6a**	1.27	87
			Conjugate 6b	1.22	89

Antibody internalization also plays a crucial role during the ADC design, since endocytosis of the conjugates is essential to release the drug into the specific cell, resulting in higher therapy efficiency. Previous not-reported studies regarding anti-CD4 and anti-CD13 internalization carried out by Pérez-Chacon at the UAM-CSIC, showed better internalization efficiency for anti-CD13 mAb. Accordingly, internalization studies were carried out in U937 cells, incubating at saturating concentrations (10 µg/mL) of anti-CD13 or the conjugates 4b, 5b and 6a at 4 ºC and 37 ºC during 3 h.

For the analysis, we considered t = 0 h of endocytosis the condition in which cells had been incubated at 4 ºC and calculated the percentage of endocytosis with respect to this condition. As shown in **Figure 31**, the naked anti-CD3 was internalized a 64%, and the percentage of endocytosis was very similar in the three conjugates studied (70, 64 and 62% for 4b, 5b and 6a, respectively). These results demonstrate that this type of bioconjugation does not affect the capacity of the antibody to internalize.



	Control	Anti-CD13		Conjugate 4b		Conjugate 5b		Conjugate 6a	
		4 ºC	37 ºC	4 ºC	37 ºC	4 ºC	37 ºC	4 ºC	37 ºC
MFI	0.72	21.2	8.18	23.0	7.41	17.6	6.77	18.9	7.54
$MFI_{control}$-MFI Ab	0	20.49	7.47	22.29	6.70	16.89	6.06	18.19	6.83
% Endocitosis	**0**	**64**		**70**		**64**		**62**	

Figure 31. Endocytosis of anti-CD13 and its conjugates in U937 cells. The average results are expressed using the MFI determined by flow cytometry. U937 cells were saturated with anti-CD13 and its conjugates under at 4 ºC and 37 ºC, at 3 h the incubated cells were processed for analysis. Table shows the MFI for conjugates 4b, 5b and 6a at 4 ºC and 37 ºC and the resulted average of internalization.

In order to test if the introduced linker to the antibody confers cytotoxicity to the conjugates, a cell viability study was carried out with anti-CD13 mAb and its conjugates. For

that, U937 cells were incubated with increasing concentrations of the antibody and its conjugates (0.05-10 µg/mL) during 72 h at 37 ºC. Then, cell viability was measured by a luminescence assay (CellTiter-Glo®, Promega Biotech). The percentage of cell viability vs the log [Ab] determine the viability curves for the studied conjugates 4-6 (**Figure 32**). As positive control we used an antibody-drug conjugate (ADC) developed by the biopharmaceutical company PharmaMar but, due to patentability interests, the ADC composition are not shown. Anti-CD13 is the antibody that composed the ADC and moreover a cytotoxic payload that is property of PharmaMar.

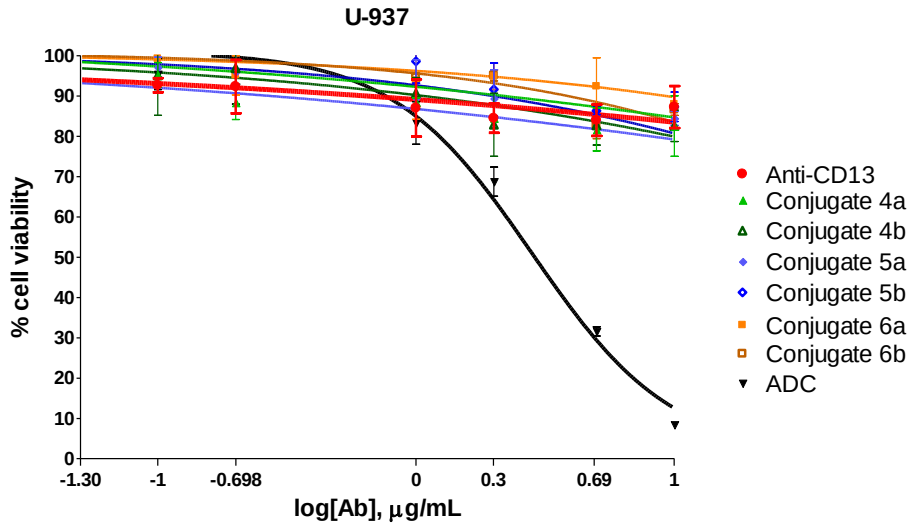


Figure 32. U-937 Cells viability studies under 72 h.

Results in showed that synthetized conjugated did not have cytotoxicity, as expected, and they worked similar to the naked antibody. In contrast, ADC killed efficiently leukemic cells, suggesting that this antigen-specific cell-killing conjugate could be a promising therapy in order to selectively remove tumor cells.

1.4. Conclusions

To sum up, we studied a kind of thiol group bioconjugation in partially reduced antibodies using some thioether-like 1,3-dibenzylic scaffolds (3c,e,g and h) as non-cleavable linker. The strong nucleofilicity of the thiol group it found the main drawback for the thiol monoalkylation of the TBMB core as polyalkylation products have found in most of the tested conditions. In this regard, an exhaustively control of the reaction conditions are needed to avoid the undesired thiol polyalkylation or the final product degradation. Reaction time, temperature and solvent play a key role in the S_N2 of thiol group into halobenzylic carbons.

The prepared conjugates were characterized via UV, HPLC, SDS-PAGE and Mass spectrometry. Moreover, in order to determine if the synthetized conjugates are biologically affected, antibody/conjugate-antigen binding affinity experiments were carried out. During the conjugation process we assume that reaction harsh conditions, such as the completely reduction of the antibody, could affect directly its properties as immunoglobulin misplacing in fact it binding affinity. However, most of the synthetized conjugates maintain their ability of binding to CD4 (conjugates 2a,b) or CD13 (conjugates 4-6), similar to the naked antibodies behavior. This fact indicates that this kind of conjugation strategy did not disrupt the antibody activity, being the 1,3-dibenzylic compounds suitable linkers for thiol bioconjugation and more specifically for ADCs.

Bearing in mind the progression of the bromobenzyl derivatives in the peptide field, we successful expand its synthetic methodologies spectrum as non-cleavable linker for the emerging field of antibodies bioconjugation.

1.5. References

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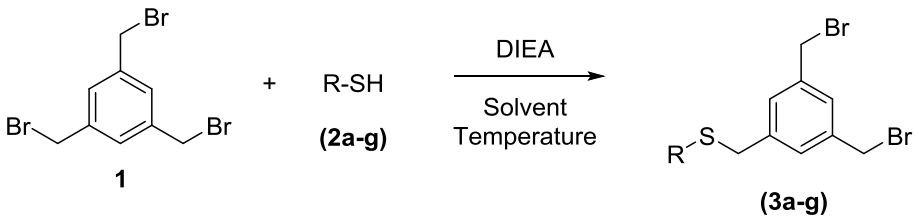
1.6. Experimental section

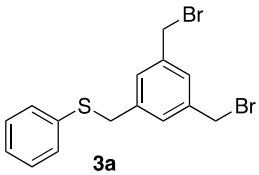
Dibromobenzyl derivatives preparation 3a-g, thiol monoalkylation studies.

General procedure



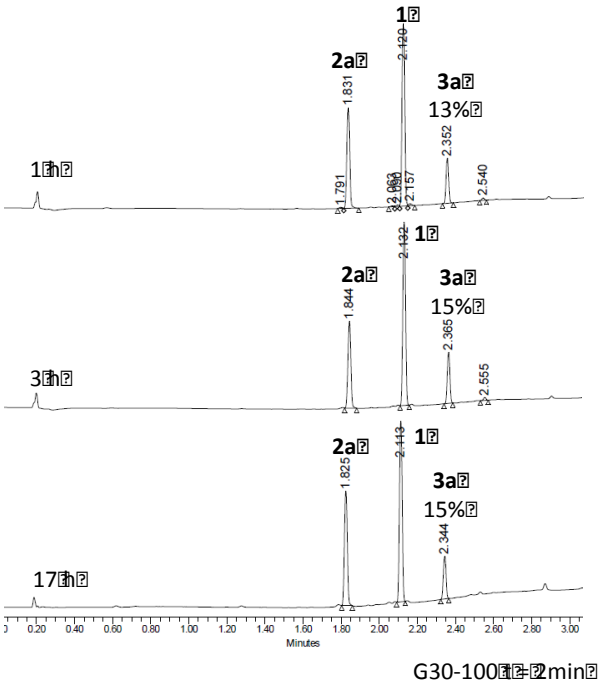
The indicated quantity (0.02 to 0.6 mmol) of the corresponding thiol (**2a-g**) was added to a solution of 1,3,5-tris(bromomethyl)benzene (**1,** 0.02 to 0.6 mmol) in the indicated solvent. Then DIEA (0.02 to 1.7 mmol) was added to the reaction and the mixture was left to react until the starting thiol disappeared. Where possible, the monoalkylated products (**3a-g**) were isolated using the appropriated chromatographic technique.

Entry 1: (3,5-bis(bromomethyl)benzyl)-1-methylthiobenzene (3a) preparation

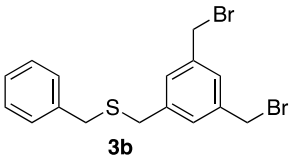


Thiophenol (14.4 µL, 0.14 mmol), 1,3,5-tris(bromomethyl)-benzene (50.0 mg, 0.14 mmol) and DIEA (30 µL, 0.17 mmol) in DCM (3 mL) were left to react in a sealed balloon during 17 h at room temperature. The reaction mixture was analyzed by HPLC at 1, 3 and 17 h in order to control the reaction progress. The product **3a** was not isolated due to the foul smelling reaction.

UPLC conversion:



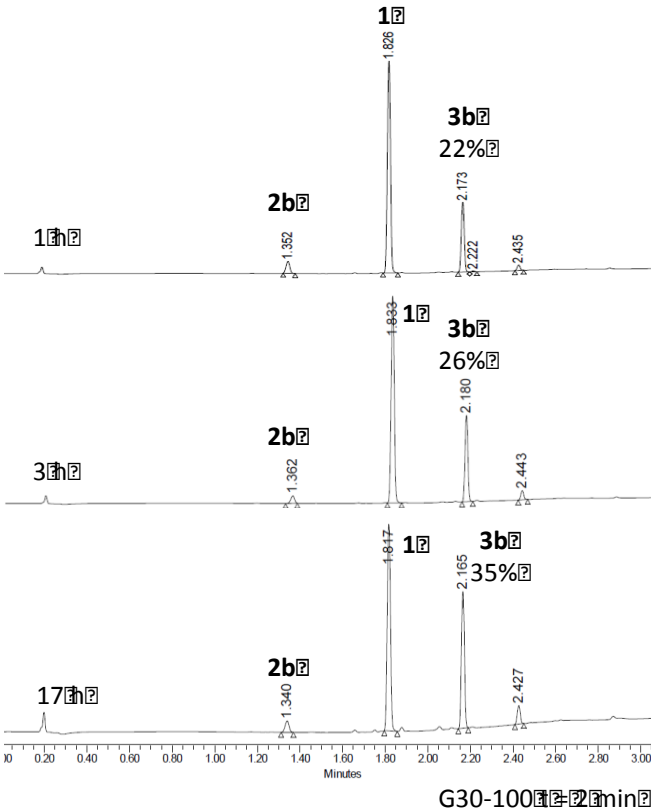
Entry 2: 3,5-bis(bromomethyl)benzyl)-1-methylthiobenzyl (3b) preparation



3b

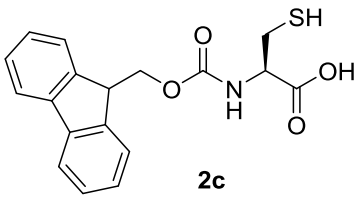
Benzyl mercaptan (16.4 µL, 0.14 mmol), 1,3,5-tris(bromomethyl)-benzene (50.0 mg, 0.14 mmol) and DIEA (30 µL, 0.17 mmol) in DCM (3 mL) were left to react in a sealed balloon during 17 h at room temperature. The reaction mixture was analyzed by UPLC at 1, 3 and 17 h in order to control the reaction progress. The product **3a** was not isolated due to the foul smelling reaction.

UPLC conversion:



Entries 3-9: Fmoc-L-Cys[bis(3,5-bromomethyl)benzene]-OH (**3c**) preparation [Fmoc-Cys(BBMB)-OH]

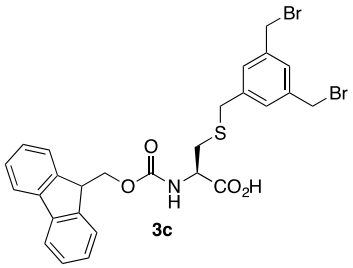
Fmoc-Cys-OH preparation (2c)



2c

First of all, Fmoc-Cys-OH (**2c**) was prepared following the described procedure.[34] Also see experimental section chapter I.2 and 3.

Fmoc-Cys(BBMB)-OH (3c)



3c

Following the general procedure, different reaction conditions were tested. The table below indicates the studied conditions for Fmoc-Cys-OH monoalkylation.

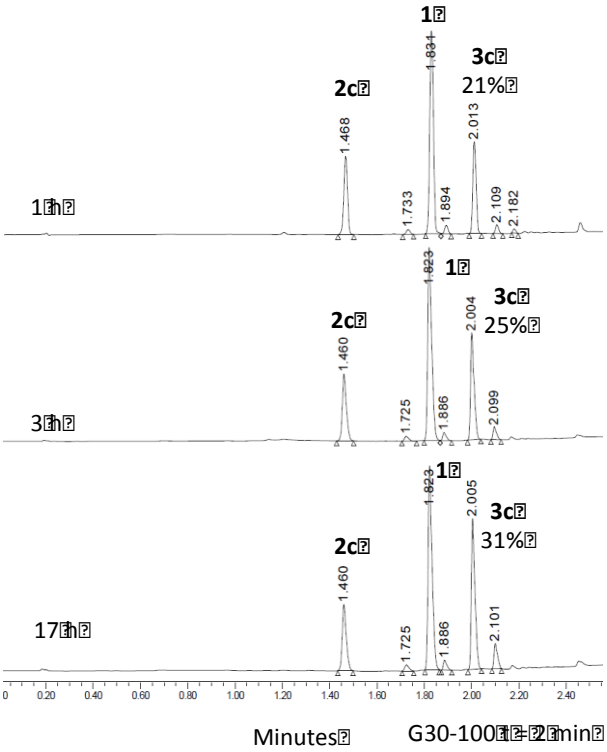
Entry	TBMB (mmol)	2c (mmol)	DIEA (mmol)	Solvent	Temp.	Reaction time (h)	HLPC Conversion	Isolated yield
3	0.05	0.05	0.05	DCM (10 mL)	rt	1	21 %	-[a]
						3	25 %	
						17	31 %	
4	0.17	0.11	0.35	DCM (6 mL)	rt	17	21 %	63 %
5	0.05	0.05	0.05	DCM (6 mL)	37 ºC	17	46 %	-[a]
6	0.56	0.56	1.68	MeCN (50 mL)	rt	0.5	33 %	-[c]
						17	8 %[c]	
7	0.29	0.29	0.87	MeCN (5 mL)	0 ºC	0.5	35 %	32 %
8	0.29	0.29	0.29	DMF (10 mL)	rt	0.5	16 %	-[b]
9	0.29	0.29	0.29	DMF (10 mL)	0 ºC	0.5	38 %	20 %

[a] Not isolated. [b] Product decomposition during Work up. ND → not determined. [c] Polialkylation decomposition.

Entry 3

Fmoc-Cys-OH (16.2 mg, 0.05 mmol), 1,3,5-tris(bromomethyl)benzene (16.8 mg, 0.05 mmol) and DIEA (8.9 µL, 0.05 mmol) in DCM (10 mL) were left to react during 17 h at room temperature. The reaction mixture was analyzed by UPLC at 1, 3 and 17 h in order to control the reaction progress. The final product was not isolated.

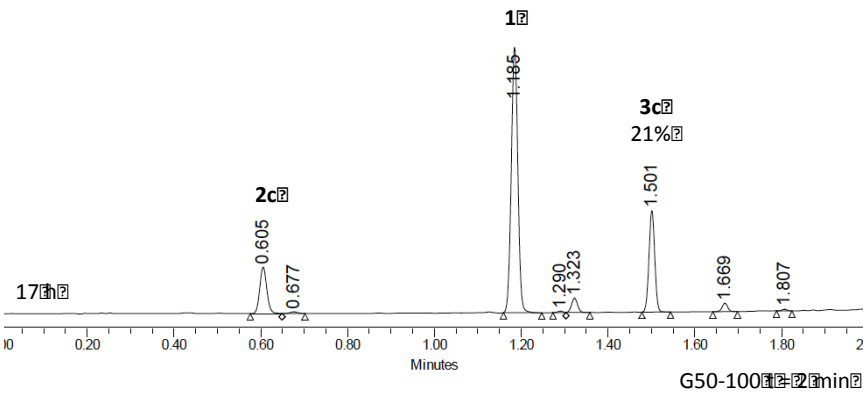
UPLC conversion:



Entry 4

Fmoc-Cys-OH (40.0 mg, 0.11 mmol), 1,3,5-tris(bromomethyl)benzene (61.0 mg, 0.17 mmol) and DIEA (60.9 µL, 0.35 mmol) in DCM (6 mL) were left to react during 17 h at room temperature. Afterwards, the reaction mixture was washed with HCl 1N (3×20 mL), brine (3×20 mL) and water (3×20 mL) and then the organic layer was dried over Na_2SO_4. Then solvent was removed under reduced pressure obtaining a white solid which was automatically purified on a pre-packed Redisep Rf Gold C18 15 g column by using H_2O/MeCN from 90:10 to 0:100 over 15 min. The collected fractions were lyophilized to obtain Fmoc-Cys(BBMB)-OH as a withe powder (45 mg, 63% of yield).

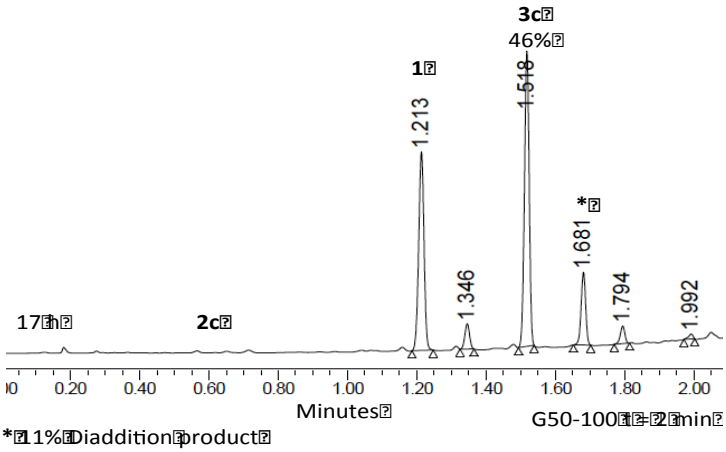
UPLC conversion:



Entry 5

Fmoc-Cys-OH (16.2 mg, 0.05 mmol), 1,3,5-tris(bromomethyl)benzene (16.8 mg, 0.05 mmol) and DIEA (8.9 µL, 0.05 mmol) in DCM (6 mL) were left to react in a sealed balloon during 17 h at 37 ºC. The reaction crude was not purified.

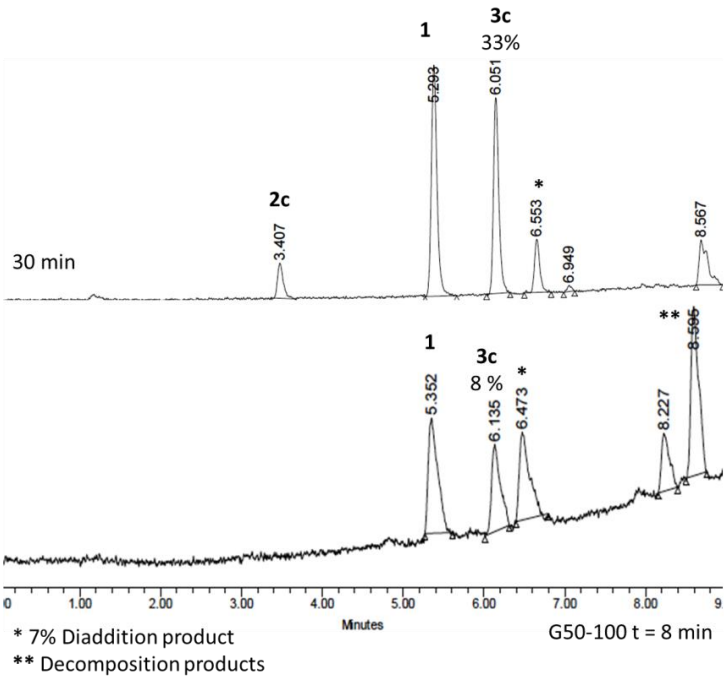
UPLC conversion:



Entry 6

Fmoc-Cys-OH (192.4 mg, 0.56 mmol), 1,3,5-tris(bromomethyl)benzene (200.0 mg, 0.56 mmol) and DIEA (293.0 µL, 1.68 mmol) in MeCN (50 mL) were left to react during 17 h at room temperature. The mixture was analysed at 1 h, and after 17 h, the HPLC analysis reveals poliaddition products in the mixture. The product was not isolated.

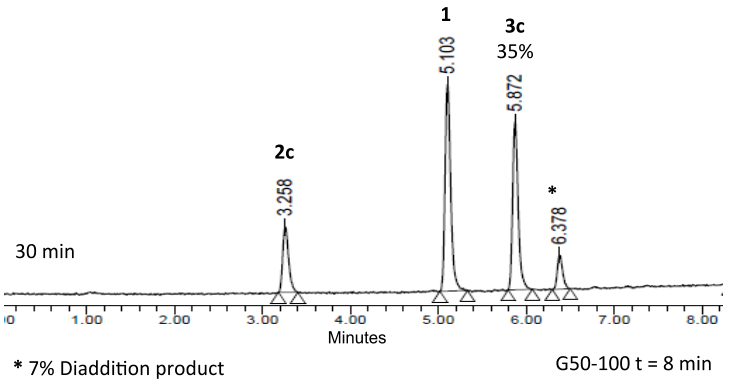
HPLC conversion:



Entry 7

Fmoc-Cys-OH (100.0 mg, 0.29 mmol), 1,3,5-tris(bromomethyl)benzene (103.9 mg, 0.29 mmol) and DIEA (152 µL, 0.87 mmol) in MeCN (5 mL) were left to react during 30 min at 0ºC. The reaction was quenched by adding HCl 1 M until pH ≈ 4. Then, solvent was removed under reduced pressure obtaining a white solid which was automatically purified on a pre-packed Redisep Rf Gold C18 15 g column by using H_2O/MeCN from 90:10 to 0:100 over 30 min. The collected fractions were lyophilized to obtain Fmoc-Cys(BBMB)-OH as a withe powder (58.2 mg, 32% of yield).

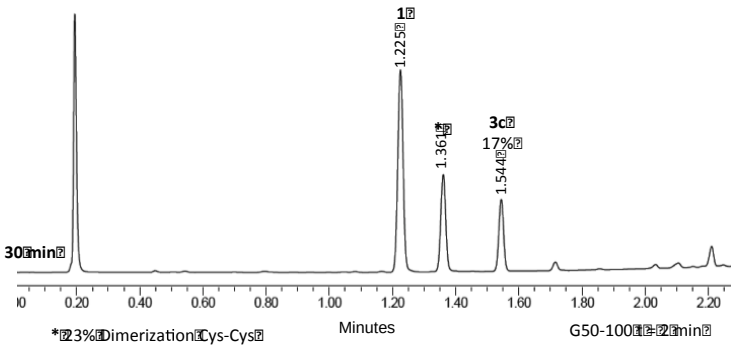
HPLC conversion:



Entry 8

Fmoc-Cys-OH (100.6 mg, 0.29 mmol), 1,3,5-tris(bromomethyl)benzene (104.5 mg, 0.29 mmol) and DIEA (51 µL, 0.29 mmo) in DMF (10 mL) were left to react during 30 min at room temperature. The reaction was quenched by adding HCl 1 M until pH ≈ 4. Then solvent was removed under reduced pressure. Unfortunately the final product decomposition took place and it was not possible to isolated.

UPLC conversion:

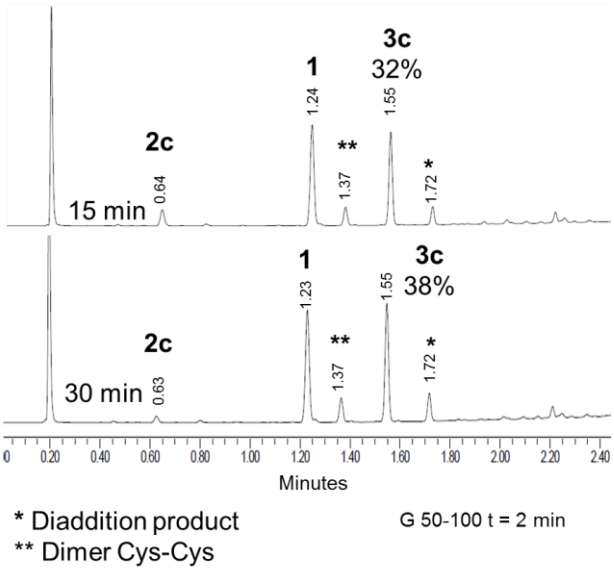


Entry 9

Fmoc-Cys-OH (100.4 mg, 0.29 mmol), 1,3,5-tris(bromomethyl)benzene (104.5 mg, 0.29 mmol) DIEA (51 µL, 0.29 mmol) in DMF (10 mL) were left to react in MeCN (5 mL) during

30 min at 0ºC. The reaction was quenched by adding HCl 1 M until pH ≈ 4. Then solvent was removed under reduced pressure obtaining a white solid which was automatically purified on a pre-packed Redisep Rf Gold C18 15 g column by using H_2O/MeCN from 90:10 to 0:100 over 30 min. The collected fractions were lyophilized to obtain Fmoc-Cys(BBMB)-OH as a withe powder (36.3 mg, 20% of yield).

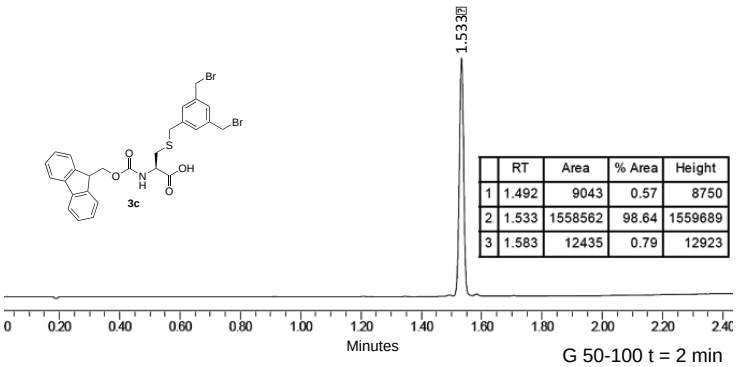
UPLC conversion:



Fmoc-Cys(BBMB)-OH (3c) characterization.

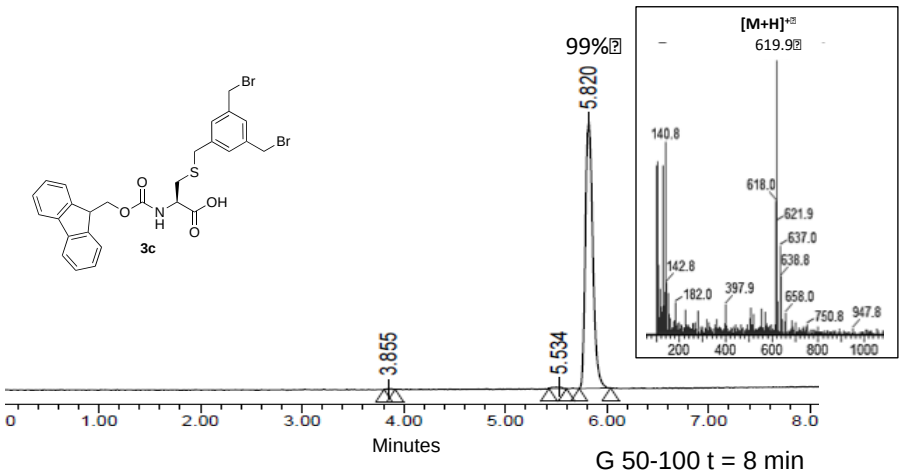
HPLC (H_2O/MeCN from 50:50 to 0:100 over 8 min): t_R: 5.8 min. UPLC (H_2O/MeCN from 50:50 to 0:100 over 2 min): t_R: 1.5 min. m/z calculated for $C_{27}H_{25}Br_2NO_4S$ = 619.3; found = 619.9 $[M+H]^+$. ^1H NMR (400 MHz, Acetone-d_6) δ 7.94 – 7.81 (m, 2H), 7.79 – 7.69 (m, 2H), 7.51 (s, 1H), 7.46 – 7.36 (m, 4H), 7.36 – 7.26 (m, 2H), 6.85 (t, *J* = 8.0 Hz, 1H), 4.66 (s, 2H), 4.61 (s, 2H), 4.55 – 4.48 (m, 1H), 4.45 – 4.31 (m, 2H), 4.30 – 4.23 (m, 1H), 3.85 (d, *J* = 15.4 Hz, 2H), 3.05 – 2.97 (m, 1H), 2.90 – 2.83 (m, 1H).

UPLC



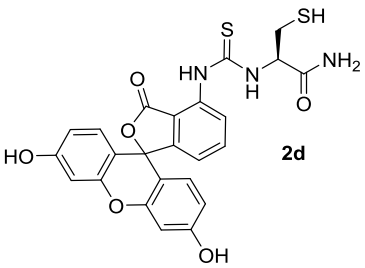
	RT	Area	% Area	Height
1	1.492	9043	0.57	8750
2	1.533	1558562	98.64	1559689
3	1.583	12435	0.79	12923

HPLC-ESI:

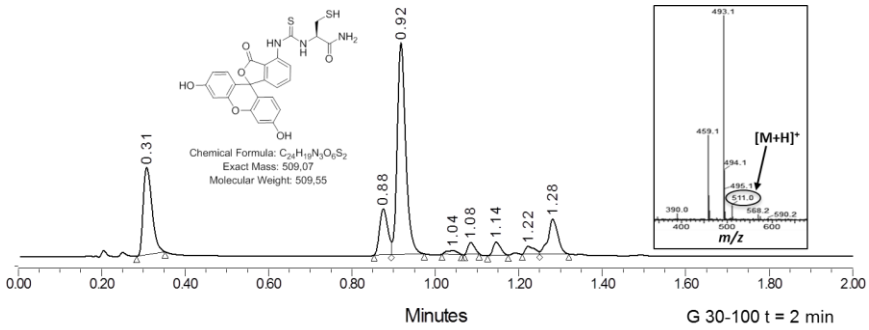


Entries 10-11: FITC-Cys(BBMB)-NH_2 (3d) preparation

FITC-Cys-NH_2 Preparation (2d)

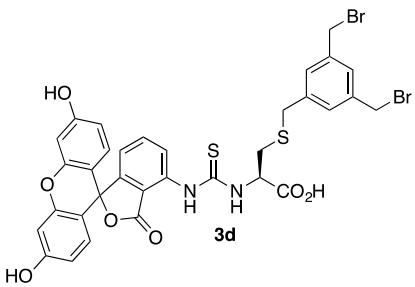


Fmoc-Rink-amide AM-polystyrene resin (1.0 g, loading capacity (□) = 0.71 mmol/g) was used for the labeled amino acid synthesis. Initially, the resin was washed with DCM (4×5 mL) and DMF (4×5 mL) and the Fmoc protecting group was removed by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 2×5 mL×5 min), and it washed with DCM (4×5 mL) and DMF (4×5 mL). Then, Fmoc-Cys(Trt)-OH (3 eq.) was coupled using DICPDI (2 eq.) and Oxyma Pure (2 eq.) in DMF with a 5 min pre-activation protocol during 1 h at 25 ºC. After that, the resin was washed with and DMF (2×5 mL). Then, Fmoc protecting group was eliminated as indicate above. Then, the fluorophore was incorporated to the H-Cys(Trt)-Resin (527.5 mg) by the corresponding treatment with FITC (1.2 eq.) and DIEA (3 eq.) in DMF. After that the resin was washed with DMF (4×5 mL) and DCM (4×5 mL), and the labeled amino acid cleavage was performed treating the resin with TFA/TIS/H_2O (95:2.5:2.5) (1×2 mL×1 h) and washing with DCM (2×2 mL). Then the filtrates were evaporated and precipitated with cold Et_2O. Then, the solid was dissolved in H_2O/MeCN (1:1) and lyophilized, obtaining the labeled Cys derivative as an orange powder (74.0 mg, 50% purity). *m/z* calculated for $C_{24}H_{19}N_3O_6S_2$ = 509.55; found = 511.0 corresponding to $[M+H]^+$.



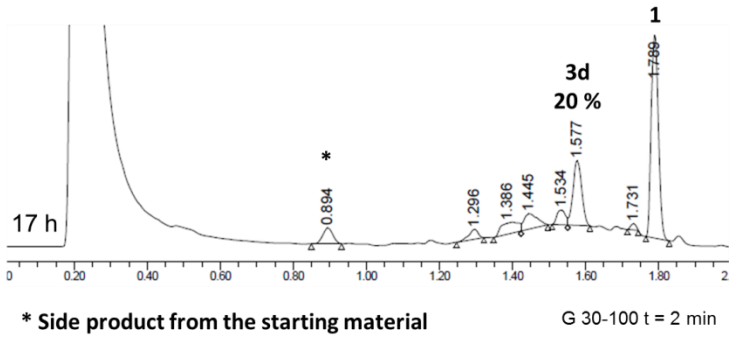
FITC-Cys(BBMB)-NH_2 (3d)

Entry 10



FITC-Cys-NH_2 (20.0 mg, 0.04 mmol), 1,3,5-tris(bromomethyl)benzene (14.0 mg, 0.04 mmol) and DIEA (8.2 µL, 0.05 mmol) in MeCN (5 mL) were left to react during 17 h at room temperature. The reaction was quenched by adding HCl 1 M until pH ≈ 4. Then, the solvent was removed under reduced pressure obtaining an orange solid which was automatically purified on a pre-packed Redisep Rf Gold C18 15 g column by using H_2O/MeCN from 90:10 to 0:100 over 30 min. The collected fractions were lyophilized. Unfortunately **3d** decomposition occurs during the work-up or purification step.

UPLC conversion:

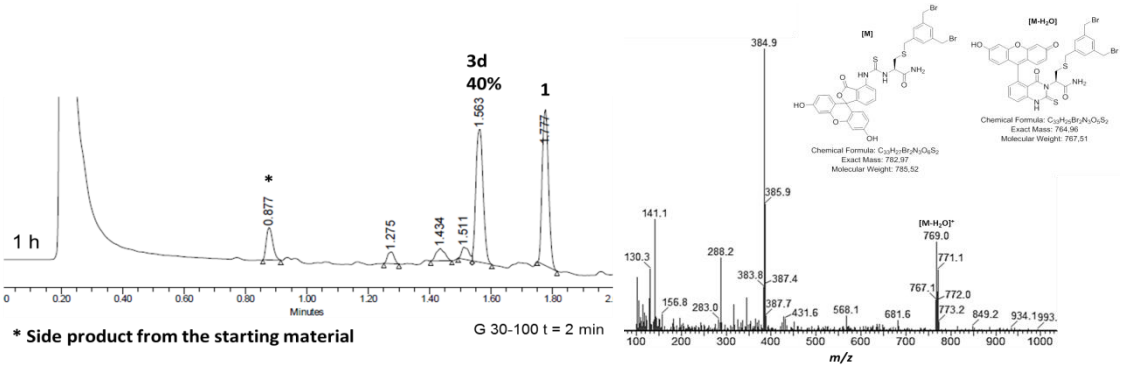


Entry 11

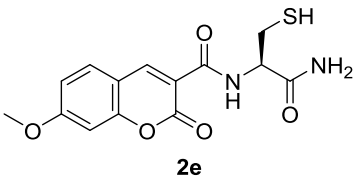
FITC-Cys-NH_2 (20.0 mg, 0.04 mmol), 1,3,5-tris(bromomethyl)benzene (14.0 mg, 0.04 mmol) and DIEA (8.2 µL, 0.05 mmol) in DMF (5 mL) were left to react during 1 h at 0ºC. The reaction was quenched by adding HCl 1 M until pH ≈ 4. Then solvent was removed under reduced pressure obtaining an orange solid which was automatically purified on a pre-packed Redisep Rf Gold C18 15 g column by using H_2O/MeCN from 90:10 to 0:100 over 30 min. The collected fractions were lyophilized. Unfortunately **3d** decomposition occurs during the work-up or purification step.

UPLC (H2O/MeCN from 70:30 to 0:100 over 2 min): tR: 1.56 min. m/z calculated for C33H27Br2N3O6S2 = 785.5; found = 769.0 corresponding to [M-H2O]+.

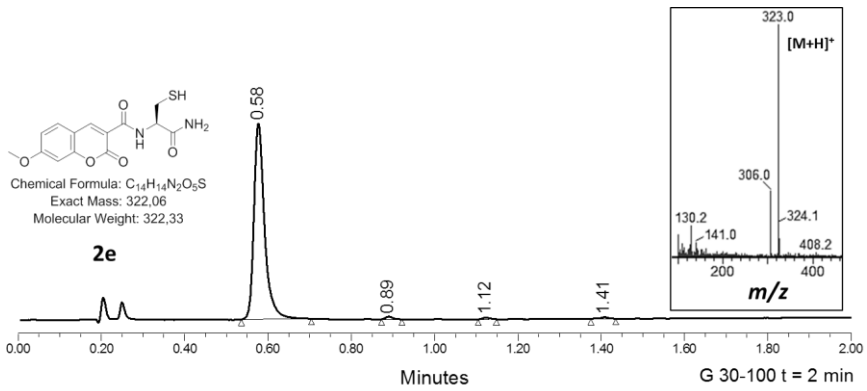
UPLC conversion and ESI:



7-Methoxy-3-carboxycoumarin-Cys-NH_2 (2e)

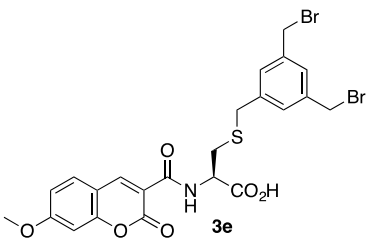


Coumarin-OSu (1.3 eq.) was incorporated in the H-Cys(Trt)-Resin (341 mg) in DMF during 1 h at room temperature. Then, the resin was washed with DMF (4×5 mL) and DCM (4×5 mL). The labeled amino acid cleavage was performed treating the resins with TFA/TIS/H_2O (95:2.5:2.5) (1×2 mL×1 h) and washing with DCM (2×2 mL). The filtrates were evaporated and precipitated with cold Et_2O. Then, the solid was dissolved in H_2O/MeCN (1:1) and lyophilized, obtaining the labeled Cys derivative as a white powder (45.4 mg, 98% purity).



Entries 12-13: 7-methoxycumarin-Cys-(BBMB)-NH_2 (**3e**) preparation

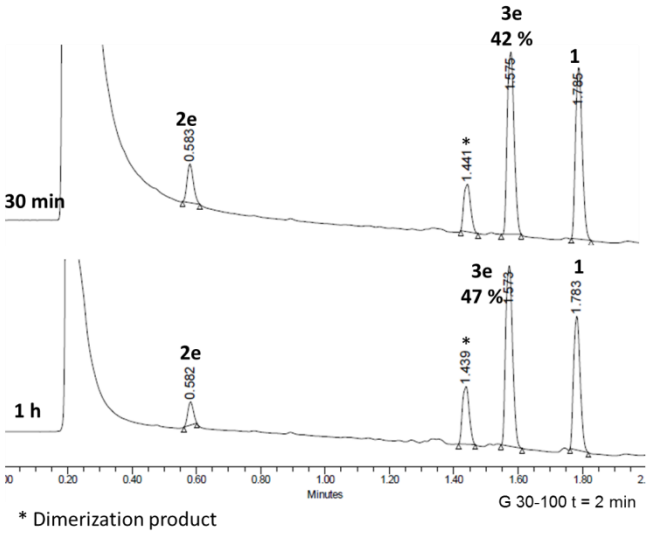
Entry 12



Coumarin-Cys-NH_2 (33.3 mg, 0.10 mmol), 1,3,5-tris(bromomethyl)benzene (36.9 mg, 0.10 mmol) and DIEA (21.6 µL, 0.12 mmol) in DMF (5 mL) were left to react during 1 h at room temperature. Then, the reaction was quenched by

adding HCl 1 M until pH ≈ 4. Then solvent was removed under but unfortunately the desired product decomposed.

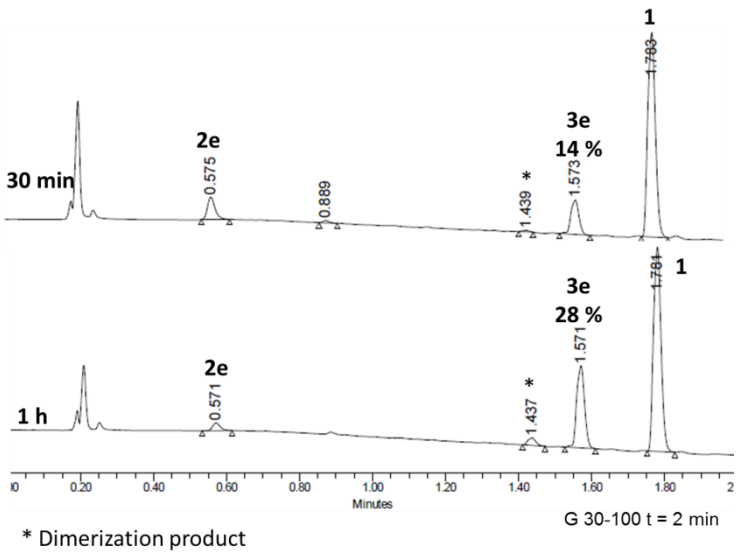
UPLC conversion:



Entry 13

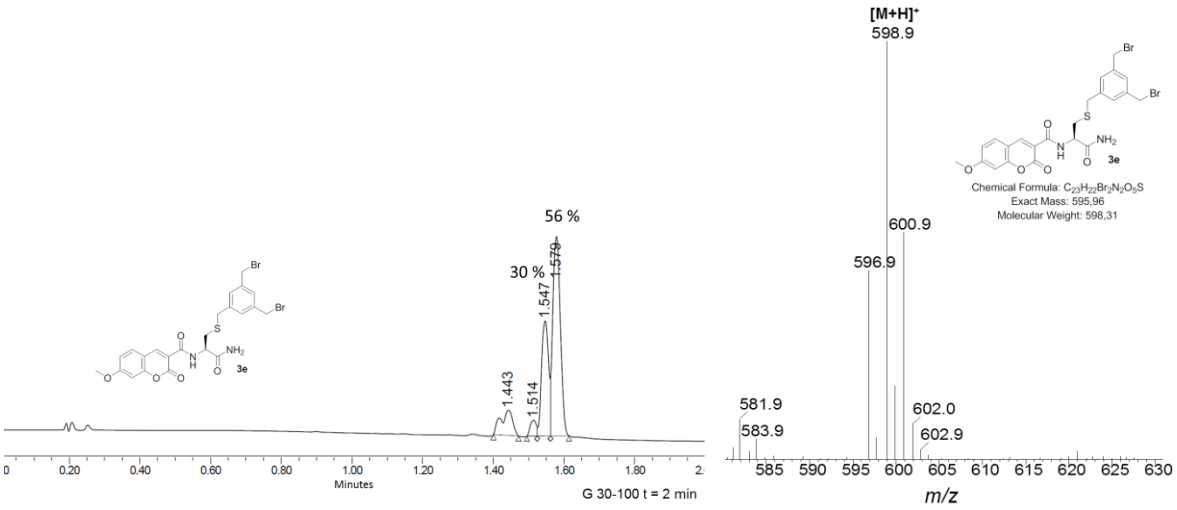
Coumarin-Cys-NH_2 (33.3 mg, 0.10 mmol), 1,3,5-tris(bromomethyl)benzene (36.9 mg, 0.10 mmol) and DIEA (21.6 µL, 0.12 mmol) in DMF (5 mL) were left to react during 1 h at 0 ºC. Then, the reaction was quenched by adding HCl 1 M until pH ≈ 4. The solvent was removed under reduced pressure obtaining a pale brown solid (101.9 mg) which was automatically purified on a pre-packed Redisep Rf Gold C18 15 g column by using H_2O/MeCN from 90:10 to 0:100 over 30 min. The collected fractions were lyophilized to obtain Coumarin-Cys(DBMB)-NH_2 as a withe powder (19.2 mg, 31% of yield).

UPLC conversion:

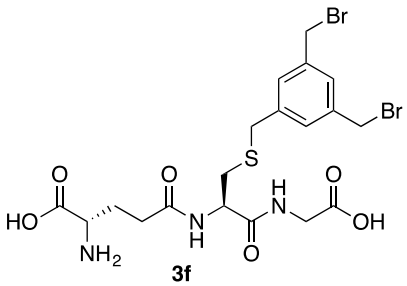


Characterization:

UPLC (H_2O/MeCN from 70:30 to 0:100 over 2 min): t_R: 1.6 min. m/z calculated for $C_{23}H_{22}Br_2N_2O_5S$ = 598.3; found = 598.9 $[M+H]^+$. Possible epimerization of the Cys α proton.



Entry 14: Glutathione-S-BBMB (3f) preparation



Following the general procedure, different reaction conditions were tested. The table below indicates the studied conditions for reduced glutathione monoalkylation.

Entry	GSH (mmol)	TBMB (mmol)	DIEA (mmol)	Solvent	Temperature	Reaction time (h)	HLPC Conversion	Isolated yield
14.1	0.33	0.33	0.33	DCM (10 mL)	rt	1	ND	-
14.2	0.33	0.33	0.33	H_2O/MeCN (1:1) (10 mL)	rt	1	ND	-
14.3	0.33	0.33	0.33	DMF/H_2O (1:1) (10 mL)	rt	0.5	ND	-

Entry 14.1

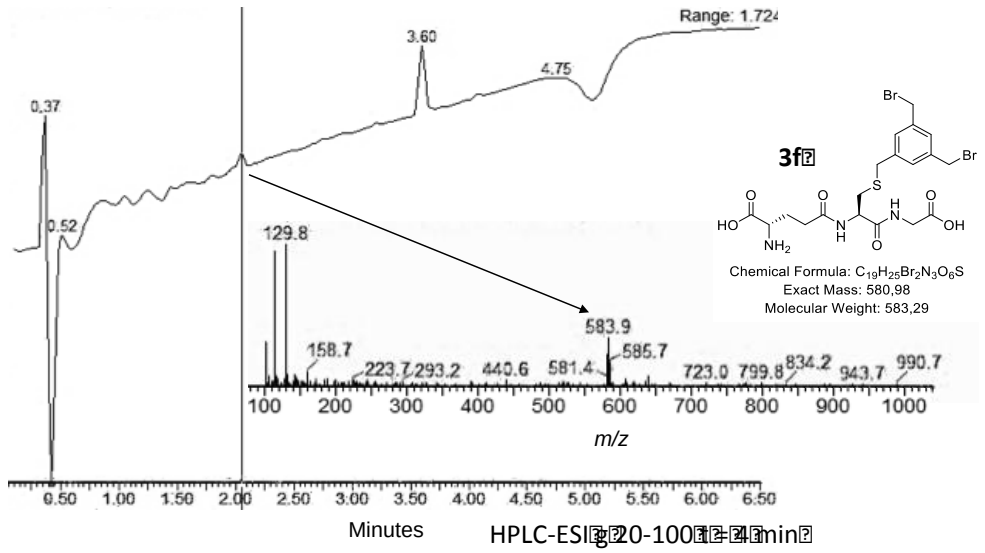
Glutathione was not soluble in DCM.

Entry 14.2

To a solution of reduced glutathione (100.0 mg, 0.33 mmol) in H_2O/MeCN (1:1) (10 mL), 1,3,5-tris(bromomethyl)benzene (116.1 mg, 0.33 mmol) and DIEA (56.7 µL, 0.33 mmol) was added. Then immediately an undesired product precipitates and the final product was not observed.

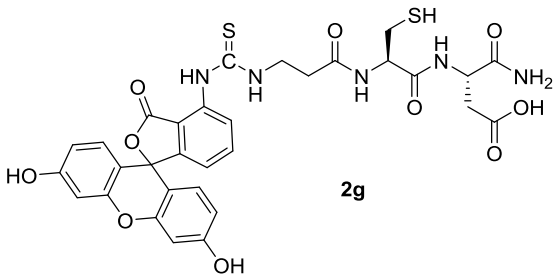
Entry 14.3

To a solution of reduced glutathione (100.0 mg, 0.33 mmol) in DMF/H_2O (8:2) (10 mL), 1,3,5-tris(bromomethyl)benzene (116.1 mg, 0.33 mmol) and DIEA (56.7 µL, 0.33 mmol) was added. Then the mixture was left to react during 30 min at room temperature. The reaction was quenched by adding HCl 1 M until pH ≈ 4. After removing part of the solvent under reduced pressure, the analysis of the product reveals the decomposition of the monoalkylation product.



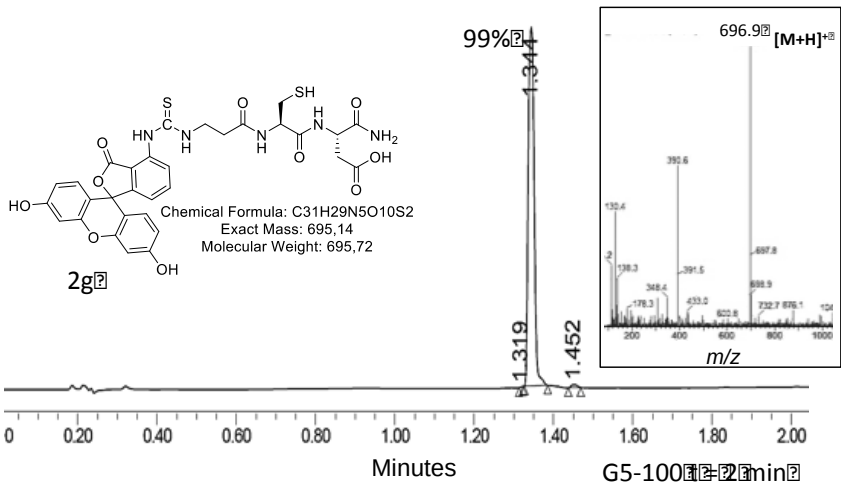
HPLC-ESI(+) 20-100% B in 4 min

FITC-βAla-Cys-Asp-NH_2 (2g) preparation

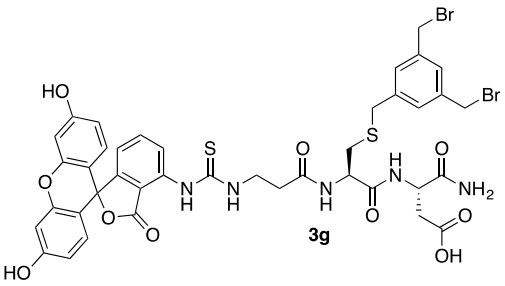


Fmoc-Rink-amide AM-polystyrene resin (500 mg, loading capacity (□) = 0.71 mmol/g) was used for peptide synthesis. Initially, the resin was washed with DCM (4×5 mL) and DMF (4×5 mL) and the Fmoc protecting group was removed by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 2×5 mL×5 min), and washed with DCM (4×5 mL) and DMF (4×5 mL). Then, the corresponding Fmoc amino acids (3 eq.) were coupled using DICPDI (2 eq.) and Oxyma Pure (2 eq.) in DMF with a 5 min pre-activation protocol and it was left to react during 1 h and 30 min at 25 ºC. After each coupling, the resin was washed with DMF (4×5 mL), DCM (4×5 mL) and DMF (2×5 mL). Fmoc protecting group was eliminated as indicate above. To the tripeptide H-βAla-Cys-Asp-NH-Resin (250 mg), the fluorophore was coupled treating the peptidyl resin with FITC (3 eq.), and DIEA (3 eq.) in

DMF during 1 h at room temperature. The peptide cleavage was performed treating the peptidyl-resins with TFA/TIS/H_2O (95:2.5:2.5) (1×2 mL×1 h) and washing with DCM (2×2 mL). Then the filtered washes were evaporated and precipitated with cold Et_2O. The resulting peptide was automatically purified on a pre-packed Redisep Rf Gold C18 43 g column by using H_2O/MeCN from 90:10 to 0:100 over 30 min. The collected fractions were lyophilized to obtain FITC-βAla-Cys-Asp-NH_2 as an orange powder (28.6 mg, 99% purity).



Entries 15-16: FITC-βAla-Cys(DBMB)-Asp-NH_2 (3g) preparation



Following the general procedure, different reaction conditions were tested. The table below indicates the studied conditions for reduced glutathione monoalkylation

Entry	**2g** (mmol)	TBMB (mmol)	DIEA (mmol)	Solvent	Temperature	Reaction time (h)	HLPC Conversion	Isolated yield
15	0.022	0.026	0.026	MeCN (2 mL)	0 ºC	1	14%	5.7%
16	0.035	0.038	0.069	DMF	0 ºC	0.5	24%	12%

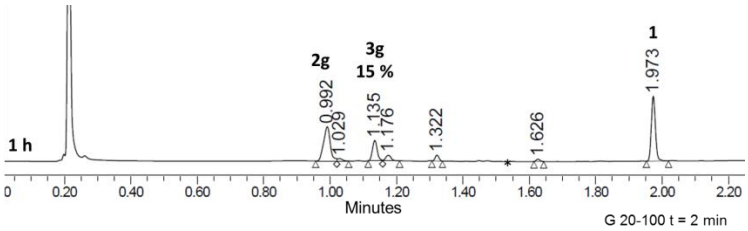
Entry 15

FITC-βAla-Cys-Asp-NH_2 (15.0 mg, 0.022 mmol) was added to a solution of 1,3,5-tris(bromomethyl)benzene (9.2 mg, 0.026 mmol) in MeCN (2 mL). Then, DIEA was added to the mixture (4.5 µL, 0.026 mmol), and it was left to react during 1 h at 0 ºC. The reaction was quenched by adding HCl 1 M until pH ≈ 4, and the solvent was removed under reduced pressure obtaining an orange solid which was automatically purified on a pre-packed Redisep Rf Gold C18 15 g column by using H_2O/MeCN from 90:10 to 0:100 over 15 min. The

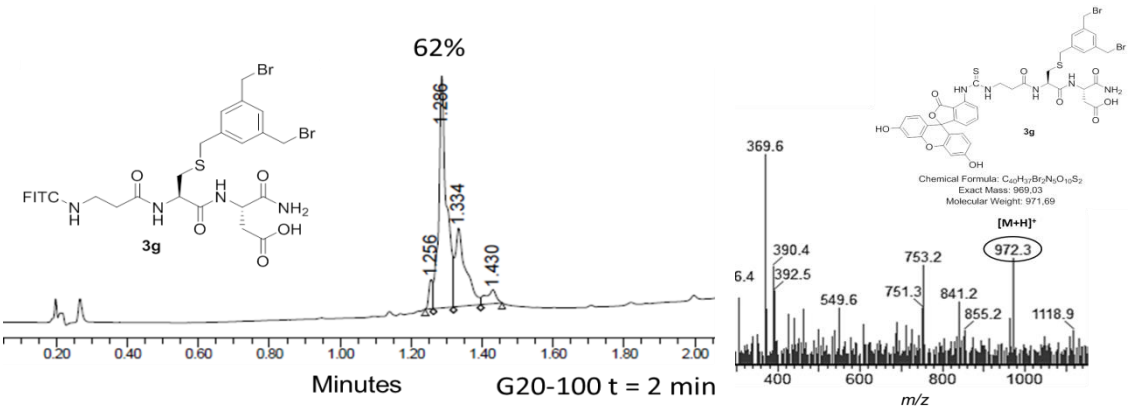
collected fractions were lyophilized to obtain FITC-βAla-Cys(BBMB)-Asp-NH_2 as an orange powder (1.1 mg, 5.7% of yield).

UPLC (H_2O/MeCN from 80:20 to 0:100 over 2 min): t_R: 1.3 min. m/z calculated for $C_{40}H_{37}Br_2N_5O_{10}S_2$ = 971.69; found = 972.3 corresponding to $[M+H]^+$.

UPLC conversion:

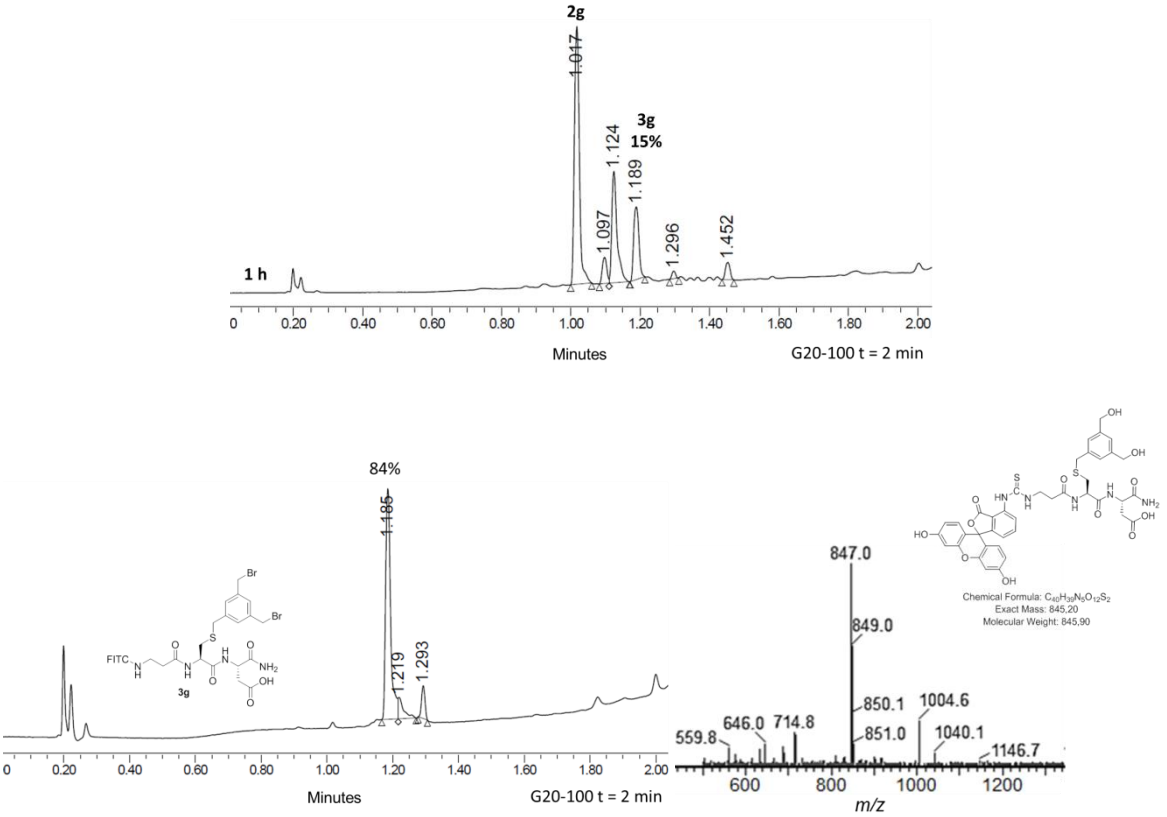


Purified compound:



Entry 16

FITC-βAla-Cys-Asp-NH_2 (24.3 mg, 0.035 mmol) was added to a solution of 1,3,5-tris(bromomethyl)benzene (13.7 mg, 0.038 mmol) in DMF (5 mL). Then DIEA was added to the mixture (12.2 µL, 0.0698 mmol), and it was left to react during 30 min at 0 ºC. The reaction was quenched by adding HCl 1 M until pH ≈ 4, and the solvent was removed under reduced pressure obtaining an orange solid, which was automatically purified on a pre-packed Redisep Rf Gold C18 13 g column by using H_2O/MeCN from 90:10 to 0:100 over 15 min. The collected fractions were lyophilized to obtain an orange powder (4.1 mg, 12% of yield). As shown in the mass chromatogram, we observed the hydrolyzed dibromobenzyl compound. It is possible that the compound decomposition occurs during the ionization.

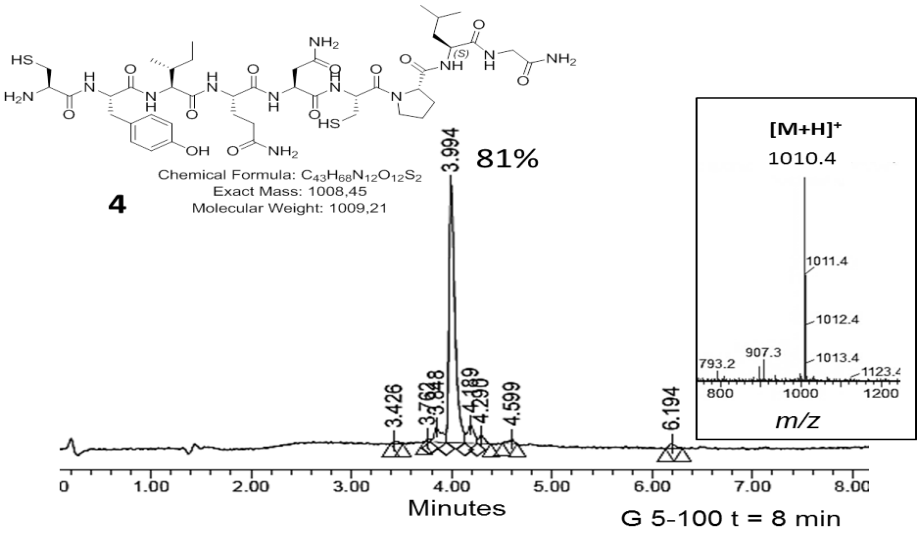


Conjugation

Reduced oxytocin (h-cys-tyr-ile-gln-asp-cys-pro-leu-gly-nh$_2$) preparation (4).

Fmoc-Rink-amide AM-polystyrene resin (loading capacity (□) = 0.71 mmol/g) was used for the peptide synthesis. Initially, the resin was washed with DCM (4×5 mL) and DMF (4×5 mL) and the Fmoc protecting group was removed by treating the resins with DMF/piperidine (4:1) (1×5 mL×1 min + 2×5 mL×5 min), and washed with DCM (4×5 mL) and DMF (4×5 mL). Then the first amino acid was manually incorporated by treating the resin with Fmoc-Gly-OH (3 eq.), DIPCDI (3 eq.) and Oxyma Pure (3 eq.) in DMF, after pre-activation of 5 min. The mixture was left to react for 1 h and 30 min. Afterwards, the resin was washed with DMF (4×5 mL) and DCM (4×5 mL). Then, the peptide chain was automatically elongated on the CEM Liberty Blue™ peptide synthesizer using the conditions described above. Fmoc-Cys(Thp)-OH was used for the peptide synthesis.[34] The peptide cleavage was performed treating the peptidyl-resin with TFA/TIS/DMB (92.5:2.5:5) (1×2 mL×1 h) and washing with DCM (2×2 mL). Then the filtrates were evaporated and precipitated with cold Et_2O. The resulting peptide was directly analyzed without further purification.

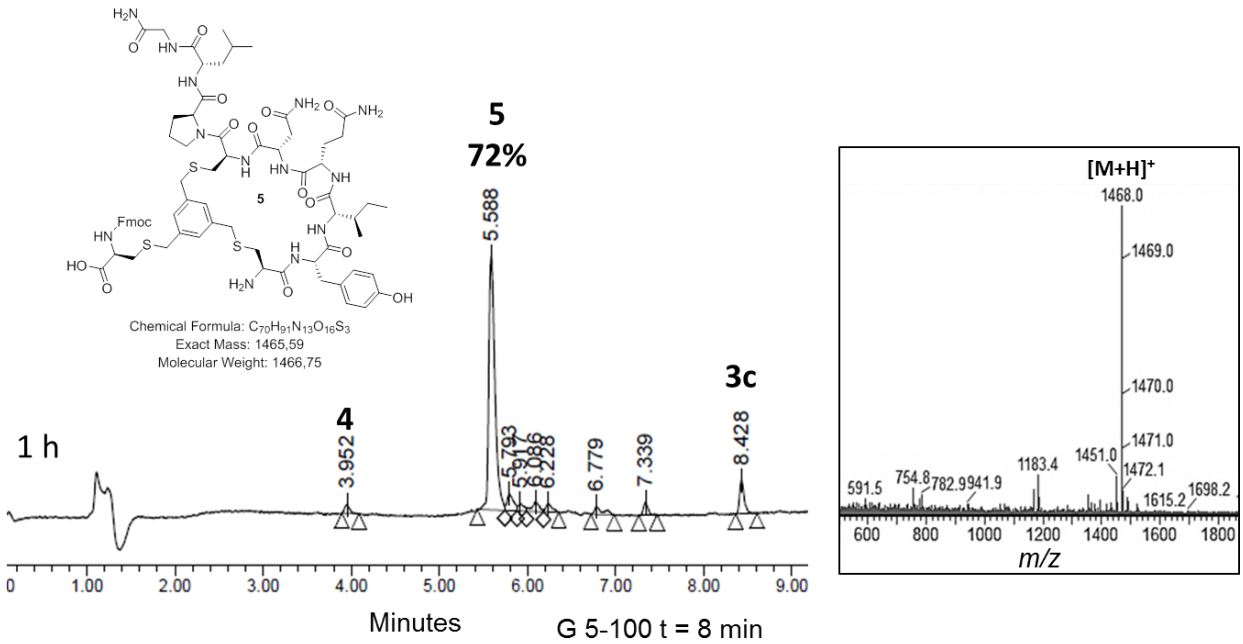
HPLC (H_2O/MeCN from 95:5 to 0:100 over 8 min): t_R: 3.99 min. m/z calculated for $C_{43}H_{68}N_{12}O_{12}S_2$ = 1009.21; found = 1010.4 corresponding to $[M+H]^+$.



Oxytocin and Fmoc-Cys(BBMB)-OH conjugation (5)
Test 1

To a solution of reduced Oxytocin (0.17 mg/mL) in $NaHCO_3$ buffer (120 µL) at pH = 8, a solution of Fmoc-Cys(DBMB)-OH in H_2O/MeCN (20 µL of 1 mg/mL) was added. The reaction was left to react during 1 h. The mixture was analyzed at 15 and 60 min by HPLC, and the disappearance of the chromatographic peak corresponding to Oxytocin (t_R =3.9 min), and the appearance of a new peak (t_R =5.6 min) corresponding to the stapled Oxytocin were observed.

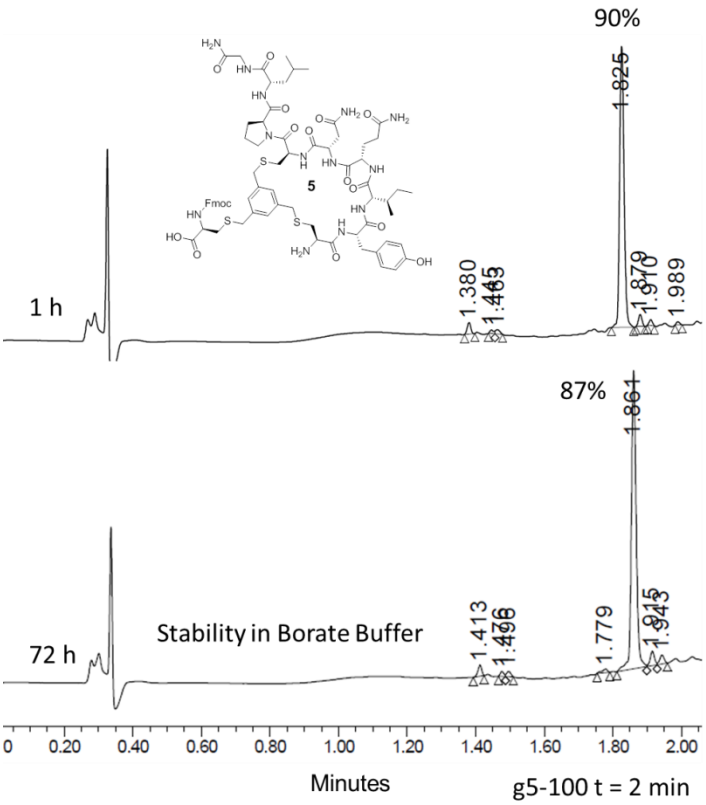
HPLC (H_2O/MeCN from 95:5 to 0:100 over 8 min): t_R: 5.59 min. m/z calculated for $C_{70}H_{91}N_{13}O_{16}S_3$ = 1466.75; found = 1468.0 corresponding to $[M+H]^+$.



Test 2

To a solution of reduced Oxytocin (10 mg, 9.9 µmol) in Borate buffer (25 mM H_3BO_3, 25 mM NaOH, 25 mM NaCl and 1 mM EDTA) at pH = 8 (5 mL), a solution of Fmoc-Cys(DBMB)-OH (6.1 mg, 9.9 µmol) in H_2O/MeCN (1:1) (1 mL) was added. The reaction was left to react during 1 h at room temperature and the mixture was analyzed by UPLC observing the completion of the reaction (90 % purity) after 1 hour. The same sample was analysed after 72 h in borate buffer compound **5** remain stable.

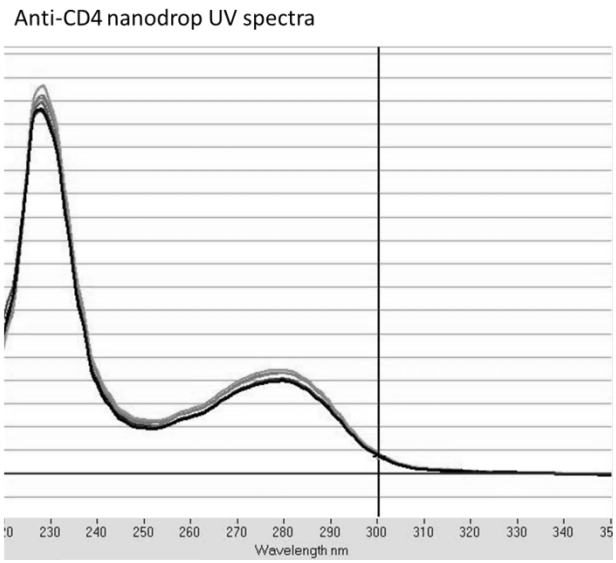
UPLC (H_2O/MeCN from 95:5 to 0:100 over 8 min): t_R: 1.8 min.



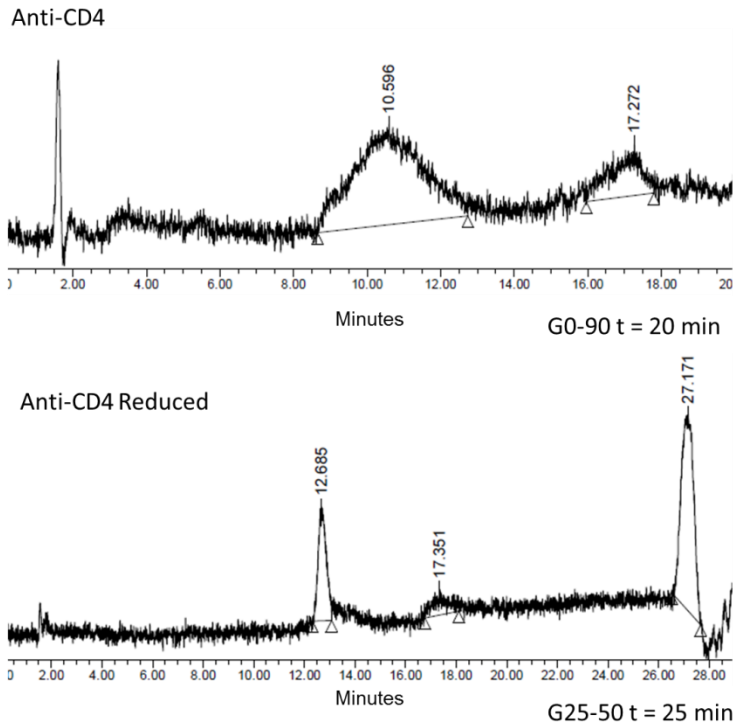
Antibodies conjugation

Anti-CD4 analysis

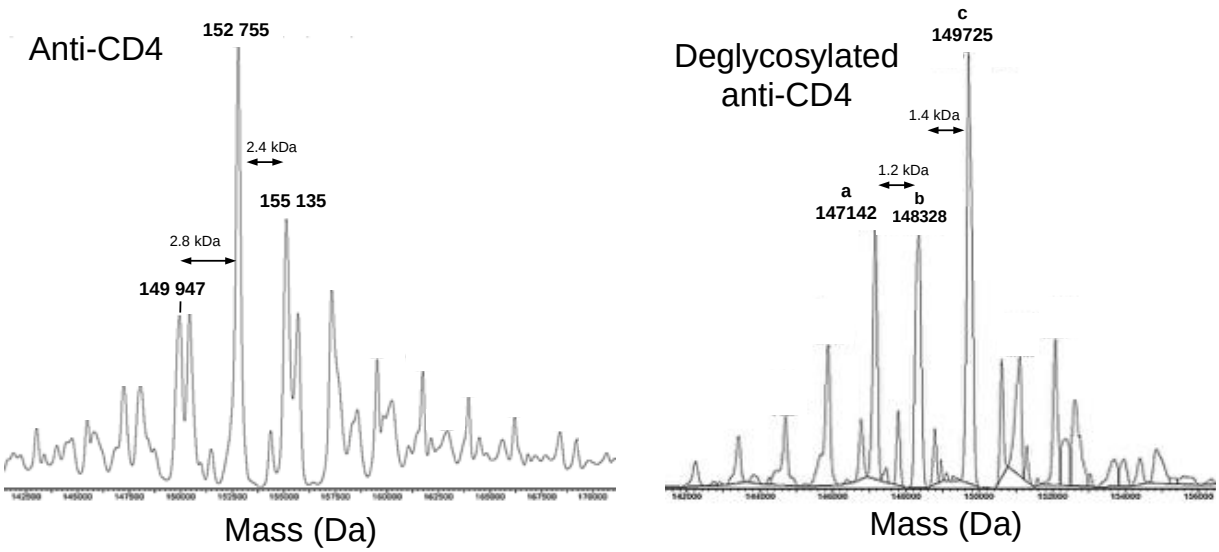
The provided Anti-CD4 solution was dialyzed over PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4), borate buffer at pH = 8 (25 mM H_3BO_3, 25 mM NaOH, 25 mM NaCl and 1 mM EDTA) or water for the antibody storage, for further reactions and for analysis, respectively.



HPLC analysis:



MS-ESI analysis



Conjugation to anti-CD4

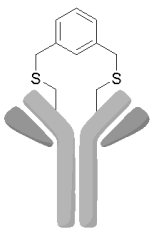
General procedure

A solution of anti-CD4 or anti-CD13 in borate buffer at pH = 8 (25 mM H_3BO_3, 25 mM NaOH, 25 mM NaCl and 1 mM EDTA) was treated with TCEP at 37 ºC during 2 h. Then, the reduced antibody was treated with a solution of the dibromobenzyl derivative solved in DMSO. The mixture was left to react during 30 min at 4 ºC. Then the crude was purified using PD Minitrap G-25 column and PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) as eluent for the antibody storage. The collected samples (approximately 200 µL) were analysed by UV nanodrop in order to determine their concentration.

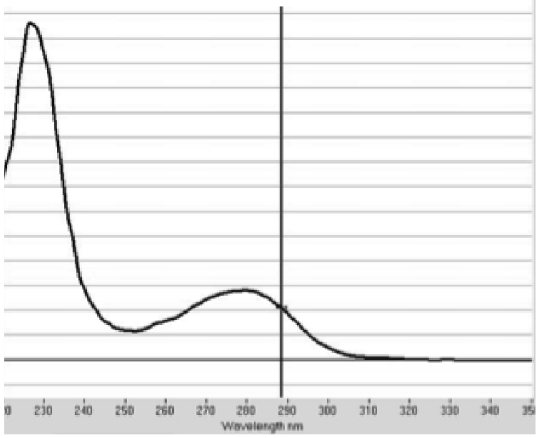
The samples were analysed by HPLC, SDS-PAGE in reducing and non-reducing conditions, and mass spectrometry. For mass analysis, an aliquot of the antibody solution in PBS (18 µL) was treated with PNGase F 500 000 U/mL (1 µL) at 37 ºC during 17 h in order to perform the conjugate deglycosilation in order to improve the mass analysis spectra.

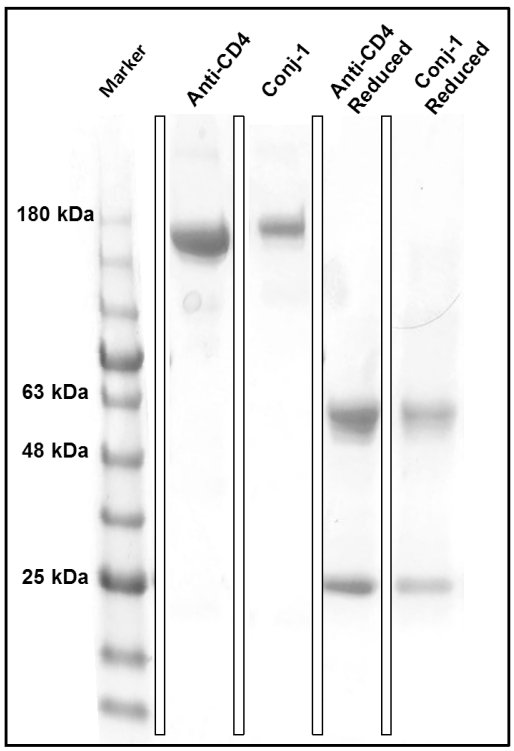
Conjugate 1: α,α′-Dibromo-*m*-xylene conjugated to anti-CD4

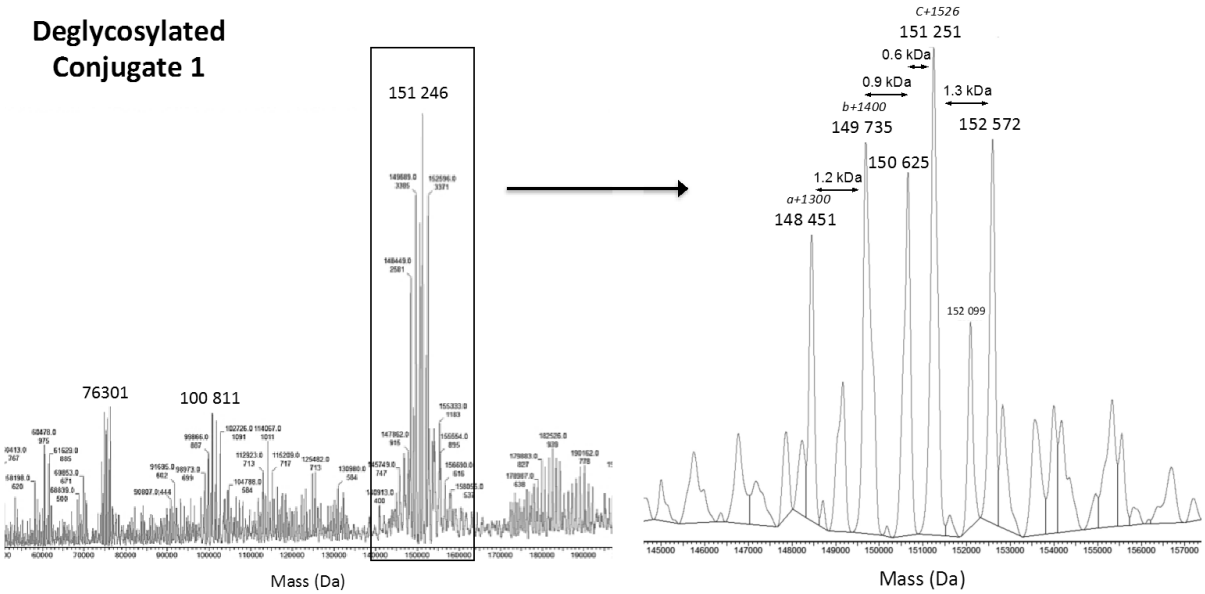
After reduction of 0.5 mL of Anti-CD4 (1.6 mg/mL, 10.7 µM) with TCEP (1.5 µg, 5.3 nmol, 10.6 µM) following the general procedure, the reduce Ab (1 mL) was treated with 5.0 µL of 1,3-dibromobenzene (0.4 mg, 1.5 µmol, 1.5 mM) solution in DMSO during 30 min at 4 ºC solution. After purification, five samples of approximately 150 µL compressed between 0.1 and 1.0 mg/mL were collected.



Conjugate 1 nanodrop UV spectra

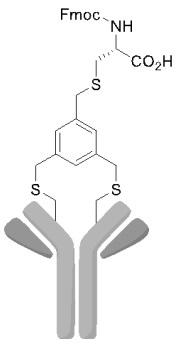




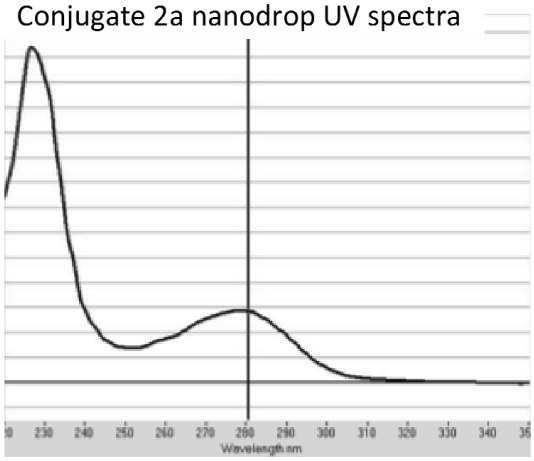


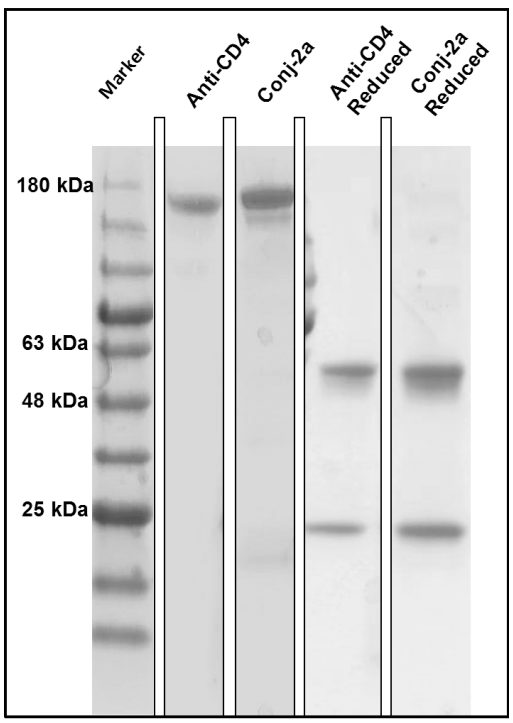
Conjugate 2a: Fmoc-Cys(DBMB)-OH conjugated to anti-CD4

After reduction of 1 mL of Anti-CD4 (0.88 mg/mL, 5.9 µM) with TCEP (1.7 µg, 5.9 nmol, 5.9 µM). following the general procedure, 0, 5 mL of the reduce Ab (1 mL) (0.88 mg/mL) was treated with 4.0 µL of Fmoc-Cys(DBMB)-OH (1.0 mg, 1.6 µmol, 1.6 mM) solution in DMSO, during 30 min at 4 ºC. After the purification, six samples of approximately 150 µL compressed between 0.1 and 1.1 mg/mL were collected.

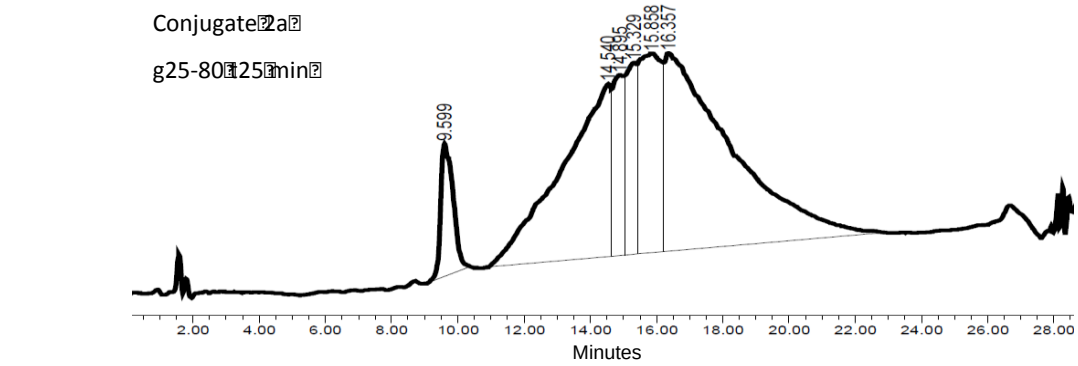


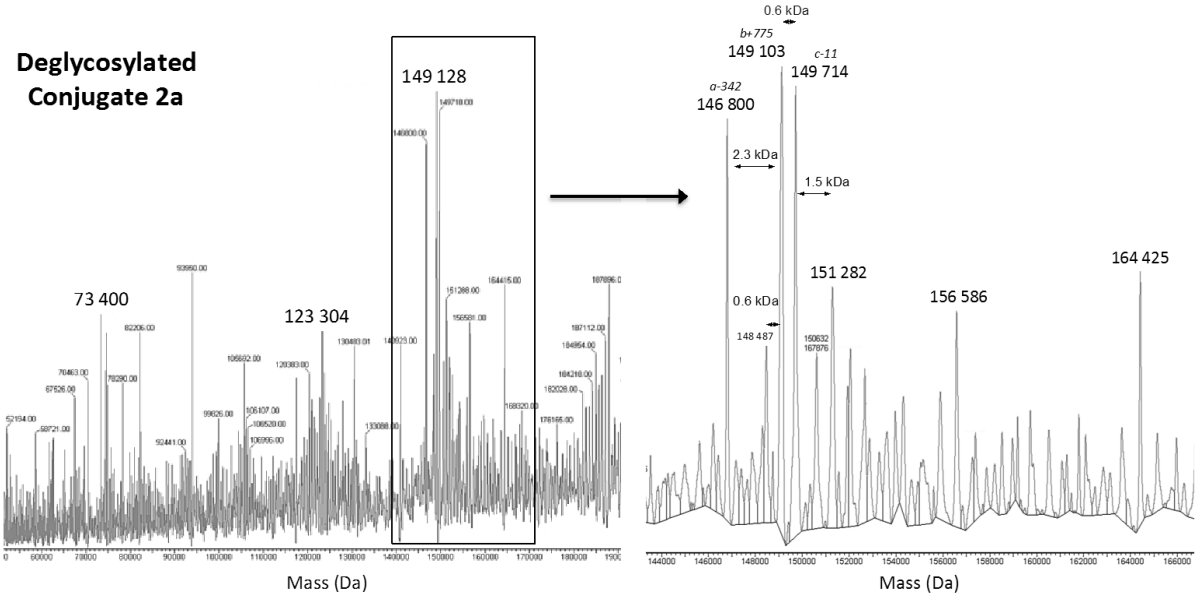
Conjugate 2a nanodrop UV spectra





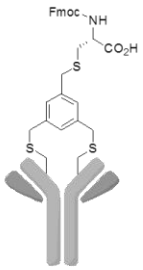
HPLC analysis:



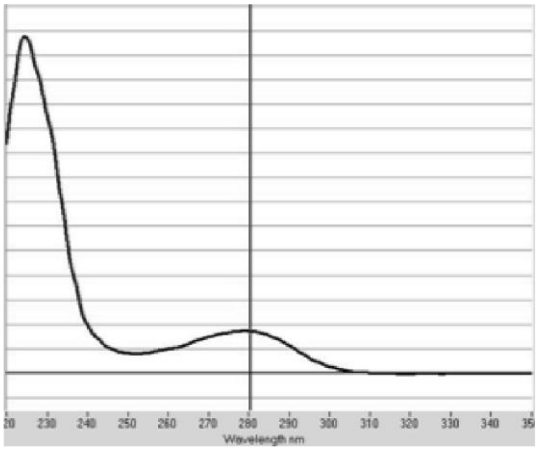


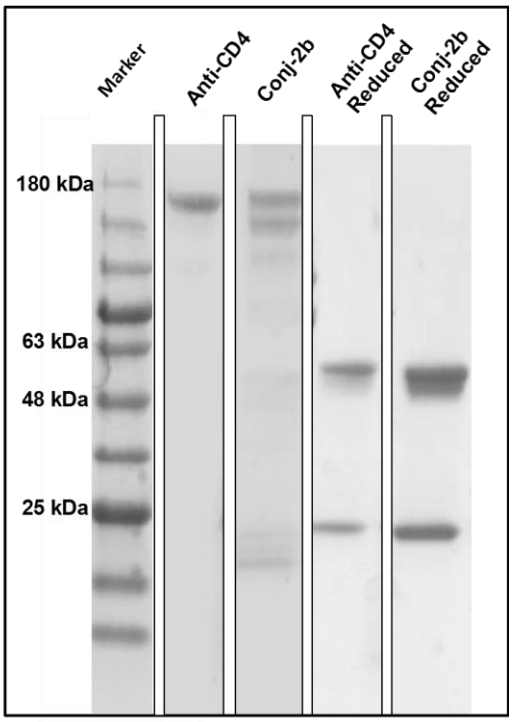
Conjugate 2b: Fmoc-Cys(DBMB)-OH conjugated to anti-CD4

1 mL of anti-CD4 (0.7 mg/mL, 4.7 µM) in borate buffer at was treated with TCEP (4.3 µg, 0.015 µmol, 0.015 mM). Then, 0.5 mL of the reduced antibody (0.67 mg/mL, 4.5 µM) was treated with 2 µL solution of Fmoc-Cys(DBMB)-OH (4 µg, 6.5 nmol, 6.5 µM) solution in DMSO. After purification, four samples of approximately 200 µL compressed between 0.2 and 0.6 mg/mL were collected.

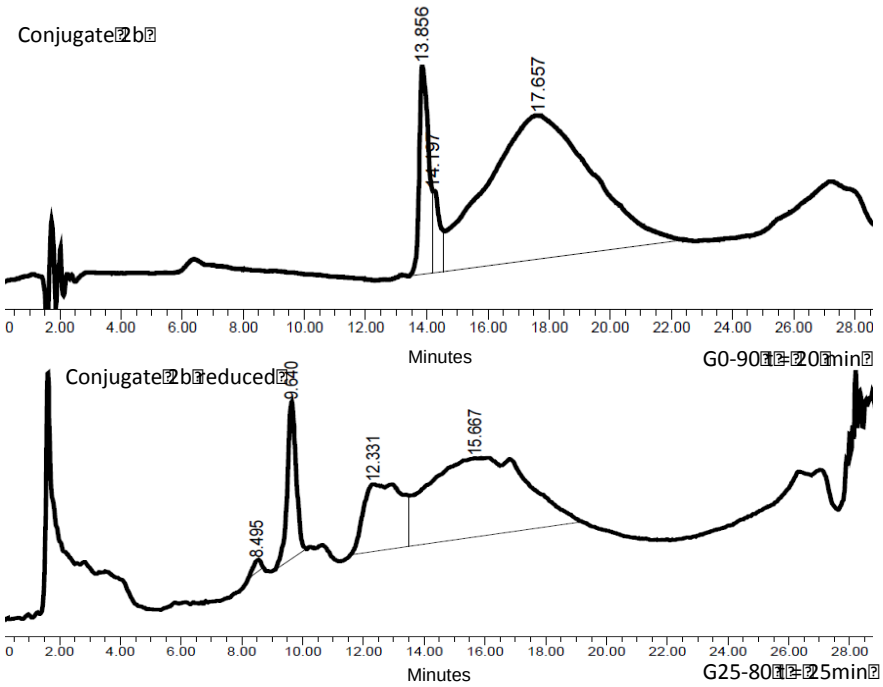


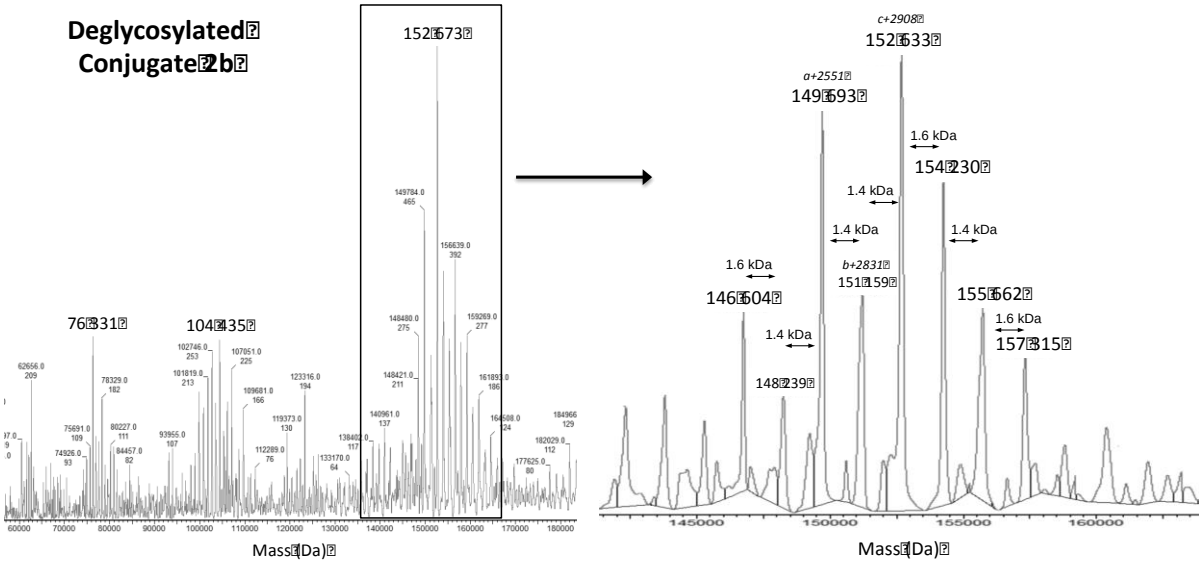
Conjugate 2b nanodrop UV spectra





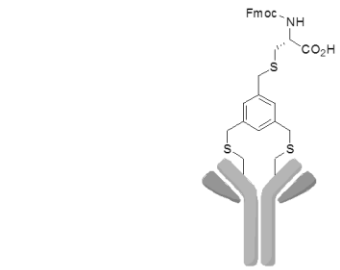
HPLC analysis:

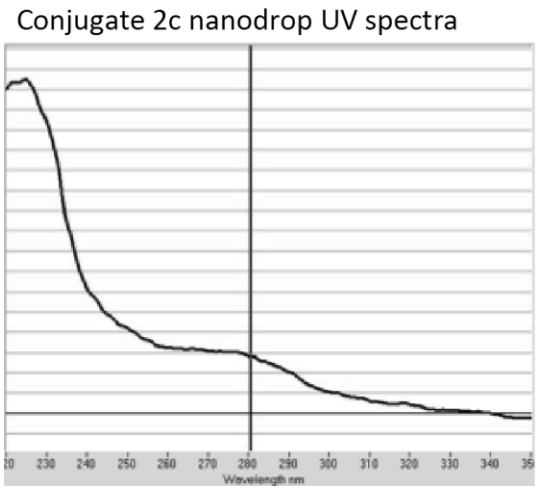


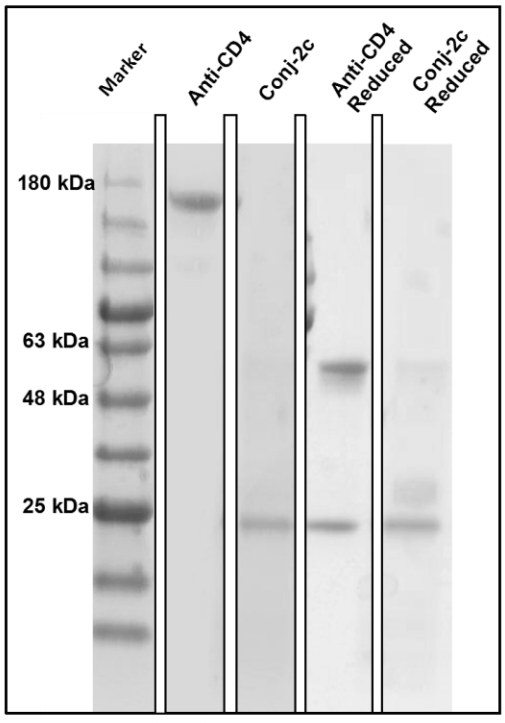


Conjugate 2c: Fmoc-Cys(DBMB)-OH conjugated to anti-CD4

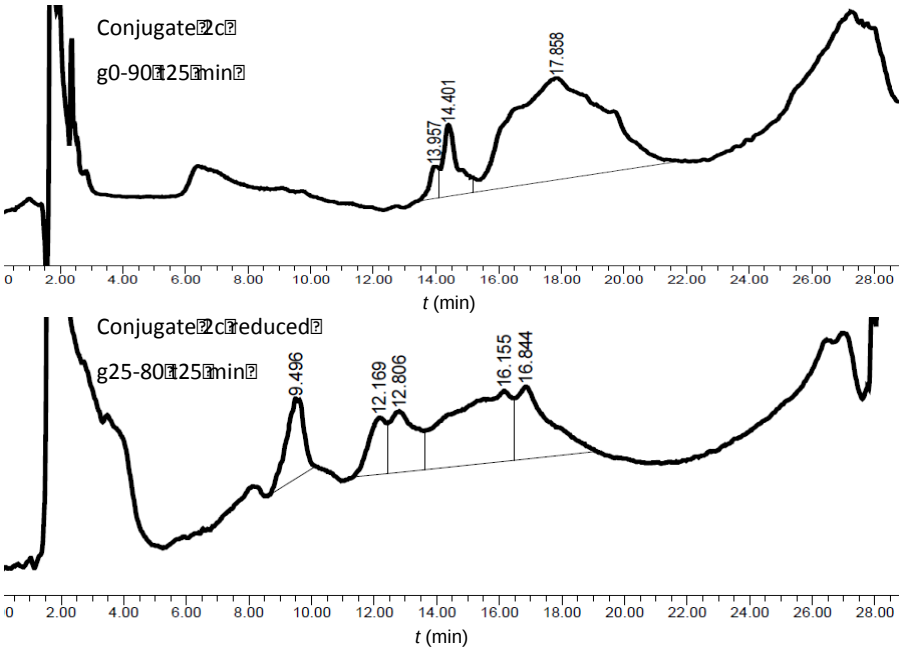
1 mL of anti-CD4 (0.69 mg/mL, 4.6 µM) in borate buffer was treated with TCEP (2.8 mg, 9.8 µmol, 10 mM). Then, 0.4 mL of the reduced antibody (0.69 mg/mL) was treated with 1 µL of a solution of Fmoc-Cys(DBMB)-OH (52 mg, 84 µmol, 84 mM) solution in DMSO. After the purification, six samples of approximately 150 µL compressed between 0.1 and 0.5 mg/mL were collected.

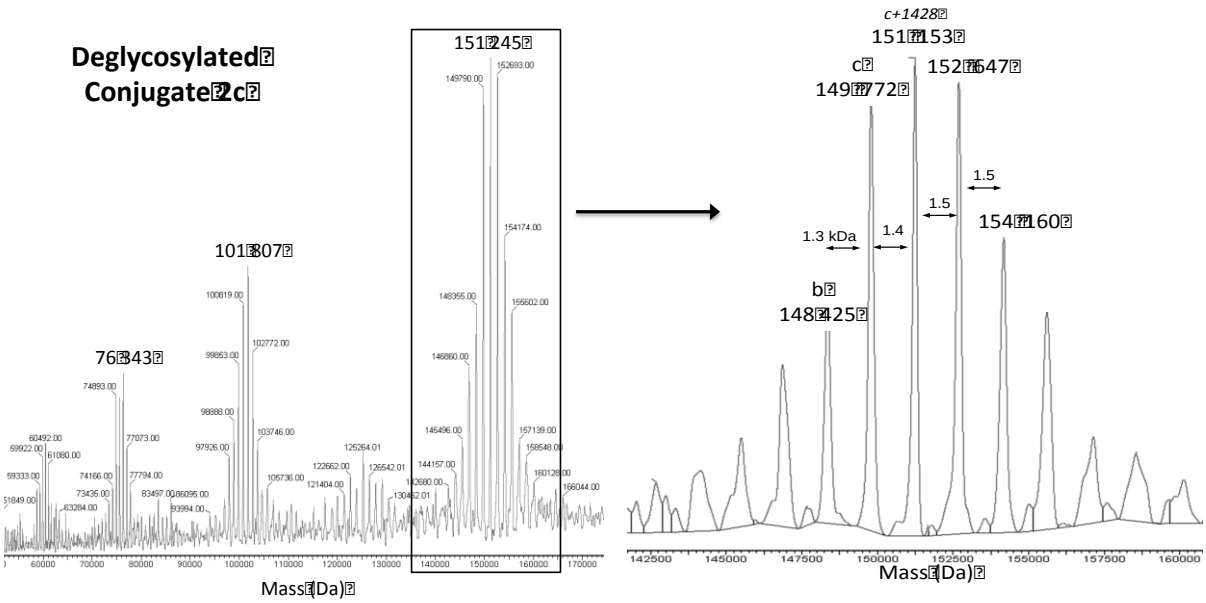






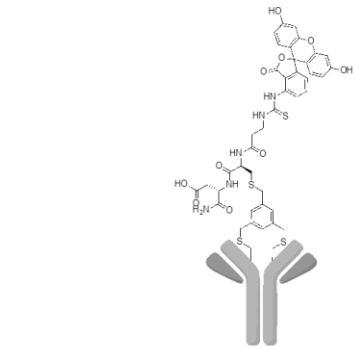
HPLC analysis:

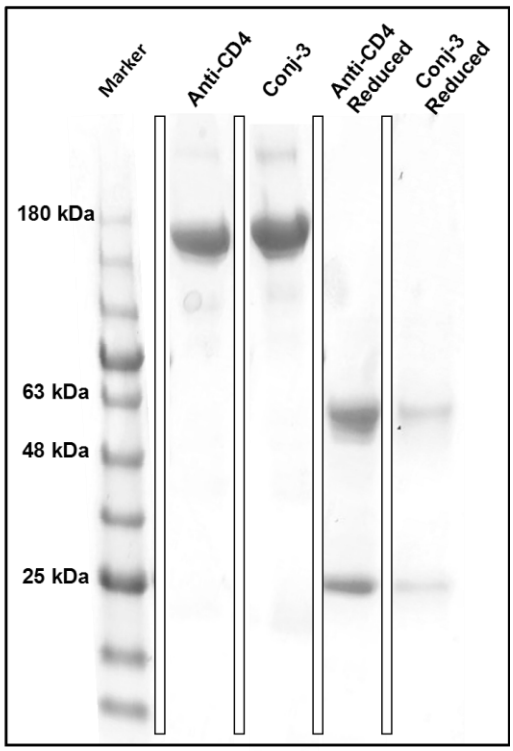




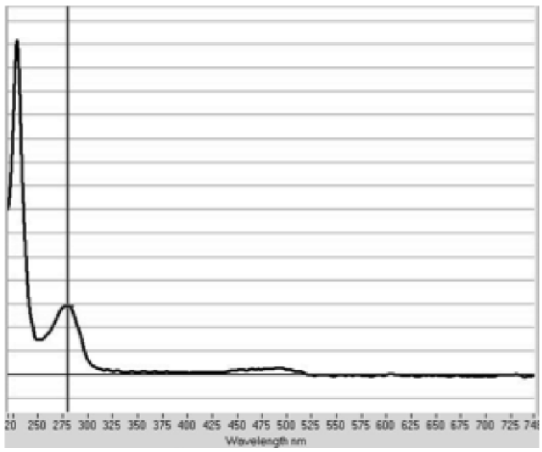
Conjugate 3: FITC-β-Ala-Cys(BBMB)-Asp-NH_2 (3g) conjugated to anti-CD4

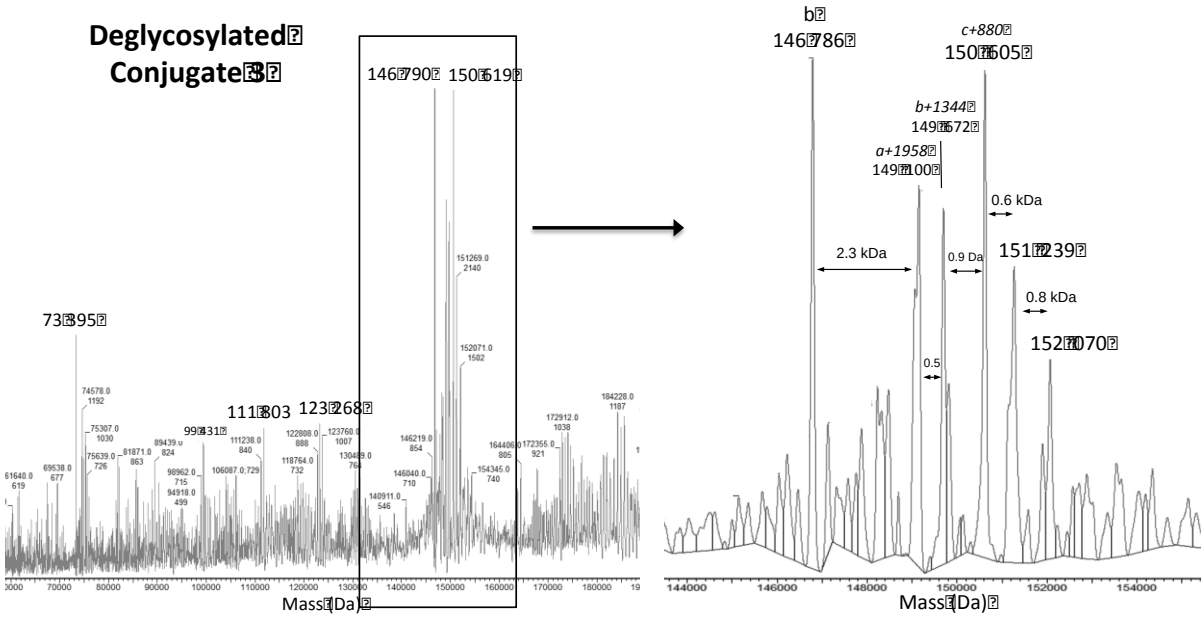
0.5 mL of anti-CD4 (1.5 mg/mL, 9.7 µM) in borate buffer was treated with TCEP (1.4 µg, 4.9 nmol, 9.7 µM). Then, 0.5 mL of the reduced antibody (1.5 mg/mL), was treated with 10 µL of a solution of FITC-bAla-Cys-Asp-NH_2 (1.2 mg, 1.2 µmol, 1.0 mM) solution in DMSO. After the purification, six samples of approximately 100 µL compressed between 0.2 and 1.0 mg/mL were collected.





Conjugate 3 nanodrop UV spectra



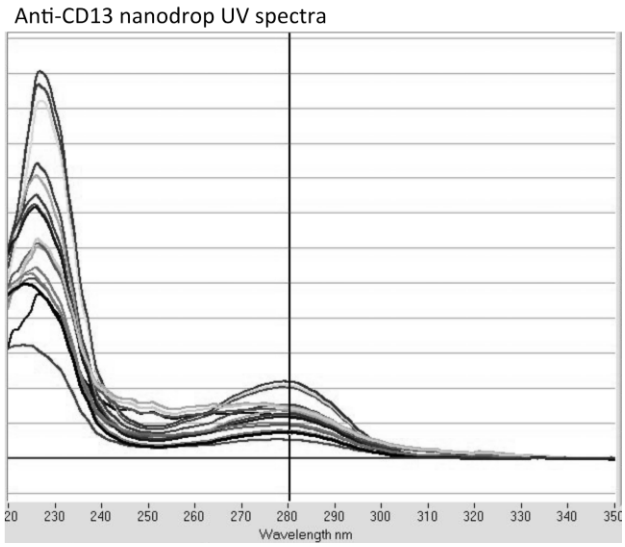


Conjugation to anti-CD13

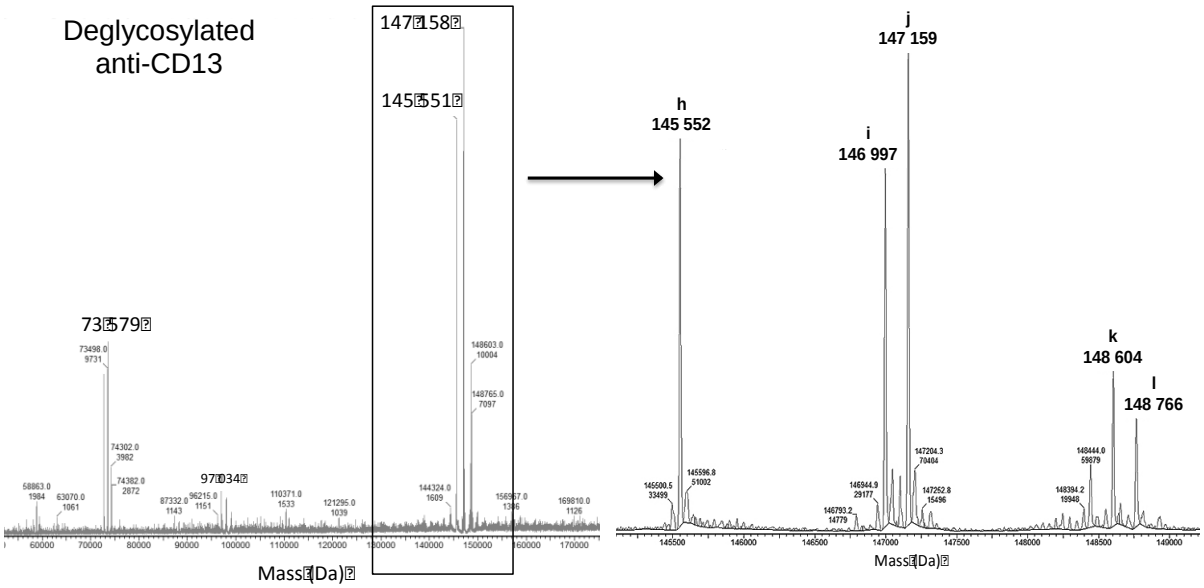
Anti-CD13 analysis

Anti-CD13 was provided in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH = 7.4) at 7.5 mg/mL. For bioconjugation anti-CD13 was diluted with borate buffer at pH = 8 (25 mM H_3BO_3, 25 mM NaOH, 25 mM NaCl and 1 mM EDTA) to

approximately 1 mg/mL of antibody. For mass analysis the provided Ab solution was dialyzed over NH_4Ac using amicon 50 KDa.

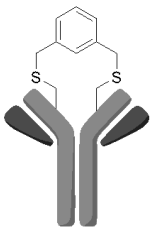


MS-ESI:

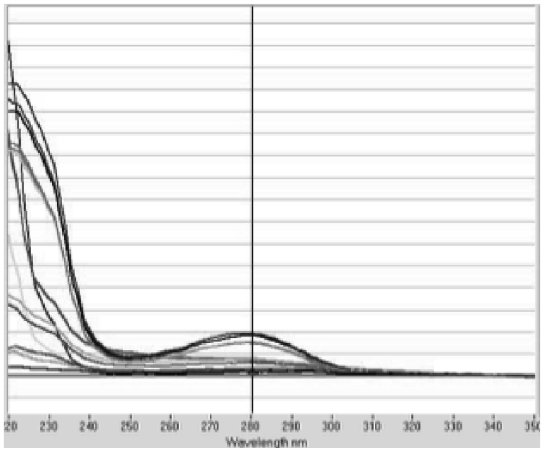


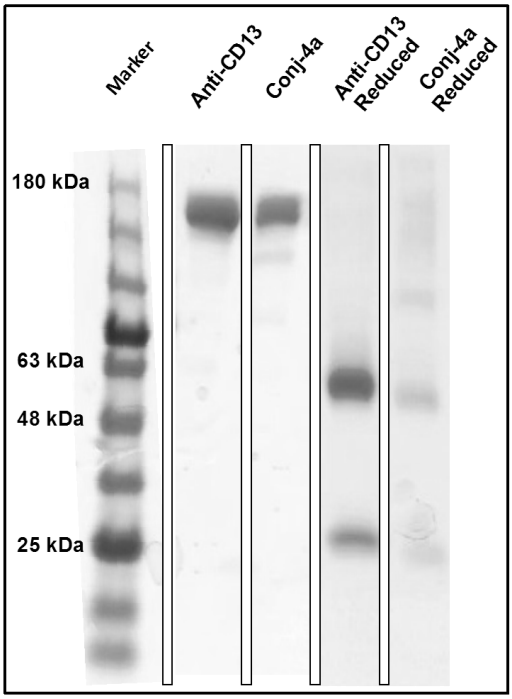
Conjugate 4a: α,α′-Dibromo-*m*-xylene (3h) conjugated to anti-CD13

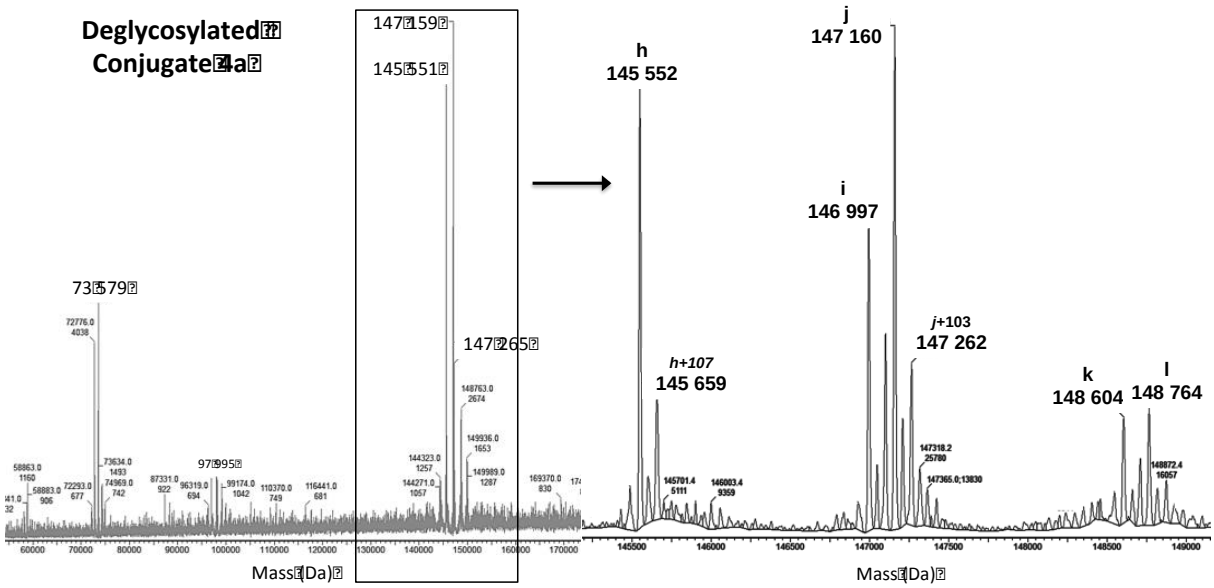
0.5 mL of anti-CD13 (0.84 mg/mL, 5.6 µM) in borate buffer was treated with TCEP (0.96 µg, 3.4 nmol, 6.7 µM). Then, 0.25 mL of the reduced antibody (0.84 mg/mL), was treated with 0.8 µL of a solution of α,α′-dibromo-*m*-xylene (**3h**) (1.1 mg, 4.1 µmol, 3.8 mM) solution in DMSO. After the purification, three samples of approximately 250 µL compressed between 0.10 and 0.34 mg/mL were collected.



Conjugate 4a nanodrop UV spectra

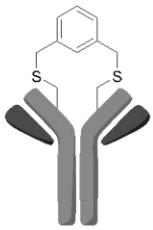


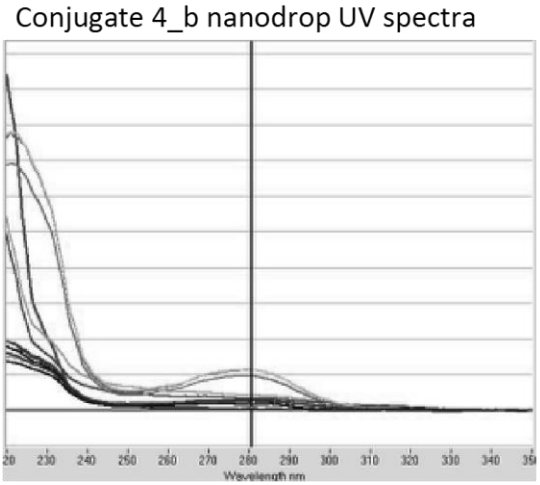


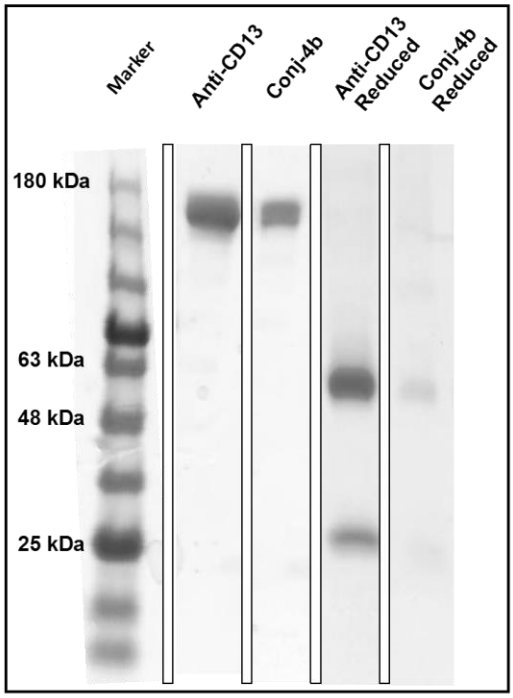


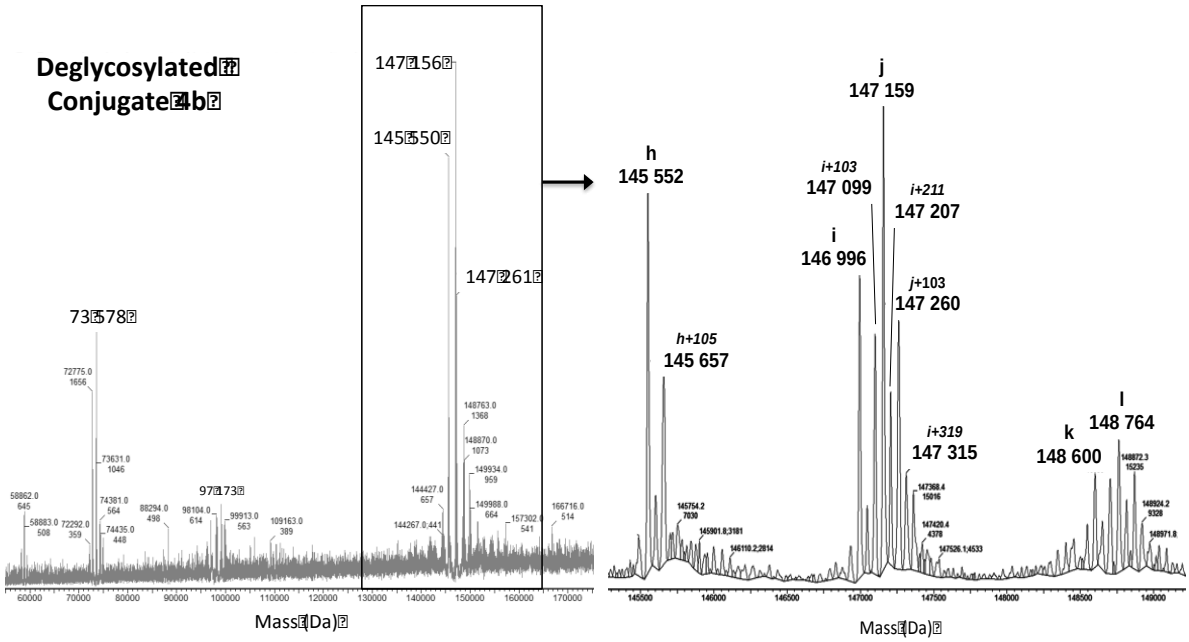
Conjugate 4b: α,α′-Dibromo-*m*-xylene conjugated to anti-CD13

0.25 mL of the reduced antibody (0.84 mg/mL) was treated with 1.6 µL of a solution of α,α′-dibromo-*m*-xylene (**3h**) (1.1 mg, 4.1 µmol, 3.8 mM) solution in DMSO. After the purification, three samples of approximately 250 µL compressed between 0.10 and 0.39 mg/mL were collected.



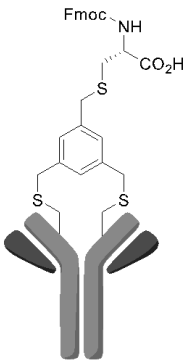


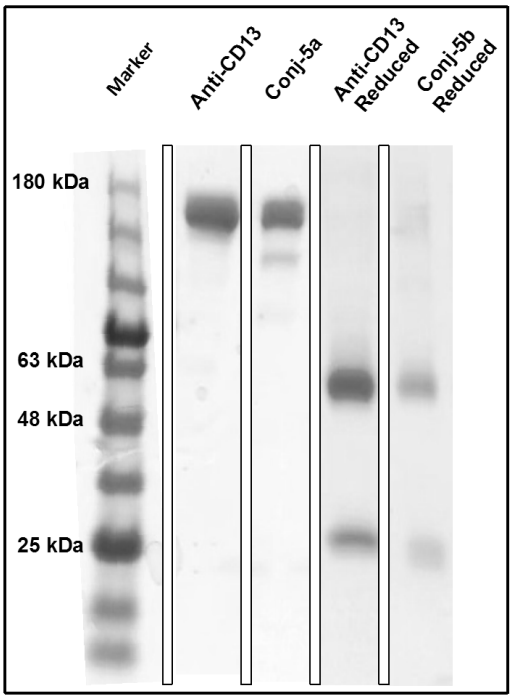




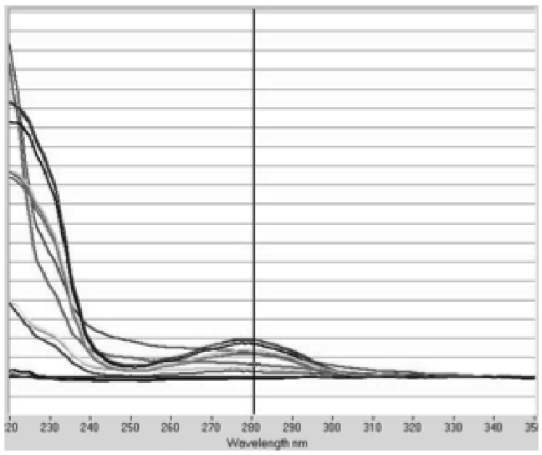
Conjugate 5a: Fmoc-Cys(DBMB)-OH conjugated to Anti-CD13

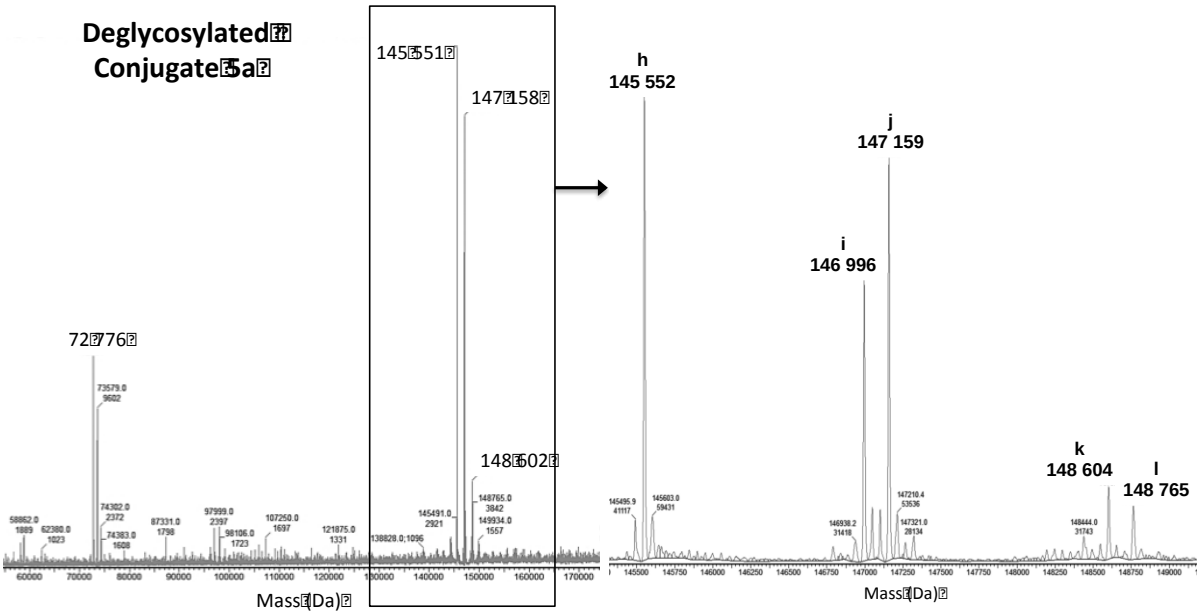
0.5 mL of anti-CD13 (0.90 mg/mL, 6.0 µM) in borate buffer was treated with TCEP (1.0 µg, 3.6 nmol, 7.2 µM). Then, 0.25 mL of the reduced antibody (0.9 mg/mL), was treated with 1.8 µL of a solution of Fmoc-Cys(DBMB)-OH (1.6 mg, 2.6 µmol, 1.6 mM) solution in DMSO. After the purification, three samples of approximately 250 µL compressed between 0.10 and 0.34 mg/mL were collected.





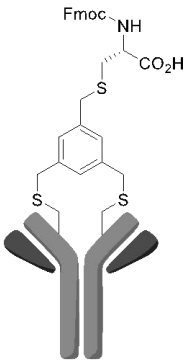
Conjugate 5a nanodrop UV spectra

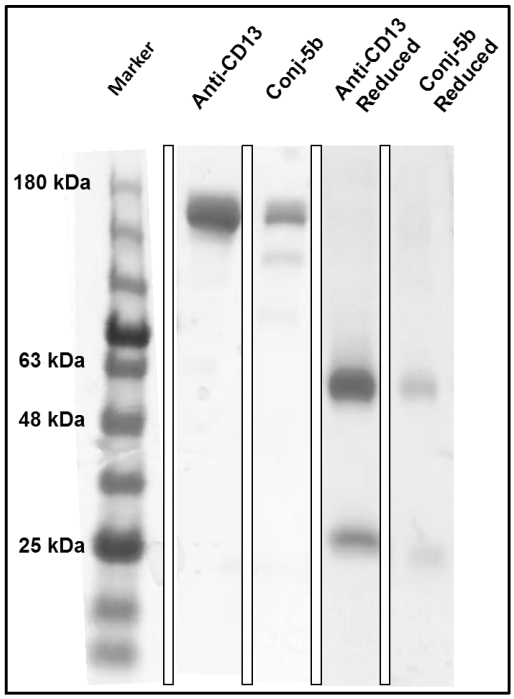




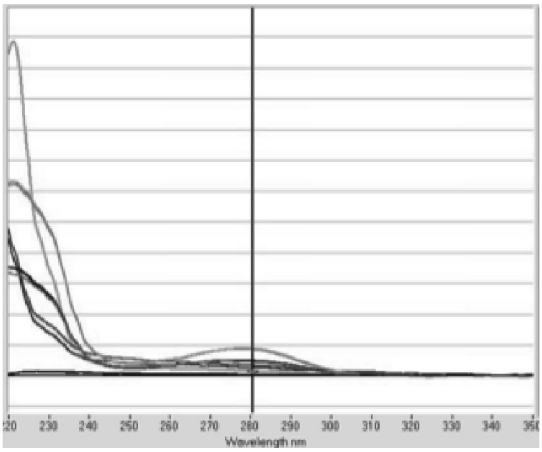
Conjugate 5b: Fmoc-Cys(DBMB)-OH conjugated to anti-CD13

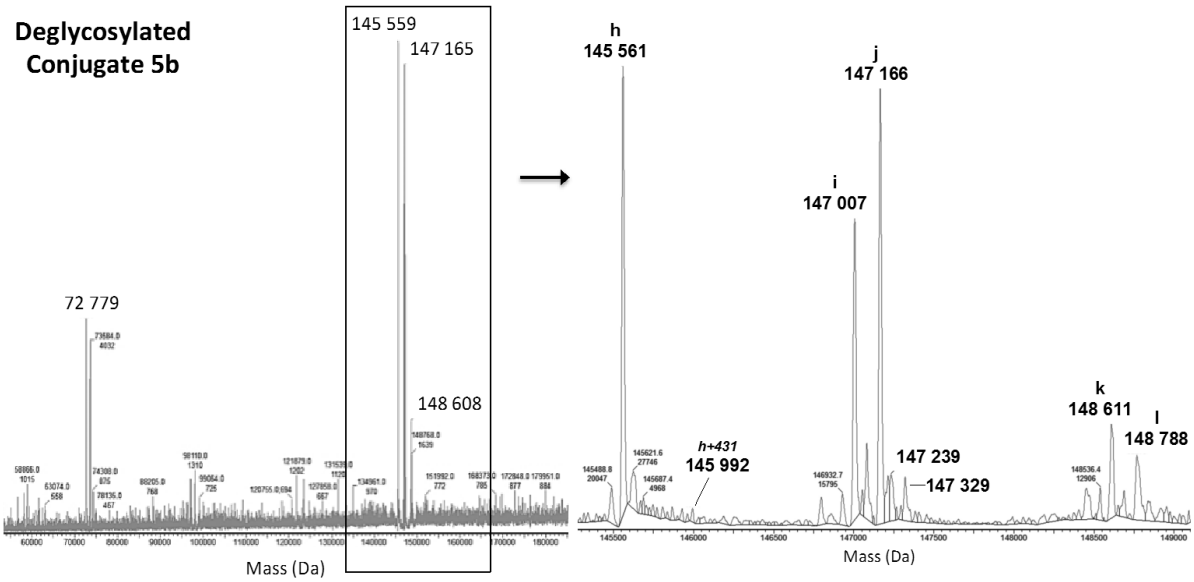
0.5 mL of anti-CD13 (0.90 mg/mL, 6.0 µM) in borate buffer was treated with TCEP (1.0 µg, 3.6 nmol, 7.2 µM). Then, 0.25 mL of the reduced antibody (0.9 mg/mL) was treated with 3.7 µL of a solution of Fmoc-Cys(DBMB)-OH (1.6 mg, 2.6 µmol, 1.6 mM) solution in DMSO. After the purification, two samples of approximately 250 µL (0.17 and 0.32 mg/mL) were collected.





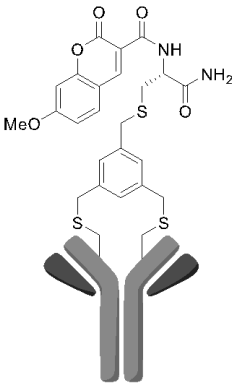
Conjugate 5b nanodrop UV spectra

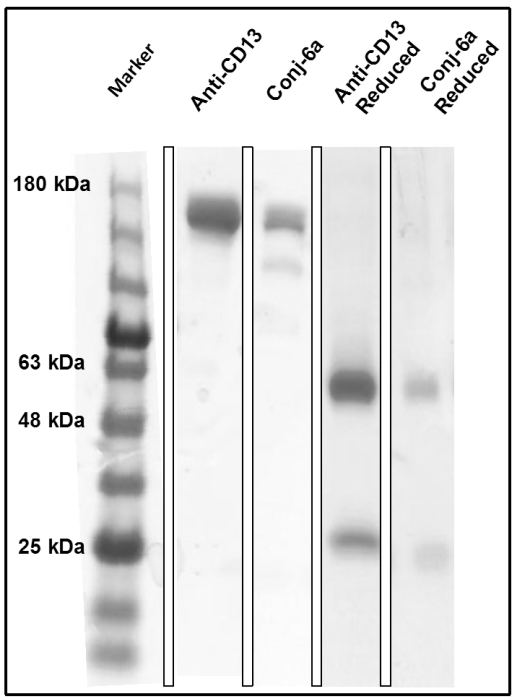




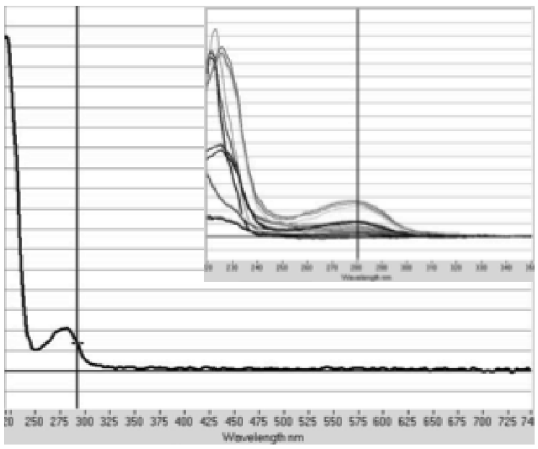
Conjugate 6a: 7-MeO-Cumarin-Cys(BBMB)-NH_2 conjugated to anti-CD13

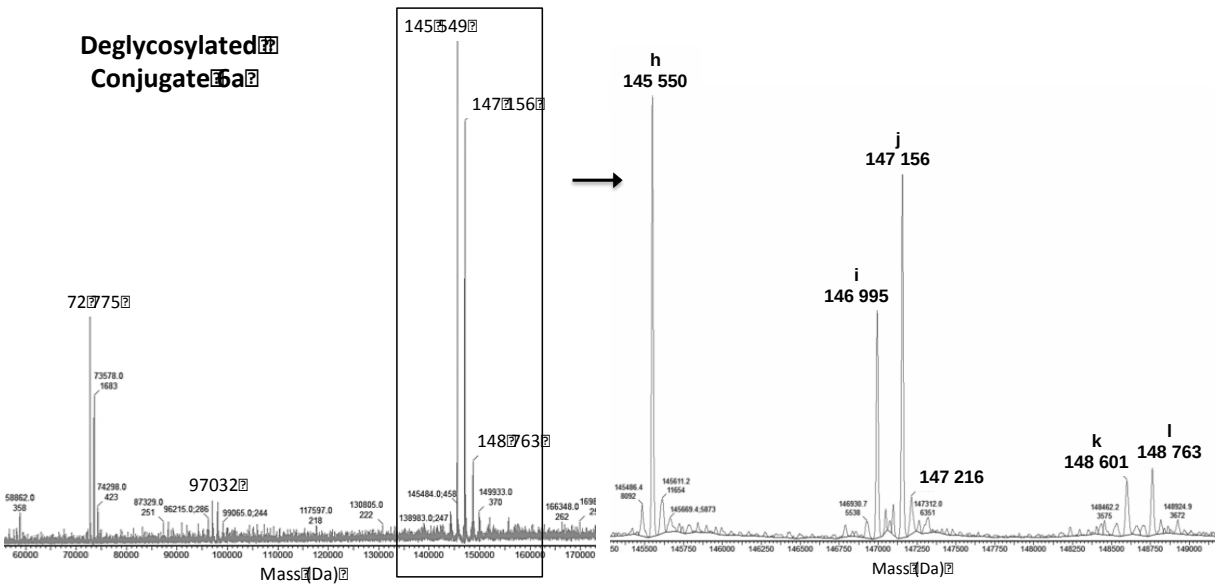
0.5 mL of anti-CD13 (0.98 mg/mL, 6.5 µM) in borate buffer was treated with TCEP (1.1 µg, 4.0 nmol, 7.8 µM). Then, 0.25 mL of the reduced antibody (0.98 mg/mL) was treated with 4.0 µL of a solution of Cumarin-Cys(DBMB)-NH_2 (0.8 mg, 1.3 µmol, 1.6 mM) solution in DMSO. After the purification, three samples of approximately 250 µL compressed between 0.06 and 0.36 mg/mL were collected.





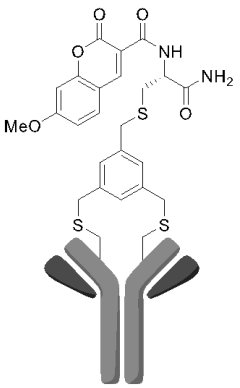
Conjugate 6a nanodrop UV spectra

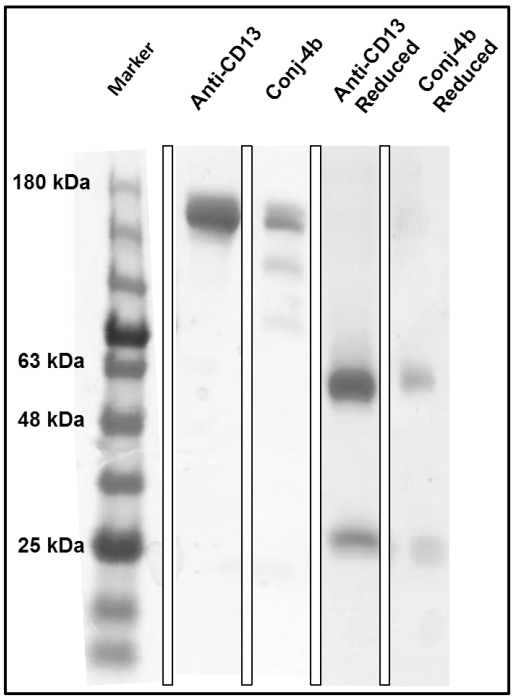




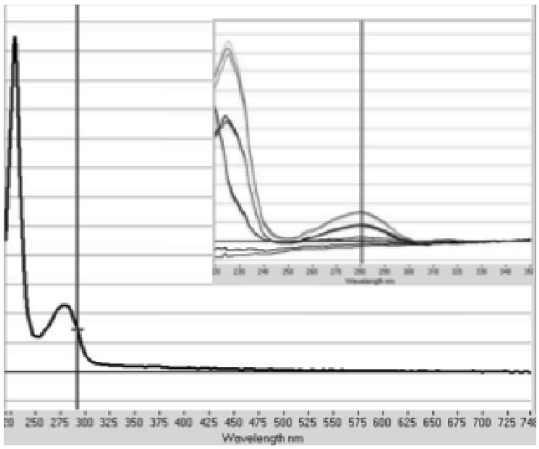
Conjugate 6b: 7-MeO-Cumarin-Cys(BBMB)-NH_2 conjugated to anti-CD13

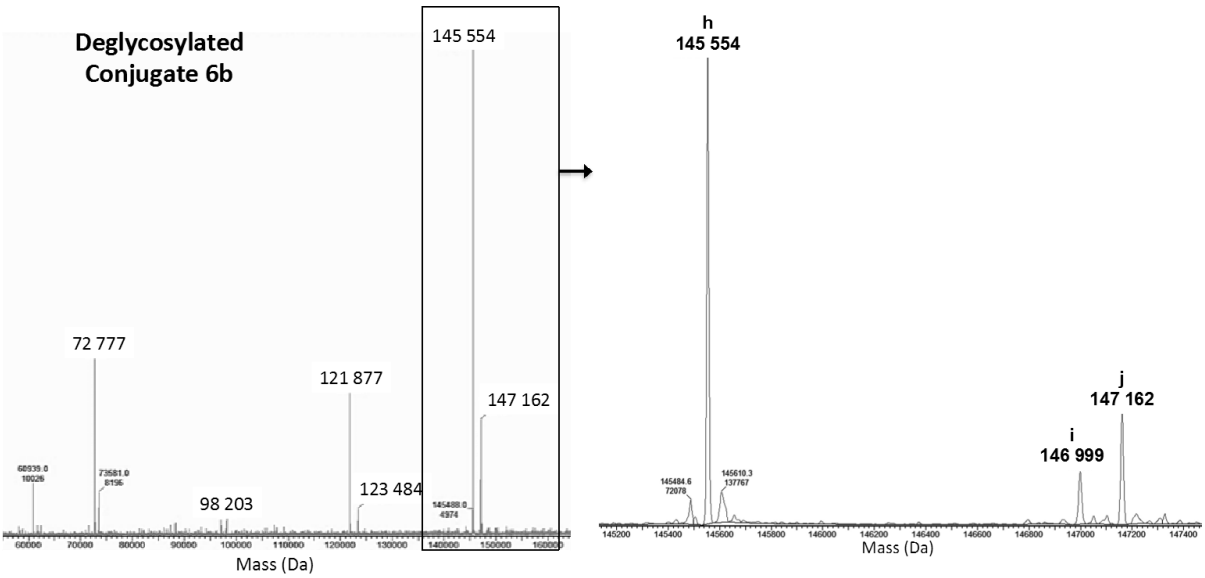
0.5 mL of anti-CD13 (0.98 mg/mL, 6.5 µM) in borate buffer was treated with TCEP (1.1 µg, 4.0 nmol, 7.8 µM). Then, 0.25 mL of the reduced antibody (0.98 mg/mL) was treated with 4.0 µL of a solution of Cumarin-Cys(BBMB)-NH_2 (0.4 mg, 0.67 µmol, 1.7 mM) in DMSO (0.4 mL). After the purification, two samples of approximately 250 µL (0.13 and 0.22 mg/mL) were collected.





Conjugate 6 nanodrop UV spectra





Binding affinity experiments:

Jurkat (5 x 10^5) and U937 (5 x 10^5) cells were incubated with the anti-CD4 HP2/6 and anti-CD13 TEA1/8 mAbs, respectively, and their corresponding conjugates at the indicated concentrations on ice for 1 h. Then, cells were washed with PBS and incubated with 10 µg/ml of FITC-labelled anti-mouse IgG antibodies (BD Biosciences) for 30 min on ice. Finally, cells were washed and resuspended in PBS for their analysis by flow cytometry using a FACSCanto II cytometer. Table shows the MFI for each conjugate and concentration tested.

Anti-CD4 results

mAb Concentration (µg/mL)	Mean Fluorescense Intensity (MFI)					
	Anti-CD4 HP2/6	Conjugate 1	Conjugate 2a	Conjugate 2b	Conjugate 2c	Conjugate 3
0.1	1.62	1.52	2.67	1.71	1.55	2.22
0.5	4.96	2.63	4.76	4.54	1.62	3.13
1.0	5.88	3.05	6.20	5.51	1.6	2.39
3.0	7.55	5.14	6.65	6.57	1.56	5.18
5.0	8.28	5.30	7.38	7.62	1.62	5.28
10.0	7.79	5.69	8.33	7.00	1.68	5.42

Anti-CD13 results

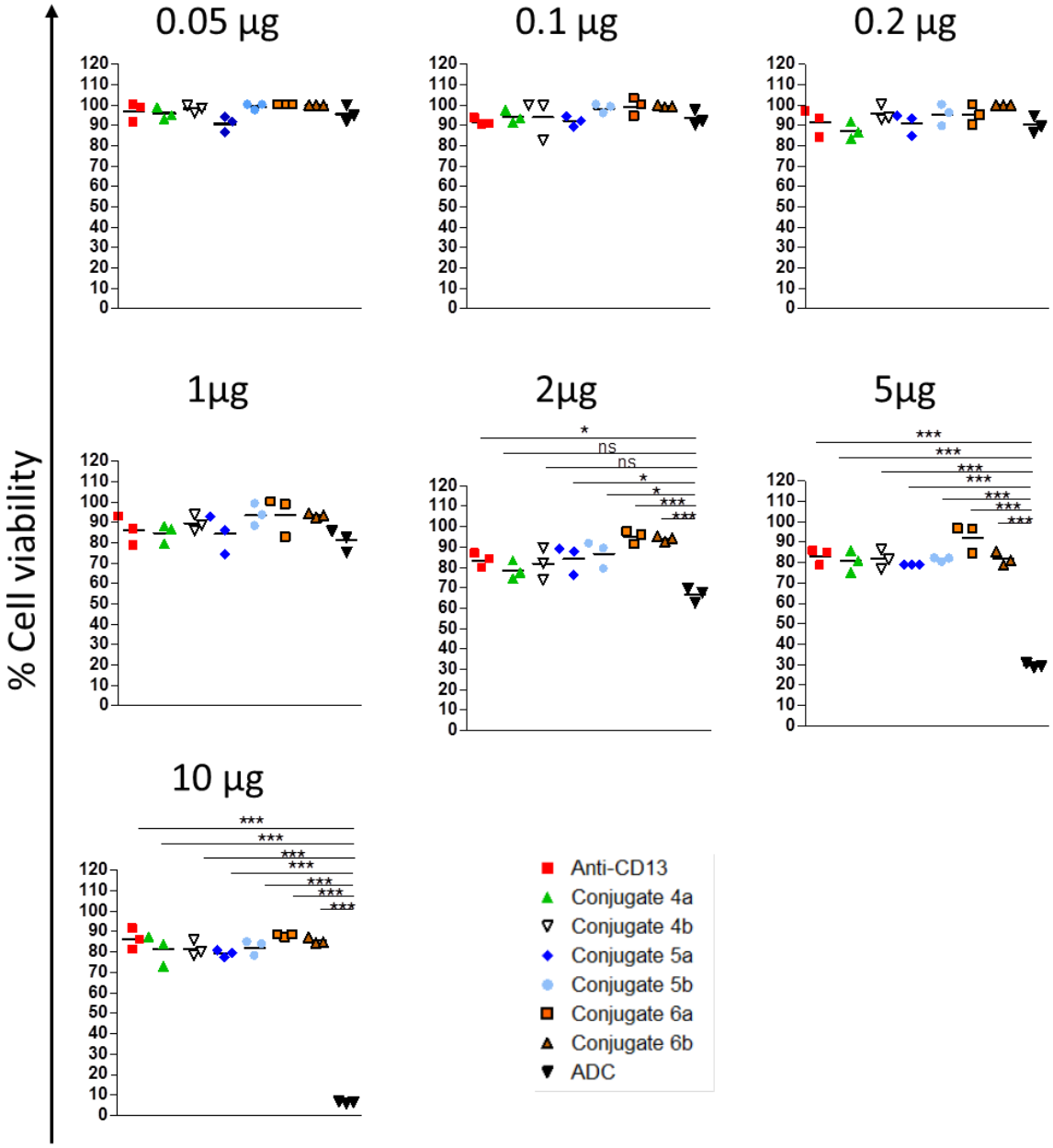
mAb Concentration (µg/mL)	Mean Fluorescense Intensity (MFI)						
	Anti-CD13 mAb	Conjugate 4a	Conjugate 4b	Conjugate 5a	Conjugate 5b	Conjugate 6a	Conjugate 6b
0.5	10.2	6.13	6.24	6.65	5.22	5.86	6.19
1.0	17.3	10.9	11.5	11.8	9.88	10.1	10.7
2.0	24.7	18.4	19.0	19.4	16.3	16.8	17.9
5.0	30.7	27.8	29.3	28.3	25.2	26.8	27.3

Internalization experiments:

U937 (5 x 10^5) cells were incubated with the anti-CD13 or the conjugates 4b, 5b and 6a at 10 µg/mL. For the time 0 of endocytosis cells were incubated at 4 ºC for 3 h. On the other hand for time 3 h, the cells were incubated at 37 ºC during 3 h. Then cells were washed with PBS and incubated with 10 µg/ml of FITC-labelled anti-mouse IgG antibodies for 30 min on ice, then the cells were washed resuspended in PBS for their analysis by flow cytometry using a FACSCanto II cytometer.

Cell viability experiments:

U937 (4.4 x 10^6) cells were incubated by triplicate alone or with the anti-CD13, with their respective conjugates or the cell-killing ADC provided from PharmaMar at the indicated concentrations (0.05-10 µg/mL) during 72 h at 37 ºC. Cell viability was measured by a luminescence assay (CellTiter-Glo®, Promega Biotech, Madrid, Spain). Analysis was performed considering the control condition as 100% of viability. The following figure shows the calculated % of cell viability for each concentration of the mAb and its conjugates.


0.05 µg
0.1 µg
0.2 µg
1µg
2µg
5µg
10 µg
% Cell viability
Anti-CD13
Conjugate 4a
Conjugate 4b
Conjugate 5a
Conjugate 5b
Conjugate 6a
Conjugate 6b
ADC

Chapter II. Non-cleavable linkers

2. Perfluoroaryl linkers

2.1. Introduction

Chemical modifications of biomacromolecules could be considered a double-edge sword because they could enhance their biological properties and strengthen both, the biocompatibility and the selectivity on them.[1–4] For this reason, bioconjugation becomes a powerful tool for the development of new compounds for biomedicine and biotechnology. The approval of new chemically-modified biomolecules by the FDA in the recent years[5] demonstrates the increasing scientific interest and potential of this field.[6]

In nature, chemical modifications occur specifically through a wide envelop of reactive amino acid side chains, thus the challenge for years has been mimic that kind of natural modifications. Regioselectivity in bioconjugation is achieved through a variety of reactive groups such as amines, thiols, alcohols or even carboxylic acids.[4] Several methods have been described for bioconjugation[7] such as lysine, aspartic or glutamic acid, and serine or threonine, but the random distribution of these amino acids over the biomolecule structure leads to heterogeneous conjugations, and as a result to obtaining a great deal of species. In this sense, cysteine (Cys) which generally founds as disulphide bond and it needs previous reduction to generate the active thiol group,[8,9] has successfully used to promote regioselective bioconjugation in peptides, proteins and antibodies.[10–12] Additionally it has shown to be one of the most effective amino acids in order to perform site-specific conjugation and in consequence generate homogeneous conjugates, which could have better stability and therapeutic index for antibody-drug conjugates (ADCs).[13,14]

The most used methodology for Cys conjugation is the well-known thiol group alkylation via Michael addition over the double bond of a maleimide ring,[15,16] haloacetyl derivatives and alkyl halides are also used for this purpose.[17] Furthermore, disulfide linkage has been tapped for bioconjugation, considering as a cleavable linker in presence of reduced glutathione.[18,19] Recently, Pentelute and co-workers published the use of perfluoroaromatic rings as peptide stappling[20] using mild conditions. To probe arylation of cysteines, they evaluated the high reactivity through S_NAr[21] under non-forcing conditions of the perfluoroaromatic molecules. Furthermore, they tested a new generation of rationally designed perfluoroaryl-based cross-linking for peptide macrocyclization based on Cys perfluoroarylation for relatively short peptides (14 amino acid residues),[22] and for longer peptides which usually the peptide macrocyclization becomes inefficient due to the large entropic penalty. Accordingly, Pentelute group have shown how glutathione S-transferase (GST) can efficiently mediate the

macrocyclization reaction of long peptides by catalysing a SNAr process between cysteine and perfluoroaromatic moieties have found that the use of enzymes becomes an efficient strategy to perform large peptides macrocyclization.[23] Additionally, following the "click" chemistry concept, Pentelute and coworkers studied the enzymatic site-specific Cys perfluoroarylation for glutathione-like peptides (*N*-terminal γ-Glu-Cys-Gly sequence).[24] In this work, the author introduced perfluoroarylated compounds as linker for protein bioconjugation. However, the enzymatic methodology requires protein engineering or post-translational modifications in order to introduce a *N*-terminal γ-Glu-Cys-Gly sequence into the biomolecules. Is important to highlight the above-mentioned procedures, due to the selectivity that the thiol group of Cys present through nucleophilic aromatic substitution (S_NAr) with perfluoro aromatic rings under mild conditions.

Thus, this set of factors involving Cys inspired us to amplify the methodological synthetic spectrum available for this kind of bioconjugation, focused on the study of the conjugation through Cys using perfluoroarylated compounds as non-cleavable linkers. As an outstanding challenge, we used antibodies in order to test the advantages that perfluoroarylated compounds offer. Furthermore, the ability to manipulate these complex proteins opens the avenue to a sort of application in biomedicine-related fields.

In this section, we studied if the perfluoroaromatic scaffolds (decafluorobiphenyl and hexafluorobenzene) are stablished as chemoselective, stable, and non-cleavable linkers for complex biomolecular systems such as antibodies. Furthermore we tried to demonstrated the suitability of these perfluoroaromatic compounds for bioconjugation despite their lack of solubility in aqueous media, and also if the bioconjugation via perfluoroaryl affects the antibody-antigen binding affinity, which is crucial into the antibody activity and efficacy. Concretely, we tested the procedure using the HP2/6 monoclonal antibody (mAb) that recognize human CD4, a transmembrane glycoprotein found in the cell surface of T helper lymphocytes, although it can also be expressed on monocytes or macrophages. CD4 is a member of the immunoglobulin superfamily that interacts with MHC class II molecules and cooperates with the TCR in T cell activation. CD4 is also responsible for HIV-1 interaction through its binding with HIV-1 viral envelope gp120.[25] The therapeutic activity of CD4 mAbs in graft versus host diseases, autoimmunity, HIV infection and inflammation has been profusely studied.[26] Indeed, a humanized anti CD4 mAb Zanolimumab has shown promising results in the treatment of rheumatoid arthritis, psoriasis, Sezary syndrome and against T cell lymphomas.[26,27]

Introduction

2.2. Objectives

- Study the thiol monoalkylation of decafluorobiphenyl and pentafluorobenzene for their functionalization and further bioconjugation.

- Test and find the optimal bioconjugation conditions for the prepared monoalkylated thioesters'.

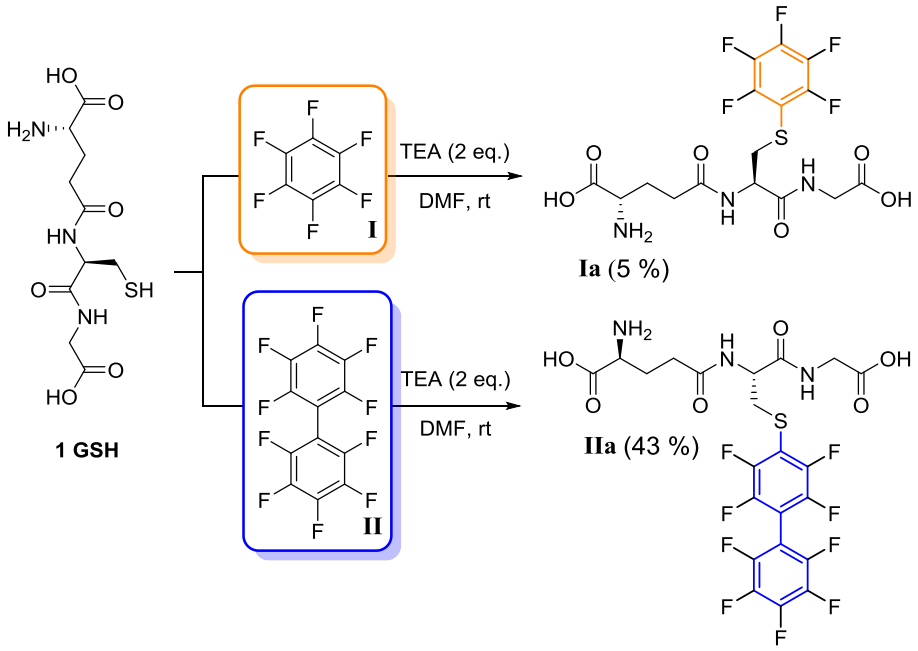
- Use dibromobenzyl compounds for antibodies bioconjugation and study if the bioconjugation process affect to the macromolecular entity.

2.3. Results and discussion

Hexafluorobenzene (**I**) and decafluorobiphenyl (**II**) were tested as linkers for bioconjugation carried out the previous preparation of the corresponding mono-cargo-derivatives **Ia** and **IIa-b** (**Scheme 25** and **Scheme 26**). Reduced glutathione (**GSH**) and a Cys containing peptide **2** were used like thiol derivatives and potential cargoes. After the linker-cargo preparation, two different bioconjugation ways were assessed using the monoclonal antibody Anti-CD4 as biomolecule (**Scheme 27**).

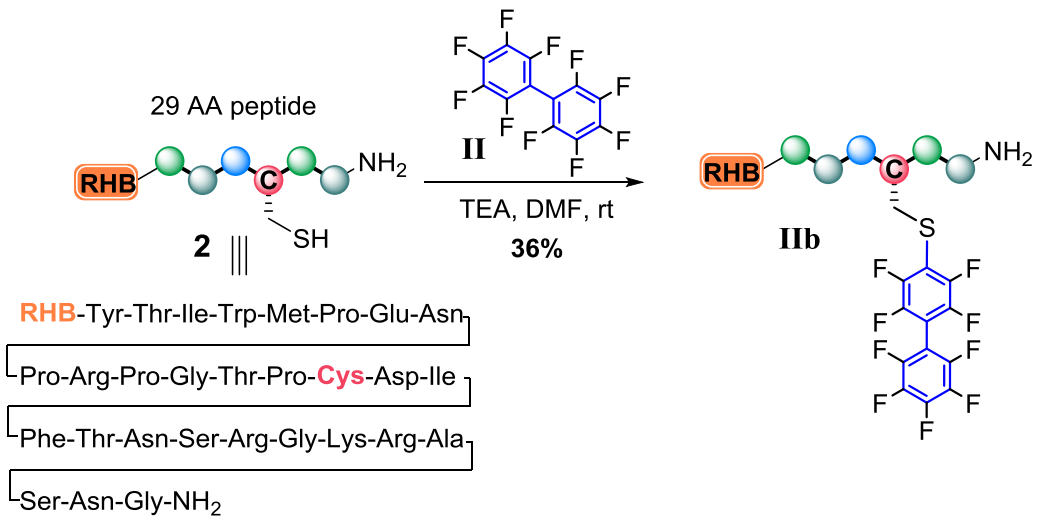
- **Linker-cargo preparation**

The perfluoroarylated compounds **I** and **II** (10 eq) were bounded to reduced glutathione (**GSH**, 1eq), which contain an available thiol group in their structure, in presence of TEA (2 eq) as base, and using MeCN or DMF as solvent (**Scheme 25**). As it was predictable for S_NAr where the solvent plays a key role,[28,29] higher yields were obtained using DMF. A rigorous control of the reaction conditions was needed in order to obtain the monoaddition products. As it was previously reported,[20] these perfluoroarylated compounds have shown high tendency to suffer double (1,4) aromatic nucleophilic substitution (S_NAr) in front of the Cys thiol group due to 1) the steric hindrance of the *ortho* position, and 2) due to the activation of the *para* position of the aromatic ring by the thioether group formed in the first instance. In consequence, without reaction time control it will lead the 1,4-bisadduct of the thiol moiety into perfluoro aromatic rings, generating unreacted *S*-disubstituted 1,4 derivatives, which lack of interest for bioconjugation, and are the main by-products during the mono-adduct preparation. The reactions were followed by RP-HPLC, as well as by RP-HPLC-ESI (See experimental section) showing a control reaction time for **I** tighter than **II** due to the rapid formation of the *S*-disubstituted 1,4-tetrafluorobenzene derivative. The desired products *S*-substituted 1-pentafluorobenzene (**Ia**) and *S*-substituted 1-nonafluorobiphenyl (**IIa**) were obtained after purification by RP column chromatography with 5 % and 43 % of yield, and higher purities of 97 and 99 %, respectively.



Scheme 25. S-Glutathione perfluoroarylation.

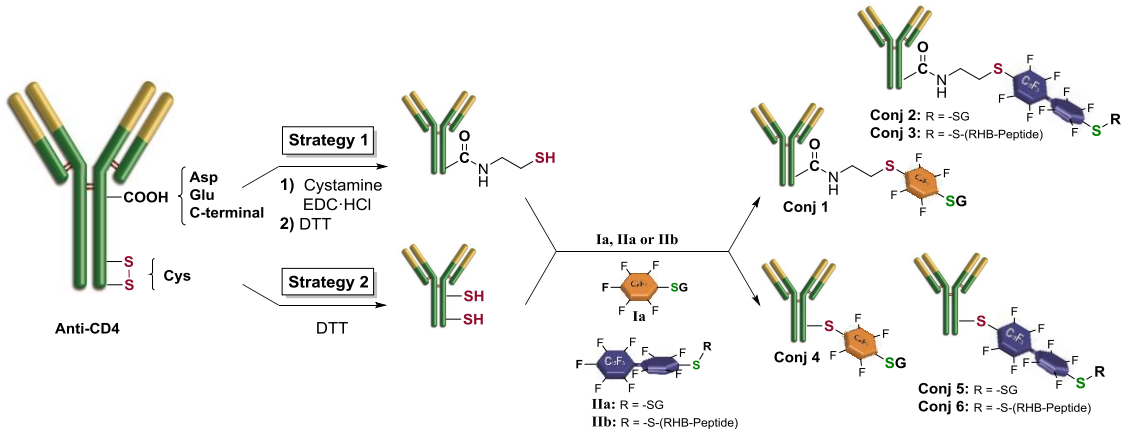
Knowing that the monoaddition using decafluorobiphenyl (**II**) was more effective than with hexafluorobenzene (**I**), we carried out the perfluoroaryl monoaddition of more complex Cys containing peptide using the perfluorobiphenyl scaffold (**II**) (**Scheme 26**). For that purpose, rodhamine B Cys containing peptide with 29 amino acid residues (**2**) was synthetized by SPPS (See experimental section); and then using the established reaction conditions for **IIa**, the perfluoarylated peptide **IIb** was yielded in 36%.



Scheme 26. Cys-containing peptide perfluoroarylation with decafluorobiphenyl (II).

- **Bioconjugation**

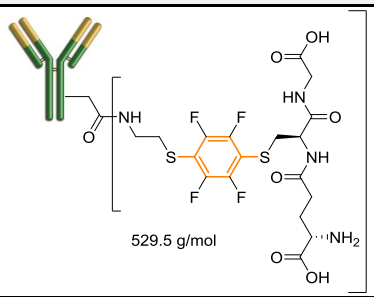
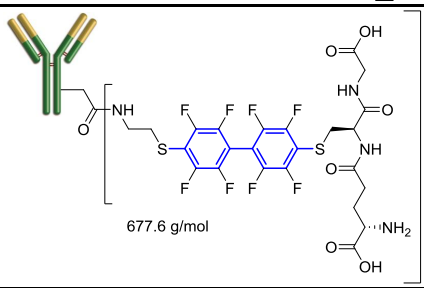
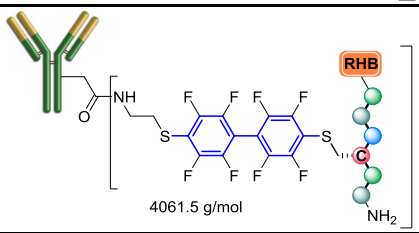
With the monosubstituted perfluoroarylated thiol-reactive compounds in hand (**Ia**, **IIa** and **IIb**), two different ways of bioconjugation trough the thiol groups present in the antibody were tackled (**Scheme 27**). First of all, the conjugation was performed through the post translational modification of the carboxylic acids presents into Anti-CD4 by an amide bond formation with a cystamine, which, previous reduced procedure with DTT, allows to obtain the thiols (–SH) functionalized mAb (**Scheme 27**, Strategy 1).

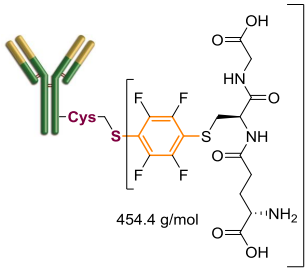
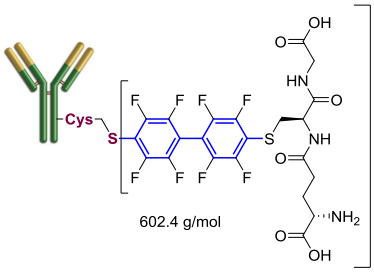
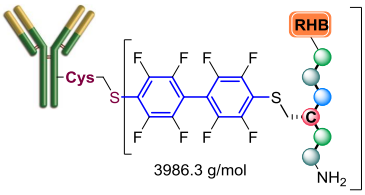


Scheme 27. Conjugation strategies.

The other way consisted of disulfide bonds reduction from the antibody to generate the sulfhydryl groups available for bioconjugation (**Scheme 27**, Strategy 2). The bioconjugation was carried out using the modified mAb´s obtaining in both strategies and the perfluorated compounds previously synthesized (**Ia**, **IIa** and **IIb**) to furnish the corresponding conjugates. After separating any unconjugated linker-cargo (See experimental section), the purified ADCs (**Table 16**) were analyzed by reducing SDS-PAGE (**Figure 33**) and mass spectrometry (**Figure 34**).

Table 16. Synthetized conjugates (1-6).

Strategy 1	
Conj	**Anti-CD4 Cysteamine-modified conjugates**
1	
2	
3	

Strategy 2	
Conj	**Reduced Anti-CD4 conjugates**
4	
5	
6	

Contrary to the conjugation via Lys, the analysis by reducing SDS-PAGE of conjugates (**Figure 33**) shown that the conjugation strategies used with the antibody took place homogeneously and moreover the light and heavy chains integrity was maintained after the bioconjugation. The low molecular weight of the conjugated molecules hampers qualitative mass analysis by electrophoresis due to the small difference between the naked and conjugated antibody.

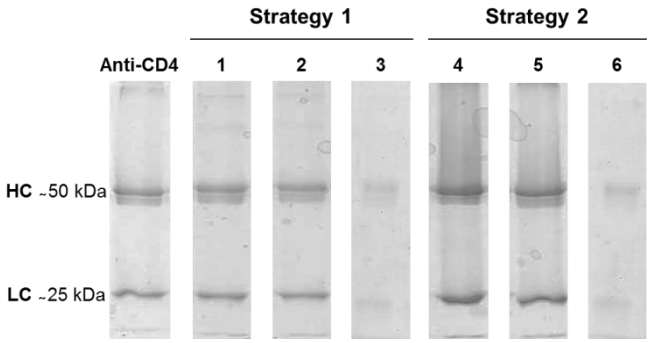


Figure 33. Reducing SDS-PAGE of conjugates (Conj 1-6, 10% Acrylamide). HC and LC correspond to Heavy and Light chain, respectively.

The integrity of the conjugate was corroborated by the intact conjugate mass analysis (**Figure 34**, conjugates **1** (B), **2** (C) and **4** (D); and experimental section). In spite of the detection of a broad isotopic envelop which hindering the accurate analysis of the bioconjugates, the major mass increment was observed with conjugates 1, 2 and 4 where all the peaks suffer an increment which is possible to attribute to the cysteamine or to the perfluorated linker incorporation. In this sense, comparing with peak c from the naked antibody in the analysis for the mentioned conjugates, a mass increment of 798, 717 and 595 Da for conjugates 1, 2 and 4 respectively, have shown. This peak increase could correspond to the attachment of one molecule of the compounds **Ia** or **IIa**. Is important to consider, that the strategy 1 (conjugates 1-3) conjugation introduce into the antibody, one cystamine or in other words, two cysteamine residues (154 Da) once the cystamine is reduced. Furthermore, a higher difference with peak d from **Figure 34**A is assessed in **Figure 34**C (1349 Da) and D (1263), indicating the possibility that at least two molecules were attached to the antibody. On contrary, the incorporation of the 29-amino acid residues peptide (**IIb**) is not detected by mass spectrometry for both tested strategies (see mass spectra in the experimental section).

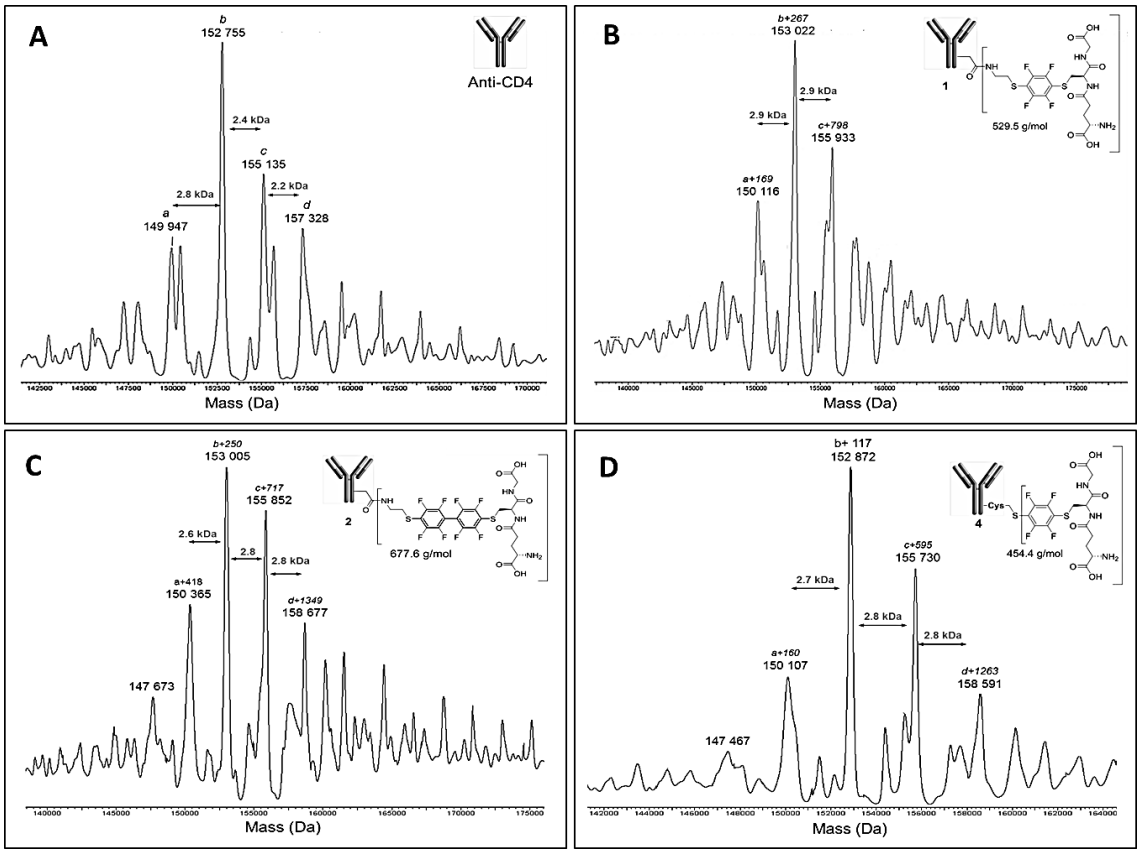


Figure 34. A,B,C,D correspond to Anti-CD4, Conjugates 1, 2 and 4 mass analysis (TOF ES^+) respectively.

Antibody-Conjugate cell binding analysis.

To determine whether the conjugation of the linkers to the anti-CD4 HP2/6 mAb has any effect on the binding to its CD4 epitope, we performed an antibody binding analysis on Jurkat T cells that naturally express CD4 on the cell surface. We used different concentrations (0.1-10 µg/ml) of the naked anti-CD4 HP2/6 mAb and of the conjugates 1-6. As shown in **Figure 35**, the naked anti-CD4 HP2/8 mAb shows the higher mean fluorescence intensity and the higher binding affinity (AC_{50} = 0.95 µg/ml, indicated as the antibody concentration (AC) needed to achieve 50% of the higher MFI value for each conjugate).

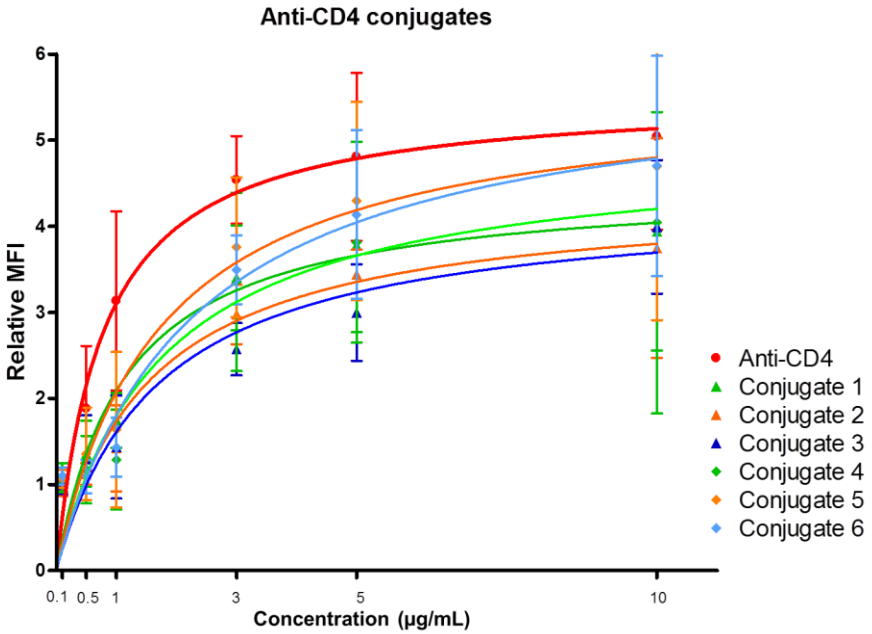


Figure 35. Analysis of the binding of anti-CD4 HP2/6 mAbs and the different conjugates to Jurkat T cells by flow cytometry. Jurkat T cells were incubated with the indicated concentrations of anti-CD4 mAb and the conjugates 1-6 followed by incubation with FITC-labelled goat anti-mouse IgG antibodies as described in Materials and Methods. Cytometry analysis was performed and the mean fluorescence intensity (MFI) ratio was calculated using as reference the MFI value or an irrelevant mAb (basal MFI value).

Conjugates 5 and 6 have reduced binding affinity compared to the anti-CD4 mAb (AC_{50} 2 µg/ml and 2.37 µg/ml, respectively) although they reach a similar maximum mean fluorescence intensity value (91 % in both cases) than that of the anti-CD4 mAb (**Table 17**). This result suggests that conjugates 5 and 6 would coat all available CD4 molecules on the cell surface. Conjugates 1, 2, 3 and 4 had AC_{50} of 1.65, 2.2, 2.65, and 2.55 µg/ml respectively, but only returned 62-75% of the maximum MIF obtained using the naked anti-CD4 mAb. However, this reduction in the maximum MFI obtained with these conjugates might simply reflect a diminished binding of the secondary FITC antibody as a result of epitope masking caused by the linkers.

Table 17. Conjugate affinity, indicated as the concentration of the conjugate required to obtain half of the maximum mean fluorescence intensity (AC_{50}) and the percentage of binding relative to that of the natural anti-CD4 mAb.

	AC_{50} (µg/ml)	**max MFI %**
Anti-CD4 mAb	0.95	100
Conjugate 1	1.65	73
Conjugate 2	2.20	62
Conjugate 3	2.65	74
Conjugate 4	2.55	75
Conjugate 5	2.02	91
Conjugate 6	2.37	91

In summary, the synthetized conjugates maintain their ability of binding to CD4 as the naked antibody showed. This fact indicates that both conjugation strategies tested did not disrupt the antibody activity, being perfluoroarylated compounds suitable linkers for ADCs.

2.4. Conclusions

To sum up, we studied the conjugation via thiol group of antibodies (one of the most complex systems in nature) using hexafluorobenzene and decafluorobiphenyl as non-cleavable linkers, and the unique regioselectivity 1,4 of the S_NAr of these perfluoroaromatic rings. Decafluorobiphenyl was found to be more effective than hexafluorobenzene for monoalkylation of decorated thiol molecules. Two strategies of thiol assisted conjugation were tackled being both effective but however, the strategy that involves partial disulfide bond reduction of the antibody (Strategy 2) allowed better site-specific conjugation. Moreover, we demonstrated that this bioconjugation procedure preserved the binding activity to CD4 of these conjugates. Even though a reduction in the binding affinity of the conjugates to the antigen was observed, compared to the naked antibody, these conjugates maintain a high binding activity to the antigen, thus highlighting the suitability of the perfluoroarylation-based bioconjugation methods for the modification of bioactive antibodies. Bearing in mind the progression of perfluoroarylated compounds in the peptide field we successful expand its synthetic methodologies spectrum as non-cleavable linker for the emerging field of antibodies bioconjugation.

2.5. References

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2.6. Experimental section

Linker-Cargo preparation

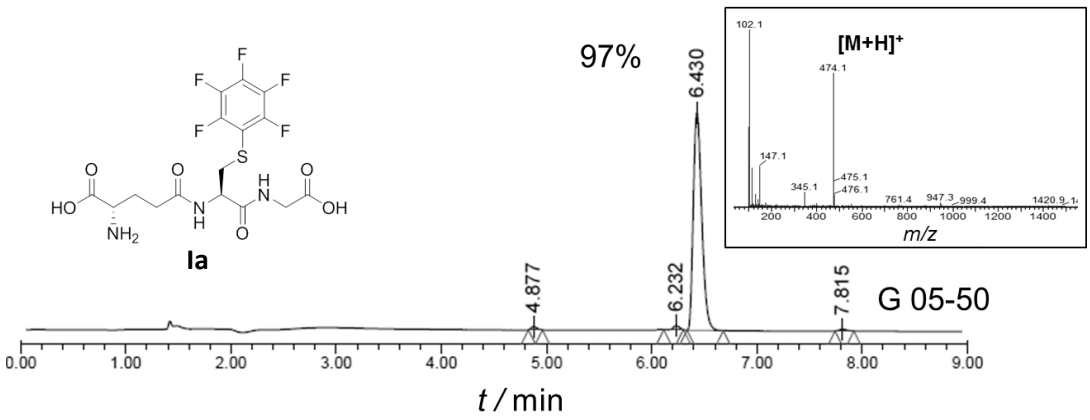
Synthesis of C_6F_5-S-Glutathione (Ia)

Test 1) To a solution of reduced gluthatione (133 mg, 0.432 mmol) in 50 mL of dry MeCN, hexafluorobenzene (1 mL, 8.64 mmol), and triethyl amine (TEA) (62 µL, 0.864mmol) were added. Then reaction crude was analysed and at 24 h of reaction, it doesn't work. So and excess of TEA was added (100 µL, 1.39 mmol) to the mixture. Then the formation of the desired product was observed by HPLC and HPLC-MS. Due to the low conversion observed in the HPLC (0.19%), the reaction crude was no purified. Other reaction conditions need to be used.

Test 2) To a mixture of reduced gluthatione (66 mg, 0.22 mmol) in 50 mL of dry DMF, hexafluorobenzene (250 µL, 2.15 mmol) and TEA (30 µL, 0.43 mmol) were added, then the reaction was left to react over 7 h at room temperature under N_2 atmosphere. Then the solvent was removed under vacuum and the reaction crude was purified on pre-packed Redisep Rf Gold C18 43 g column by using H_2O/MeCN from 100:0 to 0:100 over 25 min. The collected fractions were lyophilized obtaining 3.9 mg (5%) and 2.0 mg (2%) of the dialkylated and monoalkylated (**Ia**) products respectively.

Test 3) To a mixture of reduced gluthatione (132 mg, 0.43 mmol) in 100 mL of dry DMF, hexafluorobenzene (500 µL, 4.32 mmol) and TEA (124 µL, 1.72 mmol) were added, then the reaction was left to react over 3.5 h at room temperature under N_2 atmosphere. Then the solvent was removed under vacuum and the reaction crude was purified on a pre-packed Redisep Rf Gold C18 13 g column by using H_2O/MeCN from 100:0 to 0:100 over 40 min. The collected fractions corresponding to the desired product were lyophilized obtaining 9.8 mg (5%) of the monoalkylated product (**Ia**).

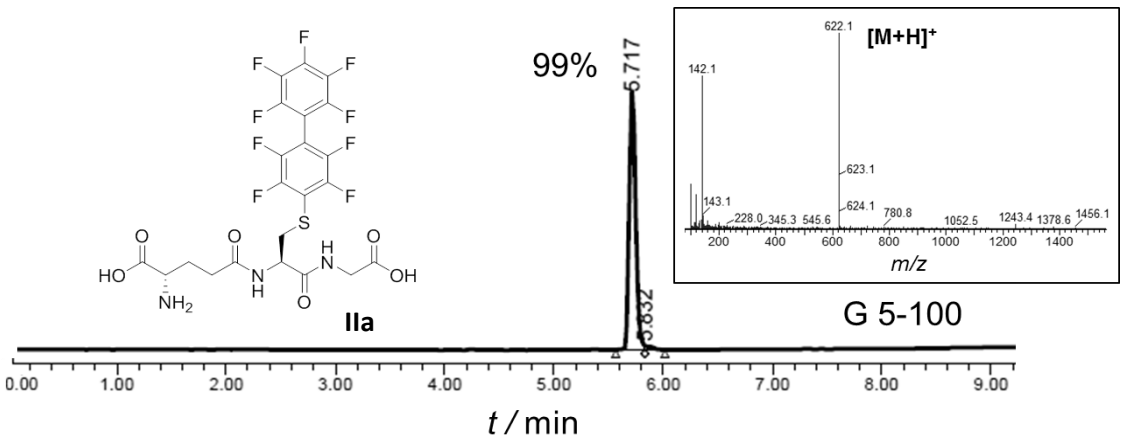
Pentafluoro-SGlutathione (Ia): HPLC (H_2O/MeCN from 95:5 to 50:50 over 8 min): t_R: 6.4 min. *m/z* calculated for $C_{16}H_{16}F_5N_3O_6S$ = 473.4; found $[M+H]^+$ = 474.1 ^{19}F NMR (376 MHz, $CDCl_3$) δ -161.75 – -162.03 (5F).



Synthesis of $C_{12}F_9$-S-Glutathione (IIa)

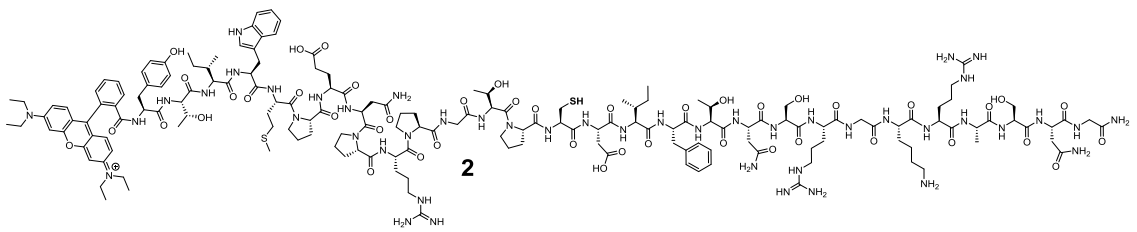
To a mixture of reduced gluthatione (23 mg, 0.075 mmol) in 50 mL of dry DMF, decafluorobiphenyl (250.5 mg, 0.75 mmol) and TEA (36 µL, 0.50 mmol) were added, then the reaction was stirred at room temperature under N_2 and it was followed by HPLC until consumption of the reduced glutathione (24h). Then the solvent was removed under vacuum and the reaction crude was purified on a pre-packed Redisep Rf Gold C18 13 g column by using H_2O/MeCN from 90:10 to 0:100 over 45 min. The collected fractions were lyophilized obtaining 20.0 mg (43%) of the monoalkylated product as a white powder.

Nonafluorobiphenyl-SGlutathione (IIa): HPLC (H_2O/MeCN from 95:5 to 0:100 over 8 min): t_R: 5.7 min. *m/z* calculated for $C_{22}H_{16}F_9N_3O_6S$ = 621.43; found $[M+H]^+$ = 622.1.



Synthesis of $C_{12}F_9$-S-[RHB-peptide] (IIb)

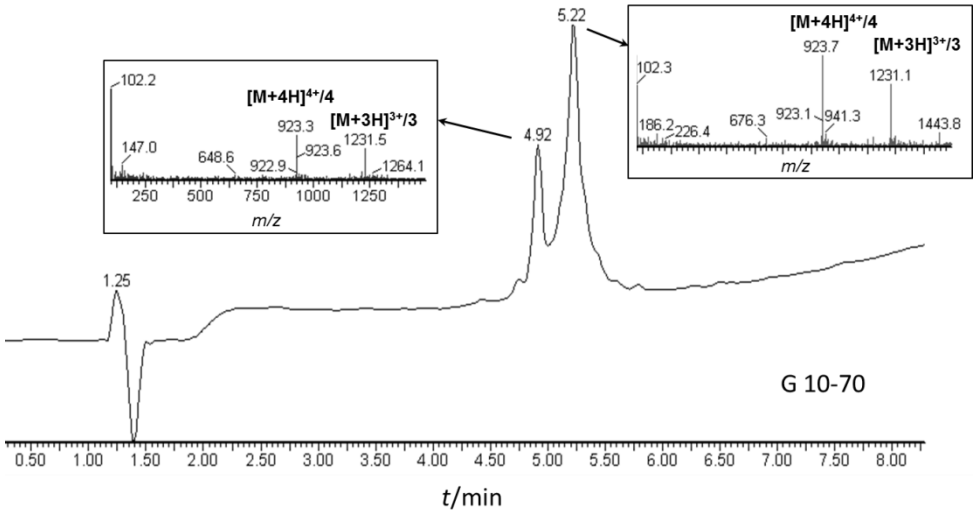
A) Synthesis of RHB-Tyr-Thr-Ile-Trp-Met-Pro-Glu-Asn-Pro-Arg-Pro-Gly-Thr-Pro-Cys-Asp-Ile-Phe-Thr-Asn-Ser-Arg-Gly-Lys-Arg-Ala-Ser-Asn-Gly-NH_2 (**2**)



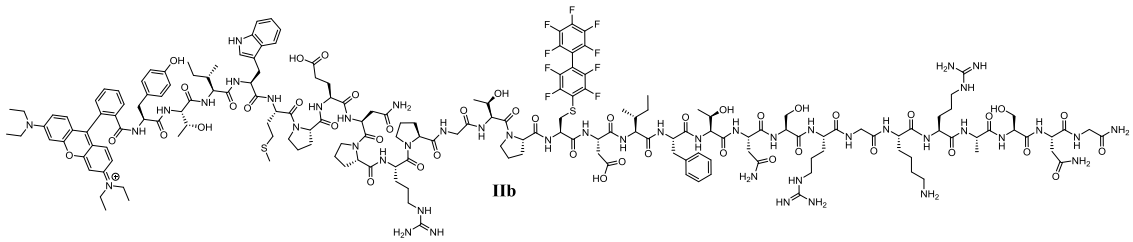
The automatically SPPS of YTIWMPENPRPGTP**C**DIFTNSRGKRASNG-NH_2 (RGV peptide) was carried out in CEM Discover Microwave Peptide Synthesizer following the Fmoc/tBu strategy and using Fmoc-Rink amide MBHA (200 mg, 0.73 mmol/g) as solid support. The peptide synthesis was monitored by RP-HPLC after the peptidyl resin cleavage of the intermediates (aliquot of 2 mg) using TFA/TIS/H_2O (95:2.5:2.5) during 1 h. When the peptide synthesis was achieved, rhodamine B (RHB) was manually conjugated to the RGV peptide. This reaction was carried out with rhodamine B (210 mg, 3 equiv), HBTU (166 mg, 3 equiv) and DIEA (152 µL, 6 equiv) in DMF for 45 min at room temperature. After completion of the synthesis, the resin was washed with dichloromethane (DCM) and the

peptide was cleaved from the solid support with the concomitant amino acid side chains protecting groups elimination using the following acidolytic mixture: TFA/TIS/H_2O (95:2.5:2.5) for 1.5 h. Then the peptide crude was purified by semi-preparative HPLC-MS using linear gradient from 90:10 to 30:70 of H_2O/MeCN from over 15 min.

The purified peptide RHB-YTIWMPENPRPGTP**C**DIFTNSRGKRASNG-NH_2 was characterized by HPLC-MS: (H_2O/MeCN from 90:10 to 30:70 over 8 min): t_R: 4.9 and 5.2 min. *m/z* calculated for $C_{169}H_{247}N_{46}O_{44}S_2^+$ = 3691.18 Da, found: $[M+3H]^{3+}/3$ = 1231.5 and $[M+4H]^{4+}/4$ = 923.3. MALDI-TOF: *m/z* calculated for $C_{169}H_{247}N_{46}O_{44}S_2^+$ = 3691.18 Da, found $[M+H]^+$ = 3693.59 Da.

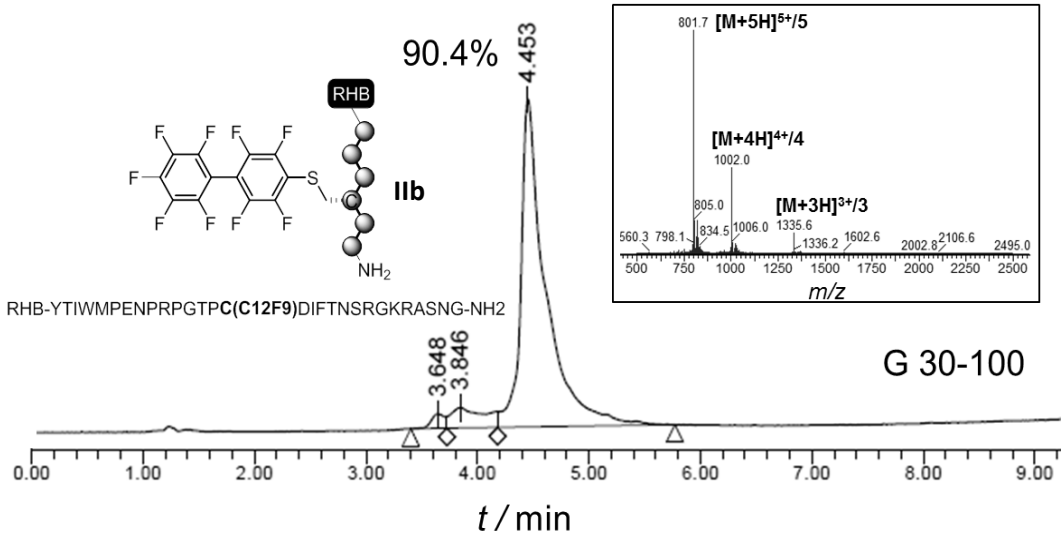


B) Synthesis of RHB-Tyr-Thr-Ile-Trp-Met-Pro-Glu-Asn-Pro-Arg-Pro-Gly-Thr-Pro-Cys(**$C_{12}F_9$**)-Asp-Ile-Phe-Thr-Asn-Ser-Arg-Gly-Lys-Arg-Ala-Ser-Asn-Gly-NH_2 (**IIb**)



To a mixture of a thiol-free 29 amino acids peptide (RHB-YTIWMPENPRPG-TPCDIFTNSRGKRASNG-NH_2) (10 mg, 2.7 µmol) in 10 mL of dry DMF, decafluorobiphenyl (9 mg, 27 µmol) and TEA (0.4 µL, 5.5 µmol) were added, and then the reaction was stirred at room temperature under N_2 during 17 h. Afterwards, the solvent was removed under vacuum and the reaction crude was purified on a pre-packed Redisep Rf Gold C18 4.3 g column by using H_2O/MeCN from 100:0 to 0:100 over 15 min. The collected fractions were lyophilized obtaining 3.9 mg (36%) of the monoalkylated product as a pale pink powder.

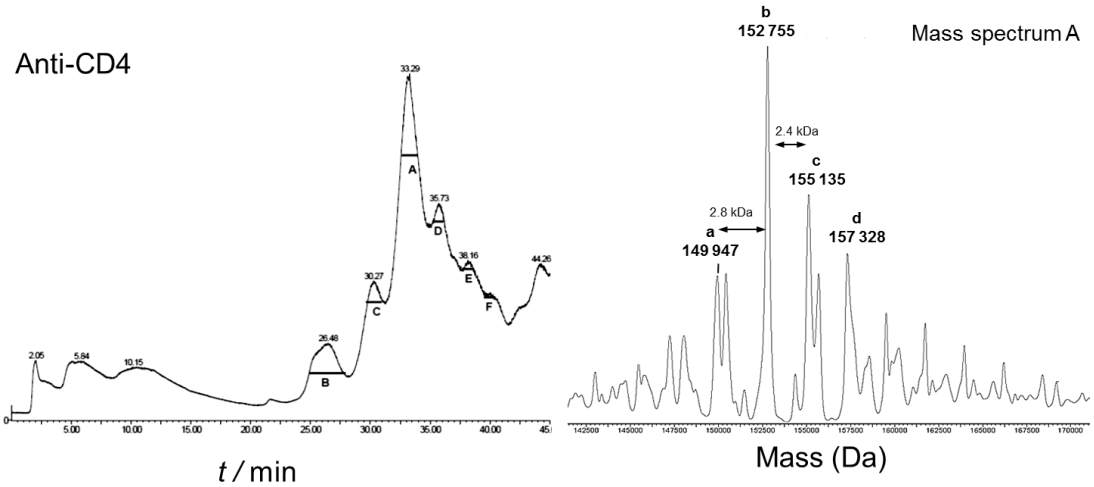
$C_{12}F_9$-S-RHB-Peptide (IIb): HPLC (H_2O/MeCN from 70:30 to 0:100 over 8 min): t_R: 4.5 min. m/z calculated for $C_{181}H_{246}F_9N_{46}O_{44}S_2$+ = 4005.3 Da; found $[M+5H]^{5+}/5$ = 801.7, $[M+4H]^{4+}/4$ = 1002.0 and $[M+3H]^{3+}/3$ = 1335.6.



Bioconjugation

Anti-CD4 characterization.

5 mL of Anti-CD4 (2 mg/mL) in saline solution antibody was dialyzed (MWCO: 12-14,000) over water and 5 mL of antibody over phosphate saline buffer (PBS). A sample of 1.20 mg/mL in water was diluted ¼ with 1% formic acid aqueous solution to 2.0 µM. Then 40 µL was injected for MS analysis.



Anti-CD4	Mass (Da)
a	149947
b	152755
c	155135
d	157328

General procedure

Strategy 1 (Conjugates 1-3)

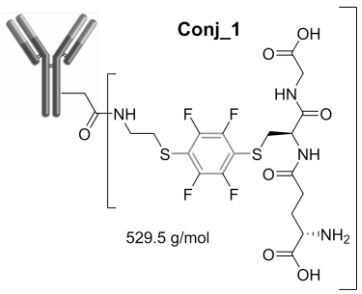
A solution of Anti-CD4 in 0.1 M Na_3PO_4, 0.15 M NaCl at pH = 7.4 was treated with cysteamine dihydrochloride (20 eq) and EDC·HCl (>100 eq) during 2 h at room temperature. Afterwards, the mixture was purified through PD-10 column and the antibody-containing fractions were combined. Then the modified antibody was treated with DTT (>100 eq) during 30 min at room temperature. Then the solvent mixture was exchanged using a PD-10 column to a solution of 50 mM of Na_2HPO_4, 0.15 M NaCl and 10 mM of EDTA at pH = 7.2.

Then the cysteamine-modified antibody was left to react with the corresponding modified perfluoryl compound (**Ia**, **IIa** and **IIb**) which was prepared following the above described procedure. After that, the mixture was purified through PD-10 column or dyalysis. Then the conjugates were analyzed by gel electrophoresis using 10% of acrylamide under reducing conditions and by mass spectrometry.

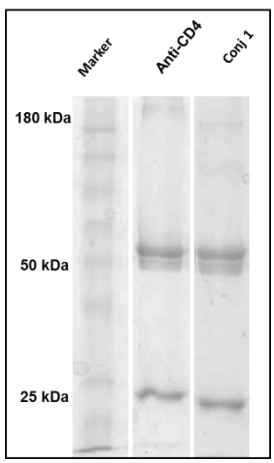
Strategy 2 (Conjugates 4-6)

A solution of Anti-CD4 in 0.1 M Na_3PO_4, 0.15 M NaCl and 10 mM EDTA at pH = 7.2 was treated with DTT (3 eq) during 90 min at 37 ºC. Then the reduced antibody was purified using a PD-10 column, eluting with the same buffer solution. Then the reduced antibody was treated with the corresponding modified perfluoryl compound (**Ia**, **IIa** and **IIb**) which was prepared following the above described procedure. After that, the mixture was purified through PD-10 column or dialysis. Then the conjugates were analyzed by gel electrophoresis using 10% of acrylamide under reducing conditions and by mass spectrometry.

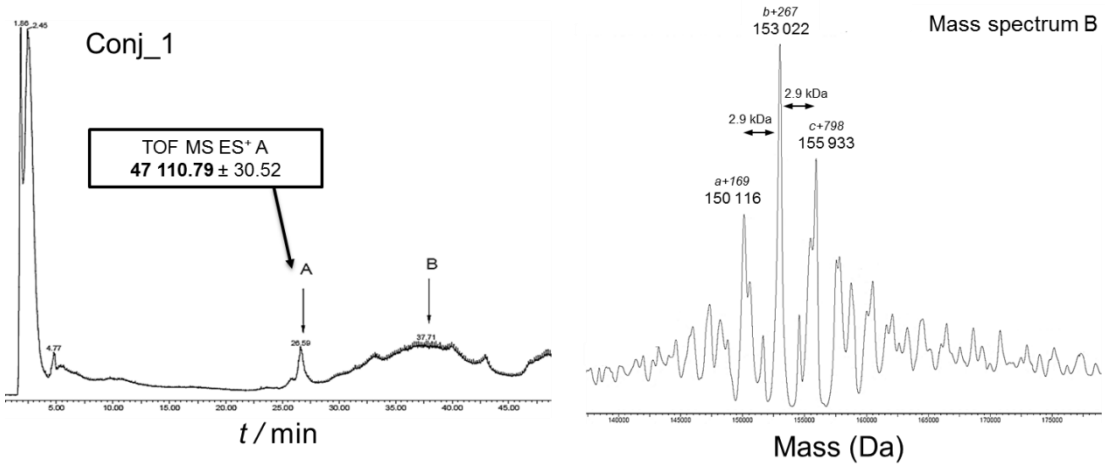
Conjugate 1. Anti-CD4-Cysteamine-C_6F_4-S-glutathione**.**



1 mL of Anti-CD4 solution (0.90 mg/mL, 6.0 µM), cystamine dihydrochloride (30 µg, 0.13 mM) and EDC·HCl (127 µg, 0.66 µmol, 0.66 mM). After PD-10 purification, 1 mL (0.85 mg/mL) of modified antibody was obtained. And then, it was treated with DTT (1 mg, 6.5 µmol). The solvent mixture was exchanged using a PD-10 column and approximately 1 mL (0.70 mg/mL) was obtained.

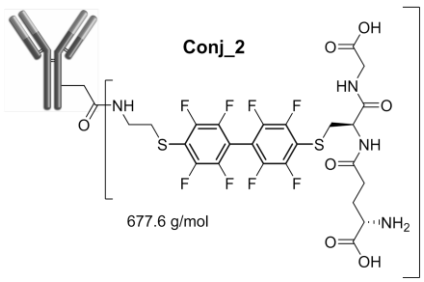


The previously synthetized C_6F_5-S-Glutathione (**Ia**) (2.0 mg, 4.2 µmol) was dissolved into DMF (50 µL) and it was added to the previously modified antibody solution (0.70 mg/mL). The reaction was left to react during 3 h at room temperature. After that, the crude was dialyzed (MWCO: 12-14,000) over PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, pH = 7.4) obtaining 1 mL of the conjugate (0.65 mg/mL). A sample of 0.64 mg/mL in water was diluted ½ with 1% formic acid aqueous solution to 2.1 µM. Then 12 µL was injected for MS analysis.

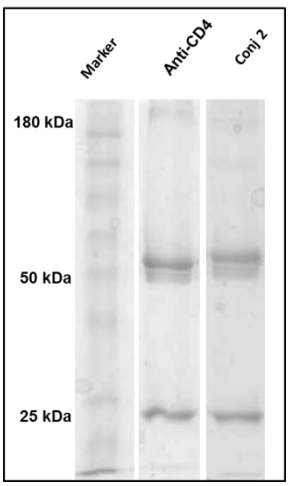


Conj_1	Mass detected (Da)	Anti-CD4 Mass (Da)	Δ_{mass} (Da)	MW	DAR
1	150116	149947	169	530	0,3
2	153022	152755	267		0,5
3	155933	155135	798		1,5

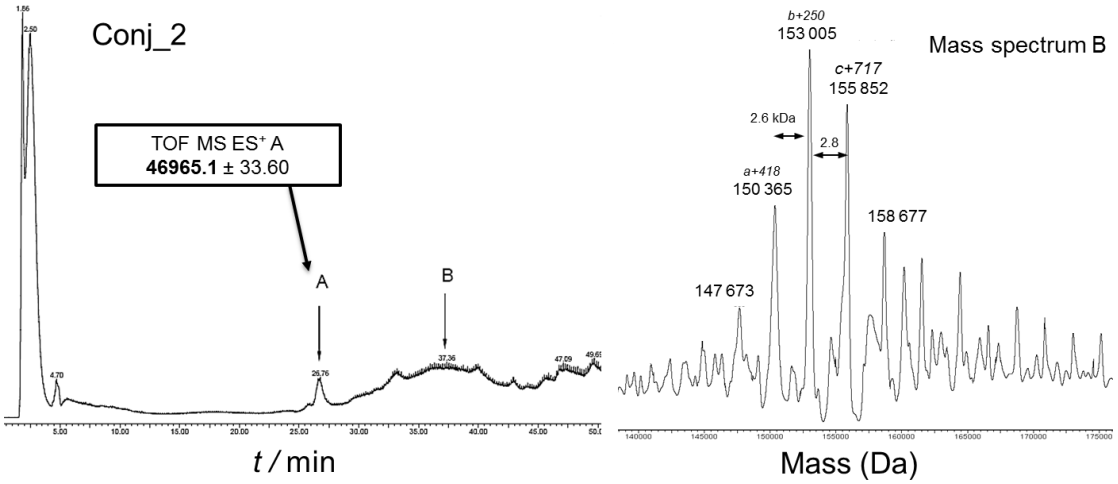
Conjugate 2. Anti-CD4-Cysteamine-$C_{12}F_8$-S-glutathione.



1 mL of Anti-CD4 solution (0.91 mg/mL, 6.0 µM), cystamine dihydrochloride (30 µg, 0.13 mM) and EDC·HCl (127 µg, 0.66 µmol, 0.66 mM). After PD-10 purification, approximately 1 mL (0.90 mg/mL) of modified antibody was obtained. And then, it was treated with DTT (1 mg, 6.5 µmol). The solvent mixture was exchanged using a PD-10 column and approximately 1 mL (0.85 mg/mL) was obtained.

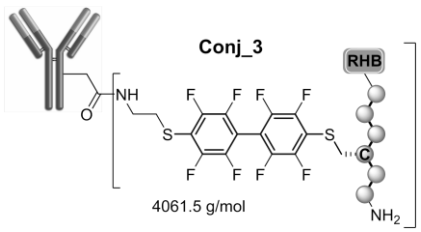


The previously synthetized $C_{12}F_9$-S-Glutathione (**IIa**) (2.0 mg, 3.2 µmol) was dissolved into DMF (50 µL) and it was added to the previously modified antibody solution (0.85 mg/mL). The reaction was left to react during 3 h at room temperature. After that, the crude was dialyzed (MWCO: 12-14,000) over PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, pH = 7.4) and over water obtaining 0.5 mL (0.64 mg/mL) and 0.5 mL (0.65 mg/mL) respectively. A sample of 0.64 mg/mL in water was diluted ½ with 1% formic acid aqueous solution to 2.1 µM. Then 12 µL was injected for MS analysis.

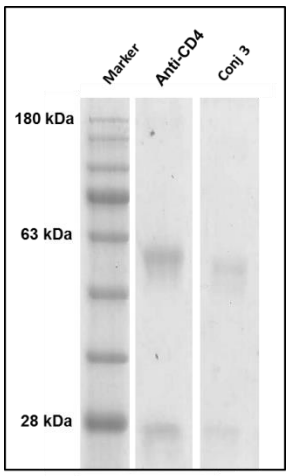


	Conj_2 Mass detected (Da)	Anti-CD4 Mass (Da)	Δ_{mass} (Da)	MW	DAR
1	150365	149947	418	677,6	0,6
2	153005	152755	250		0,4
3	155852	155135	717		1,1

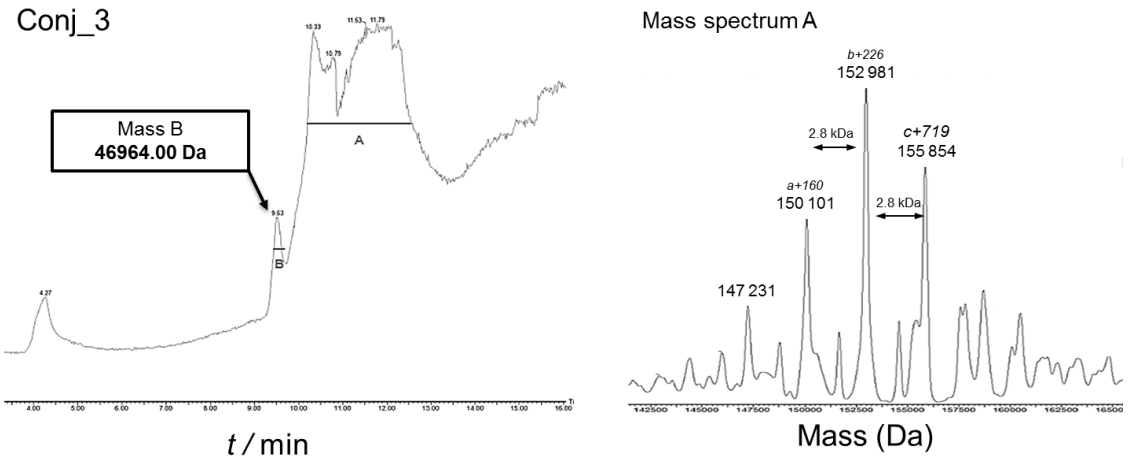
Conjugate 3. Anti-CD4-Cysteamine-$C_{12}F_8$-S-RHB-Peptide.



1 mL of the non-reduced cystamine modified antibody (0.60 mg/mL, 4.0 µM) was treated with DTT (1 mg, 6.5 µmol). The solvent mixture was exchanged using a PD-10 column and approximately 1.5 mL (0.44 mg/mL) was obtained.

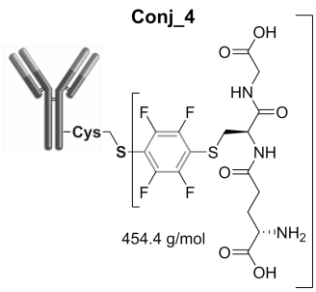


The previously synthetized $C_{12}F_9$-S-RHB-peptide (**IIb**) (1.0 mg, 0.25 µmol) was dissolved DMF (80 µL) and TEA (2 µL, 0.02 µmol) and it was added to 1 mL of the previously modified antibody solution (0.44 mg/mL). The reaction was left to react during 3 h at room temperature. After that, the crude was dialyzed (MWCO: 12-14,000) over PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, pH = 7.4) obtaining approximately 1.5 mL of the conjugate (0.49 mg/mL). Moreover, 0.5 mL of sample was dialyzed (MWCO: 12-14,000) in front of water in order to perform their mass analysis (0.33 mg/mL). A sample of 0.33 mg/mL in water was diluted ½ with 1% formic acid aqueous solution to 1.1 µM. Then 12 µL was injected for MS analysis.

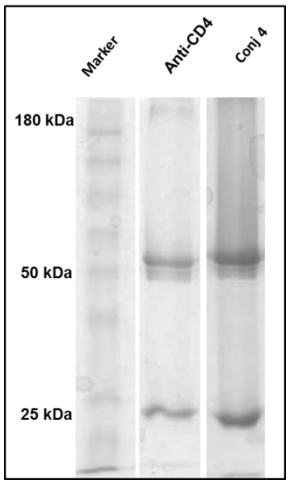


Conj_3	Mass detected (Da)	Anti-CD4 Mass (Da)	Δ_{mass} (Da)	MW	DAR
1	150101	149947	154	4081,5	0,0
2	152981	152755	226		0,1
3	155854	155135	719		0,2

Conjugate 4. Anti-CD4-S-C_6F_4-S-Glutathione.

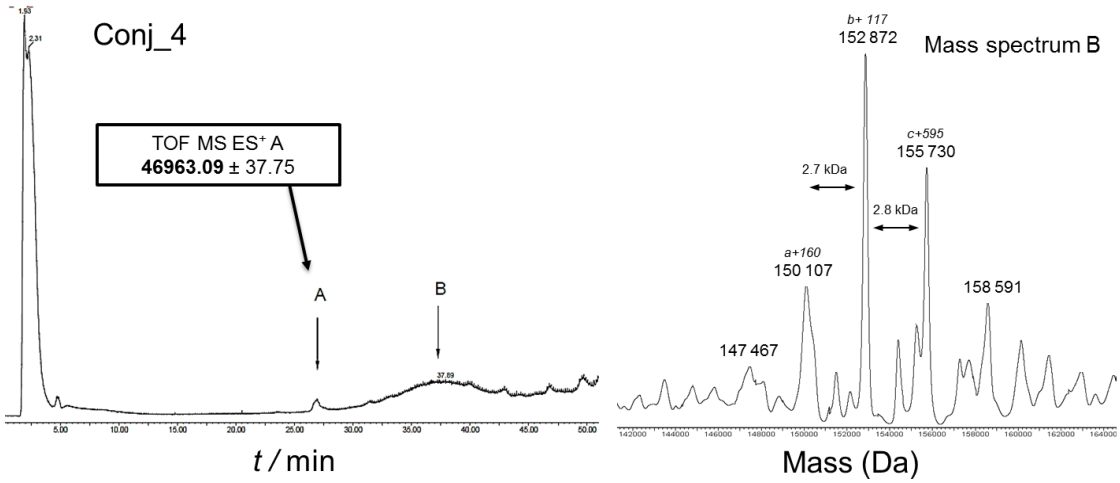


1 mL of Anti-CD4 solution (1.21 mg/mL, 8.1 µM) was treated with DTT (4 µg, 0.026 µmol). After PD-10 purification, 1 mL of the reduced antibody (1.08 mg/mL) was treated with a solution of C_6F_5-S-Glutathione (**3**) (2.0 mg, 4.2 µmol) in DMF (50 µL). The reaction was left to react during 3 h at room temperature. After PD-10 purification, approximately 1 mL of the modified antibody was obtained (0.91 mg/mL).

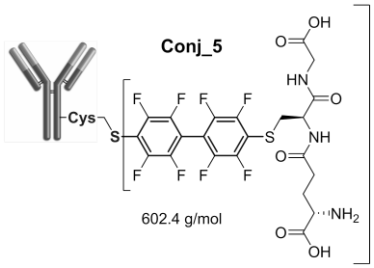


A sample of 0.78 mg/mL in water was diluted 1/5 with 1% formic acid aqueous solution to 1.0 µM. Then 12 µL was injected for MS analysis.

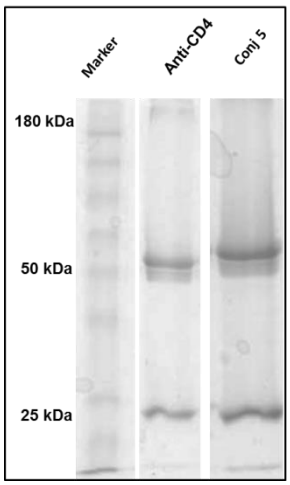
Conj_4	Mass detected (Da)	Anti-CD4 Mass (Da)	Δ_{mass} (Da)	MW	DAR
1	150107	149947	160	454,4	0,4
2	152872	152755	117		0,3
3	155730	155135	595		1,3



Conjugate 5. Anti-CD4-S-$C_{12}F_8$-S-Glutathione.

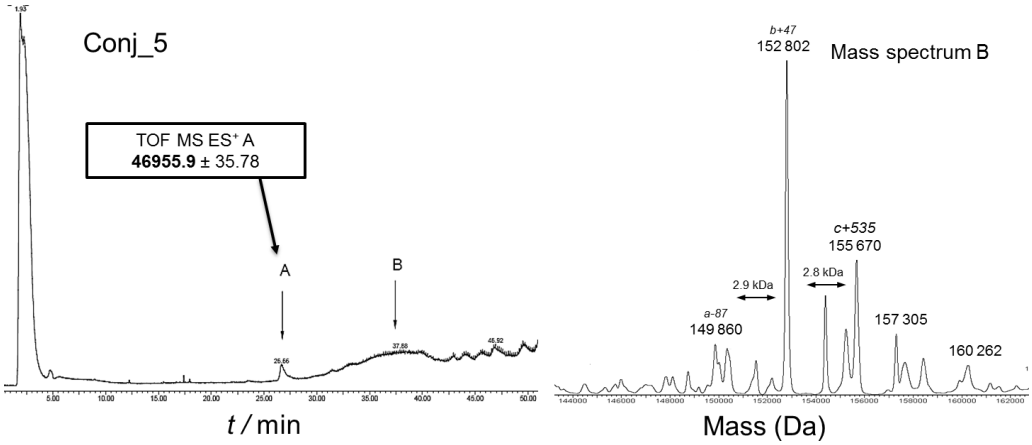


1 mL of Anti-CD4 solution (1.27 mg/mL, 8.5 µM) was treated with DTT (4 µg, 0.026 µmol). After PD-10 purification, 1 mL of the reduced antibody (0.95 mg/mL) was treated with a solution of $C_{12}F_9$-S-Glutathione (**IIa**) (2.0 mg, 3.2 µmol) in DMF (50 µL). The reaction was left to react during 3 h at room temperature. After PD-10 purification, approximately 1 mL of the modified antibody was obtained (0.80 mg/mL).

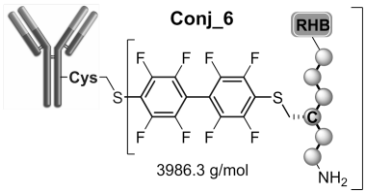


A sample of 0.80 mg/mL in water was diluted 1/5 with 1% formic acid aqueous solution to 1.1 µM. Then 12 µL was injected for MS analysis.

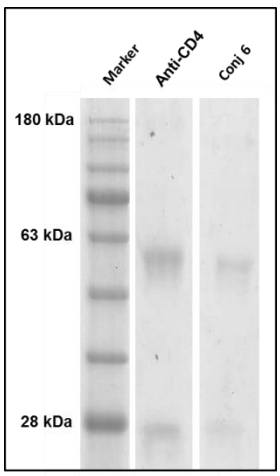
Conj_5	Mass detected (Da)	Anti-CD4 Mass (Da)	Δ_{mass} (Da)	MW	DAR
1	149860	149947	-87	602,4	-0,1
2	152802	152755	47		0,1
3	155670	155135	535		0,9

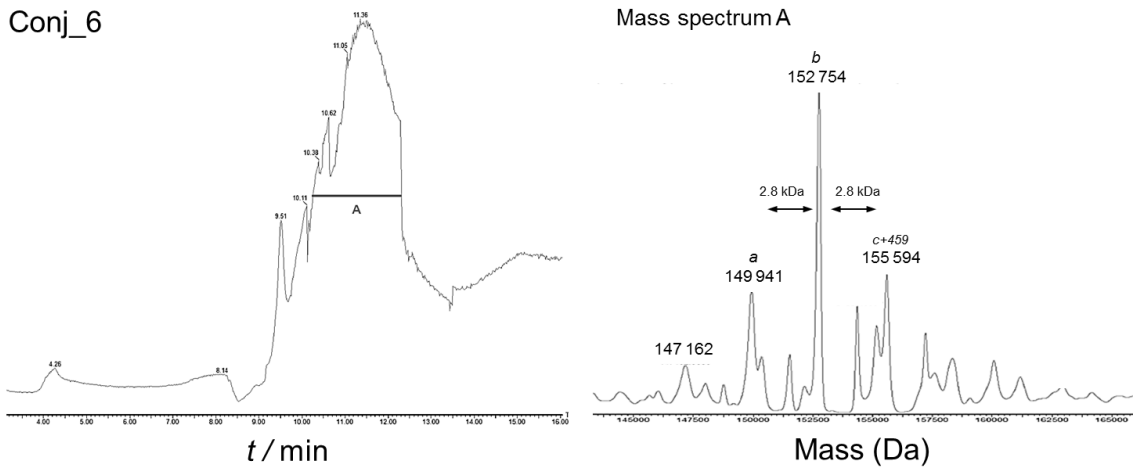


Conjugate 6. Anti-CD4-S-$C_{12}F_8$-S-RHB-Peptide.



1 mL of Anti-CD4 solution (2.0 mg/mL, 8.5 µM) was treated with DTT (4 µg, 0.026 µmol). After PD-10 purification, 1 mL of the reduced antibody (1.15 mg/mL) was treated with a solution of $C_{12}F_9$-S-RHB-peptide (**IIb**) (1.0 mg, 0.25 µmol) dissolved in DMF (80 µL) and TEA (2 µL, 0.02 µmol). The reaction was left to react during 1 h at 37 ºC. After that, the crude was dialyzed (MWCO: 12-14,000) over PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, pH = 7.4) obtaining two fractions of 0.5 mL of conjugate (0.88 and 0.33 mg/mL). Moreover, 0.5 mL of the crude was dialyzed (MWCO: 12-14,000) in front of water in order to perform their mass analysis (0.34 mg/mL). A sample of 0.33 mg/mL in water was diluted ½ with 1% formic acid aqueous solution to 1.1 µM. Then 12 µL was injected for MS analysis.





Conj_6	Mass detected (Da)	Anti-CD4 Mass (Da)	Δ_{mass} (Da)	MW	DAR
1	149941	149947	-6	3986,3	0,0
2	152754	152755	-1		0,0
3	155594	155135	459		0,1

Binding affinity experiments:

Jurkat T cells (5 x 10^5) were incubated with the anti-CD4 HP2/6 mAb and with the anti-CD4 HP2/6 conjugates under study (conjugates 1-6) at the indicated concentrations and incubated on ice for 1 h. Then cells were washed twice with PBS (137 nM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) and incubated with 10 µg/ml of FITC-labelled anti-mouse IgG antibodies (BD biosciences) for 30 min on ice, then the cells were washed twice in PBS and resuspended in PBS for their analysis by flow cytometry using a FACSCanto II (BD biosciences). Table shows the MFI ± SD for each conjugate and concentration tested. Results are expressed as the mean +/- SD of 3 independent experiments.

mAb (µg/ml)	Mean Fluorescence Intensity (MFI) ± SD						
	Anti-CD4 mAb	conj 1	conj 2	conj 3	conj 4	conj 5	conj 6
0,1	1.02 ± 0.15	1.04 ± 0.07	0.99 ± 0.11	1 ± 0.11	1.08 ± 0.16	1.06 ± 0.1	1.1 ± 0.1
0,5	1.88 ± 0.73	1.35 ± 0.38	1.12 ± 0.12	1.29 ± 0.51	1.17 ± 0.39	1.35 ± 0.54	1.09 ± 0.19
1	3.13 ± 1.04	1.74 ± 0.32	1.42 ± 0.5	1.43 ± 0.59	1.29 ± 0.57	1.64 ± 0.9	1.3 ± 0.34
3	4.54 ± 0.51	3.34 ± 0.61	2.97 ± 0.34	2.57 ± 0.3	3.35 ± 1.03	3.76 ± 0.81	3.49 ± 0.4
5	4.81 ± 0.97	3.78 ± 1.01	3.44 ± 0.28	3 ± 0.56	3.81 ± 1.16	4.3 ± 1.14	4.14 ± 0.98
10	5.04 ± 1.08	3.94 ± 1.38	3.75 ± 1.27	3.99 ± 0.77	4.04 ± 2.21	4.69 ± 1.78	4.70 ± 1.28

Resumen

Conectores para bioconjugación

i. Introducción

La bioconjugación se considerada la unión entre dos moléculas en la que al menos una, está considerada una biomolécula. Propiamente dicho, una biomolécula se considera aquella presente en diferentes organismos vivos y que está basada principalmente por átomos de carbono, hidrógeno, oxígeno y nitrógeno. Hasta la fecha, un gran número de biomoléculas han sido aisladas o sintetizadas exitosamente, siendo los amino ácidos, péptidos, hormonas, ácidos nucleicos y proteínas los ejemplos más destacados.[1] Con el fin de realizar la mencionada bioconjugación entre dos entidades moleculares, en la presente tesis doctoral se han estudiado diferentes métodos de conjugación a péptidos, proteínas y anticuerpos para contribuir en el campo de la bioconjugación.

El los últimos años, la bioconjugación ha tenido un papel muy importante en el descubrimiento de fármacos eficientes, selectivos, biocompatibles y sin efectos secundarios. Diversos aspectos tales como las propiedades físico-químicas, comportamiento y estabilidad biológicas tanto como inmunogenicidad se han de considerar para el desarrollo de este tipo de biomedicinas.[2]

Conectores para bioconjugación

Toda modificación química o conjugación a péptidos o proteínas tiene lugar mediante la reacción entre los grupos funcionales tanto de biomolécula como de la molécula a conjugar. Esta unión covalente entre dos entidades moleculares se realiza mediante un conector (del ingles *linker*) que ha de cumplir una serie de características para poder ser eficaz en la bioconjugación. En función a los grupos reactivos presentes en los conectores se conocen dos tipos. Por una lado los conectores homobifuncionales que presentan en su estructura dos grupos reactivos idénticos (por ejemplo dos grupos maleimido reactivos frente a tioles) y por otro lado los conectores heterobifuncionales, que a su vez disponen de dos grupos reactivos diferenciados (por ejemplo un grupo maleimido reactivo a tioles y un éster succínico, reactivo a aminas).

Propiedades de los conectores

Para un diseño eficiente de conectores para la bioconjugación, se ha de hacer especial hincapié en sus propiedades físico-químicas. En función a la estabilidad de los mencionados conectores, se distinguen dos grandes grupos en los cuales se centran los estudios de la presente tesis doctoral: conectores escindibles o hidrolizables (***cleavable linkers)*** y conectores no-escindibles (***non-cleavable linkers)***.

- Conectores hidrolizables:

Se engloban aquellos que contienen uno de los extremos sensibles a cambios fisiológicos tales como pH, cambio de concentración de glutatión o un incremento de enzimas hidrolíticas.

De esta forma, permite la liberación del compuesto incorporado a transportador biológico (péptidos, nanopartículas, proteínas, etc.). Los conectores hidrolizables han de ser estables durante la preparación y a nivel fisiológico, pero idealmente han de liberar el compuesto incorporado en la cercanía o el interior de las células o tejidos a tratar.[3] Además de ser hidrolizables en condiciones suaves, han de permitir obtener elevados rendimientos en la conjugación, así como reaccionar selectivamente (bioortogonalidad) y permitir la eliminación del material no reaccionado en la conjugación.[4] Hasta la fecha, un gran número de estos compuestos han sido desarrollados exitosamente y hoy día son comúnmente usados en bioconjugación **(Figura 1**). De las diversas vías que promueven la hidrólisis de los conectores escindibles, cabe destacar la hidrólisis selectiva por acción enzimática,[5–8] así como por un incremento del pH[9–18] en los alrededores de un tumor por la generación excesiva de ácido láctico derivado del metabolismo de glucosa en un crecimiento celular anómalo,[19–21] o incluso la hidrólisis que tiene lugar en los enlaces disulfuro[22–26] por mediación de elevada concentración de glutatión intracelular en celular tumorales.[27–29]

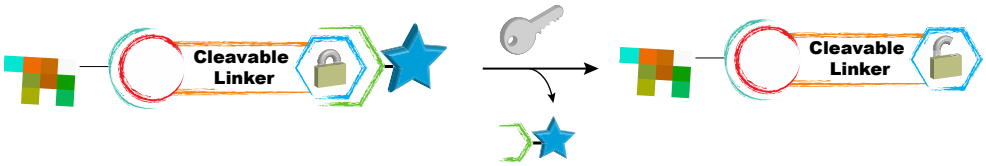
- Conectores no hidrolizables:

Por otro lado, los espaciadores no-escindibles han disponer de las previamente mencionadas características tales como ser estables durante la formación de la unión entre las entidades moleculares, han de ser selectivos y eficientes en el proceso de bioconjugación y han de presentar estabilidad en condiciones fisiológicas. Este tipo de conectores se caracterizan por tener una estabilidad adicional en relación a los conectores hidrolizables previamente mencionados. Los agentes citotóxicos utilizados con conectores no hidrolizables presentan actividad incluso estando unidos a los conectores. Su principal función es mantener unidas ambas entidades moleculares de interés, fármacos y transportadores. Pese a considerarse conectores no escindibles, los procesos metabólicos intracelulares acaban degradando el conjunto macromolecular a metabolitos sencillos para su excreción.[33]

Hasta la fecha se han desarrollado un gran número de conectores estables en condiciones fisiológicas utilizando para ello uniones tipo alquilo (C-C), éter (C-O), tioéter (C-S) o amino (C-N).[34,35]

Conjugación regio-selectiva

La conjugación regio-selectiva supone uno de los principales problemas con los que nos encontramos a la hora de enlazar conjuntos macromoleculares tales como proteínas debido a la diversidad de grupos funcionales presentes en las mismas.



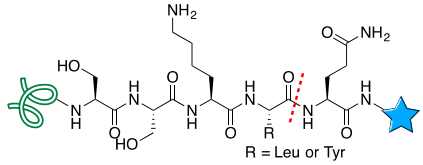
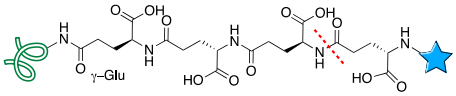
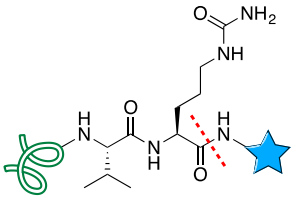
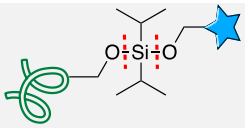
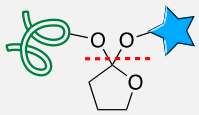
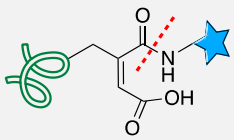
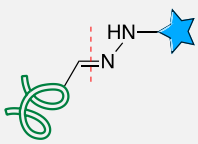
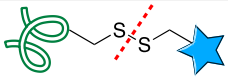
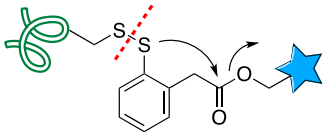
Estructura del conector	**Mediador (tipo)**	**Nombre del conector**[Reference]
	PSA (Enzimático)	SSKL/YQ[30–32]
	PSMA (Enzimático)	Poly-γ-Glutamated[5]
	Cathepsin B (Enzimático)	Val-Cit[6–8]
	H^+ (Acido)	silil eter[9,10]
		ortoester[11]
		cetal/Thp[12–14]
		cis-aconitilo[15]
		Hidrazona[16,17]
	Glutatión (Reducción de disulfuro)	Enlaces disulfuro[22–25]
		Disulfuro autodestruible[26]

Figura 1. Conectores hidrolizables para bioconjugación y sus mediadores de hidrolisis.

- Amino ácidos

La bioconjugación tiene lugar principalmente sobre las cadenas laterales reactivas de ciertos aminoácidos naturales y se pueden diferenciar en aminas, tioles, alcoholes, amidas y ácidos carboxílicos (**Figura 2**)

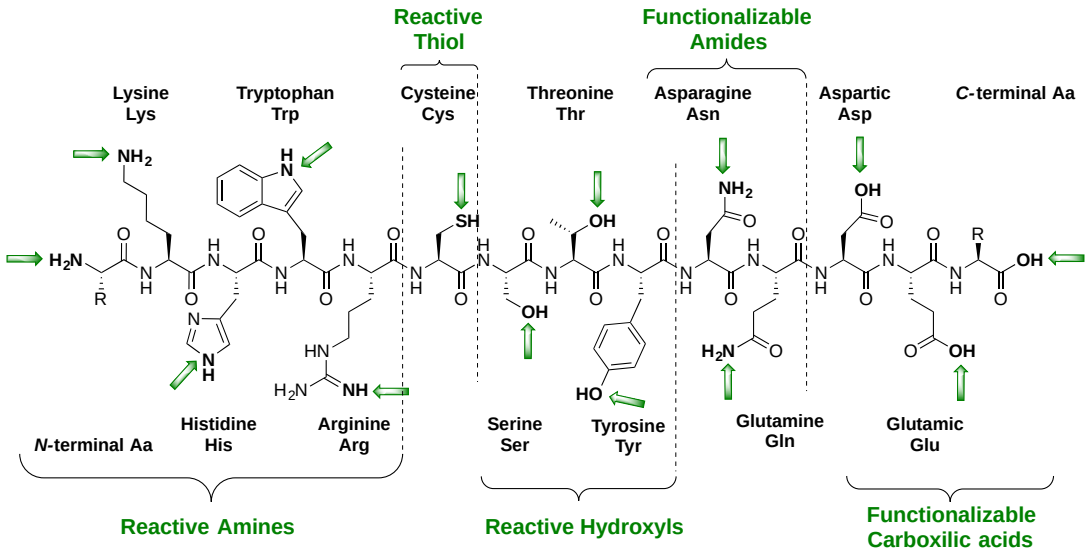


Figura 2. Regiones reactivas de los aminoácidos.

- Aminas reactivas

Están presentes en el extremo N-terminal de péptidos y proteínas y en las cadenas laterales de los aminoácidos lisina (Lys), histidina (His), triptófano (Trp) y arginina (Arg). En su mayoría son responsables de conferir carga positiva a las macromoléculas siendo responsable de la solubilidad de las mismas. Sobre estos aminoácidos la conjugación se lleva a cabo mediante la formación de un enlace alquílico o mediante acilación de las aminas. Por su nucleofília en el grupo ε-amino y debido a su abundancia natural, la Lys es el aminoácido más utilizado dentro de este grupo para la bioconjugación.[36]

- Tioles reactivos

El aminoácido cisteína (Cys) es la única fuente natural de sulfhidrílo (-SH) reactivo. En la naturaleza se encuentran como puentes disulfuro para estabilizar la estructura y conformación de péptidos y proteínas.[2,37–39] Para la liberación en forma de tiol reactivo es necesaria su previa reducción mediante glutatión reducido (forma natural) u otros agentes reductores sintéticos tales como TCEP o DTT. [40–44]

La perfecta combinación de escasa presencia en péptidos y proteínas (<2%), de su elevada nucleofília y de su selectividad en determinadas reacciones, hacen de la conjugación vía tiol de Cys la más eficiente y utilizada en el campo de la bioconjugación.[45–50] La alquilación de tioles con haluros de alquilo[35,51] o mediante adición de Michael sobre el anillo de

maleimida,[47,52–65] así como la formación de nuevos puentes disulfuro[25,66] son algunas de las metodologías comúnmente utilizadas para la conjugación vía tiol de la Cys **(Figura 3).**

- Hidroxilos reactivos

Generalmente, los grupos hidroxilos de serina (Ser), treonina (Thr) y tirosina (Tyr) en biomacromoléculas son los puntos de fosforilación o unión de carbohidratos.[67] La conjugación vía hidroxilo puede tener lugar mediante la formación de éteres o esters.[34] Sin embargo debido a la falta de regio selectividad la conjugación mediante las cadenas laterales de estos aminoácidos está en desuso. Diversas estrategias han sido desarrolladas para este propósito, siendo la conjugación sobre tirosina mediante substitución electrofilia del anillo aromático, la más exitosa y utilizada.[68–71]

- Amidas modificables

Se consideran amidas modificables aquellas que forman parte de la cadena lateral de la asparagina (Asn). Principalmente se encuentra en forma de *N*-glucosamidasamida[83] y su principal metodología de conjugación descrita se basa en la modificación del enlace amida de los *N*-glicósidos vía enzimática utilizando transglutaminasas.[84–89]

- Ácidos carboxílicos modificables

Los ácidos carboxílicos presentes en biomacromoléculas pueden ser modificados en condiciones suaves mediante la formación de un enlace covalente tipo amida. Un gran número de metodologías han sido descritas para la formación de enlaces amidas,[90] sin embargo, las más utilizadas involucran el uso de agentes de acoplamiento solubles en medios acuosos tales como la carbodiimida (EDC·HCl),[91,92] y la *N,N'*-carbonildiimidazole (CDI).[93]

- Introducción de nuevos grupos reactivos

En el caso de querer mejorar la regioselectividad en la bioconjugación, es posible introducir nuevo grupos reactivos en las macromoléculas con el fin de conjugar de forma selectiva un determinado compuesto. El 2-iminotiolano conocido como el reactivo de Traut's,[94,95] que permite la reacción con aminas liberando simultáneamente un grupo sulfhidrílo o la cysteamina[96] que en reaccionar con los ácidos carboxílicos mediante un enlace amida permite introducir un grupo tiol reactivo en la macromolécula son ejemplos muy utilizados en bioconjugación.

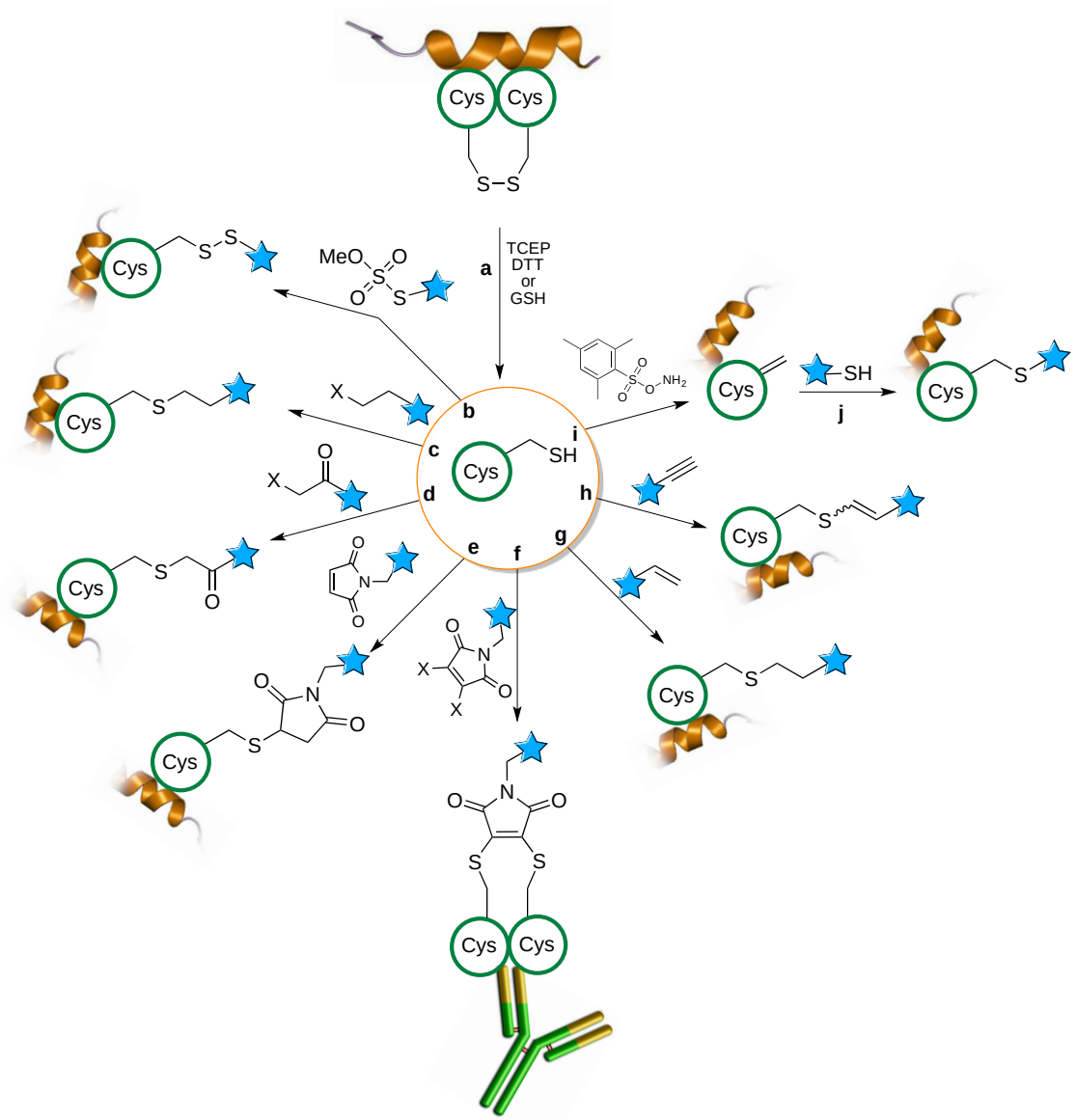


Figura 3. Conjugación a través de cisteína. a) reducción disulfuro, b) formación disulfuro[72] c) haloalquilación[73] d) alquilación α-haloacetil [74] e) alquilación maleimida[47,54–58] f) bisalquilación con bromomaleimida[47,75] g) Reacciones "Click" tiol-ene[76,77] y h) tiol-yne[78] i) formación dehidroalanina con *O*-mesitilenesulfonilo-hidroxilamina seguido de j) adición tio-Michael y aquilación.[79–82]

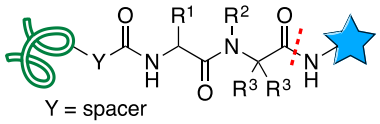
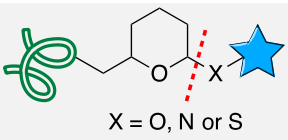
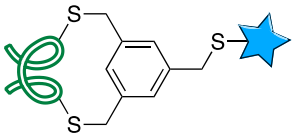
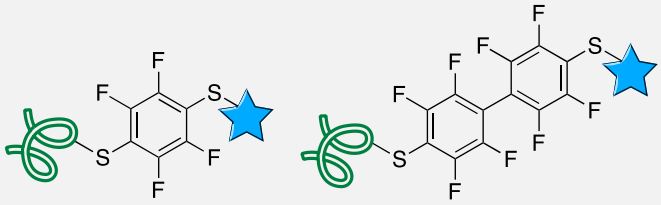
i. Contenido de la tesis

El cáncer está considerado como una de las principales causas de mortalidad a nivel mundial. Y ello conlleva elevados esfuerzos socio-económicos para tratar y curar esta enfermedad a pesar de disponer un amplio número de agentes anticancerígenos descritos, la obtención de nuevos agentes terapéuticos con mejor eficacia y menores efectos secundarios continua siendo a día de hoy, un reto científico a lograr.

Con el fin de mejorar estos tratamientos, se han desarrollados diversos sistemas de liberación controlada de fármacos. Un ejemplo interesante son, los sistemas conocidos como ADCs (*Antibody-Drug Conjugates*)[97–100] que consisten en un anticuerpo que permite direccionar el fármaco a célula diana. Dicha unión fármaco-anticuerpo se produce mediante un conector o *linker* que pueden ser no hidrolizables (*Non-cleavable linkers*) o hidrolizables (*Cleavable Linkers).*

La presente tesis centra su objeto de estudio en el diseño de este tipo de conectores (no hidrolizables y hidrolizables) de una forma más general para bioconjugación dado que para la utilización de fármacos citotóxicos se precisan condiciones de trabajo especiales de las que no se disponen en nuestros laboratorios. Se han estudiado diferentes tipos de conectores (**Tabla 1**): hidrolizables (capítulo 1) y no hidrolizables (capítulo 2), para ser utilizados en biomoléculas tales como péptidos, proteínas y anticuerpos.

Tabla 1. Conectores para bioconjugación estudiados en la presente tesis.

Conectores Hidrolizables (*Cleavable Linkers*)	
Aductos de Ugi Dialquilglicinas *N*-alquiladas (Sistemas 1,4-dicarbonilicos)	
Tetrahidropiranilo (Thp) (enlace acetal)	
Conectores no Hidrolizables (*Non-Cleavable Linkers*)	
Tris(metilbenceno) (Unión tipo tioeter bencílico)	
Compuestos perfluoroarilados (Unión tipo tioeter aromático)	

Proyecto colaborativo, MarinMab

Este proyecto nace precisamente a raíz de un proyecto de colaboración multidisciplinar con la empresa PharmaMar, la Universidad Autónoma de Madrid, el CSIC y el IRB/UB denominado MarinMab que tiene como fin el desarrollo de nuevas terapias basadas en conjugados anticuerpo-fármaco. El proyecto ha estado financiado por el Ministerio de Economía y competitividad (MINECO) y el Fondo Europeo de Desarrollo Regional (FEDER). Debido a un contrato de confidencialidad con la empresa biofarmacéutica PharmaMar, no es posible explicar en detalle todos los protocolos o estructuras químicas de la presente tesis.

Precedentes del grupo de investigación

El grupo de investigación liderado por el Dr. Fernando Albericio cuenta con una dilatada experiencia en el campo de la síntesis de péptidos y las metodologías utilizadas para este fin tales como agentes de acoplamiento, conectores y grupos protectores para la síntesis de péptidos en fase sólida. Con el presente proyecto de tesis el grupo de investigación pretende abordar un campo multidisciplinar con un enorme potencial como es el de la bioconjugación y de esta forma contribuir con los conocimientos adquiridos durante años en química la química orgánica y la química de péptidos para el desarrollo de agentes terapéuticos con un elevado interés farmacéutico para el tratamiento del cáncer.

ii. Objetivos

Capítulo 1. Conectores hidrolizables

II. Estudiar los aductos de Ugi para su utilización como conectores hidrolizables en bioconjugación.
   - Funcionalización
   - Estabilidad
   - Labilidad
   - Bioortogonalidad en la bioconjugación
   - Preparación y caracterización de los bioconjugados
   - Caracterización de los ADCs

II.1. Estudiar el tetrahidropirano (Thp) como conector hidrolizable para bioconjugación.
   - Funcionalización
   - Estabilidad
   - Labilidad

III.2. Estudiar el tetrahidropirano (Thp) como grupo protector para la cisteína en síntesis de péptidos en fase sólida.
   - Funcionalización de la Cys
   - Estabilidad
   - Labilidad
   - Síntesis y escisión de péptidos con el aminoácido Fmoc-Cys(Thp)

Capítulo 2. Conectores no hidrolizables

III. Estudiar la monotioalquilación del 1,3,5-tris(bromometil)benceno (TBMB) para su posterior uso en bioconjugación
   - Estabilidad
   - Bioortogonalidad en la bioconjugación
   - Preparación y caracterización de los bioconjugados
   - Caracterización de los ADCs

IV. Estudiar la monotioalquilación de decafluorobifenilo y el pentafluorobenceno para su uso en bioconjugación
   - Preparación de tioéteres monoalquilados
   - Estabilidad
   - Bioortogonalidad en la bioconjugación
   - Preparación y caracterización de los bioconjugados, ADCs

Capítulo I. Conectores hidrolizables

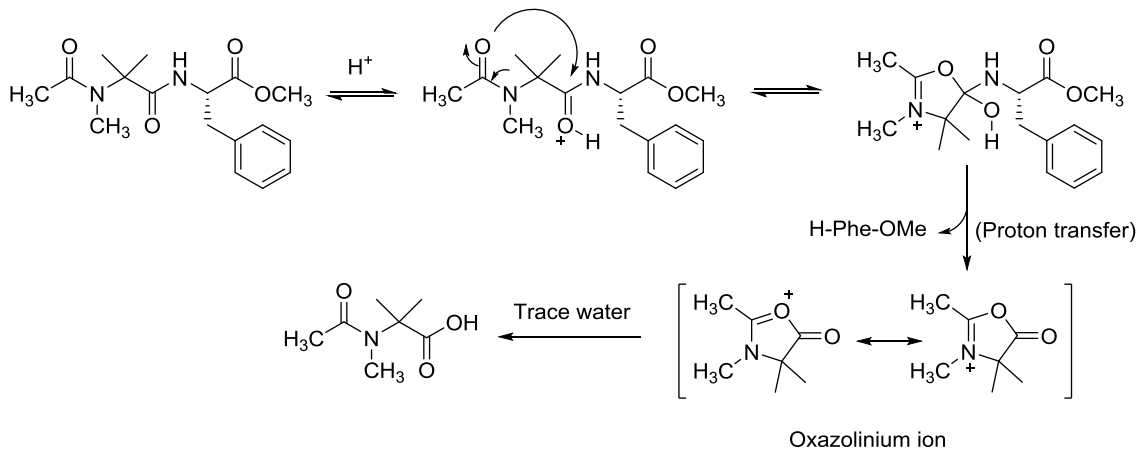
I.1. Tipo Ugi

Introducción

En el presente capítulo se estudian sistemas que pueden ser propensos a sufrir hidrolisis en el interior o las proximidades de células liberando así el compuesto conjugado. Se han estudiado dos tipos de conectores hidrolizables, los sistemas basados en compuestos 1,4-dicarbonilicos y los sistemas basados en el anillo de tetrahidropirano como se detalla a continuación.

En ciertas ocasiones, los químicos nos encontramos con reacciones secundarias indeseadas que reducen drásticamente los rendimientos de la reacción para la obtención de un producto deseado. Sin embargo, dichas reacciones han sido una fuente de inspiración para muchos científicos a la hora de desarrollar sus investigaciones.[101]

En química la hidrólisis de amidas de forma incontrolada ha supuesto un problema a la hora la obtención de un producto final. Sacando provecho de esto y con el fin de diseñar nuevos sistemas que permitan una hidrólisis de un determinado enlace para su uso como conector hidrolizable, se han estudiado los1,4-dicarbonílicos compuestos por dos amidas trialquiladas (*N*-trialquilglicinamidas). Estos sistemas impedidos estéricamente por la presencia de un carbono alfa tetrasustitudo, presentan una acidólisis selectiva en el extremo C-terminal en ser tratadas condiciones ácidas mediante la formación de un anillo de oxazolona (**Esquema 1**).[102,103]

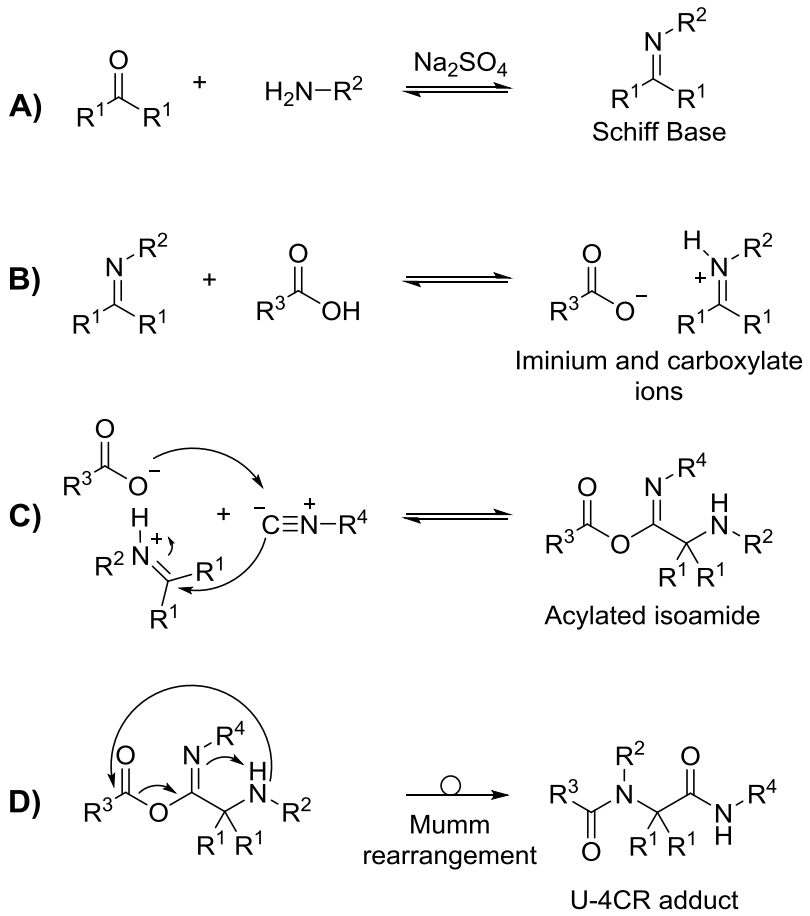


Esquema 1. Mecanismo de ruptura del enlace amida C-terminal de la *N*-metil α,α-dimetil glicina vía un intermedio de oxazolona.

Dicha hidrólisis selectiva de un enlace amida llamó nuestra atención para el diseño de sistemas 1,4-dicarbonilicos basados en estas estructuras y utilizarlos como conectores para bioconjugación que sean hidrolizables en condiciones ácidas.

Síntesis de las dialquil glicinas *N*-alquiladas

Pese a inicialmente haberse descubierto otras vías sintéticas para la preparación de péptidos que contienen dialquil glicinas *N*-alquiladas,[104–107] la preparación de estos compuesto más explotada, utilizada es mediante la reacción multicomponente de Ugi[108] en la que una cetona, una amina primaria, un ácido carboxílico y un isonitrilo se condensan para formar en una sola etapa de reacción las deseadas trialquil glicinas con rendimientos muy elevados (**Esquema 2**).



Esquema 2. Mecanismo de la reacción multicomponente de Ugi (U-4CR).

Dado que el principal objetivo de la presente tesis es el desarrollo de nuevos conectores, tanto hidrolizables como no hidrolizables, para bioconjugación y que para la obtención de este tipo de conectores se precisa obtenerlos en elevados rendimientos, que se puedan funcionalizar y que permitan modificaciones de los mismos. Los sistemas 1,4-dicarbonilicos obtenidos a partir de la reacción de Ugi pueden tener un elevado potencial en su uso como conectores hidrolizables para bioconjugación y es por ello que se ha realizado un estudio concienzudo en la presente tesis.

Objetivos

- Estudiar la preparación y evaluar acidólisis de los aductos obtenidos a partir de la reacción de Ugi.
- Mejorar la velocidad de ruptura del enlace amida de los compuestos de Ugi preparados hasta la fecha.
- Encontrar las condiciones idóneas para la funcionalización de los extremos N- y C-terminal de los dipéptidos obtenidos a partir de la reacción de Ugi.
- Utilizar las α,α-dialquilglicinas *N*-alquiladas funcionalizadas para conjugar a diferentes biomoléculas complejas.
- Estudiar la afinidad antígeno-anticuerpo de los conjugados con anticuerpos preparados.

Resultados y discusión

Con el fin de estudiar los aductos de Ugi como posibles conectores hidrolizables, se preparó un librería de α,α-dialquilglicinas *N*-alquiladas mediante la mencionada reacción de Ugi utilizando diferentes aminas, cetonas y ácidos carboxílicos (**Figura 4**). La robusta reacción de Ugi permite la síntesis con unos rendimientos desde moderados (13%) a elevados (77%) a partir de la condensación de una gran diversidad de reactivos de partida en tiempos que van desde muy cortos (1 hora) hasta más prolongados (3 semanas).

La versatilidad de la reacción de Ugi ha permitido la obtención de 15 dipéptidos (**1a-o**) que presentan una gran diversidad de grupos funcionales tanto en cadenas laterales como unidos directamente al átomo de nitrógeno que forma el enlace amida central.

La mencionada biblioteca de compuestos se fue construyendo en base a las velocidades de acidólisis que presentaban los análogos que se iban preparando (**Figura 5**). De esta forma se consiguió afinar la velocidad de ruptura del enlace amida en condiciones ácidas por la modificación de los grupos funcionales involucrados directamente en el mecanismo de acidólisis vía el intermedio de oxazolona.

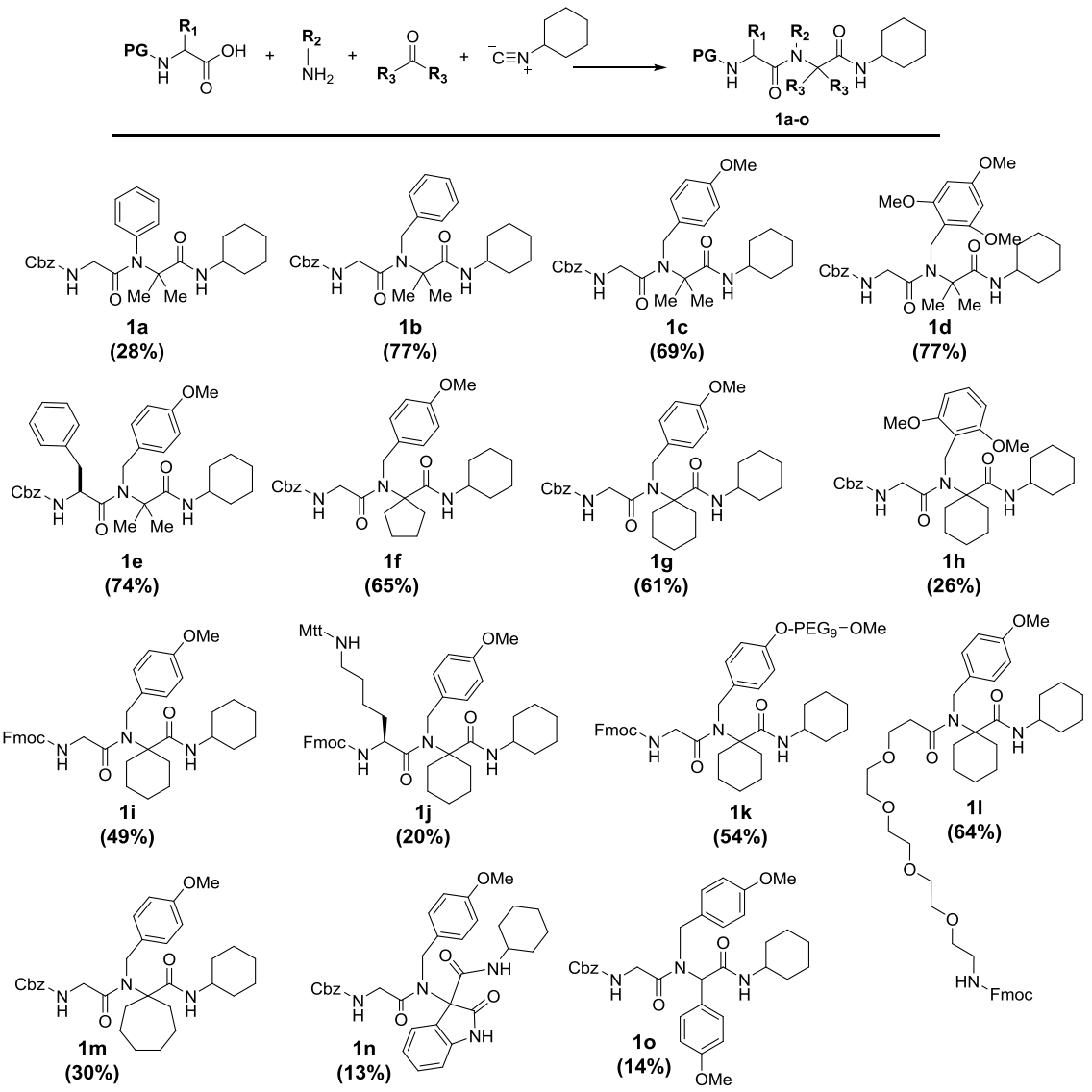


Figura 4. Reacción, estructuras y (rendimientos) de las α,α-dialquilglicinas *N*-aciladas sintetizadas mediante la reacción de Ugi.

Así mismo, los derivados más interesantes en cuanto a tiempos de ruptura del enlace amida del extremo C-terminal (**Figura 5 y Figura 6**), resultaron los compuestos que contenían dialquilglicinas cíclicas, debido a la restricción conformacional que el ciclo confiere a la molécula. Disponiendo así el oxígeno carbonílico de la amida central a una menor distancia del carbono, carbonílico de la amida C-terminal sobre la que tiene lugar el ataque nucleófilo que promociona la ruptura de la amida en este extremo.

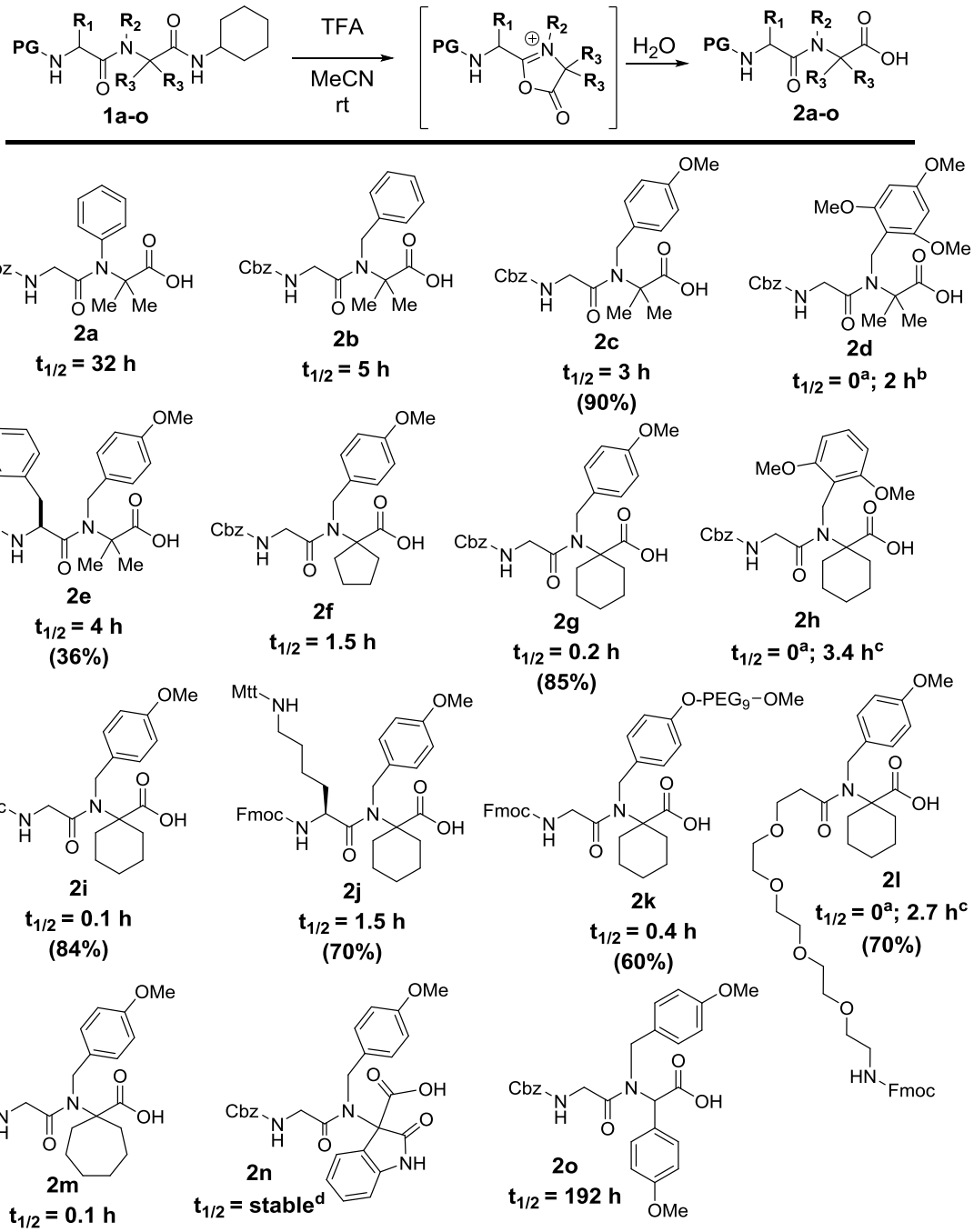


Figura 5. Estudio de acidolysis y los resultantes ácidos carboxílicos. $t_{1/2}$ fue determinado por la linealización de las curvas de acidólisis. [a] Descomposición complete del producto. De forma general la acidólisis se realizó tratando los compuestos con 1% TFA en MeCN excepto los marcados con [b] que fueron tratados con 0.1% de TFA en MeCN. [c] Acidólisis con 1% TFA en H_2O/MeCN (1:1). [d] El compuesto 1n resultó completamente estable en 1% TFA en MeCN.

Los compuestos sintetizados se sometieron a la acidólisis del enlace amida del extremo C-terminal en tratarlos con una mezcla de ácido trifluoroacético (TFA) al 1% en acetonitrilo (MeCN), obteniéndose los correspondientes ácidos carboxílicos (**2a-o**). Algunos de los compuestos presentaron una reactividad tal, que a esa concentración de ácido sufrían

degradación completa del compuesto, por ello se estudió también su acidólisis a una concentración menor de TFA (0.1%).

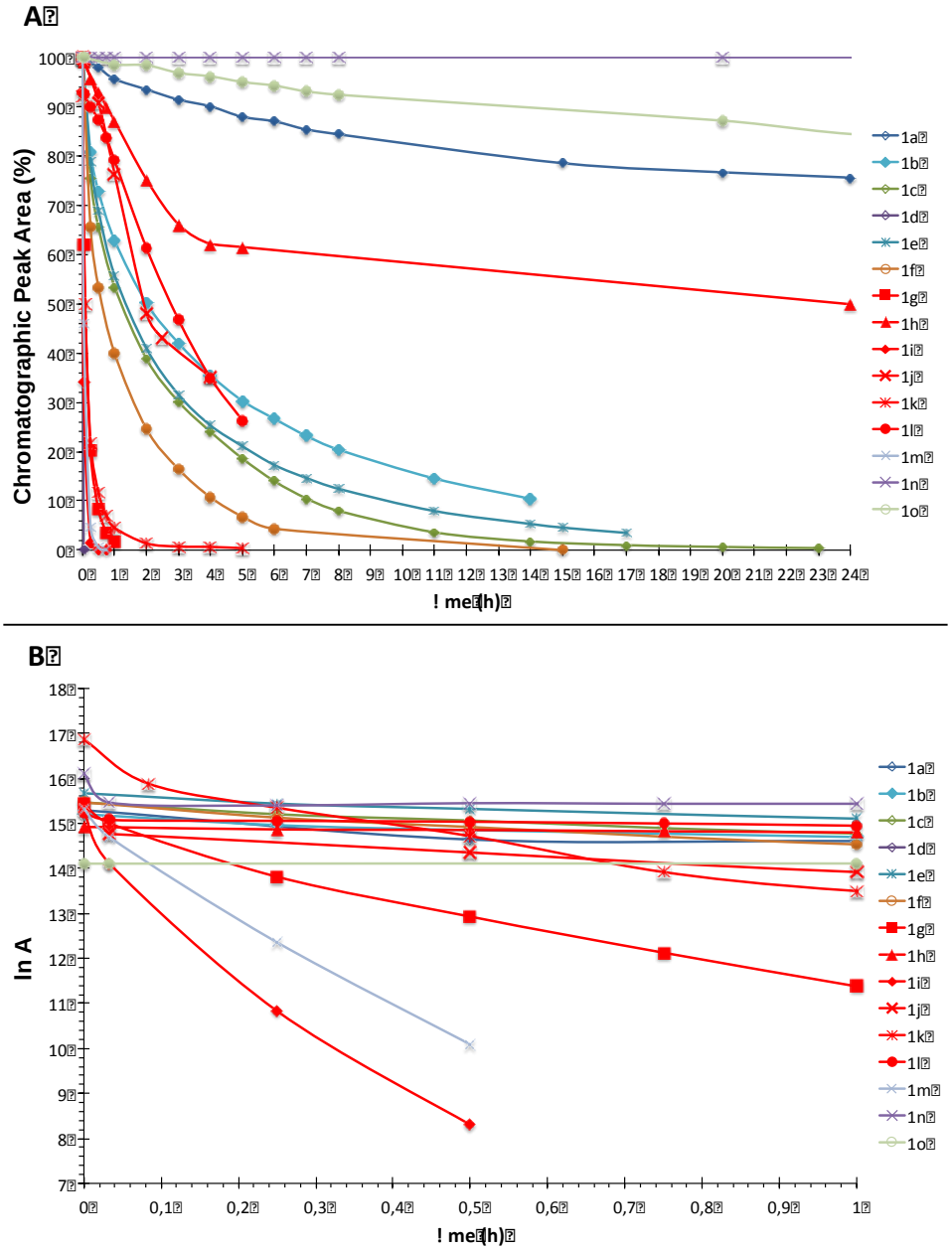


Figura 6. Estudio de acidólisis de los compuestos 1a-o a una concentración de 1 mg/mL en TFA:MeCN (1:99). A) Área del pico cromatográfico (%) correspondiente a la degradación de la amida en el tiempo indicado y B) linealización de la curva de acidólisis donde A corresponde a el área del pico cromatográfico de las amidas 1a-o a el tiempo de tratamiento indicado.

El estudio de la acidólisis de las amidas en el extremo C-terminal ha ayudado a entender y determinar que grupos funcionales están involucrados en la liberación de la amina. En este sentido, tanto la electronegatividad del anillo unido la amina central, tanto como el volumen de los sustituyentes en la posición alfa del amino ácido del extremo C-terminal, juegan un rol importante en la velocidad de acidólisis para los compuestos estudiados. Como se indicó

anteriormente, la combinación del grupo *p*-metoxibenzilo junto al anillo de ciclohexilo en la posición alfa (homocicloleucinas), resultaron ser unas de las combinaciones óptimas en cuanto a tiempo de acidólisis y por lo tanto se decidió estudiarlos como posibles conectores hidrolizables en bioconjugación.

Funcionalización del extremo C-terminal:

Con el fin de probar la viabilidad como conector de los sistemas de Ugi, en primer lugar se procedió a la modificación del extremo C-terminal. Para ello se utilizaron agentes de acoplamiento convencionales para la química en solución (EDC·HCl y Oxyma Pure®, obteniéndose nuevas amidas funcionalizadas con amidas de diferente índole tales como alifáticas y aromáticas (**Figura 7**). Paralelamente la empresa biotecnológica PharmaMar preparó conjugados anticuerpo-fármaco utilizando los conectores aquí descritos.

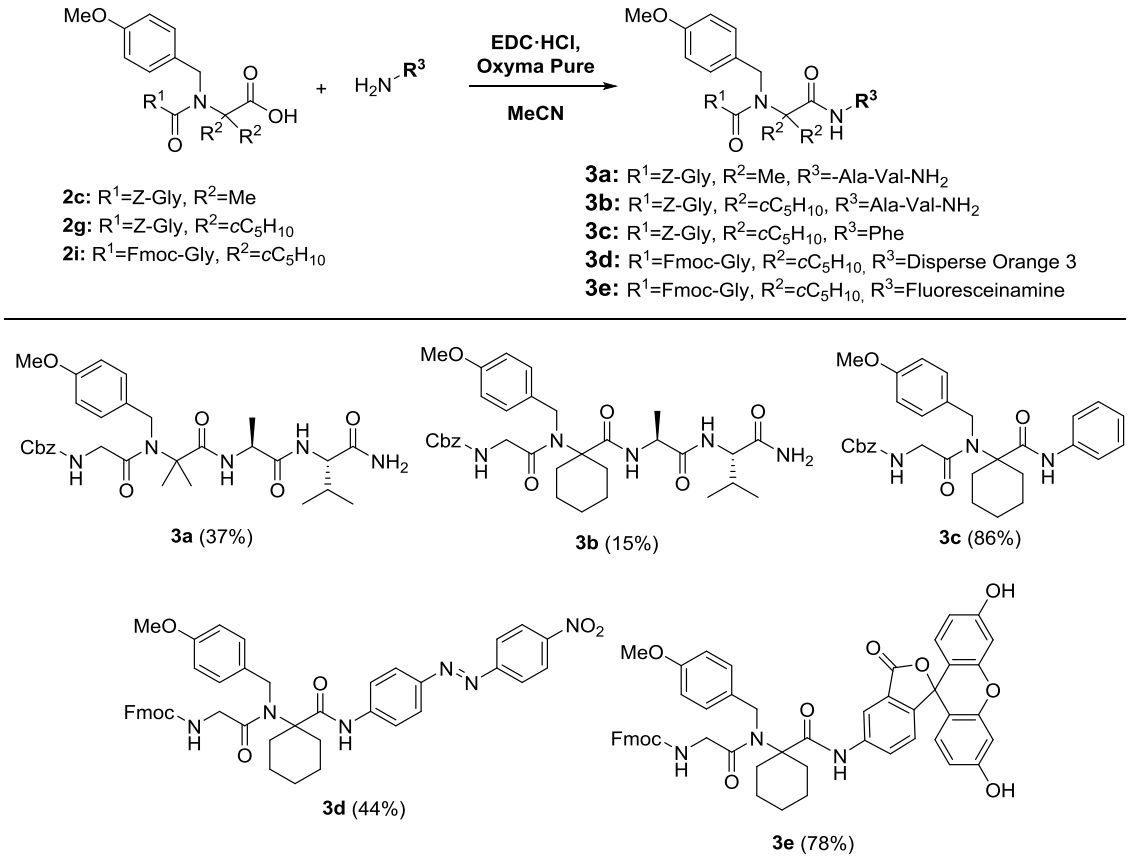
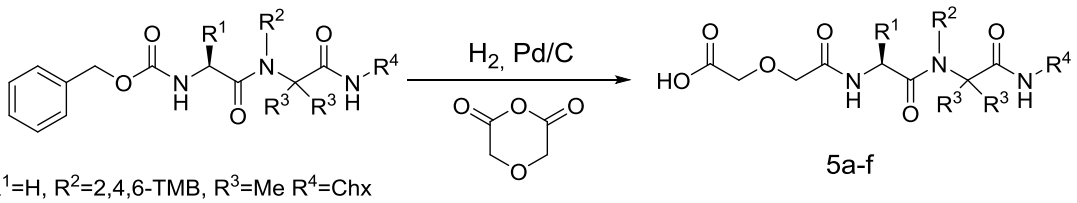


Figura 7. Funcionalización del ácido carboxílico terminal de los aductos de Ugi 2c, 2g y 2i. (Rendimiento)

Funcionalización del extremo N-terminal:

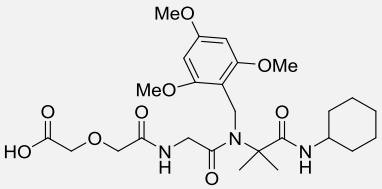
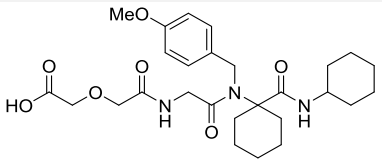
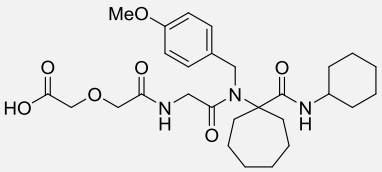
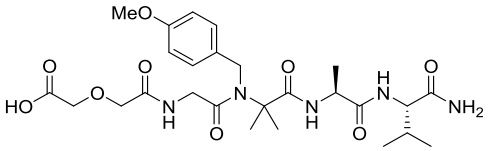
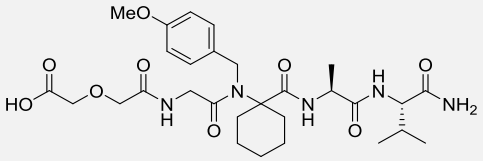
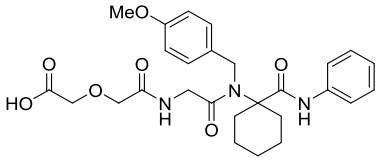
Para la funcionalización del extremo N-terminal con grupos afines para la activación y la posterior bioconjugación, se precisa la previa eliminación de los grupos protectores. Los compuestos preparados de Ugi que contenían el grupo protector benziloxicarbonilo (Cbz) fueron desprotegidos mediante hidrogenación catalizada por paladio con rendimientos generalmente elevados. Dado que la manipulación de estos dipéptidos podía evolucionar en la formación de la diketopiperazina (DKP) inactivando el sistema por la formación irreversible de un enlace amida e imposibilitando la conjugación, se mejoró esta estrategia de desprotección añadiendo un anhídrido cíclico en la mezcla de hidrogenación. De esta forma en ser liberada la amina del extremo N-terminal, *in situ* está misma sufre una acilación por el anhídrido diglicolico presente en la mezcla, liberando a su vez un ácido carboxílico (**Esquema 3, Tabla 2**). Este procedimiento ha permitido la obtención, en elevados rendimientos y en un número menor de etapas, de los compuestos de Ugi funcionalizados con un ácido carboxílico para su conjugación vía el grupo amino de las cadenas laterales de lisinas en péptidos, proteínas y anticuerpos.



1d: R^1=H, R^2=2,4,6-TMB, R^3=Me R^4=Chx
1g: R^1=H, R^2=PMB, R^3=cC_5H_{10}, R^4=Chx
1m: R^1=H, R^2=PMB, R^3=cC_6H_{10}, R^4=Chx
3a: R^1=H, R^2=2,4,6-TMB, R^3=Me R^4=Ala-Val-NH_2
3b: R^1=H, R^2=PMB, R^3=cC_5H_{10}, R^4=Ala-Val-NH_2
3c: R^1=H, R^2=PMB, R^3=cC_5H_{10}, R^4=Phe

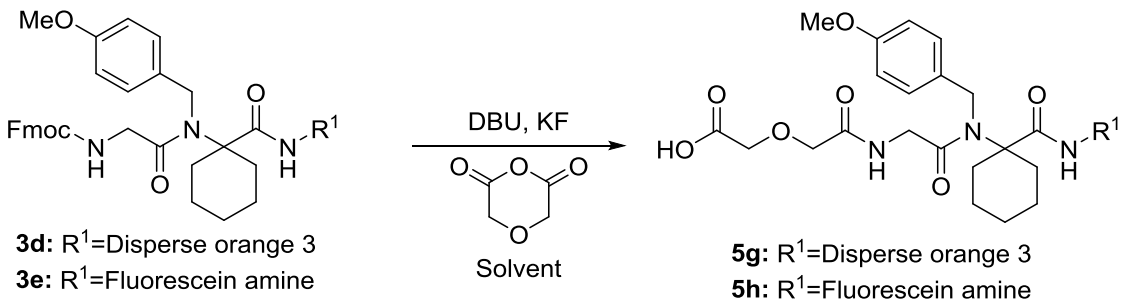
Esquema 3. Eliminación del grupo Cbz mediante hidrogenación e incorporación del ácido diglicolico simultaneas.

Tabla 2. Productos obtenidos y rendimientos de la eliminación del grupo Cbz mediante hidrogenación e incorporación del ácido diglicólico simultaneas

Entrada	Compuesto protegido	Producto final		Rendimiento (%)	Pureza (%)[a]
1	1d	5a		97	96
2	1g	5b		98	97
3	1m	5c		90	94
4	3a	5d		97	83
5	3b	5e		99	93
6	3c	5f		97	98

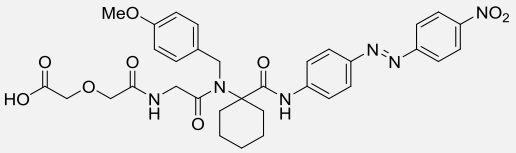
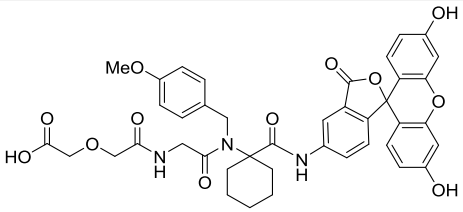
[a] Determinado por el área de los picos cromatográficos del HPLC del producto aislado sin purificación.

De la misma forma los compuestos que contenían el grupo Fmoc se consiguió llevar a cabo la desprotección y la acilación simultanea de la amina liberada (**Esquema 4, Tabla 3**).



Esquema 4. Eliminación del grupo Fmoc y acilación simultanea de la amina liberada.

Tabla 3. Productos obtenidos y rendimientos de la eliminación del grupo Fmoc y acilación simultánea**.**

Entrada	Compuesto protegido	Disolvente	Producto final		Rendimiento (%)
1	3d	THF	5g		9
2	3e	DMF	5h		30

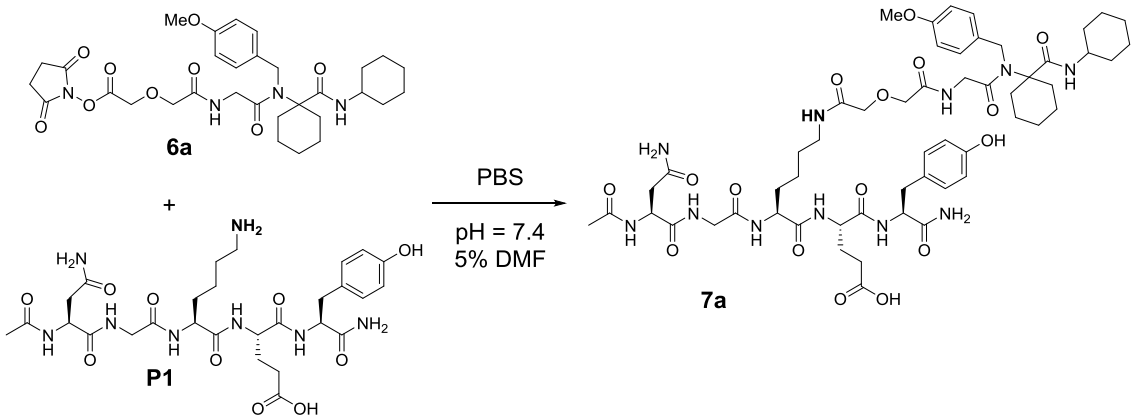
De la misma manera que los compuestos **1a-o**, los compuestos **5a-h** fueron tratados con mezclas acidolíticas (1% TFA en MeCN) mostrando los mismos $t_{1/2}$ que sus precursores protegidos. La incorporación del ácido diglicolico permitió el estudio en medios acuosos debido a que mejoraba la solubilidad respecto a los compuestos protegidos. Los compuestos presentaron estabilidad en suero humano y además en condiciones de pH lisosomal (4.8), cosa que deja entrever que los compuestos serán estables en las condiciones de degradación.

Activación para la bioconjugación:

De los ácidos carboxílicos preparados se seleccionaron el 5b, d, f, g y h para su activación en forma de éster succínico. La activación más efectiva resultó utilizando diciclohexil carbodiimida (DCC) como agente de acoplamiento en presencia de *N*-hidroxi succinimida (HOSu) en AcOEt:dioxano (1:1) como disolvente. La reacción cuantitativa permitió la obtención de los derivados activos listos para la bioconjugación con aminas reactivas.

Bioconjugación:

En primer lugar se abordó la unión a del compuesto **7a** sobre el pentapéptido Ac-Asn-Gly-Lys-Glu-Tyr-NH_2 **(P1)** sintetizado manualmente en soporte polimérico mediante la estrategia Fmoc/tBu (**Esquema 5**). Pasadas 17 h de tratamiento, se consiguió un 73% de conjugación determinado por HPLC (**Figura 8**)



Esquema 5. Bioconjugación vía lisina a un pentapéptido.

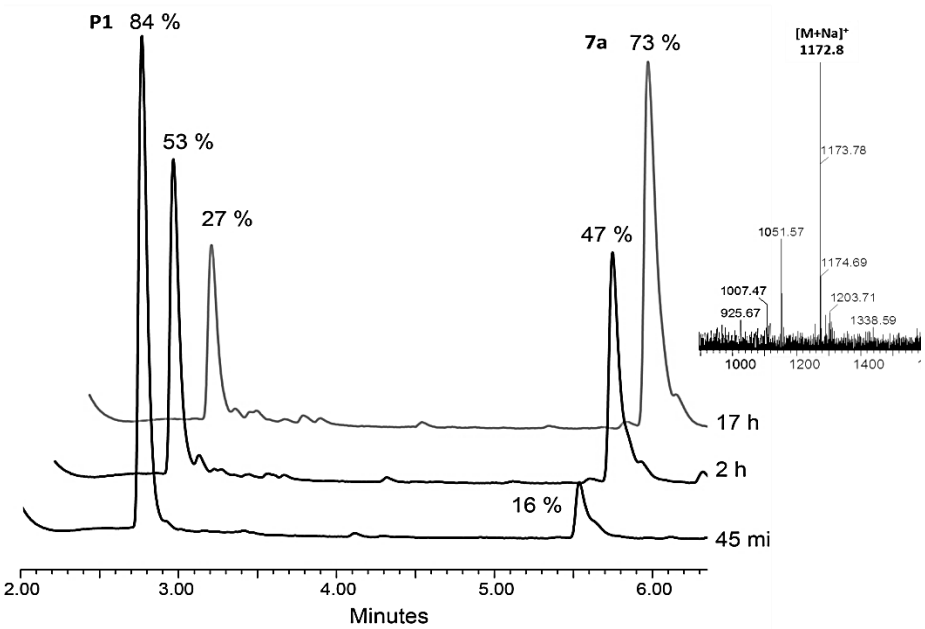
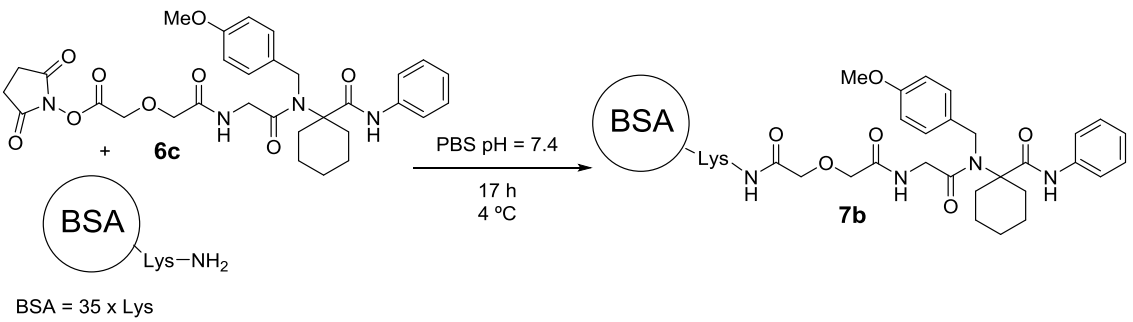


Figura 8. HPLC y ESI de la formación del bioconjugado peptídico 7a.

Viendo el éxito de la bioconjugación en péptidos, se procedió a realizar la bioconjugación en proteína. Para ello compuesto activado 6c se trató con albumina bovina (BSA) en PBS como solución amortiguadora (**Esquema 6**). El análisis por HPLC y MALDI reveló que la conjugación había tenido lugar y que se consiguió anclar aproximadamente 16 moléculas del compuesto a la proteína (**Figura 9**).



Esquema 6. Conjugación vía lisina de BSA al aducto activado de Ugi 6c.

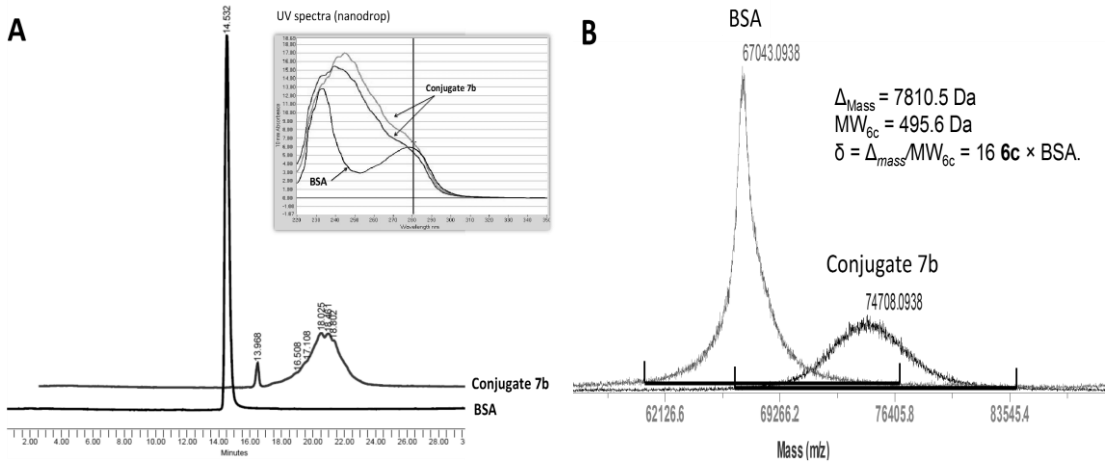
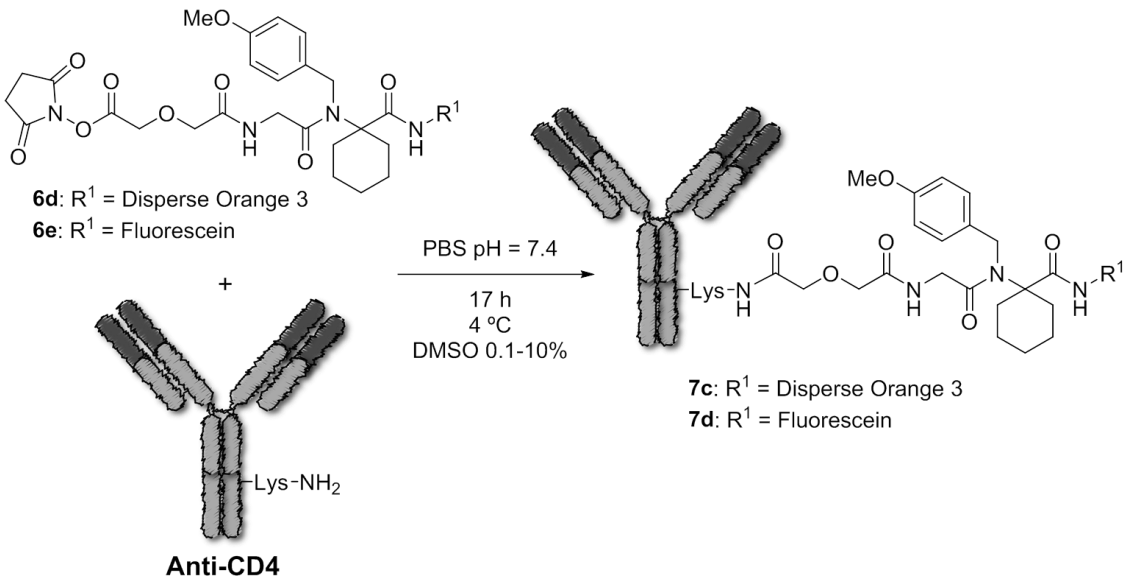


Figura 9. BSA y su conjugado 7b **A**) HPLC y análisis de UV y **B)** MALDI**.**

Finalmente se procedió a la conjugación a un anticuerpo monoclonal también vía lisina con el fin de ensayar la conjugación sobre un sistema muy complejo y poder ver los efectos de este tipo de bioconjugación a nivel de reconocimiento celular. Para ello, los compuestos **6d** y **6e**, que contienen un fluoróforo, fueron incorporados al anticuerpo Anti-CD4 (**Esquema 7, Tabla 4**).

Los conjugados fueron convenientemente caracterizados utilizando diferentes técnicas tales como gel de electroforesis (SDS-PAGE), análisis de UV y además las moléculas conjugadas por anticuerpo, se determinaron utilizando espectrometría de masas para compuestos de elevado peso molecular (ESI-TOF).

Para finalizar se estudió si la afinidad del anticuerpo sobre el antígeno, se veía afectada en conjugarle alguna molécula. Para ello se llevaron a cabo estudios mediante citometría de flujo incubando los conjugados con células tipo Jurkat que presentan una sobreexpresión de la proteína CD4 con el fin la cantidad de conjugados con anticuerpo que son capaces de unirse su antígeno específico. Como era de esperar, el anticuerpo sin conjugar presentó mayor afinidad de unión al antígeno y mayor capacidad de unión. Los conjugados ensayados en cambio disminuían estas dos variantes pero sin perder la actividad completa del anticuerpo.



Esquema 7. Bioconjugación vía lisina a Anti-CD4 con los compuestos activados **6d y 6e.**

Tabla 4. Condiciones de conjugación para Anti-CD4 y los compuestos **6d y 6e.**

Entrada	Compuesto conjugado (Eq.)	DMSO (%)	Conjugado obtenido	Densidad de Conjugación (δ)
1	6d (3.7)	2	7c	2
2	6e (4.2)	9	7d	2/3

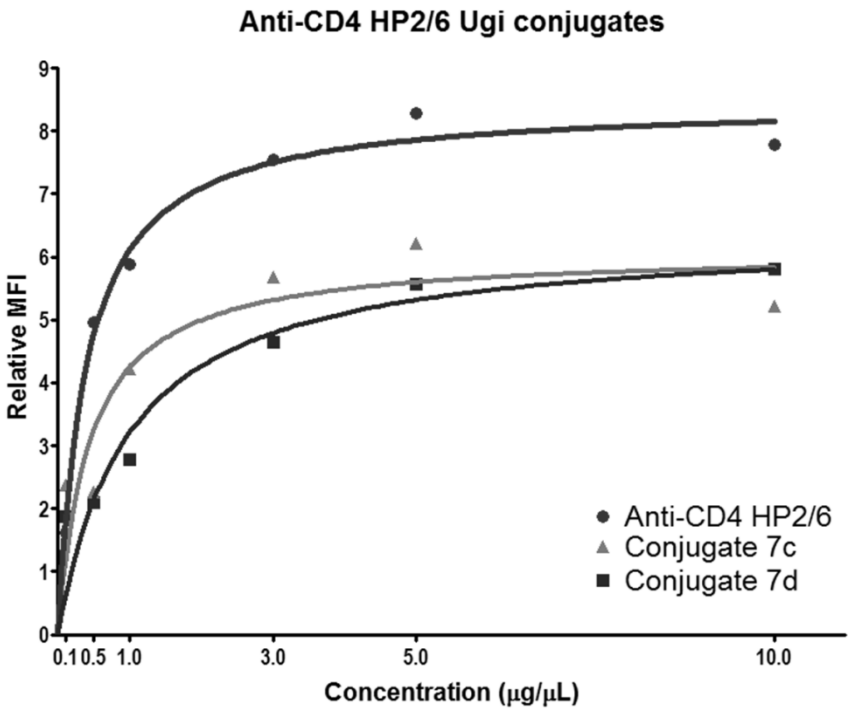


Figura 10. Análisis mediante citometría de flujo de la unión celular de los anticuerpos conjugados a las células Jurkat. La línea celular fue incubada con las concentraciones indicadas de conjugados, seguida de la incubación con anti-mouse IgG marcado con FITC. MFI indica las cuentas de fluorescencia detectadas por el citómetro.

I.1 Conectores tipo Ugi

Tabla 5. AC_{50} y Max MFI (%) para los mAb anti-CD4 y sus conjugados (7c-d). AC_{50}, calculada como la concentración de anticuerpo necesaria para alcanzar el 50% de la máxima MFI para cada conjugado.

	AC_{50} (μg/mL)	Max MFI (%)
Anti-CD4	0.38	100
Conjutate 7c	0.53	59
Conjugate 7d	0.57	58

A su vez, la empresa biofarmacéutica PharmaMar abordó la síntesis de conjugados anticuerpo-fármaco citotóxico (ADCs) utilizando el anticuerpo comercial Trastuzumab, que reconoce la proteína HER2 sobre-expresada en células mamarias tumorales. Los resultados para los conjugados preparados con unos conectores X preparados fueron prometedores ya que consiguieron muy buena selectividad respecto células que no expresan dicho antígeno ($HER2^-$) (**Tabla 6**)

Tabla 6. Valores de IC_{50} para los ensayos de viabilidad celular en líneas celulares $HER2^+/HER2^-$.

HER2 expression	**Cell line**	**IC_{50} (nM)**	
		ADC A	**ADC B**
+	HCC1954	20,3	173,3
	SK-BR3	15,3	133,3
-	MCF7	80	333
	MDA-MB-231	120	333

I.2. Tipo Thp

Introducción

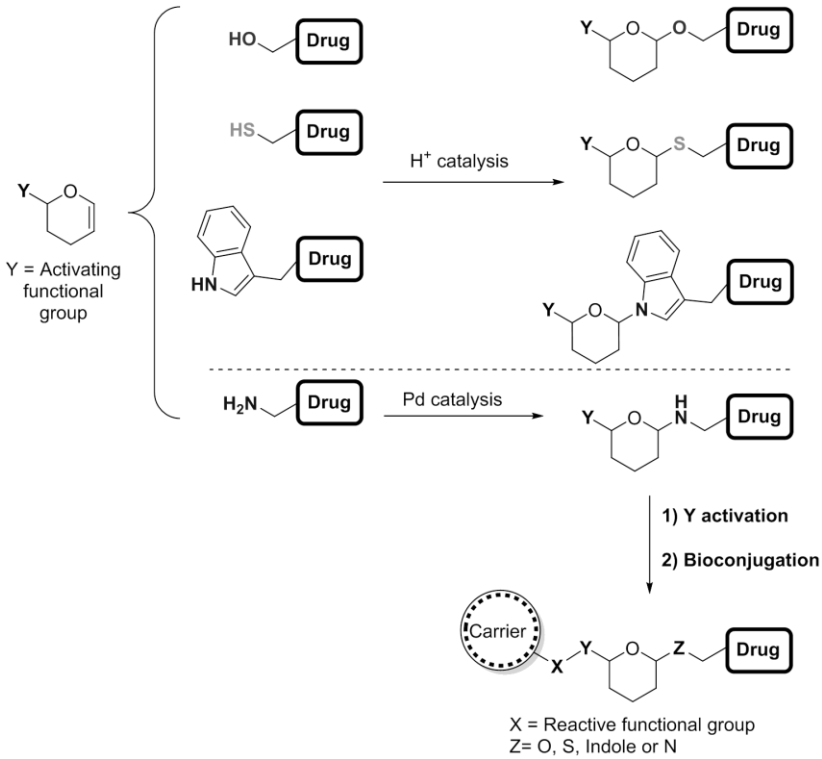
En esta sección se estudió el anillo de tetrahidropirano (Thp) como posible conector hidrolizable. Su uso está ampliamente descrito como grupo protector de hidroxilos en síntesis orgánica por su bajo coste, estabilidad y la facilidad para eliminarlo.[109] El enlace formado para la protección de alcoholes en forma de acetal presenta inestabilidad en condiciones ácidas acuosas,[110] y es por ello que decidimos estudiar este tipo de enlace como punto de unión escindible de un conector.

Curiosamente tanto la formación del enlace acetal que protege el grupo hidroxilo, como la eliminación del mismo tienen lugar en condiciones ácidas. En este sentido numerosas metodologías para protección y desprotección han sido desarrolladas a lo largo del tiempo.[109,111–113] Además de utilizarse como protector de hidroxilos, el anillo de Thp también resultan estables para la protección de tioles[114],[115] y aminas.[116–121]

La eliminación del grupo protector tiene lugar en presencia de ácidos tanto próticos como de Lewis.[113,122–125] Este echo nos animó a probar esta estructura privilegiada como conector que presenta hidrolisis en condiciones ácidas.

Además de ser usado como grupo protector, la estructura de tetrahidropirano ha sido utilizada como conector para la síntesis de péptidos en fase sólida,[126–132] y además se utilizó vagamente como conector para conjugados anticuerpo-fármaco.[13,14] De esta forma el Thp juega más puntos a nuestro favor a la hora de ser utilizado como conector hidrolizable para bioconjugación.

En esta sección se abordó el estudio de la utilización del Thp como conector hidrolizable para hidroxilos, tioles y aminas (**Esquema 8).**



Esquema 8. Funcionalización del anillo de 2,3-dihidropirano (Dhp) para su posterior bioconjugación**.**

Objectivos

- Estudiar y optimizar la introducción de tioles y aminas al anillo de tretrahidropirano para su posterior bioconjugación.
- Estudiar la estabilidad y condiciones de eliminación del Thp para utilizarlo como conector hidrolizable.
-

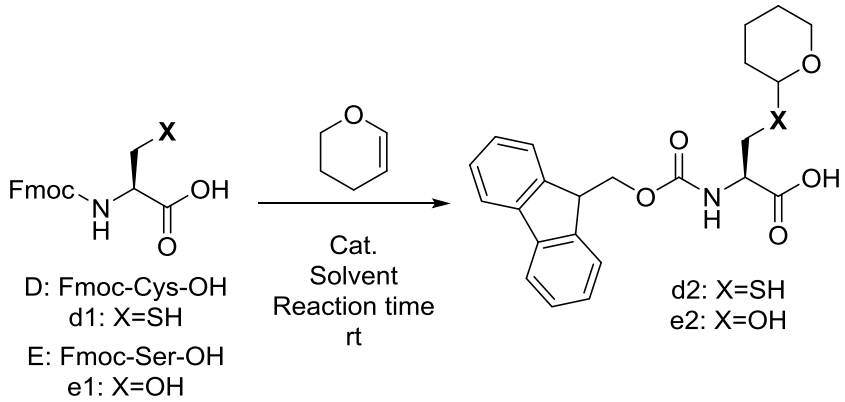
Resultados y discusión

Inicialmente se abordó la unión del anillo de dihidropirano a cisteína (Cys), serina (Ser) y fenilalanina (Phe) para comprobar si dicha unión es factible. En los dos primeros casos, la unión tenia lugar a los átomos de la cadena lateral, sin embargo en el caso de la Phe la reacción no tubo lugar.

Estos resultados preliminares nos animaron, por una parte a llevar a cabo la protección de los derivados protegidos de Cys y Ser para poder estudiar la labilidad de los mismos en condiciones ácidas. Y por otra, para intentar unir el anillo de dihidropirano a aminas utilizando otras estratégias descritas en la bibliografía.

En el caso de Cys y Ser, la unión se llevó a cabo utilizando acido *p*-toluensulfonico en DCM como disolvente, obteniéndose ambos productos protegidos con el anillo de Thp en elevados rendimientos (**Esquema 9**). Para la Phe y otras aminas (tanto secundarias, como aromáticas) se intentó sin éxito la unión mediante catalizadores de Pd, además de diferentes

ácidos y diferentes precursores del Thp como el 2-clorotetrahidropiranilo o glucosa debido a esto se decidió abandonar la estrategia de unión del anillo del Thp a aminas de diferente índole.



Esquema 9. Protección con el Thp de la Fmoc-Cys-OH y la Fmoc-Ser-OH.

Entrada	Compuesto	Catalizador	Disolvente	Tiempo de Reacción	Rendimiento (%)
1	D	PPTS (0.1 eq)	DCM	5 days	72
2	D	*p*-TsOH (0.1 eq)	DCM	1 h	87
3	E	*p*-TsOH (0.1 eq)	DCM	17 h	89

Los estudios de labilidad continuaron con la Fmoc-Cys(Thp)-OH y con la Fmoc-Ser(Thp)-OH. El anillo de tetrahidropirano confería mejor solubilidad en medios acuosos a diferencia de otros grupos protectores para estos aminoácidos (tritilo o *t*Bu) por lo que se pudo realizar los ensayos de hidrólisis directamente en condiciones ácidas acuosas (**Tabla 7**).

Tabla 7. Estudio de la desprotección de Fmoc-Cys(Thp)-OH y Fmoc-Ser(Thp)-OH.

Entrada	Compuesto	Cocktail	tiempo	Cys desprotegida, (%)
		Composición		
1	d2	MES[a] (100 mM)	48 h	0
2	e2	pH = 4.8	40 h	50
3	d2	MES/NaCl/KCl (100:137:2.7 mM)	48 h	0
4	e2	pH = 4.8	20 h	25
5	d2	MES/TIS (0.1 M:1.5%) pH = 4.8	48 h	0
6	d2	PBS		0
7	e2	PO_4^{3-}/KCl/NaCl (10:2.7:137 mM) pH = 7.4	120 h	0
8	d2	TFA/H_2O/DCM (1:1:98) pH ≈ 1	48 h	29
9	d2	TFA/H_2O/DCM (10:1:89) pH ≤ 1	48 h	94

I.2 Conectores tipo Thp

[a] Ácido 2-(*N*-morfolino)etansulfonico

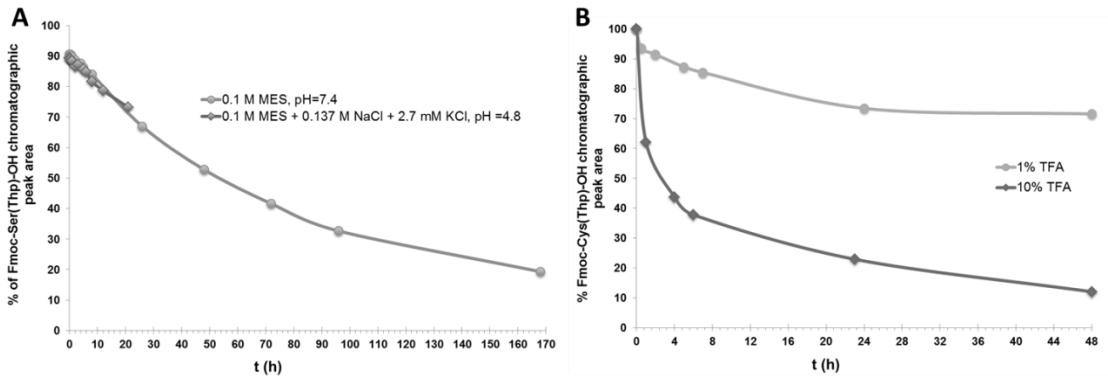


Figura 11. A) Estudio cinético de hidrólisis para la Fmoc-Ser(Thp)-OH y **B)** Fmoc-Cys(Thp)-OH

.

El estudio de hidrólisis determinó que el enlace tipo acetal permitía una liberación controlada del hidroxilo (en este caso Fmoc-Ser-OH) en condiciones similares a las presentes en medios lisosomales (pH = 4.8) y presentaba estabilidad a pH ligeramente más elevados (PBS). Como se había indicado, los conectores para este tipo de sistemas se habían estudiado tanto en síntesis de péptidos en fase sólida, así como en conjugados de anticuerpo fármaco. Es por ello que no se decidió explotar más el estudio para utilizar el tetrahidropirano con hidroxilos pese a los buenos resultados obtenidos.

Por otro lado, el enlace tioacetal (Fmoc-Cys-OH) resultó estable a pH = 4.8 con lo que su uso como conector para bioconjugación se desestimó. Sin embargo, un estudio acidolítico en condiciones ácidas de mayor concentración permitió la eliminación del grupo Thp en tiempos cortos. Este estudio nos dio la idea de utilizar el Thp como protector de Cys para su uso en síntesis de péptidos en fase sólida. Los detalles de este estudio se detallan en la sección siguiente.

I.3 Thp como grupo protector

Introducción

Hasta la fecha la química de péptidos ha sufrido grandes cambios desde sus inicios gracias a aportaciones científicas en el campo. Hoy en día la síntesis de péptidos en fase sólida (SPPS del inglés *solid phase Peptide synthesis*) está considerada la forma más eficiente para la preparación de estos compuestos. Infinidad de metodologías para formación de enlace amida, grupos protectores ortogonales, conectores o soportes poliméricos son utilizados para tal fin aunque todavía a día de hoy continúan apareciendo reacciones secundarias que dificultan es aislamiento del producto final.

Dada la dilatada experiencia con la que el grupo de investigación cuenta para la síntesis de péptidos en fase sólida, en esta sección se ha estudiado el uso del tetrahidropirano como grupo protector de la cisteína en SPPS debido a los resultados descritos en la sección previa.

La racemización de aminoácidos continua siendo uno de los principales problemas para determinados aminoácidos, siendo la cisteína uno de los más problemáticos debido a su característica composición.[133–135] Un gran número de grupos protectores han sido desarrollados hasta la fecha para la Cys (**Figura 12**), pero continúan dando algunos problemas de racemización[135] o β-eliminación del tiol formando la dehidroalanina.[136]

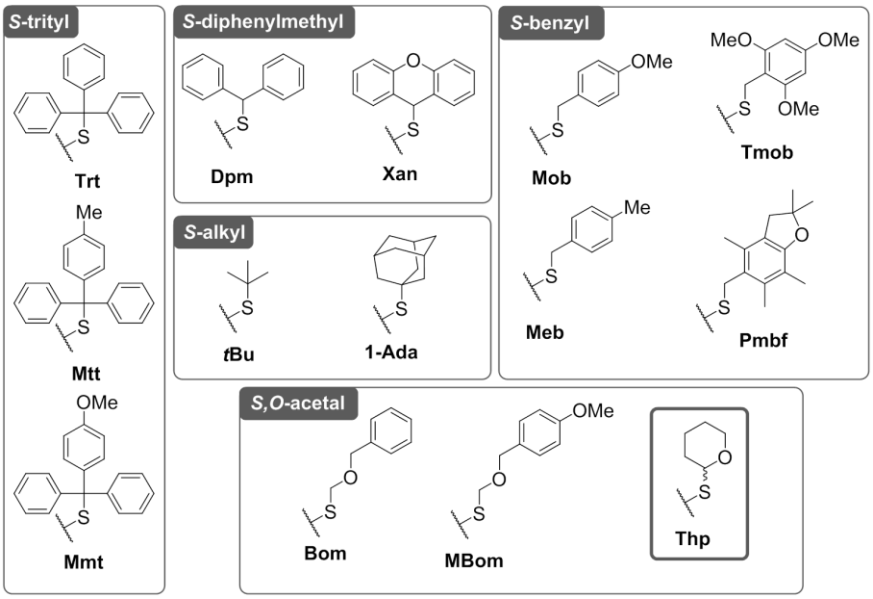


Figura 12. Grupos protectores de Cys labiles a ácido.

En la sección anterior se estudió la incorporación del Thp a la Fmoc-Cys-OH para estudiar la posibilidad de utilizar la estructura de tetrahidropirano como posible conector para bioconjugación. El echo que su estabilidad no permitiera su uso para estas aplicaciones nos

hizo pensar estudiar su posible aplicación como grupo protector. Dado que el grupo Thp no ha sido extensamente estudiado y que la gran mayoría de grupos para este amino ácido presentan grandes grupos aromáticos y poco solubles en medios acuosos, se decidió aplicar su uso en la estrategia más utilizada en SPPS a día de hoy, la de Fmoc/*t*Bu.

Resultados y discusión

La protección del tiol de la cisteína se realizó a partir de la reacción entre la Fmoc-Cys-OH y el 3,4-dihidropirano en presencia de *p*-TsOH catalítico. Su eliminación es estudió ligeramente en la sección anterior y se amplificó el estudio de labilidad de forma más focalizada a las condiciones estándares de la SPPS. La utilización de ácido trifluoroacético en presencia de triisopropilsilano (TIS) en DCM a la concentración de 10:1.5:88.5 resultó la más eficiente en tiempos cortos de reacción.

Para evaluar la eficiencia del Thp como protector, se prepararon tripéptidos que contenían la Cys protegida con el Thp en el extremo C-terminal y en centro de la secuencia peptídica utilizando los conectores Sieber y Rink amida; y 2-clorotritilo. Como agentes de acoplamiento y aditivos la diisopropilcarbodiimida (DIC) y la Oxyma Pure®. Además se compararon los crudos peptídicos con otros grupos protectores de Cys comerciales una vez escindidos de la respectivas resinas **(Figura 13**).

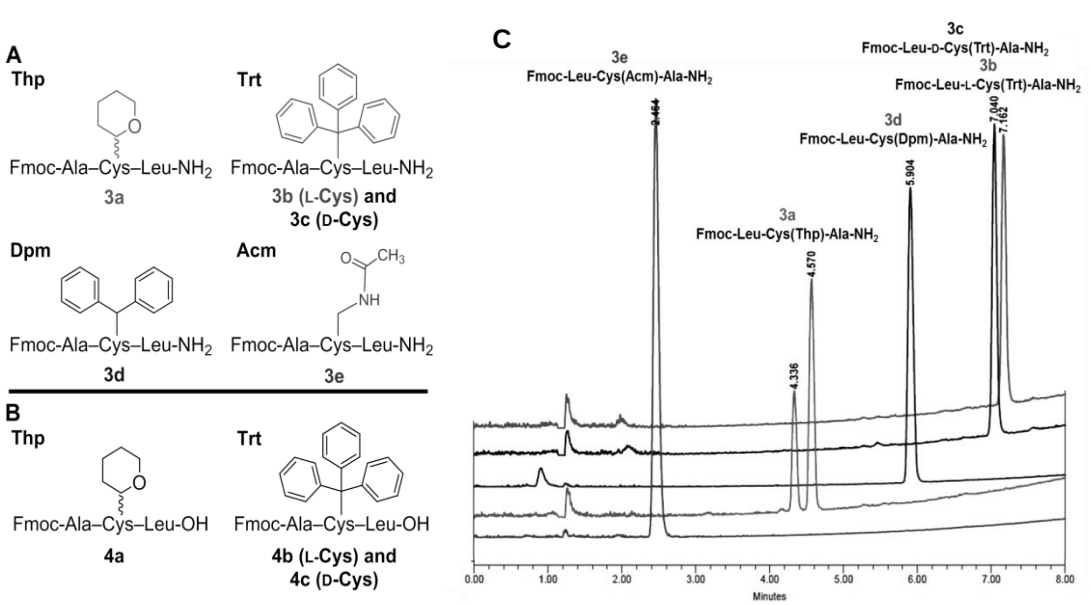


Figura 13. Tripéptidos de Cys completamente protegidos. Sintetizados en: **A)** Resina Sieber amida **y B**) resina 2-Clorotritilo. **C)** RP-HPLC Cromatogramas de los tripéptidos sintetizados 3a-e. Gradiente lineal H_2O/MeCN de (50:50) a (0:100) en 8 min.

Pese a observarse un cromatograma con dos productos debido a la introducción de un nuevo centro quiral por la adición del tetrahidropirano y al ser un grupo protector temporal, su eliminación completa con concentraciones más elevadas al 1% de TFA permitieron la

obtención de un único producto como en el caso de los otros tripéptidos preparados. Además se determinó la racemización y como se muestra a continuación en el caso de los tripéptidos sintetizado con Thp resultó ser menor que los compuestos preparados con los grupos tritilo o difenilmetil (**Tabla 8).**

Tabla 8. Estudios de racemización de Cys para los tripéptidos Ala-Cys-Leu**.**

Resina	Cys GP	Compuesto	% Racemización[a]
Sieber Amida	Thp	3a	0.7
	Trt	3b	3.3
	Dpm	3d	6.8
Rink Amida	Thp	3a	0.5
	Trt	3b	2.3
	Dpm	3d	1.6
2-Clorotritilo	Thp	4a	0.2
	Trt	4b	0.7

a Calculado como (Área del pico del D-péptido/ Área del pico del L-péptido)x100

Con el fin de determinar la compatibilidad con tratamientos prolongados de piperidina que promueven la formación de la dehidroalanina y la consiguiente adición de la piperidina, se prepararon tripéptidos con la Cys protegida en el extremo C-terminal para las resinas tipo Wang y 2-clorotritilo y se analizó mediante HPLC en grado de racemización de la Cys para los grupos protectores Thp y Trt (**Figura 14).**

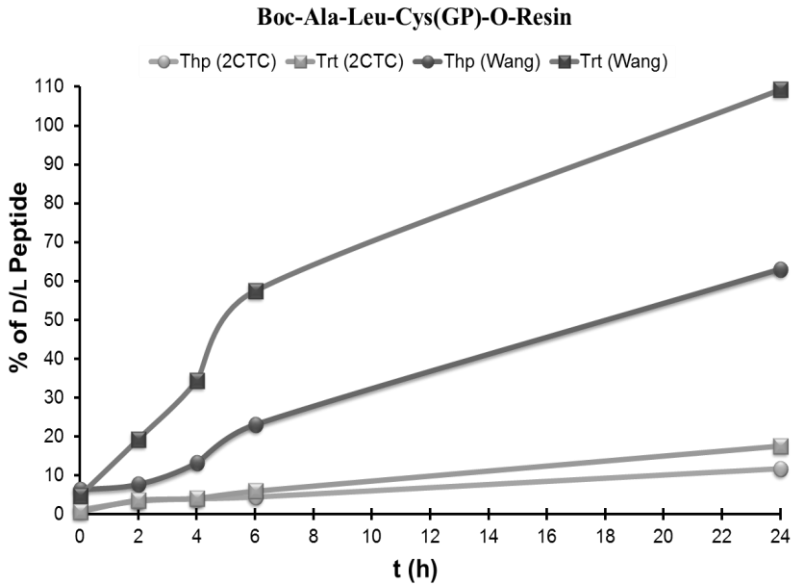


Figura 14. C-terminal cysteine tripeptide racemization studies for Boc-Ala-Leu-Cys(PG)-O-Resin (2CTC and Wang resins), using piperidine/ DMF (1:4) for 24 h. Racemization ratio was defined as (AUC to 5b / AUC to 5a)×100.

En ambos casos, el uso de tetrahidropirano como protector de la Cys presenta menor grado de racemización. Así mismo, se determinó también el grado de formación de el aducto

de piperidina indeseado, observándose nuevamente menor formación para los compuestos que se prepararon utilizando el Thp como protector de Cys.

Finalmente se preparó un péptido (oxitocina reducida) que contiene dos residuos de Cys, estando uno de ellos ubicado en el extremo N-terminal. De esta forma pudimos comprobar que a diferencia de otros grupos protectores del tipo S,O-acetal que precisan un tratamiento extra de desprotección como el MBom, el grupo Thp permite la liberación del tiol de la Cys en el último paso de escisión del péptido de la resina. En este experimento también utilizamos condiciones de microondas para comprobar la compatibilidad de este tipo de equipos con nuestro grupo protector. Tras la síntesis y la escición del péptido de la resina, se obtuvo el péptido deseado con una pureza del 80%, demostrando nuevamente la robustez del grupo Thp para la Cys.

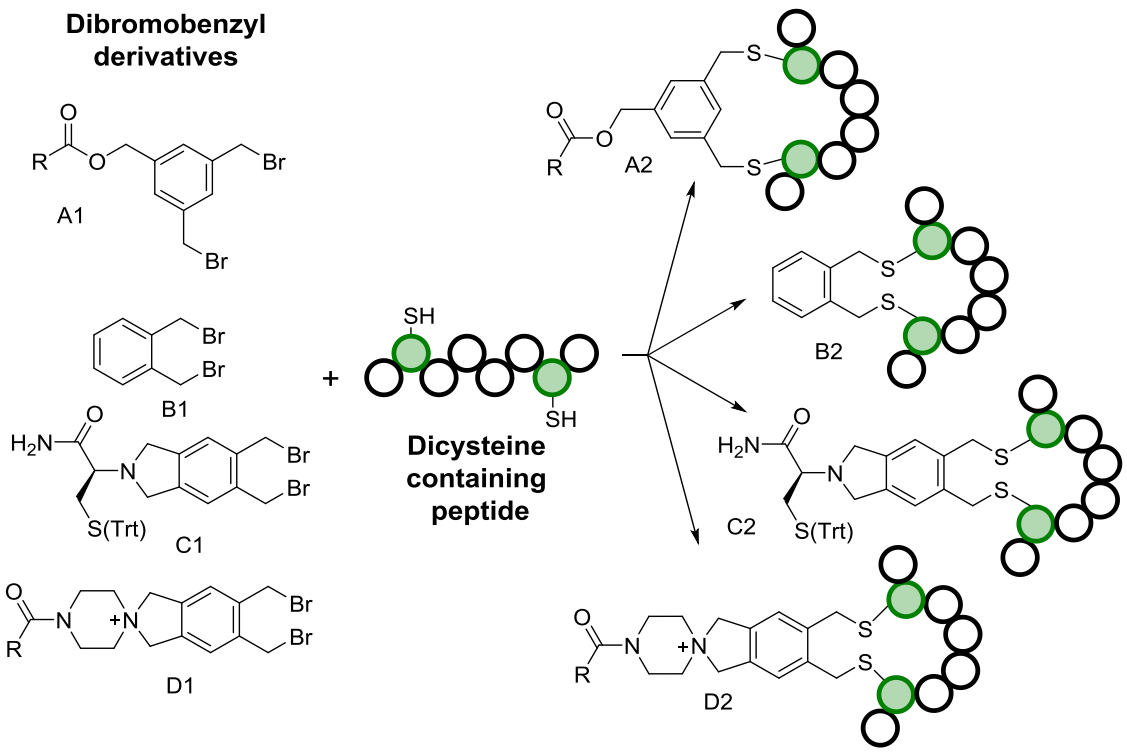
Capítulo II. Conectores no hidrolizables

II.1. Tipo Mesitileno

Introducción

Para el desarrollo de nuevos conectores no hidrolizables en esta sección se estudio la estructura tipo mesitileno. Este tipo de compuestos ha sido anteriormente utilizado en la preparación de péptidos conformacionalmente restringidos a partir de formación de ciclos intramoleculares utilizando el 1,3,5-tris(bromometil)benceno (TBMB) que reacciona prioritariamente con los tioles de la cadena lateral de las cisteínas.[137–140]La formación de estos ciclos permite la obtención de estructuras privilegiadas que favorecen la interacción con receptores moleculares y además mejoran la estabilidad a proteasas de los mismos.[141–145]

Además el grupo de Hartman ha estudiado estos sistemas como conectores bidentados para péptidos a partir de la formación de un ciclo entre dos cisteínas simulando un puente disulfuro para unirlos a ácidos carboxílicos (**Esquema 10**).[146]



Esquema 10. Derivados de dibromobencilo como conectores para péptidos.

La regioselectividad y elevada reactividad que presentan los dibromobenzilos frente a los sulfhidrílos nos animó a amplificar el uso de este tipo de conectores sobre sistemas más complejos como lo son los anticuerpos.

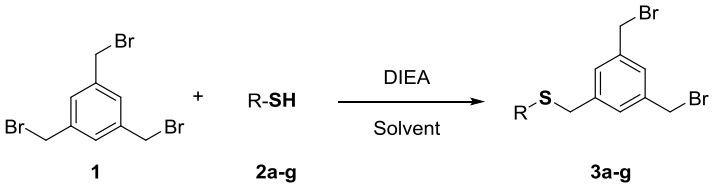
Objetivos

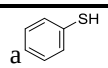
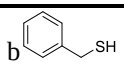
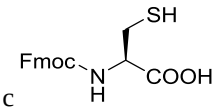
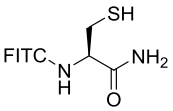
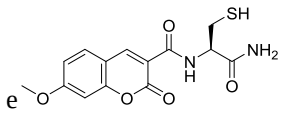
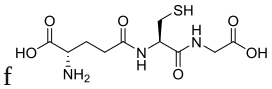
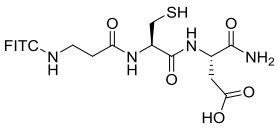
- Estudiar la monotioalquilación sobre el 1,3,5-tris(bromometil)benceno (TBMB) para su funcionalización para la posterior bioconjugación.
- Encontrar las condiciones óptimas de bioconjugación para los derivados de 1,3-bis(bromometil)benceno preparados.
- Usar los compuestos de 1,3-bis(bromometil)benceno para la conjugación en anticuerpos y estudiar si el proceso de conjugación afecta a la entidad macromolecular.

Resultados y discusión

En primer lugar se procedió al estudio de la monotioalquilación del TBMB. Para ello el TBMB fue tratado con tioles alifáticos y aromáticos en presencia de base, resultando los alifáticos los que presentaban una mayor reactividad sobre los sistemas. Se ensayó la reacción en diversos disolventes orgánicos pero debido a la reactividad del producto final, el aislamiento de los compuestos monotioalquilados resultó ser de gran dificultad, consiguiéndose el producto esperado con rendimientos relativamente bajos (**Tabla 9)**.

Tabla 9. Monotioalquilación del 1,3,5-tris(bromometil)benceno.



Entrada	R-SH (2)	Disolvente	Tiempo	Conversion HPLC	Rendimiento (3a-g)
1	 a	DCM	1 h	13%	-[a]
2	 b	DCM	1 h	22%	-[a]
3	 c	DCM	1 h	21%	×[b]
4		DCM	17 h	21%	63%
5		DCM	17 h 37 ºC	46%	×[b]
6		MeCN	17 h	8 %	×[b]
7		MeCN	30 min 0 ºC	35%	32%
8		DMF	30 min	17%	×[b]
9		DMF	30 min 0 ºC	38%	20%
10	 d	MeCN	17 h	20%	×[b]
11		DMF	1 h 0 ºC	40%	×[b]
12	 e	DMF	1 h	47%	×[b]
13		DMF	1 h 0 ºC	28%	31%
14	 f	DMF	30 min 0 ºC	ND[c]	×[b]
15	 g	MeCN	1 h	15%	5.7%
16		DMF	30 min 0 ºC	24%	12%[d]

[a] No aislado. [b] Descomposición del producto durante el aislamiento [c] ND → no determinado. [d] La masa detectada para el producto aislado corresponde a la monotioalquilación pero con la hidrólisis de los bromos.

Bioconjugación

De los compuestos que se consiguieron aislar se procedió directamente a la bioconjugación sobre la oxitocina reducida, un peptido que contiene dos cisteinas en su estructura, en una solución amortiguadora a pH = 8.

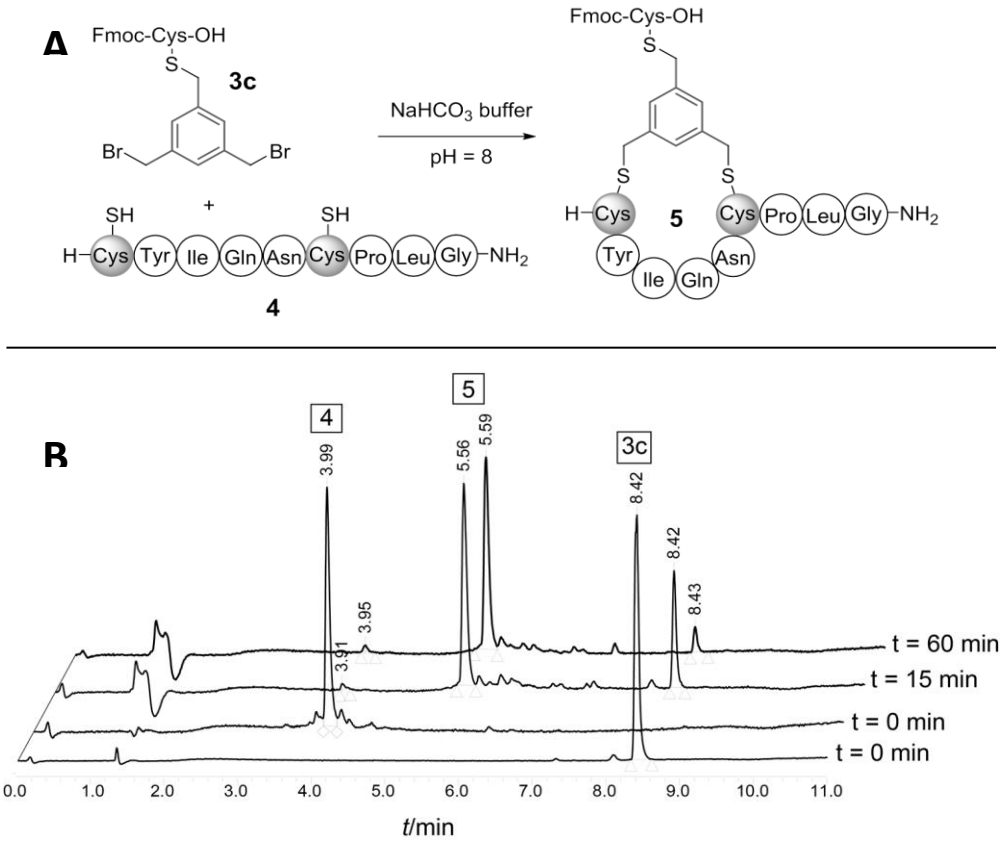
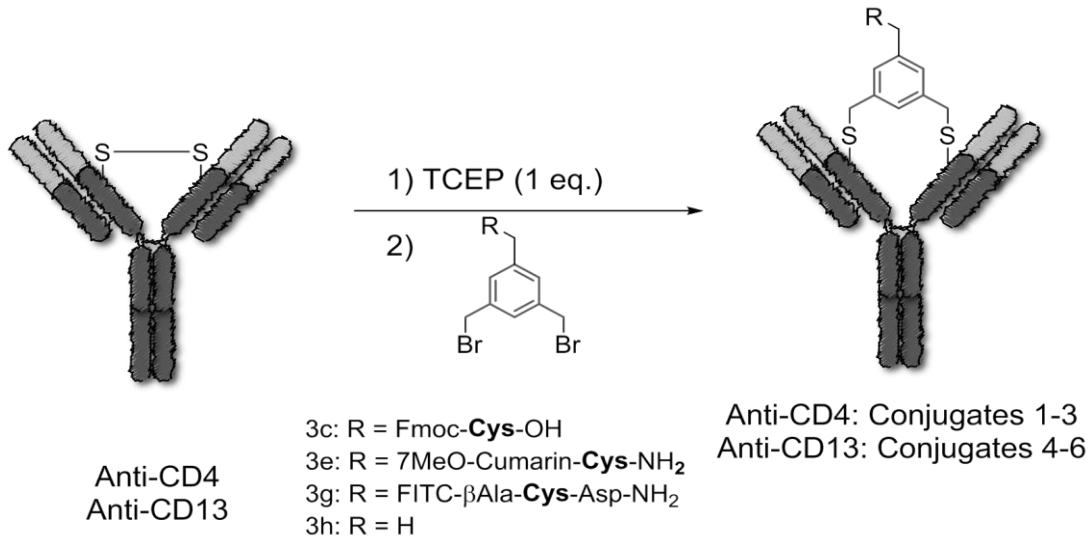


Figura 15. A) Conjugación del derivado de bis(bromometil)benceno 3c a la oxitocina reducida**. B)** Cromatogramas de HPLC de los materiales de partida y el producto final a diferentes tiempos de reacción**.**

Los resultados obtenidos demuestran que la reacción de unión al péptido de forma irreversible tiene lugar en tiempos muy cortos y de forma completa, por lo que se presentan como un tipo de conjugación muy efectiva.

Para la conjugación a anticuerpos se precisa la previa reducción parcial de los puentes disulfuro con el fin de liberar los sulfhidrilos reactivos. Para ello se optimizó la reducción parcial del anticuerpo con TCEP, resultando la más idonea el uso de un equivalente del agente reductor con el fin de no alterar la integridad del anticuerpo.

Sobre las soluciones de los anticuerpos reducidos (anti-CD4 y anti-CD13) se añadieron los conectores preparados **3c, 3e**, y **3g** además del compuesto comercial 1,3-bis(bromometil)benceno **(Esquema 11**) .



Esquema 11. Bioconjugación de los derivados de 1,3-bis(dibromometil)benceno sobre anticuerpos.

Conjugado	Anticuerpo	Compuesto Conjugado	R	eq. de R
1	Anti-CD4	3h	H	1
2a		3c	Fmoc-**Cys**-OH	2
2b				6
2c				46
3		3g	FITC-βAla-**Cys**-Asp-NH_2	2
4a	Anti-CD13	3h	H	2
4b				4
5a		3c	Fmoc-**Cys**-OH	2
5b				4
6a		3e	Cumarin-**Cys**-NH_2	4
6b				4

Los conjugados preparados fueron convenientemente caracterizados vía UV, SDS-PAGE, HPLC y espectrometría de masas (ESI). Desafortunadamente no se consiguió observar conjugación en todos los compuestos y la conjunción de técnicas utilizadas lo corroboró. Sin embargó en los conjugados que sí se observaba conjugación se pudo determinar la cantidad de moléculas introducidas en el anticuerpo. Los ejemplos más claros resultaron los conjugados 3 y 4b, donde se consiguió introducir una molécula del tripéptido FITC-βAla-Cys-Asp-NH_2 y del o-xileno para los conjugados 3 y 4b respectivamente (**Figura 16**).

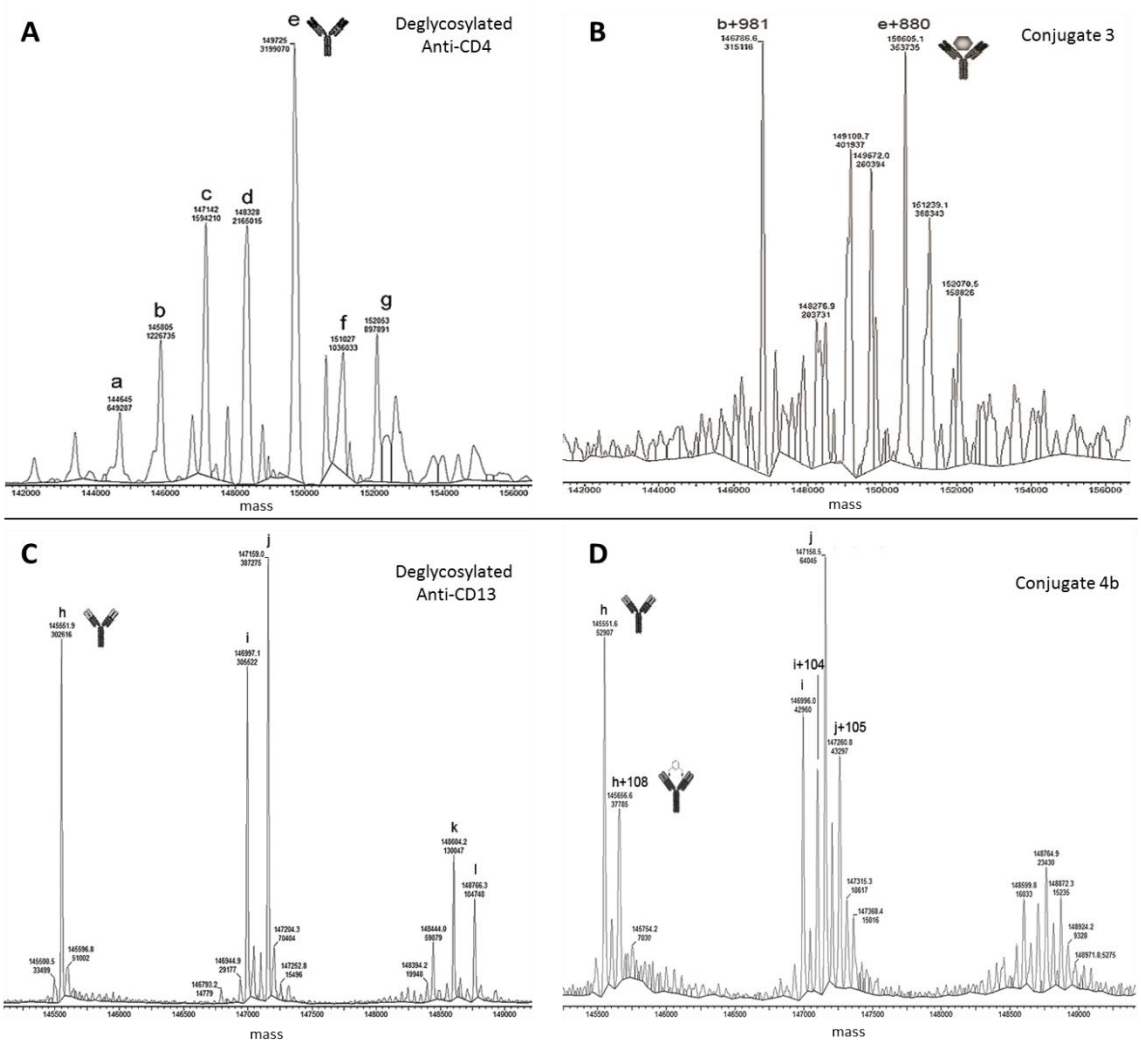


Figura 16. TOF ESI$^+$ para A) anti-CD4, B) conjugado 3, C) anti-CD13 y D) conjugado 4b todos ellos deglicosilados. Para los conjugados 3 y 4b el peso molecular de los compuestos conjugados 3g y 3h son 813.9 y 104.2 Da respectivamente.

Para determinar si este tipo de conjugación vía cisteína afectaba a los anticuerpos, se estudió la afinidad que los conjugados de anticuerpos preparados presentaban frente a células que sobreexpresan el antígeno que los anticuerpos reconocen (CD4: Jurkat Cells y CD13: U937 cells). Las curvas de cuentas de intensidad relativa de fluorescencia (MFI) frente a la concentración de anticuerpo/conjugado, muestran que los anticuerpos solos llegan a un nivel de saturación máximo antes que los conjugados (AC_{50} menor), indicando los conjugados son capaces de unirse ligeramente menos que los anticuerpos solos. Cuando se realiza una reducción completa del anticuerpo antes de la conjugación (conjugado 2c) se observa que este pierde completamente su actividad (**Figura 17, Tabla 10**).

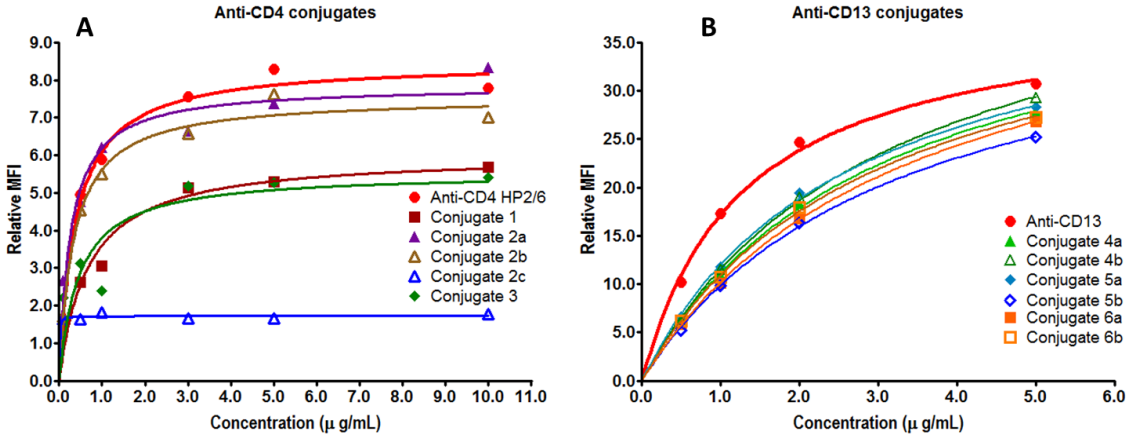
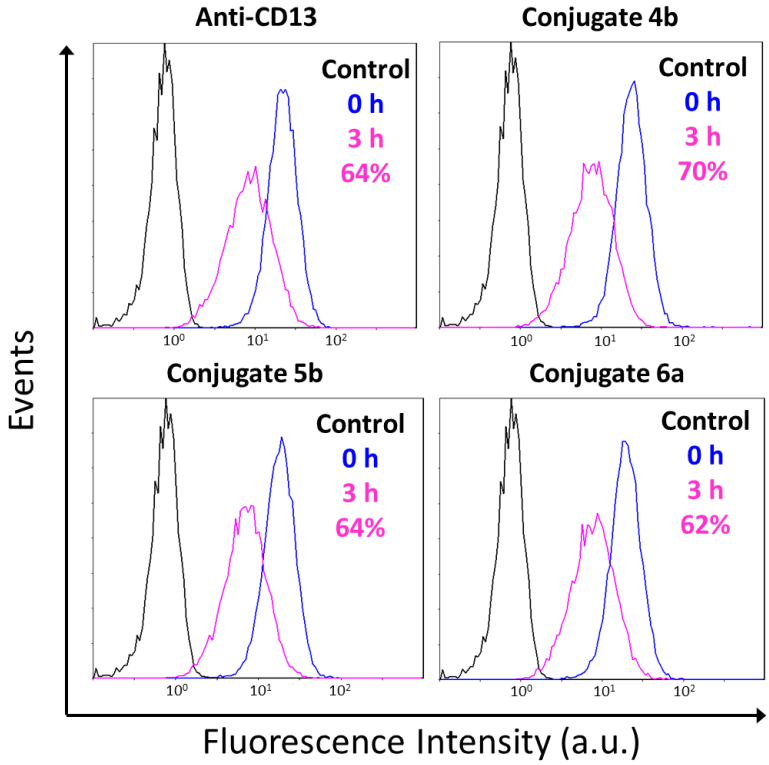


Figura 17. Analisis de afinidad de unión para los conjugados de anticuerpo determinado por citometría de flujo para A) anti-CD4 y sus conjugados 1-3 a células Jurkat y B) anti-CD13 y conjugados 4-6 a las células U937. Ambas líneas celulares fueron incubadas con las concentraciones indicadas de los anticuerpos y sus respectivos conjugados, seguido de la incubación con un anticuerpo marcado con FITC que reconoce la parte constante de los anticuerpos.

Tabla 10. AC_{50} y Max MFI (%) para los anticuerpos anti-CD4 y CD13 y sus respectivos conjugados. (1-6). AC_{50}, calculada como la concentración de anticuerpo necesaria para lograr el 50% del la MFI más elevada para cada conjugado.

	AC_{50} (µg/mL)	Max MFI (%)		AC_{50} (µg/mL)	Max MFI (%)
Anti-CD4	0.38	100	**Anti-CD13**	0.82	100
Conj 1	0.49	57	**Conj 4a**	1.23	90
Conj 2a	0.30	71	**Conj 4b**	1.24	95
Conj 2b	0.36	89	**Conj 5a**	1.16	92
Conj 2c	-	1	**Conj 5b**	1.25	82
Conj 3	0.31	46	**Conj 6a**	1.27	87
			Conj 6b	1.22	89

Además, se estudió la internalización de los conjugados de anti-CD13 con el fin de determinar si el tipo de conjugación además afecta a la función del anticuerpo pasando tras el reconocimiento del antígeno por la endocitosis del complejo anticuerpo-antígeno. Para ello se incubaron los conjugados con la misma línea celular U937 durante 3 h a 37 ºC. Para los conjugados analizados 4b, 5b y 6a se observaron unos resultados de internalización idénticos a los del anticuerpo solo, por lo tanto la internalización no se veía afectada por la vía de conjugación.



	Control	Anti-CD13		Conj 4b		Conj 5b		Conj 6a	
		4 ºC	37 ºC	4 ºC	37 ºC	4 ºC	37 ºC	4 ºC	37 ºC
MFI	0.72	21.2	8.18	23.0	7.41	17.6	6.77	18.9	7.54
$MFI_{control}$-MFI Ab	0	20.49	7.47	22.29	6.70	16.89	6.06	18.19	6.83
% Endocitosis	**0**	**64**		**70**		**64**		**62**	

Figura 18. Endocitosis de anti-CD13 y sus conjugados en la línea celular U937. El promedio de resultados está expresado utilizando la MFI determinada por citometría de flujo. Las células U937 fueron saturadas con anti-CD13 y sus conjugados y se incubaron a 4 ºC y a 37 ºC durante 3 h, donde fue procesado el análisis. La tabla muestra los resultados de MFI para los conjugados 4b, 5b y 6a a 4 ºC y a 37 ºC y el resultante promedio de internalización.

Finalmente para determinar si el conector introducido presenta algún tipo de citotoxicidad, se realizó un estudio de viabilidad celular con los conjugados del anticuerpo anti-CD13 mediante un ensayo de luminiscencia (**Figura 19**) . Para ello las células U937 fueron incubadas durante 72 h a 37 ºC con concentraciones crecientes de anticuerpo y sus conjugados y además se añadió un conjugado anticuerpo-fármaco proporcionado por la empresa PharmaMar.

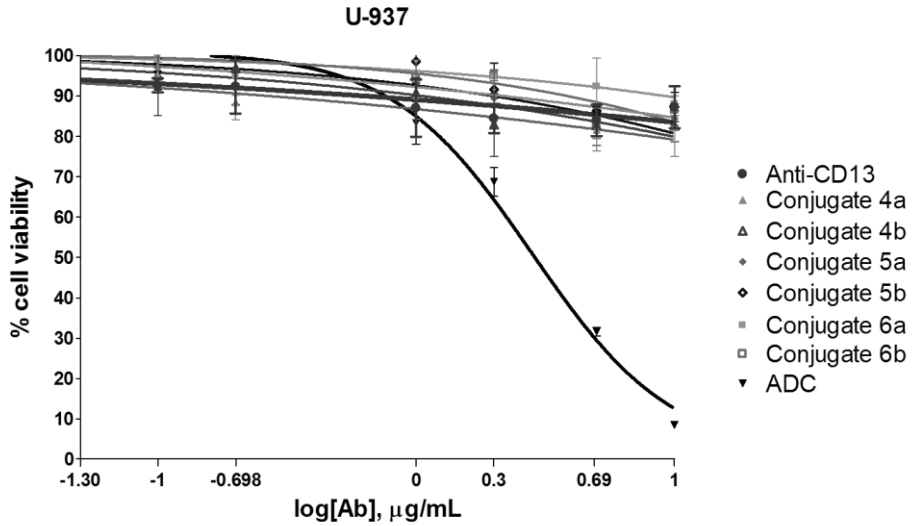


Figura 19. Estudios de viabilidad celular sobre células U-937 durante 72 h.

Como era de esperar, los conjugados preparados de antiCD-13 (4-6) presentaron el mismo comportamiento que el anticuerpo sin conjugar, con lo que nuestros conectores no presentan citotoxicidad. Sin embargo, el conjugado desarrollado por PharmaMar presentó muerte celular en un rango de más del 90%. Aquí se puede demostrar la efectividad de este tipo de conjugados, que resultan ser compuestos anticancerígenos con un elevado potencial farmacológico.

II.1 Conector tipo mesitileno

II.2. Tipo Perfluoroarilo

Introducción

En esta sección se estudiaron las estructuras tipo perfluoroarilos como posibles conectores para bioconjugación. Este tipo de compuestos son característicos por su quimioselectividad frente a sulfhidrílos vía substitución nucleófila aromática (S_NAr) en condiciones suaves,[147] siendo destacable la notoria estabilidad del enlace tipo tioéter aromático formado. Recientemente el grupo de Pentelute realizó interesantes estudios con estos compuestos acerca de la formación de péptidos cíclicos tipo grapa (*stappled peptides*)[148–150] mediante macrociclación peptídica de estos compuestos a partir de las cadenas laterales de cisteínas. Además el mismo grupo estudió su uso como conector siguiendo los principios de la química click (regio y quimioselectividad y fácil formación de enlace) aplicado a proteínas mediante vía enzimática.[51]

Todos estos acontecimientos en relación a los anillos perfluoroaromaticos y a las cadenas laterales de las cisteínas cumplen los requisitos de estabilidad y selectividad para ser utilizados como conectores no hidrolizables en bioconjugación. Especialmente nos resultaron interesantes para aplicarlo a sistemas complejos como lo son los anticuerpos.

Objectivos

- Estudiar la monotioalquilación del decafluorobifenilo y del pentafluorobenceno para su funcionalización y su posterior bioconjugación.
- Ensayar y encontrar las condiciones óptimas de bioconjugación de los tioéteres monoalquilados.
- Usar los tioéteres monoalquilados para la conjugación con anticuerpos y estudiar si las condiciones de conjugación afectan a la integridad del anticuerpo.

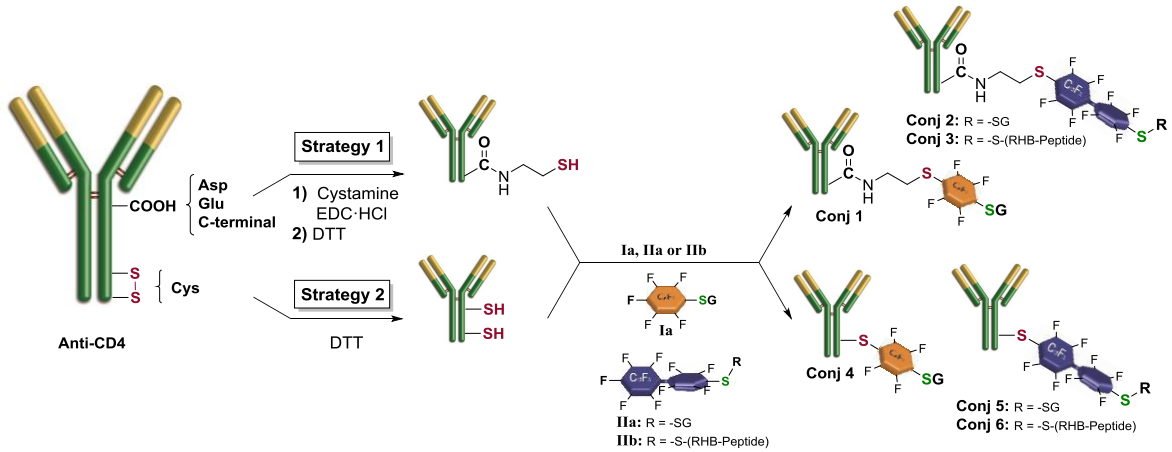
Resultados y discusión

Inicialmente se llevó a cabo la preparación de los correspondientes conectores unidos a la molécula a conjugar. La reacción se llevó a cabo en condiciones básicas utilizando los anillos de hexafluorobenceno y decafluorobifenilo como conectores y la glutatión reducida como molécula a conjugar. En ambos casos la reacción tuvo lugar y se consiguieron aislar los productos monoalquilados pero para el caso del hexafluorobenceno el producto de diadición fue muy mayoritario. Asimismo se preparó un nuevo conector-cargo utilizando el decafluorobifenilo y un péptido de 29 residuos marcado con rodamina.

- **Bioconjugación**

Los compuestos monoalquilados preparados fueron utilizados mediante dos vías diferentes de bioconjugación que involucran grupos sulfhidrilos (**Esquema 12**). La primera de ellas se basa en la previa funcionalización con cisteamina del anticuerpo (anti-CD4) para la

introducción de un grupo tiol. La segunda estrategia conlleva la previa reducción del anticuerpo para la liberación de los grupos sulfhidrilos.



Esquema 12. Estrategias de conjugación de derivados perfluoarilados a anticuerpos.

Los 6 conjugados preparados se caracterizaron mediante UV, SDS-PAGE y espectrometría de masas. Lamentablemente, se observaron unos indicios poco consistentes de que la bioconjugación había tenido lugar. El análisis por masas revelaba un incremento respecto al anti-CD4 sin conjugar, pero no era la masa correspondiente al compuesto conjugado (**Figura 20**).

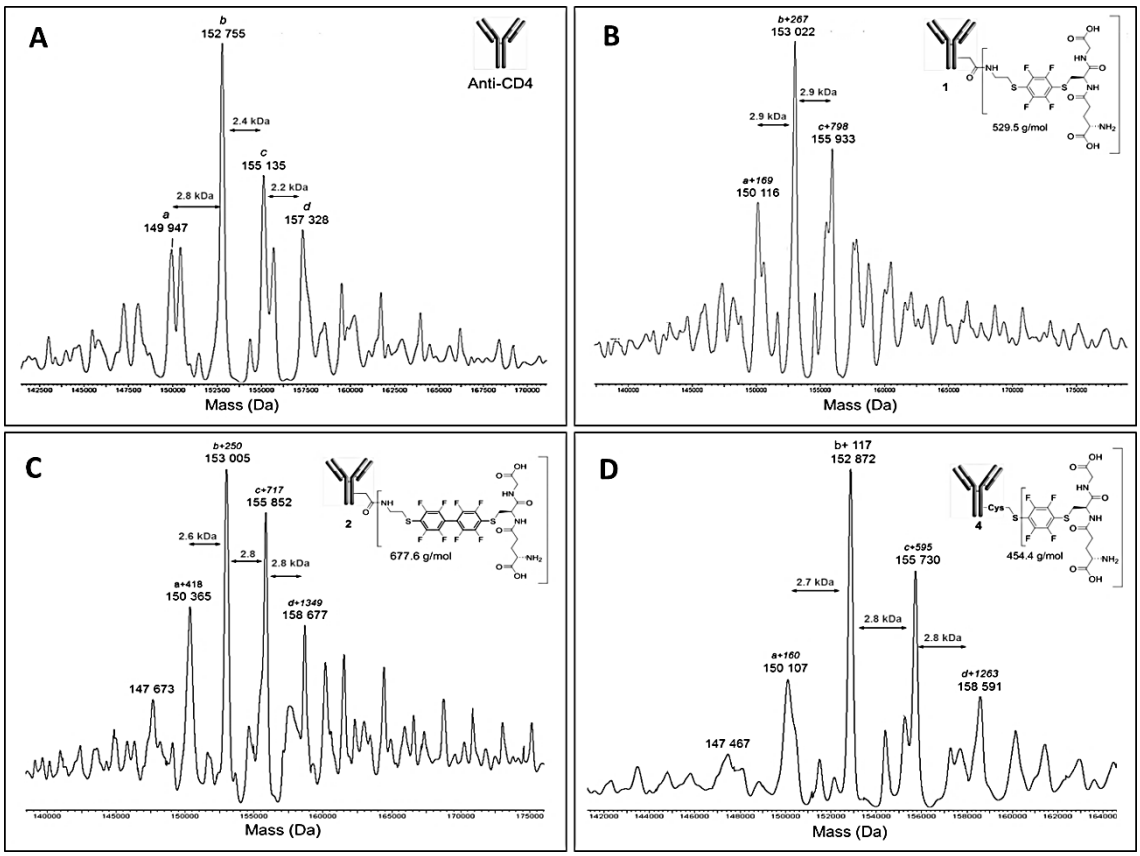


Figura 20. A,B,C,D corresponde a el análisis de masas (TOF ESI^+) de anti-CD4, conjugados 1, 2 y 4 respectivamente.

Pese a no observarse claramente la bioconjugación, se decidió realizar los ensayos de afinidad de antígeno-anticuerpo con los conjugados preparados con el fin de determinar si dicho tratamientos anulaban o no el efecto de los conjugados. Para ello se incubaron los conjugados a diferentes concentraciones con células del tipo Jurkat que sobreexpresan CD4 en superficie y se analizó mediante citometría de flujo (**Figura 21**). Tal y como muestran los resultados obtenidos mediante citometría de flujo la afinidad de los conjugados no se ve totalmente afectada por las modificaciones realizadas para la conjugación. Los valores de AC_{50} (siendo el de anti-CD4 el de menor valor) muestran que los conjugados no pierden su actividad, sino que simplemente reducen ligeramente su afinidad (**Tabla 11**).

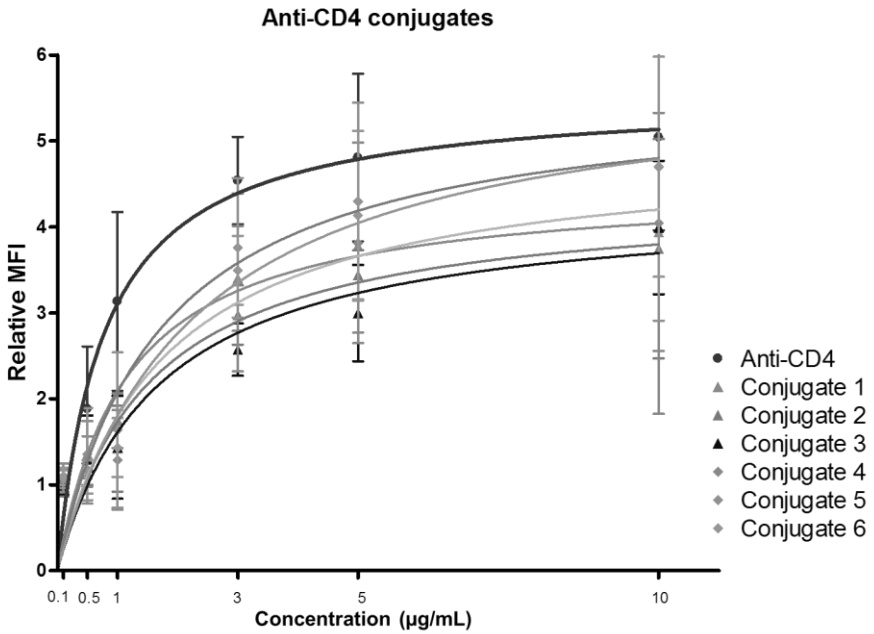


Figura 21. Análisis de la unión antígeno-anticuerpo anti-CD4 HP2/6 y los diferentes conjugados en células tipo Jurkat T por citometría de flujo. Las células Jurkat T fueron incubadas con las concentraciones indicadas de los conjugados 1-6, seguido de la incubación con anticuerpos marcados con FITC del tipo goat anti-mouse IgG. Seguidamente se analizó mediante citometría de flujo y se calculó la relación de cuentas de intensidad de fluorescencia.

Tabla 11. Afinidad de los conjugados, indicado como concentración de conjugado requerida para obtener la mitad del máximo de la máxima intensidad de fluorescencia (MFI) y el porcentaje de unión relativa al anti-CD4.

	AC_{50} (µg/ml)	**max MFI %**
Anti-CD4 mAb	0.95	100
Conjugado 1	1.65	73
Conjugado 2	2.20	62
Conjugado 3	2.65	74
Conjugado 4	2.55	75
Conjugado 5	2.02	91
Conjugado 6	2.37	91

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Conclusiones Generales

Conclusiones Generales

Capítulo I. Conectores hidrolizables

1. Conectores tipo Ugi

Se consiguió sintetizar un número elevado de compuestos de Ugi para su posterior estudio de labilidad en condiciones ácidas.

Se logró la modificación del extremo C-terminal utilizando aminas primarias de diferente naturaleza con éxito, la cual cosa permitirá la unión de fármacos citotóxicos que contengas grupos amino reactivos.

La optimización de las desprotecciones del extremo N-terminal ha permitido la obtención de ácidos carboxílicos con elevados rendimientos y crudos de elevada pureza para su preparación, en forma de éster activo, con el fin de utilizarlos en la bioconjugación vía amina.

Se estudió la estabilidad y labilidad de los complejos de Ugi preparados y además se logró exitosamente la conjugación a sistemas biológicos tales como péptidos, proteínas y anticuerpos.

Los conjugados preparados con anticuerpos no presentaron alteraciones biológicas de reconocimiento ya que se observó que no perdían la actividad por el antígeno del cual los anticuerpos son selectivos (Anti-CD4). Asi mismo, PharmaMar consiguió preparar ADCs con un elevado potencial para tratamientos contra el cáncer debido a la selectividad que estos complejos presentan *in vitro e in vivo.*

2. Conectores tipo Thp

Se abordó la introducción del tetrahidropirano sobre alcoholes, tioles y aminas. Exitosamente se optimizó la incorporación sobre el tiol y el hidroxilo de la cisteína y de la serina, respectivamente. Además se estudió la labilidad de los enlaces tipo acetal y tioacetal en diferentes medios ácidos. La desprotección del Thp en Ser tuvo lugar en condiciones acidolíticas idóneas para su uso en bioconjugación pero el echo de que ya haya sido estudiado con anterioridad nos hizo abandonar su utilización para tal fin. El enlace tipo tioacetal presentó estabilidad en condiciones acidolíticas suaves y resultó escindible en condiciones ácidas más enérgicas. De esta forma se decidió desestimar su uso como conector en bioconjugación y centrarnos en su desarrollo como potencial grupo protector de Cys en química de péptidos.

3. Thp como protector de cisteína

La protección de la Cys en forma de tioacetal con el tetrahidropirano fue posible con rendimientos muy elevados. Este protector presenta estabilidad en concentraciones muy bajas de ácido (1% TFA) y es lábil a mayor concentraciones de ácido en presencia de capturador de carbocationes. El uso del tetrahidropirano como protector de la cisteína, permitió con muy buenos resultados la preparación de tripéptidos con la Cys ubicada en el extremo C-terminal o en el interior de una secuencia, así mismo en el extremo N-terminal para péptidos de mayor tamaño. Los niveles de racemización obtenidos en la preparación de tripéptidos resultan mucho menores que los grupos protectores convencionales tales como el Trt y el Dpm. A su vez, la formación del aducto indeseado con la piperidina tras la formación de la dehidroalanina también se veía reducida en utilizar el Thp en la cadena lateral de la Cys. El Thp resultó idóneo para su uso como protector de Cys en síntesis de péptidos en fase sólida.

Capítulo II. Conectores no-hidrolizables

1. Conectores tipo mesitileno

Se abordó la monotioalquilación del 1,3,5-tris(bromometil)benceno con tioles de diversa índole. Las conversiones resultaron aceptables, pero en el aislamiento del producto final la descomposición parcial del producto tenía lugar disminuyendo los rendimientos finales.

Los compuestos tipo 1,3-bis(bromometil)benceno que se consiguieron aislar, se conjugaron a la oxitocina reducida y a anticuerpos monoclonales reducidos exitosamente.

Finalmente sobre los conjugados preparados se evaluó si el tipo de conjugación afectaba a diversos aspectos biológicos tales como la afinidad antígeno-anticuerpo, la internalización de los anticuerpos y la viabilidad celular.

Los estudios preliminares realizados con los conectores tipo mesitileno, los posicionan como unos conectores prometedores para la bioconjugación en general y para los conjugados anticuerpo-fármaco en particular.

2. Conectores tipo perfluoroarilo

Se estudió la conjugación a anticuerpos mediante dos vías que involucran tioles reactivos con perfluoroarilos (hexafluorobenceno y decafluorobifenilo). En el caso del decafluorobifenilo la preparación del conector-cargo tuvo lugar con mejor rendimiento que

para el hexafluorobenceno en el que como producto mayoritario se obtiene producto de diadición.

De los compuestos preparados se abordaron dos metodologías de bioconjugación, observándose cambios en los conjugados pero sin poder determinarse claramente la cantidad de compuesto conjugado a los anticuerpos por mala resolución de los cromatogramas. Ambas metodologías abordadas no presentaron perdida de actividad de los conjugados mediante estudios de citometría de flujo.

Annexes

ANNEXES

Annex I. Materials and methods

Pruducts and comercial source: Commercial products and solvents were used as received without further purification. A widespread part of products were from Sigma-Aldrich. All the Fmoc and Boc-amino acids and resins were purchased from Iris Biotech (Marktredwitz, Germany). Solvents for HPLC and Peptide synthesis were from Scharlau SDS (Peypin, France). SDS-PAGE solvents and stants were from Bio-Rad laboratories GmbH (München, Germany). Dialysis membranes were from Spectrum Laboratories, Inc. (Los Angeles, CA). Pre-packed Shephadex™ G-25 containing columns were from GE Healthcare Europe GmbH (Freiburg, Germany). PNGase F was from New England BioLabs Inc.

The HP2/6 hybridoma producing anti-CD4 mAb (IgG2a) was produced in Professor Francisco Sanchez Madrid laboratory's (Hospital Universitario de la Princesa, Madrid, Spain) and it was obtained in a fusion with splenocytes of mice immunized with the human T cell leukemia cell line JM, as previously described.[1] The anti-CD4 HP2/8 hybridoma and the Jurkat cell line were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; Life Technologies, Madrid, Spain), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 ºC in a humidified atmosphere containing 5% CO_2.

Anti-CD13 was provided from Dr. J.M. Dominguez (PharmaMar Company) in PBS solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH = 7.4) at 7.5 mg/mL.

Solid-phase peptide synthesis (SPPS): 2, 5, 10 and 20 mL polypropylene syringes with a porous disc-containing were used for the solid-phase peptide synthesis as reactors. At short reaction times (2 – 5 min), the reactions were manually stirred with a Teflon stick, meanwhile for long reactions times (>30 min) the reactions were stirred on a Unimax 1010 shaker from Heidolph instruments. Solvents were removed from the reactor by vacuum suction. All the reactions were carried out at room temperature (~25 ºC). The Fmoc protecting group elimination was performed by three treatments of piperidine/DMF (1:4) (1×1 min + 2×5 min). Every reaction step was followed of washings of the peptide-resin with DMF (4×1 min) and DCM (4×1min). Peptides were precipitated by ultracentrifugation using an Allegra™ 21 R centrifuge from Beckman Coulter™.

Automatic SPPS was carried out in CEM Liberty Blue™ Microwave Peptide Synthesizer. The coupling reactions were carried out with Fmoc-*L*-amino acids (0.2 M), *N*,*N*,*N*',*N*'-tetramethyl-*O*-(1H-benzotriazol-1-yl)uranium hexafluorophosphate (HBTU) (0.5 M) and *N*,*N*-diisopropylethylamine

(DIEA) (2 M) in DMF. First of all the coupling reaction was heated at 75 ºC (170 W) during 20 s and then at 90 ºC (50W) during 3 min. The Fmoc group was removed by treatments with piperidine/*N*,*N*-dimethylformamide (DMF) (20:80, *v/v*) first 30 s at 75 ºC (155 W) and then 90 s at 90 ºC (30 W).

Analysis and purification: HPLC reversed-phase column Xbridge™ BEH130 C18 3.5 μm (4.6×100 mm), from Waters (Ireland) was used to analyze the synthetized peptides. The analytical HPLC was carried out on Waters instrument comprising two solvent-delivery pumps (Waters 1525), an automatic injector (Waters 717 auto sampler), diode array wavelength detector (Waters 2487) and linear gradients of MeCN (+0.036% TFA) into H_2O (+0.045% TFA) were run at flow rate of 1 mL/min. The average in the chromatograms was determined by the area integration of the chromatographic peaks at λ = 220 nm.

Acquity UPLC BEH C18 Column, 130 Å, 1.7 μm, 2.1 mm x 100 mm) from Waters was used for UPLC analysis. The analytical UPLC was carried out on Aquity H-class System from Waters comprising a Quaternary solvent manager, a sample manager with Flow-Through Needle (SM-FTN) design and Photodiode array (PDA) eλ detector. Linear gradients of MeCN (+0.036% TFA) into H_2O (+0.045% TFA) were run at 0.61 mL/min over 2 min. The integration of the chromatographic peaks at λ = 220 nm determined the average in the chromatograms.

HPLC-ESMS reversed-phase column SunFire™ C18 3.5 μm (2.1×100 mm) was from Waters (Ireland) and it was performed on a Waters instrument comprising two solvent-delivery pumps (Waters 2695 separation module) with an automatic injector and photodiode array wavelength detector (Waters 2998) and Waters Micromass ZQ spectrometer. Linear gradients of MeCN (+0.07% formic acid) into H_2O (+0.1% formic acid) were run at flow rate of 0.3 mL/min. The solvents for HPLC were H_2O Milli-Q, which was filtered through a Milli-Q Plus (Millipore) system with 0.45 μm of pore (resistivity over to 18 $M\Omega \cdot cm^{-1}$), and MeCN (HPLC quality).

Semi-preparative HPLC-MS colum SunFire™ Prep C18 OBD™ 5 μm (19×100 mm) reversed-phase HPLC analytical column was acquired from Waters (Ireland). Semi- preparative HPLC-MS was performed on Waters instrument comprising a 2545 gradient module equipped with a sample manager module with automatic injector and fraction collector (Waters Alliance 2767). The UV detection was performed on Waters 2487 UV-visible dual λ absorbance detector connected to Waters Micromass ZQ mass detector. Linear gradients of MeCN (+0.1% TFA) into H_2O (+0.1% TFA) were run at a flow rate of 25 $mL \cdot min^{-1}$ over 15 min.

I. Materials and methods

Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF) was carried out on a 4700 proteomics analyzer from Applied Biosystems.

Electro spray ionization mass analysis (ESI-MS): The conjugates mass analyses were performed in Micromass LCT-Premier mass spectrometer from Waters equipped with an Acquity UPLC Binary Sol MGR chromatograph with an Acquity UPLC Autosampler MO from Waters Corporation. Previously to the mass detection, the samples were separated into BioSuite pPhenyl 1000RPC 2.0 x 75 mm; 10µm column from Waters. Linear gradients from 95:5 to 20:80 of H_2O/MeCN were run during 60 min using MeCN UPLC quality (+1% formic acid) into MiliQ H_2O (+1% formic acid) at a flow rate of 100 µL/min. The column outlet was directly introduced into the electrospray ionization (ESI) source (Micromass LCT-Premier).

NMR spectra was recorded on a Varian Mercury-400 (^{1}H 400 MHz, ^{13}C 100 MHz and ^{19}F 376 MHz). ^{1}H is reported as follows: chemical shift (δ ppm), [integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (*J* in Hz)]. Data for ^{13}C NMR are reported in terms of chemical shift. NMR spectra were referenced by tetramethylsilane (TMS) and it took as an internal reference the signal for the deuterated solvent that was used in each particular case.

Anti-CD4 and conjugates concentration was determined by **NanoDrop ND-1000** (Thermo-Fischer) spectrophotometer analyzing 1 µL of sample at λ = 280 nm.

The **automatic purification** was performed on CombiFlash® Rf TELEDYNE ISCO by using pre-packed Redisep® Rf Gold 50 g HPC18 column from Teledyne Technology Company. Solid samples were charged into C18 silica and eluted into the column by linear gradients of MeCN (+0.036% TFA) into H_2O (+0.045% TFA) which were run at a flow rate of 40 mL/min. UV detection was at 220 nm.

Antibody purification: The antibody conjugates was purified by dialysis when the solution contain more than 1 mL of buffer and by Shephadex™ columns when the quantity is lower. The dialysis was carried out introducing the solution of protein to purify into Spectra/Por® Dialysis Membrane MWCO: 12-14,000 and dialyzing during 3 days in front of 2 L of the desired buffer in which we want the purified protein, in that time, the buffer was changed twice a day. The following pre-packed Shephadex™ G-25 containing columns from GE Healthcare were used for antibody purification and/or antibody buffer exchange: PD-10 Desalting Columns (1-2.5 mL of sample), PD MiniTrap™ (0.1-0.5 mL). For antibody conjugate purification, the convenient pre-packed Shephadex™ G-25 column was equilibrated with the indicated buffer or water. Then the conjugate crude (0.1-1 mL) was charged into the column and by gravity the sample was eluted through the column with the corresponding buffer (1-3.5 mL). Then the fractions was collected into Eppendorfs (0.25-0.5 mL) and

then the fractions concentration was determined by UV analysis using Nanodrop. When is needed, the proteins were concentrated using Vivaspin 500 of 50 kDa MWCO from GE Healthcare Europe GmbH (Freiburg, Germany) during 15 min at 5 ºC and 13.000 rpm

Gel Electrophoresis: The conjugates were characterized on 4-20% or 10% polyacrylamide precast Mini-PROTEAN® TGX™ Gels (SDS−PAGE). Previously, the samples (5 µL, 1 mg/mL approx.) were treated with reducing loading buffer (5 µL) and heated at 95 °C for 5 min and, after that, the gel was run at 150 V. The reduced antibody analysis results in two distinct bands corresponding to light and heavy chains. PageRuler™ Unstained Protein Ladder from Bio-Rad (10-250 kDa) was used as standard.

Deglycosilation: Antibody deglycosylations were performed by treating the corresponding antibody-conjugate (1-20 µg) with PNGase F (1 µL, 500 000 U/mL) in PBS at pH = 7.4 and incubating at 37 ºC during 17 h. After that time, the samples were dialyzed with Slide-A-Lyzer™ MINI Dialysis Device, 10K MWCO over $NH_4(C_2H_3O_2)$ (50 mM) and then were analyzed by mass spectrometry.

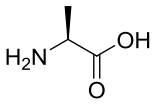
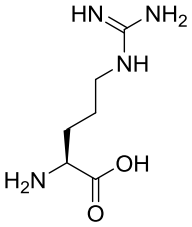
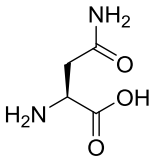
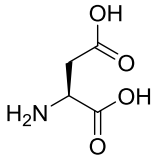
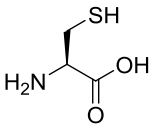
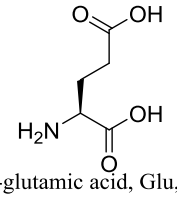
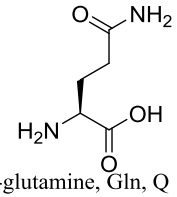
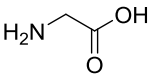
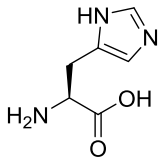
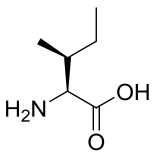
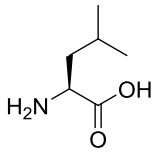
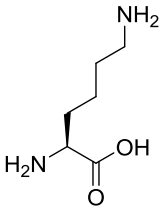
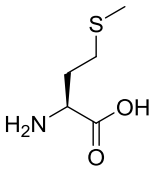
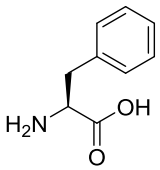
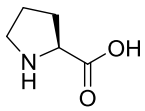
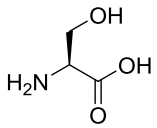
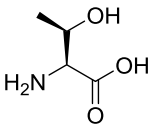
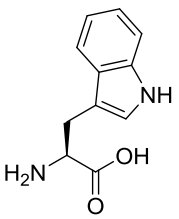
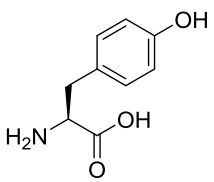
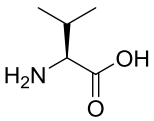
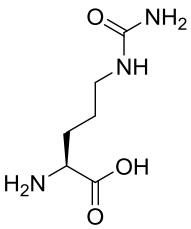
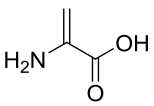
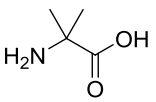
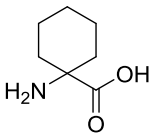
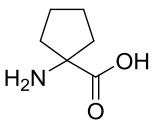
Flow cytometry analysis: Flow cytometry analysis was performed in a FACSCanto II cytometer and data were analyzed on the FACSDiva software (both from BD Biosciences). For data acquisition, 488 nm wavelength excitation laser was used and 10 000 events for each sample were collected.

Hybridoma supernatant production and antibody purification: The CD4 mAb HP2/6 hybridoma cells were cultured at high density in a CELLine CL 350 bioreactor (Integra) in RPMI 1640 medium containing 10% fetal calf serum (FSC) 2 mM glutamine, 50 U/ml penicillin and 50 µg/m streptomycin at 37 ºC in a humidified atmosphere containing 5% CO_2. Hybridoma culture supernatants were collected and the HP2/6 mAb was purified using protein-G sepharose affinity chromatography using standard protocols. Anti-CD4 was stored into saline solution (2 mg/mL) and it was dialyzed in front of the buffer in which the conjugation was performed.

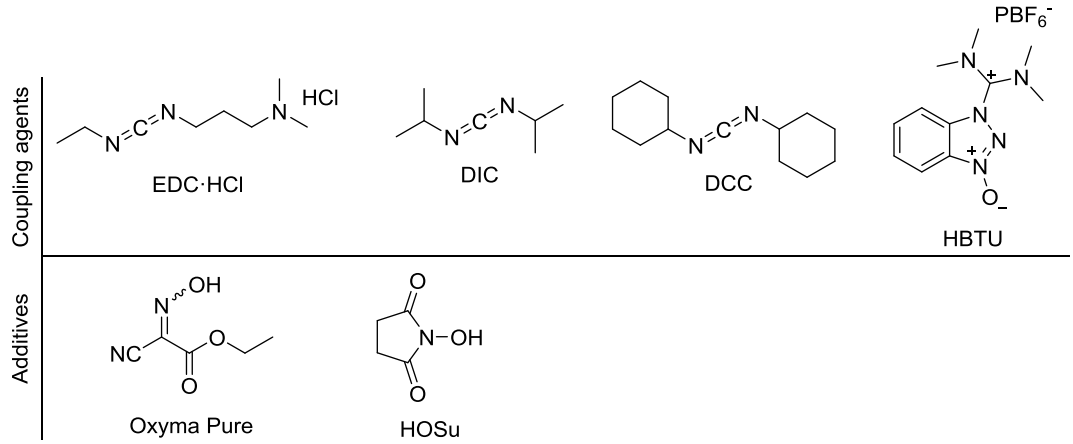
Flow cytometry analysis

The experiments were analyzed by fluorescence-activated cell sorting (FACS) using a FACSCanto™ II cytometer (BD biosciences). Data were analyzed on FACSDiva software (BD biosciences). For data acquisition, 488 nm wavelength excitation laser and 10.000 events for each sample were collected.

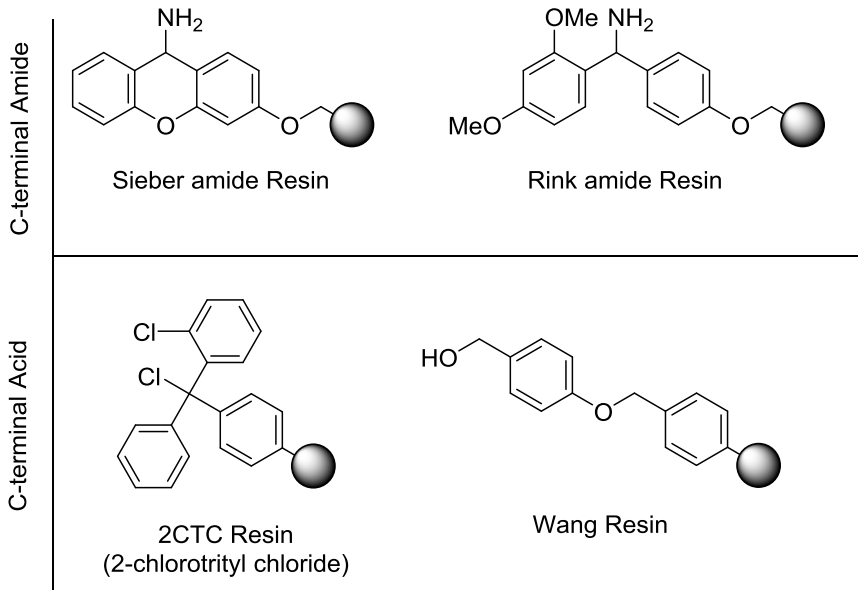
Annex II. Amino acids

 L-alanine, Ala, A	 L-arginine, Arg, R	 L-asparagine, Asn, N	 L-aspartic acid, Asp, D	 L-cysteine, Cys, C
 L-glutamic acid, Glu, E	 L-glutamine, Gln, Q	 glycine, Gly, G	 L-histidine, His, H	 L-isoleucine, Ile, I
 L-leucine, Leu, L	 L-lysine, Lys, K	 L-metionine, Met, M	 L-phenylalanine, Phe, F	 L-proline, Pro, P
 L-serine, Ser, S	 L-threonine, Thr, T	 L-tryptophan, Trp, W	 L-tyrosine, Tyr, Y	 L-valine, Val, V
 L-citrulline, Cit	 dehidroalanine, Dha	 aminoisobutiric acid, Aib	 homocycloleucine, ACHC	 cycloleucine, *c*Leu

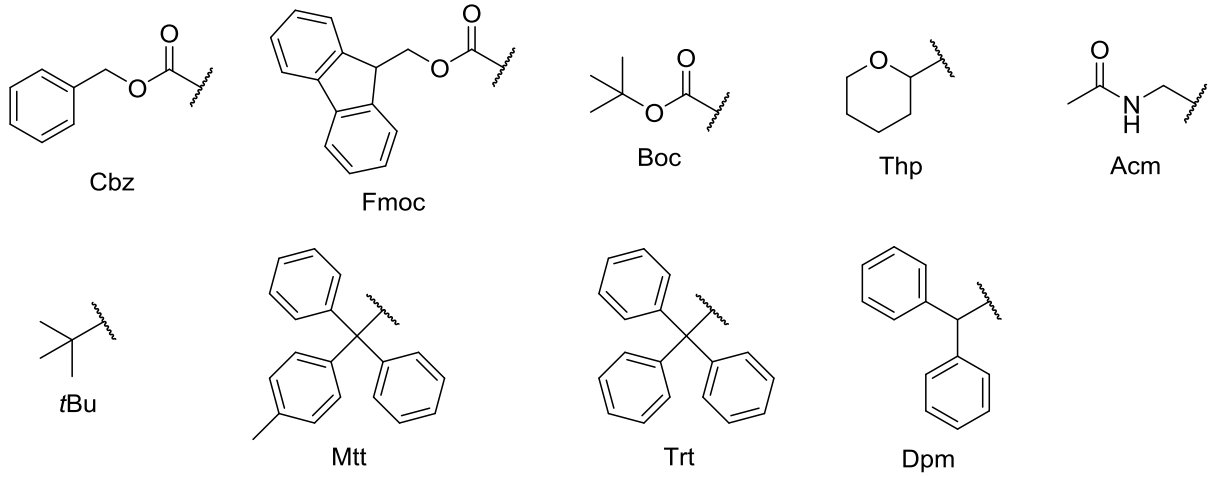
Annex III. Coupling Reagents



Annex IV. Resins and linkers



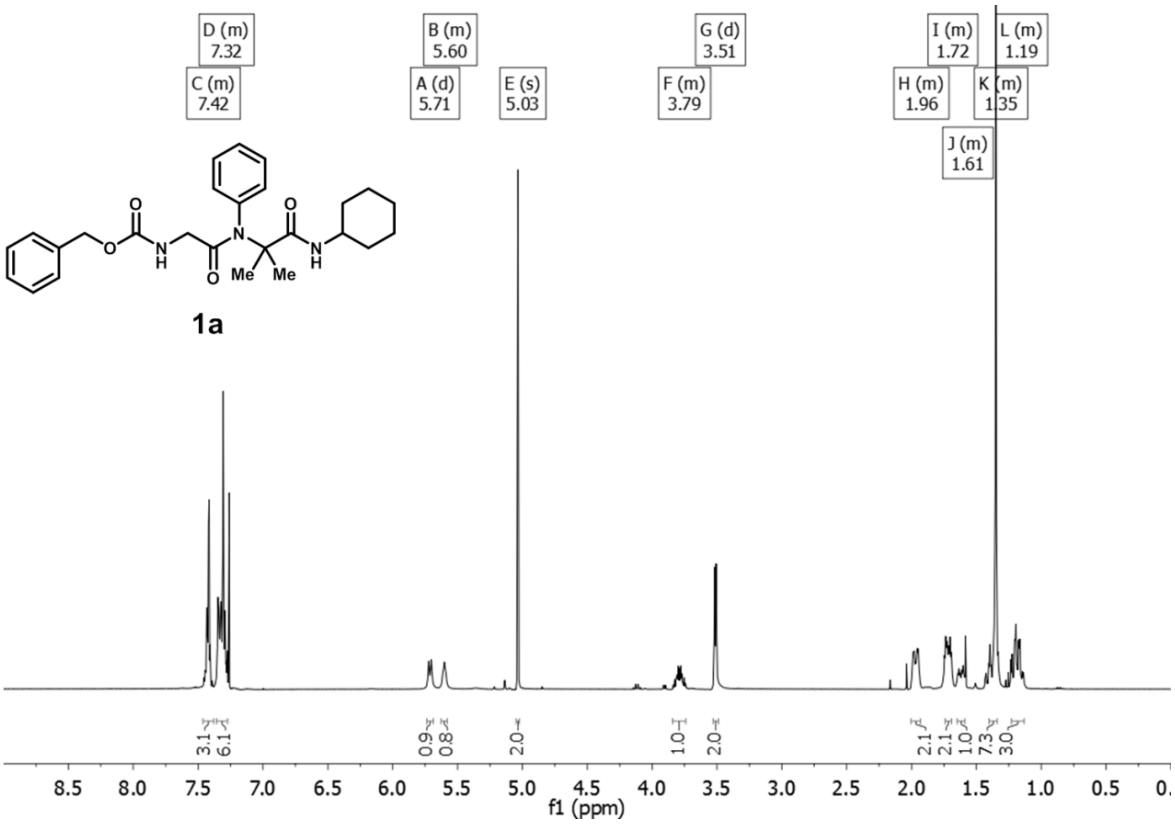
Annex V. Protecting groups



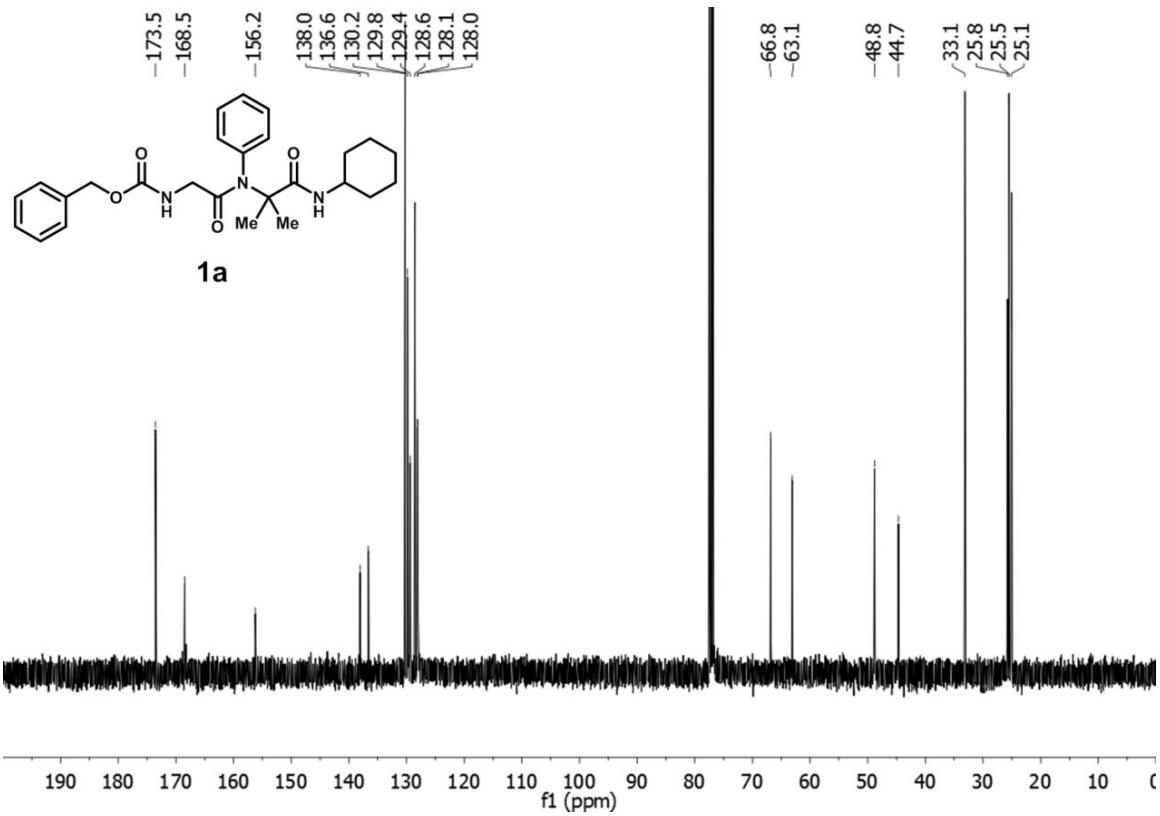
NMR data

Chapter I.1 Ugi Linkers

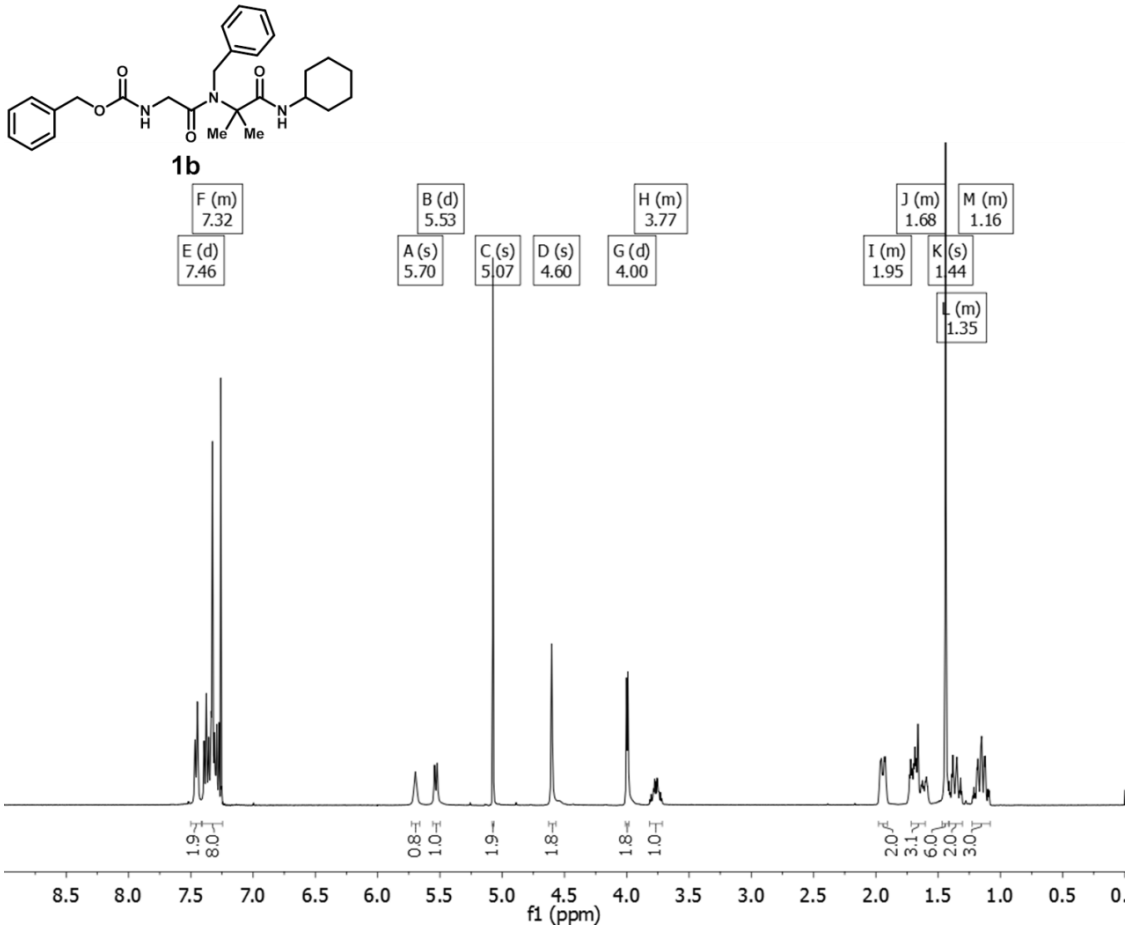
^{1}H NMR (400 MHz, $CDCl_3$): **1a**



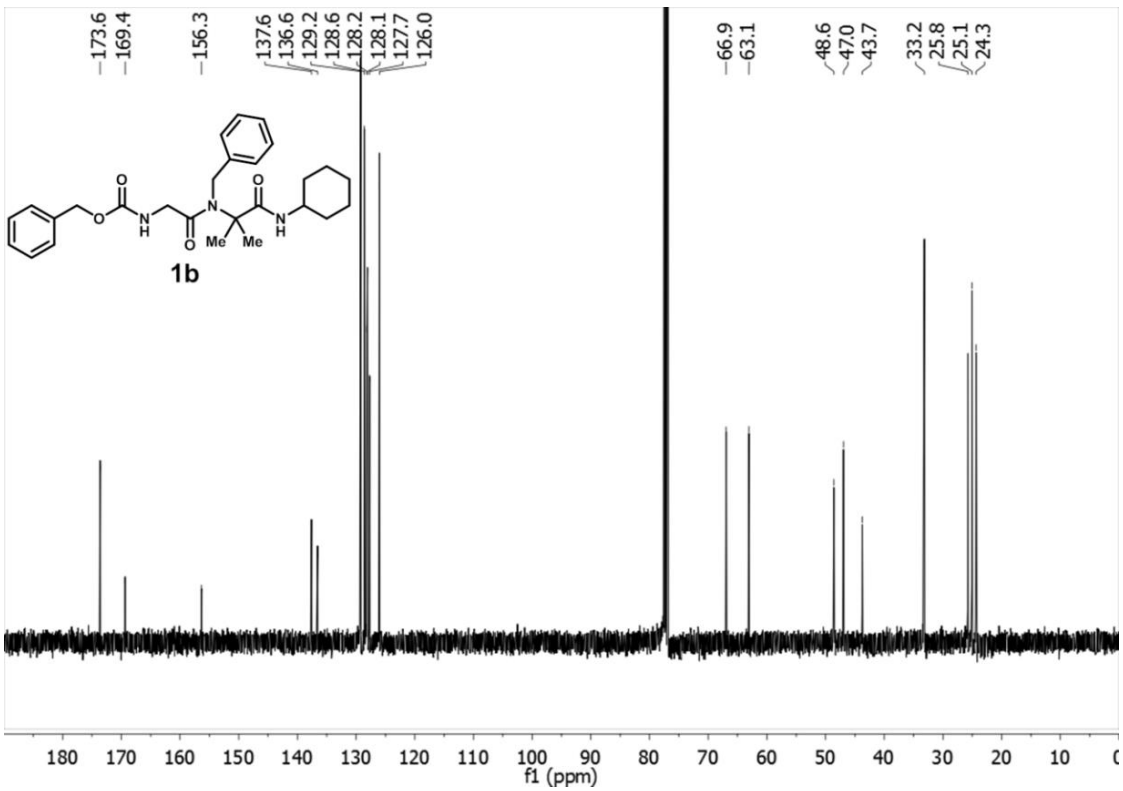
^{13}C NMR (100 MHz, $CDCl_3$): **1a**



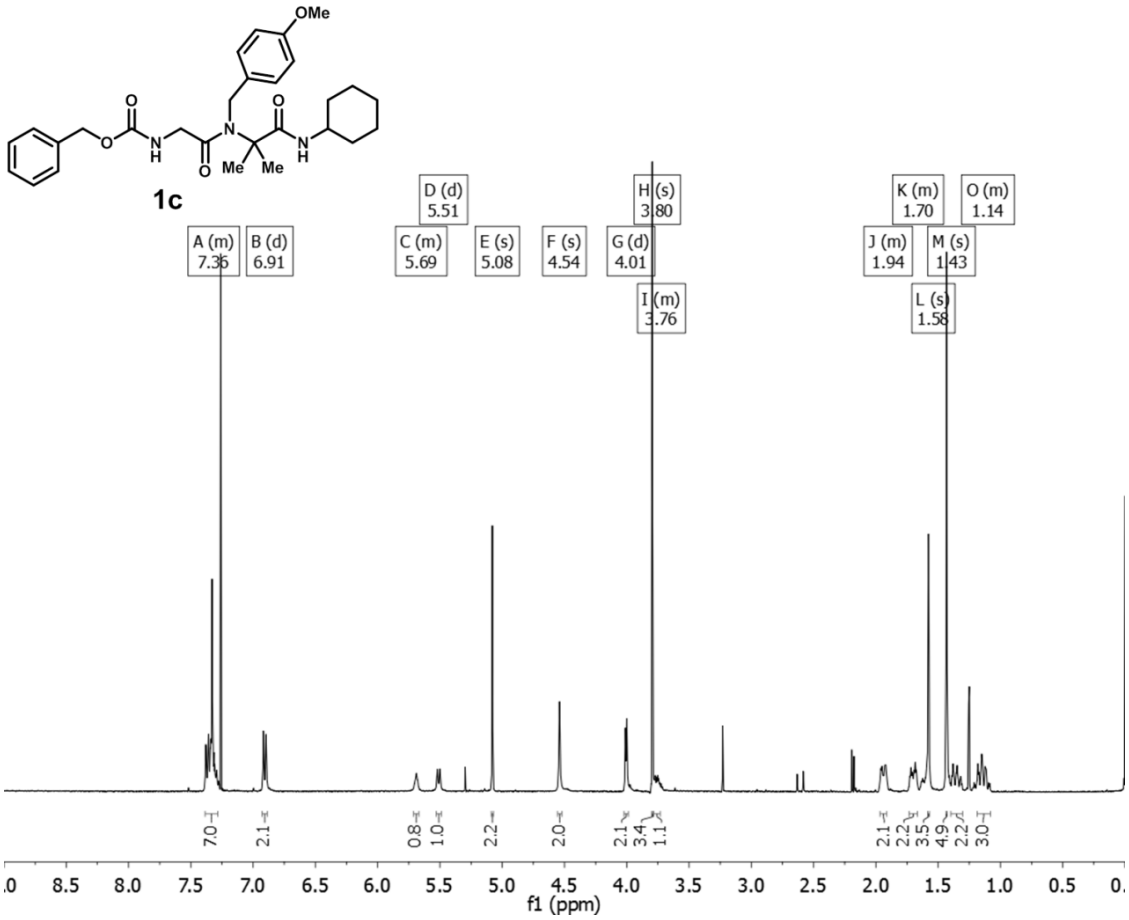
^{1}H NMR (400 MHz, $CDCl_3$): **1b**



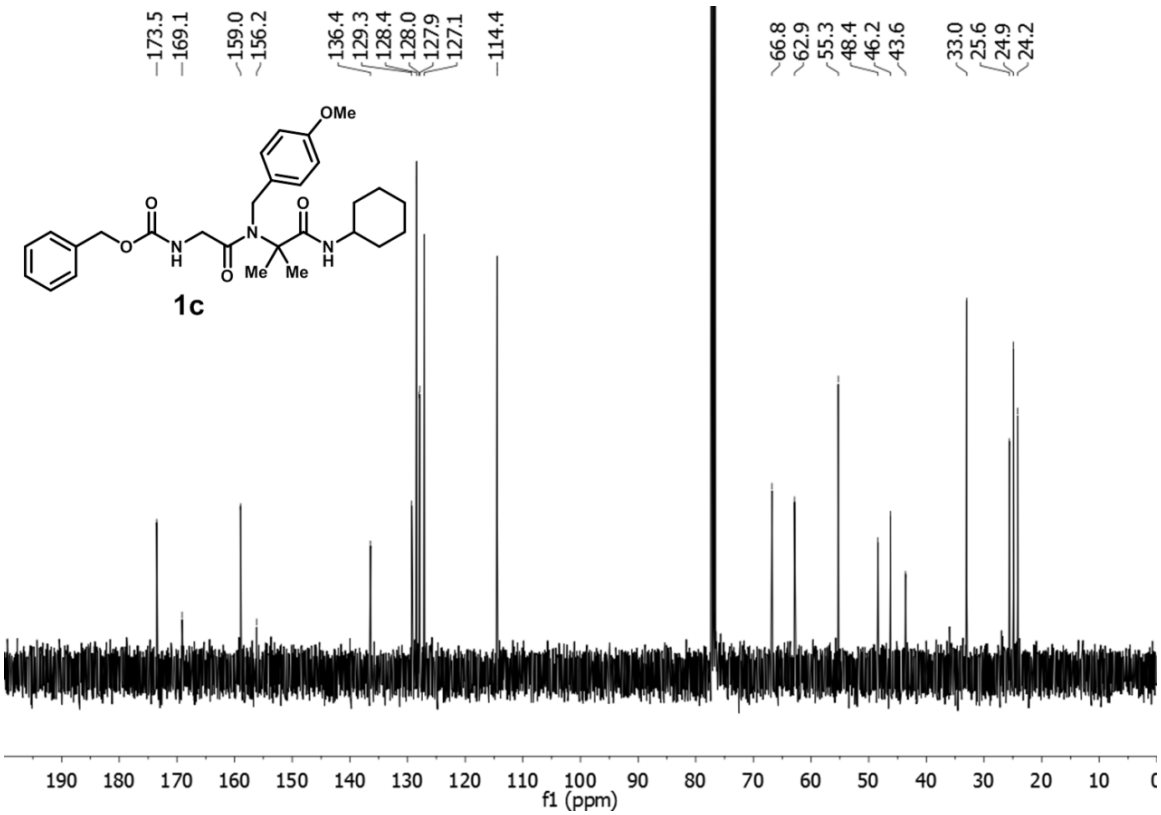
^{13}C NMR (100 MHz, $CDCl_3$): **1b**



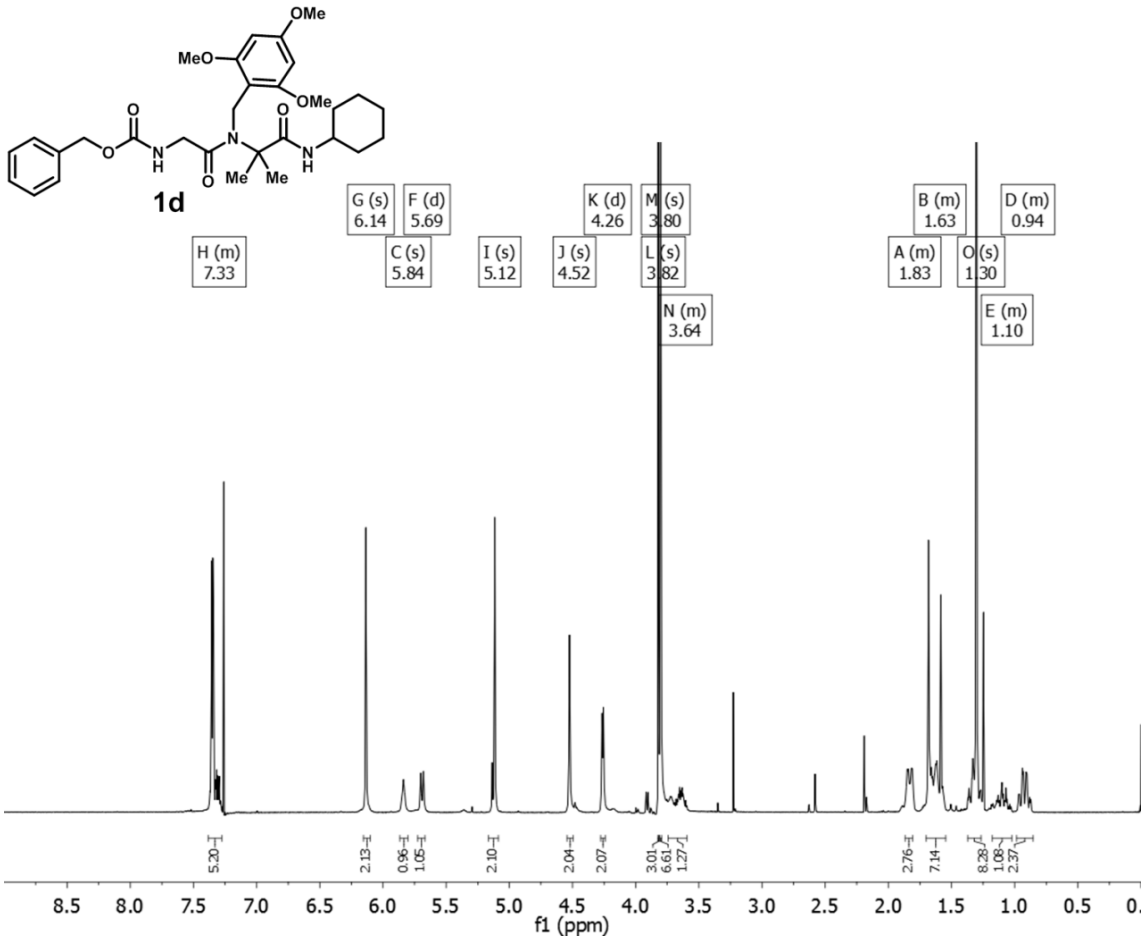
^{1}H NMR (400 MHz, $CDCl_3$): **1c**



^{13}C NMR (100 MHz, $CDCl_3$): **1c**



^{1}H NMR (400 MHz, $CDCl_3$): **1d**



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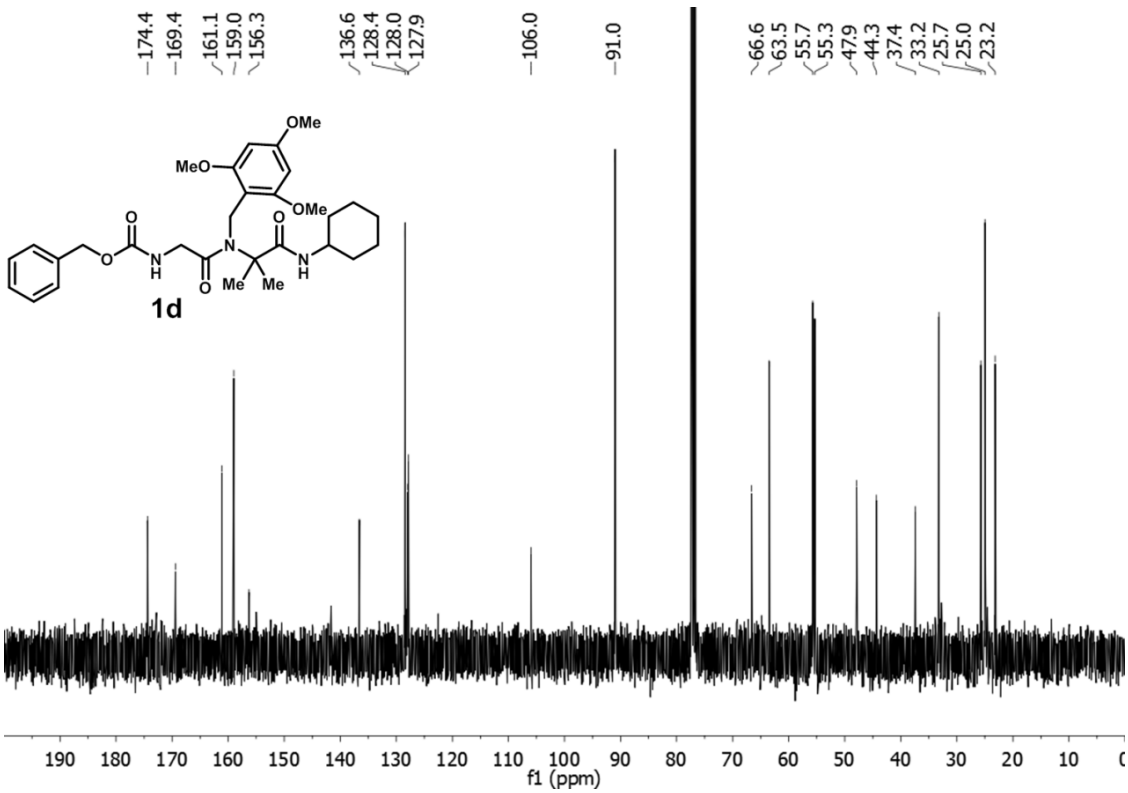

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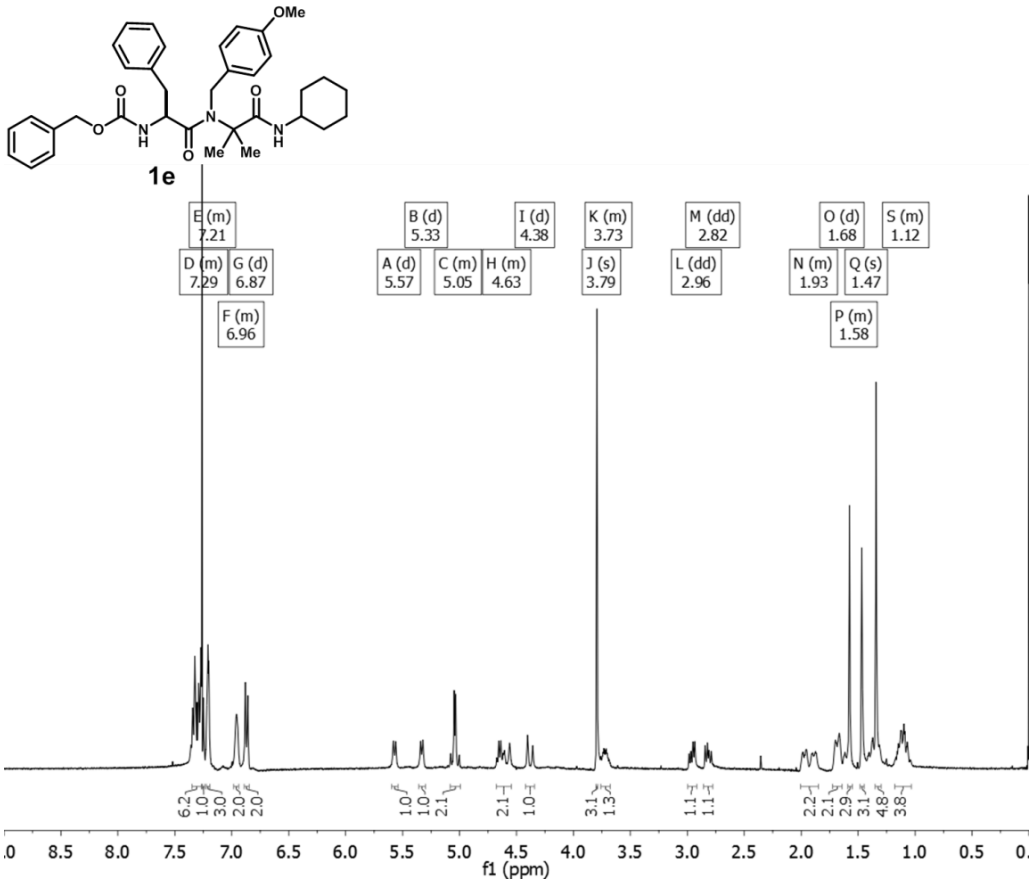

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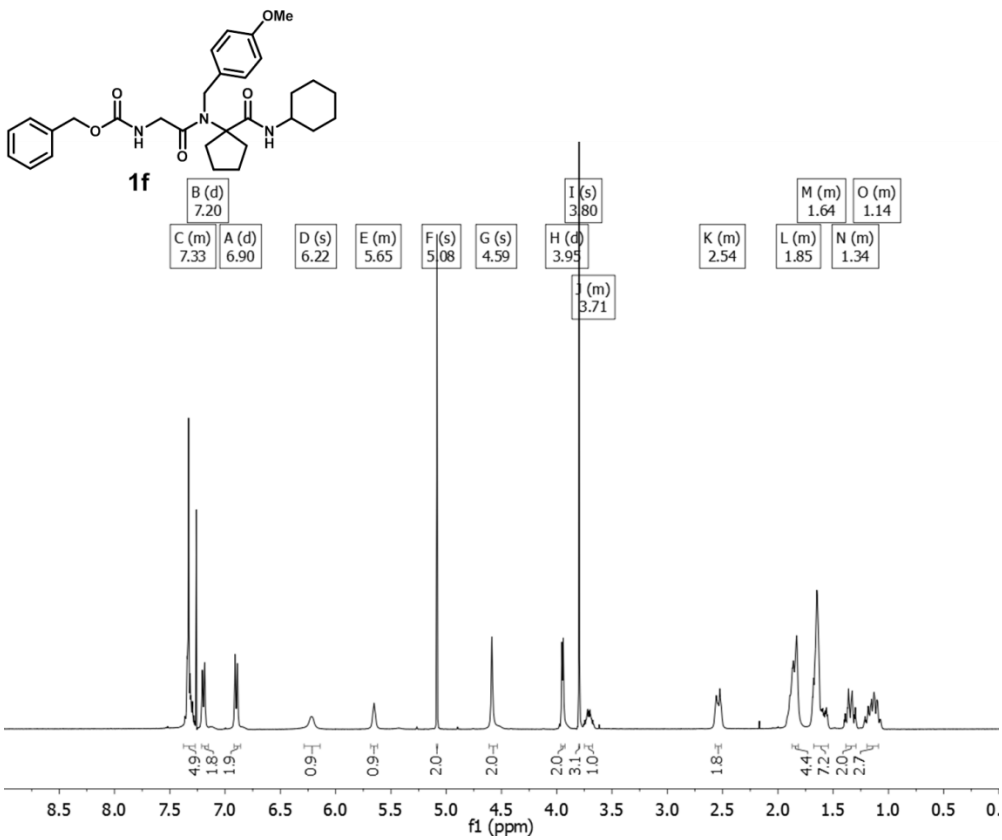
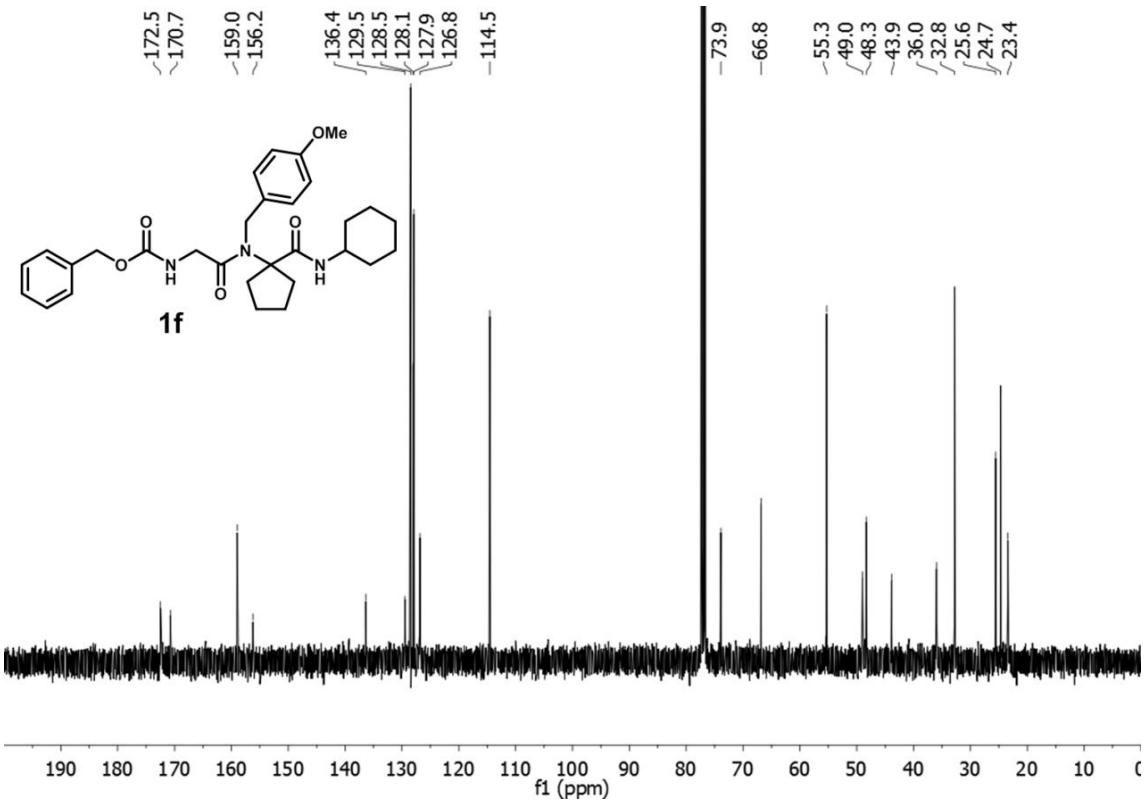
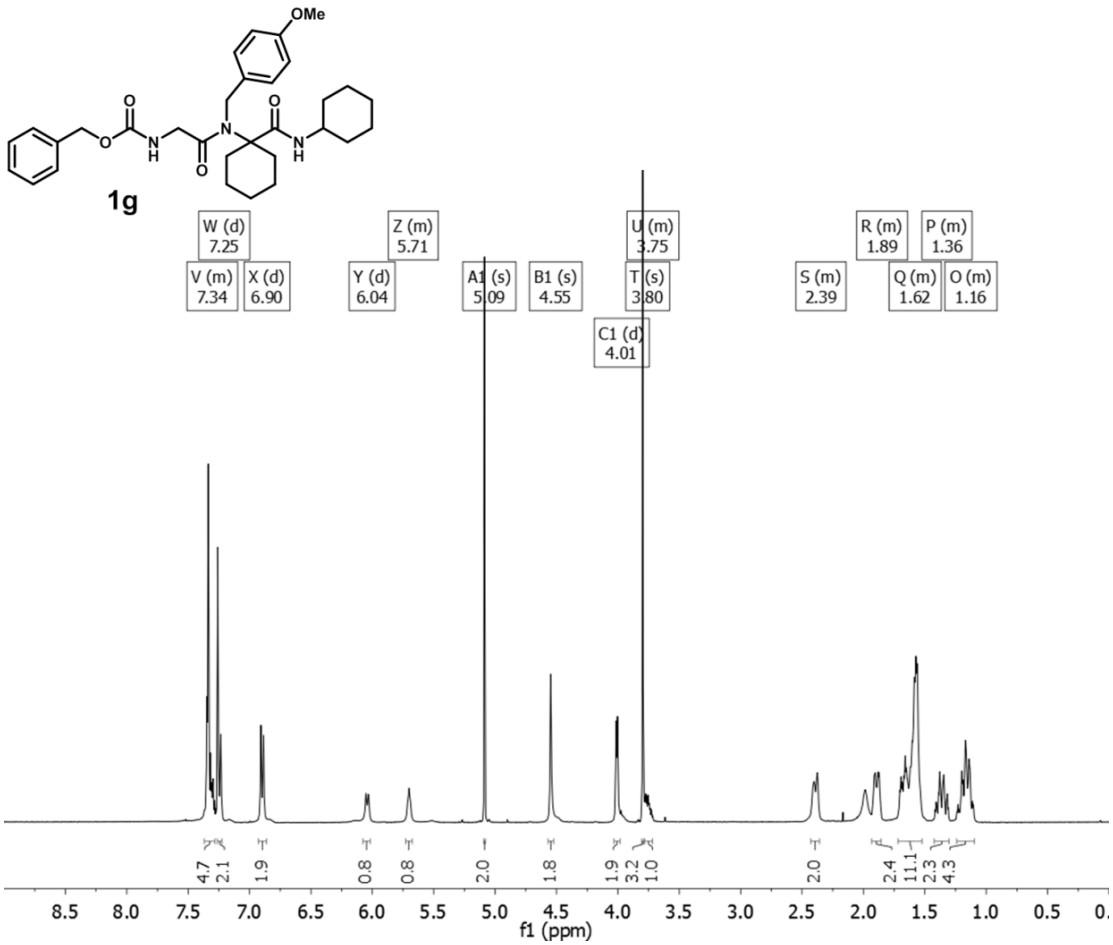


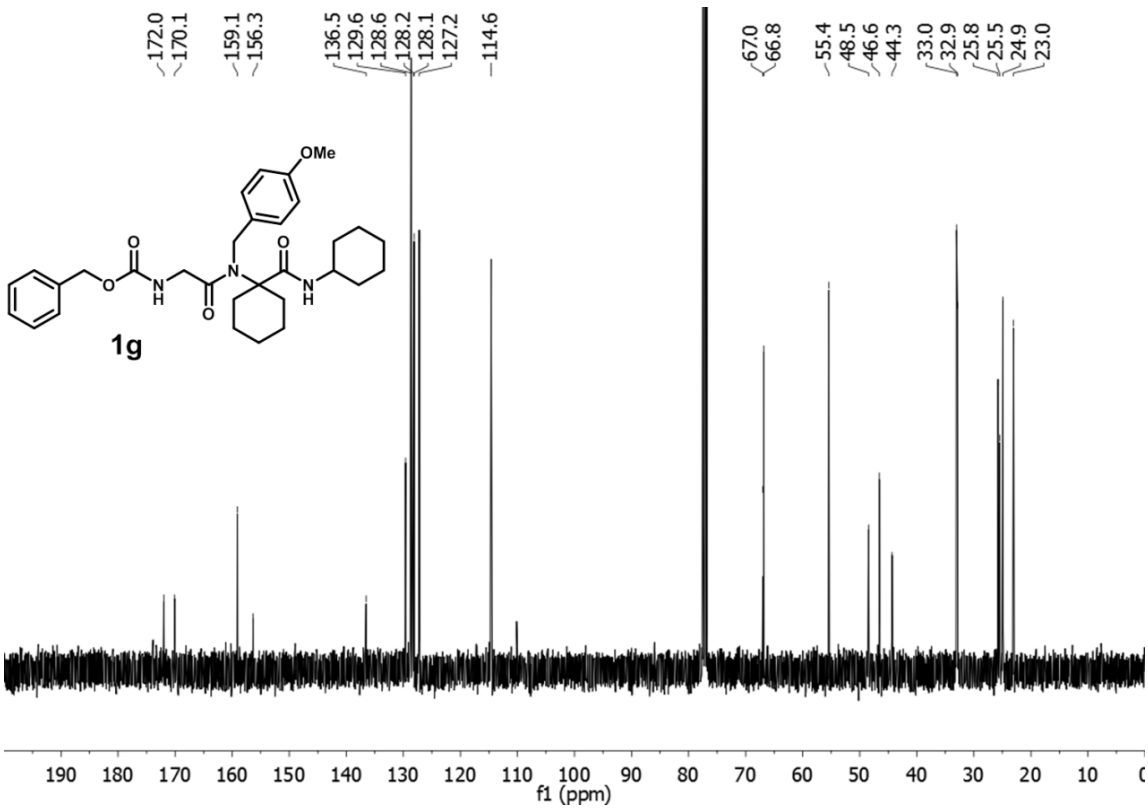
^{13}C NMR (100 MHz, $CDCl_3$): **1f**



^{1}H NMR (400 MHz, $CDCl_3$): **1g**



^{13}C NMR (100 MHz, $CDCl_3$): **1g**



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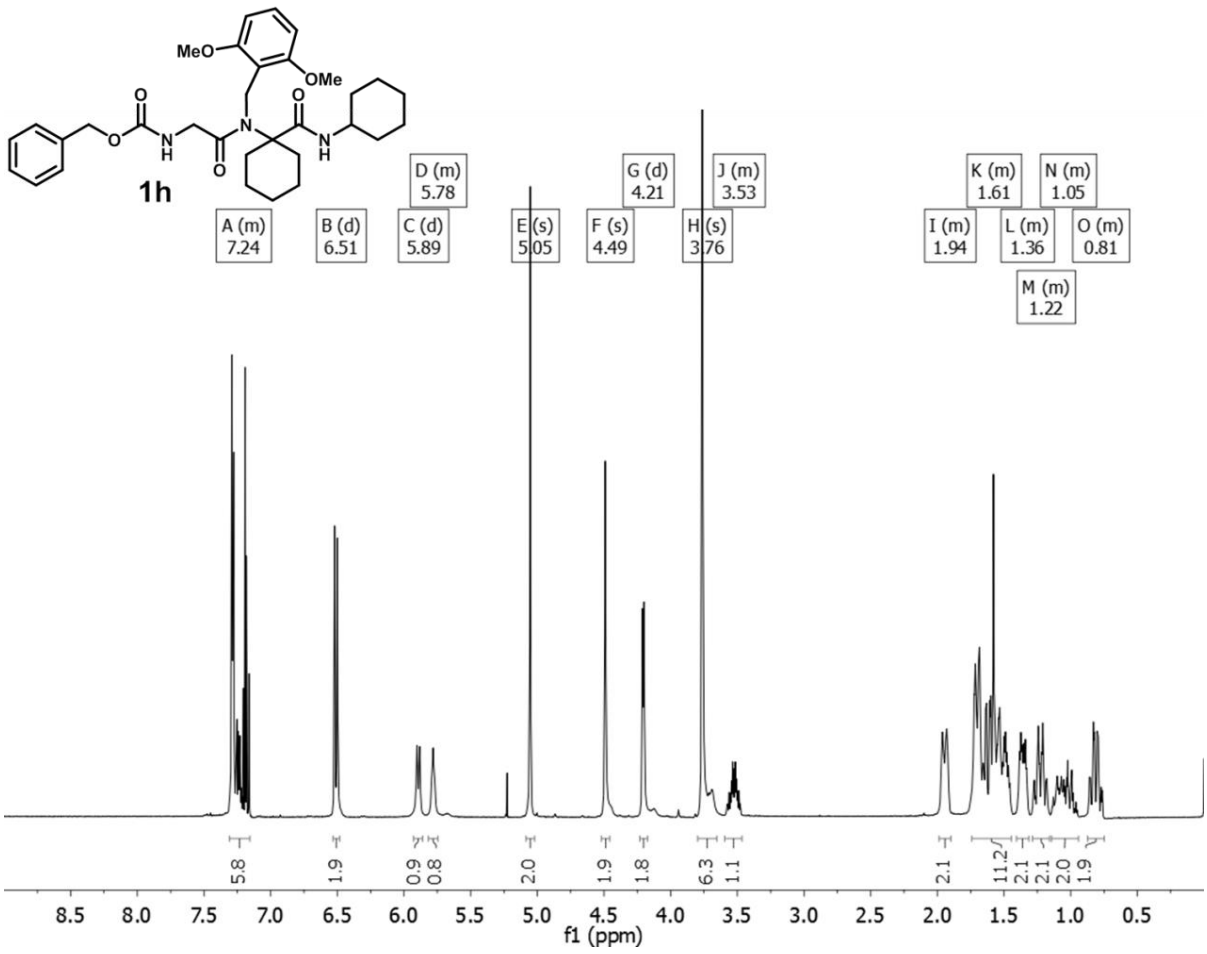
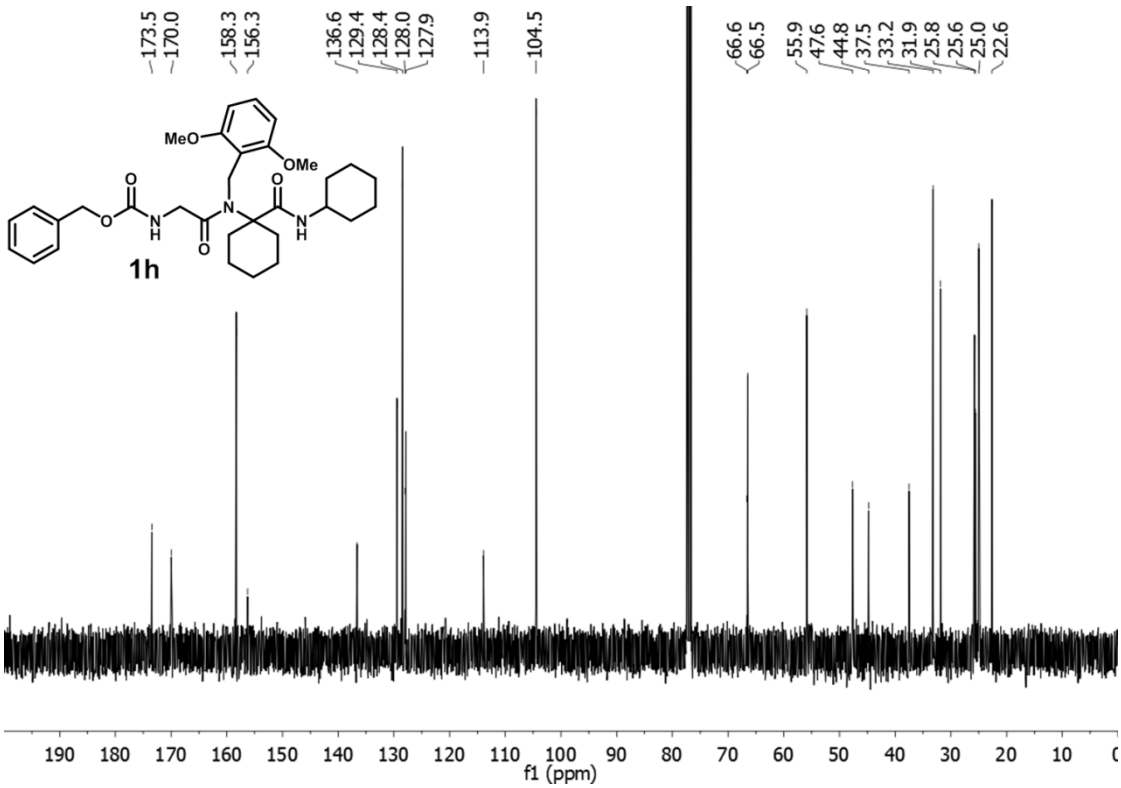
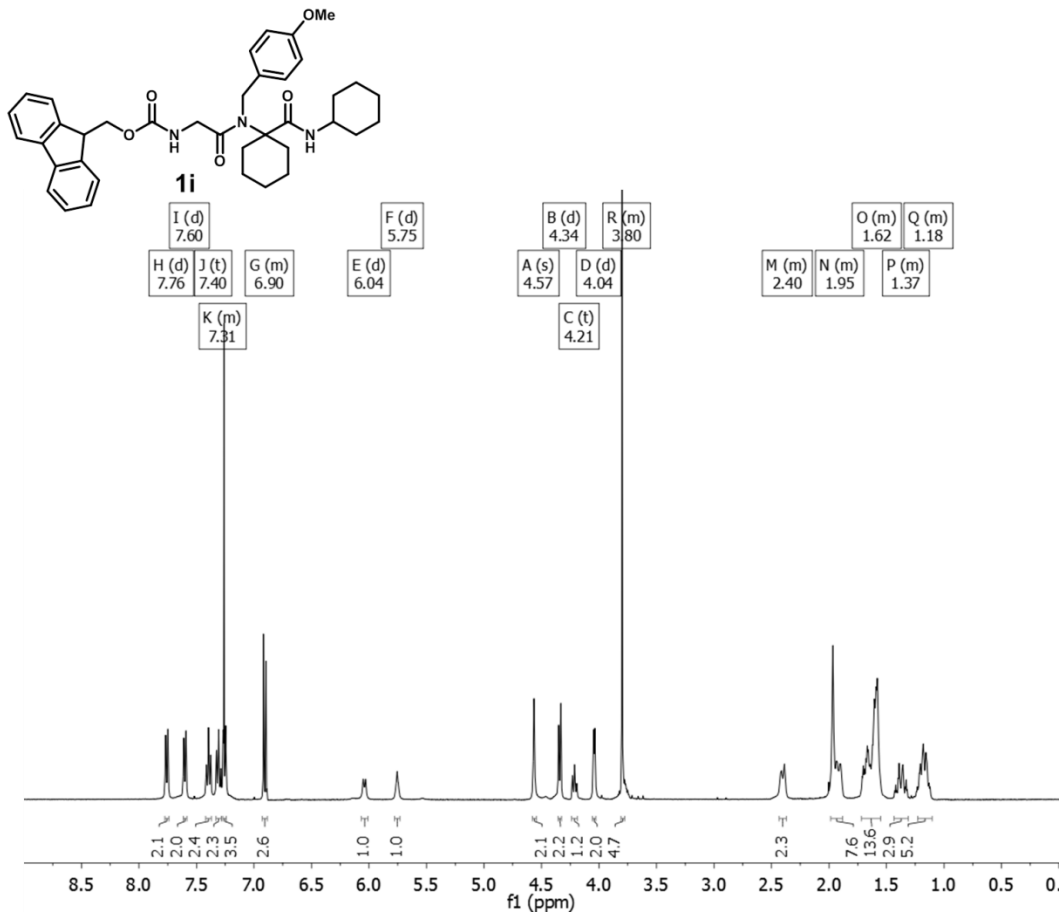


^{13}C NMR (100 MHz, $CDCl_3$): **1h**



^{1}H NMR (400 MHz, $CDCl_3$): **1i**



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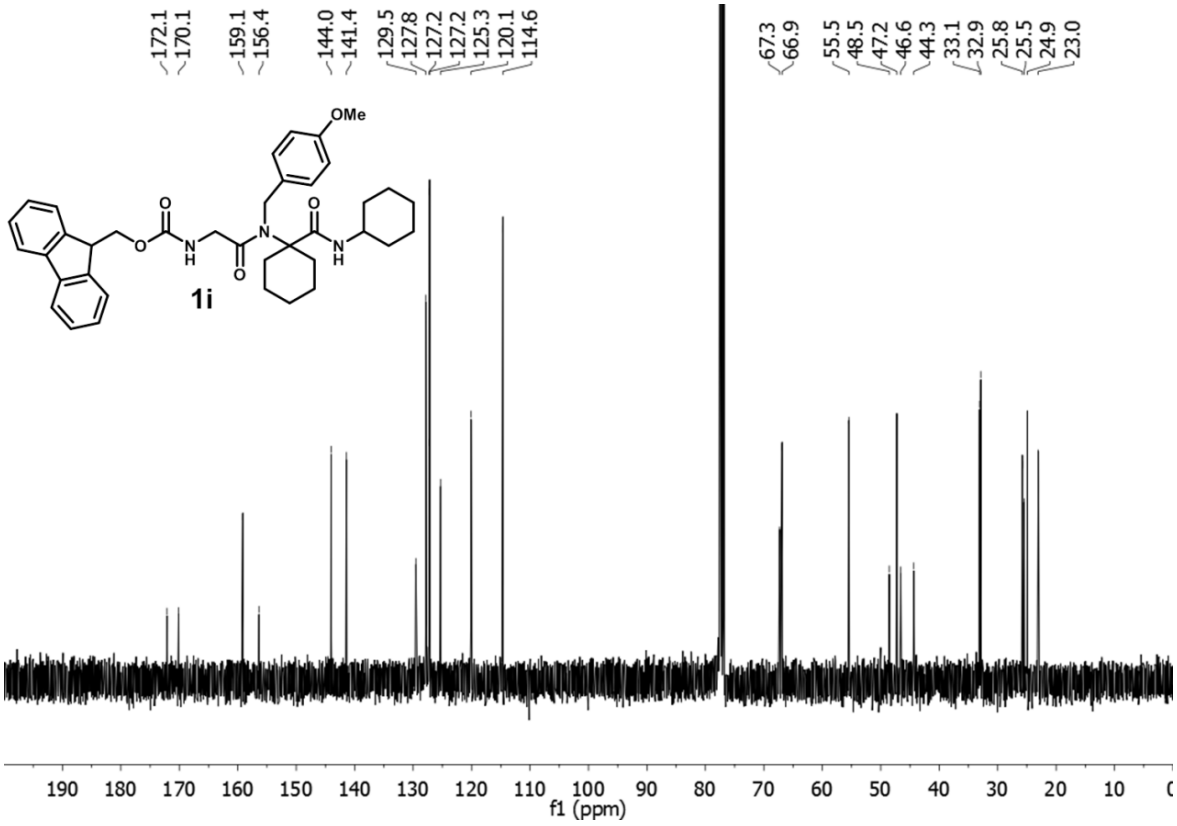

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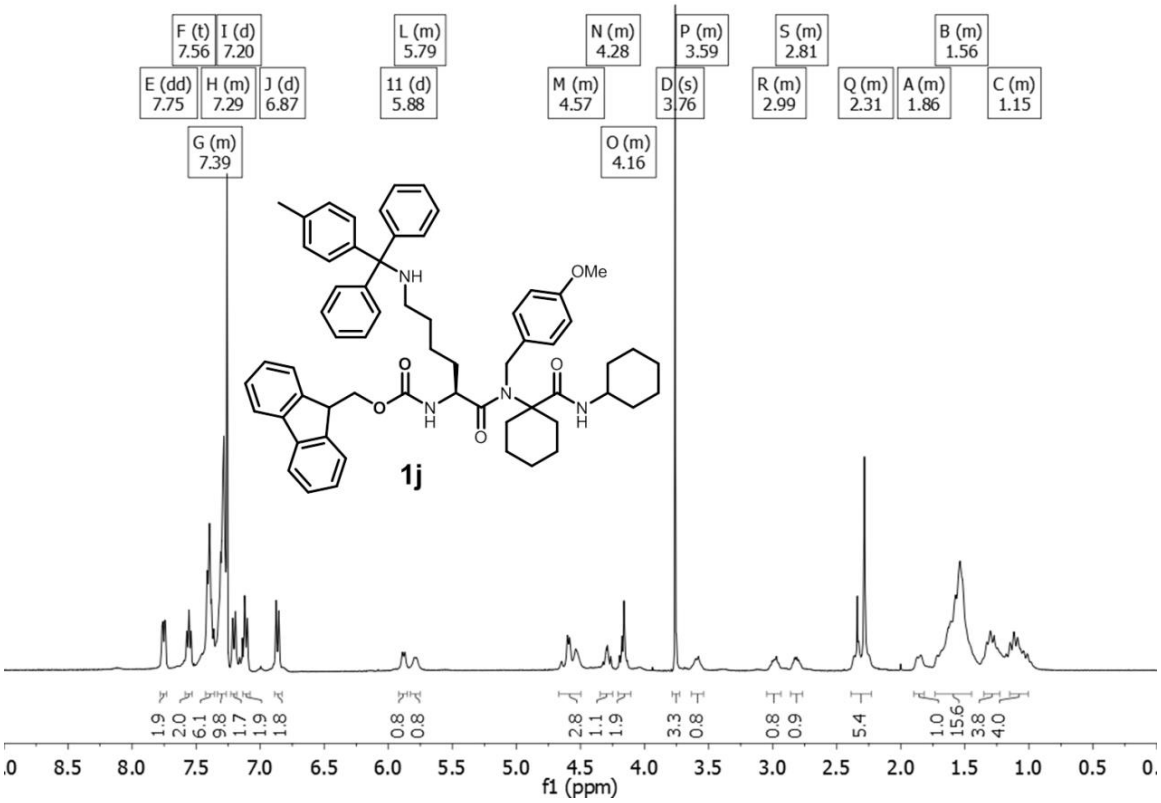

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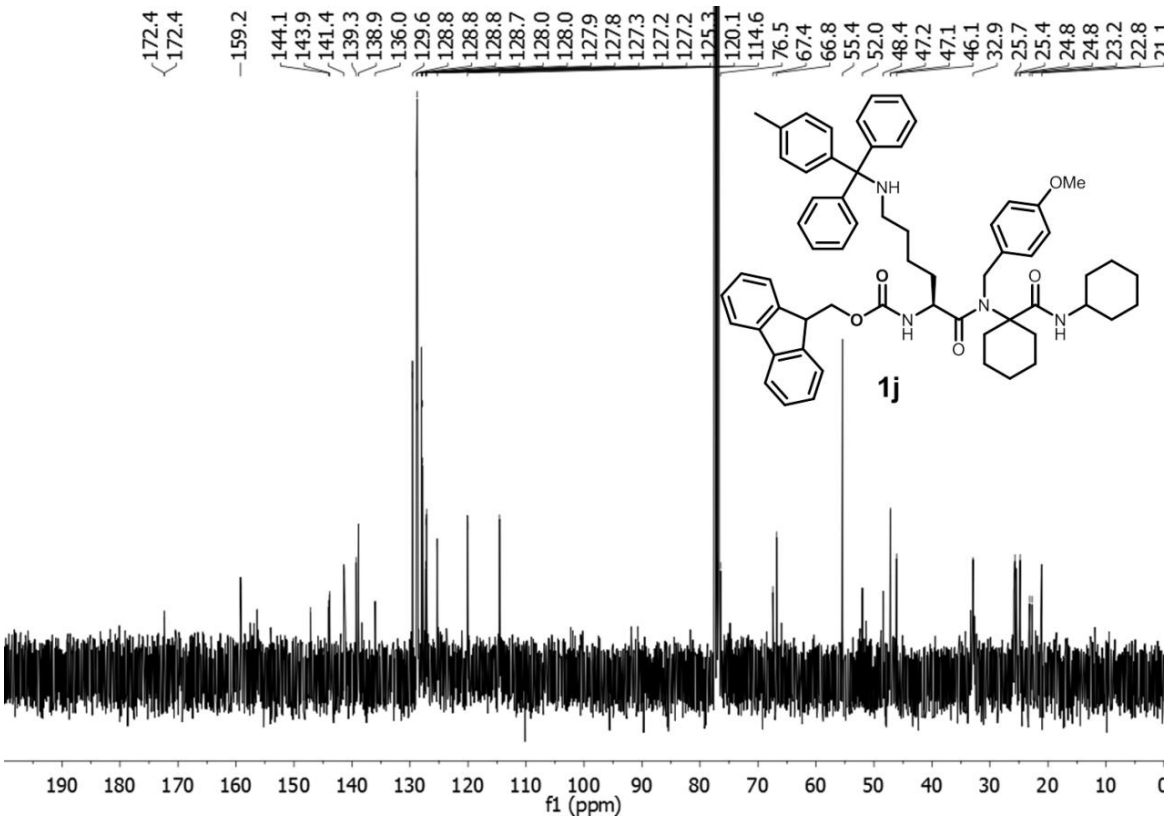
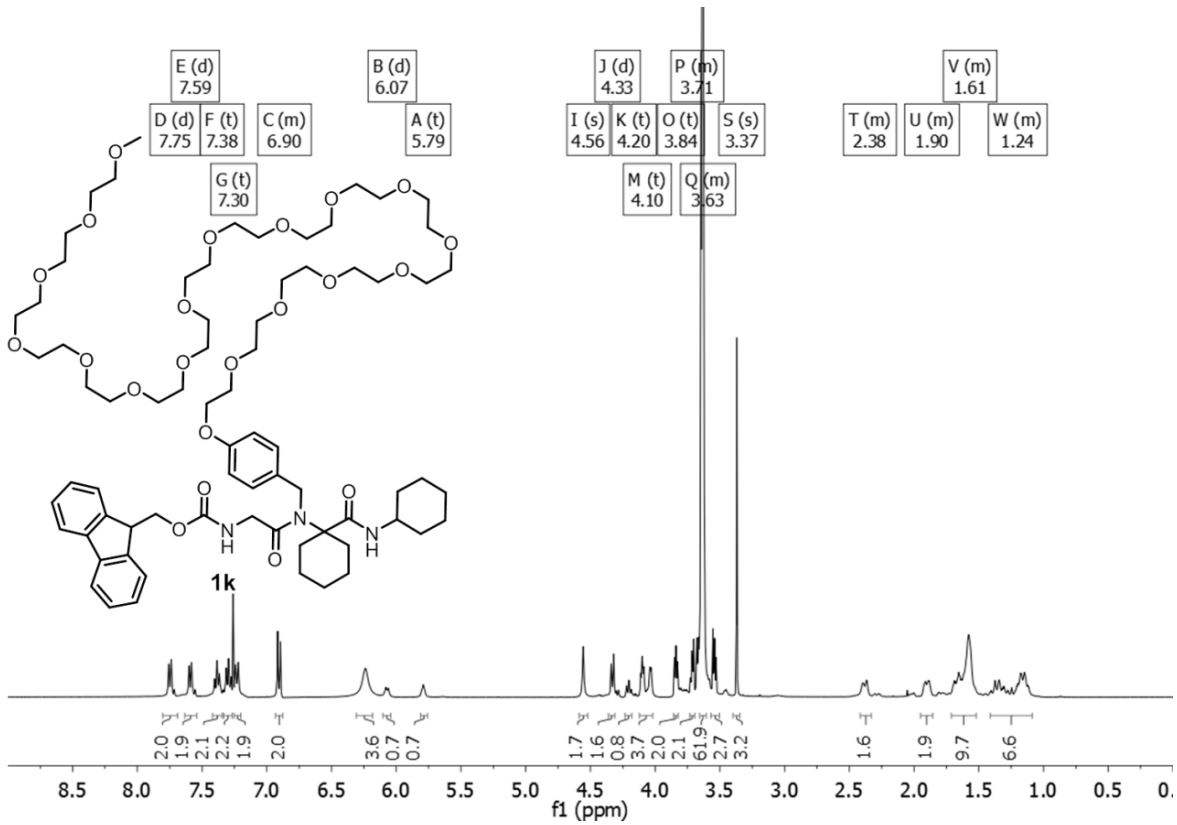
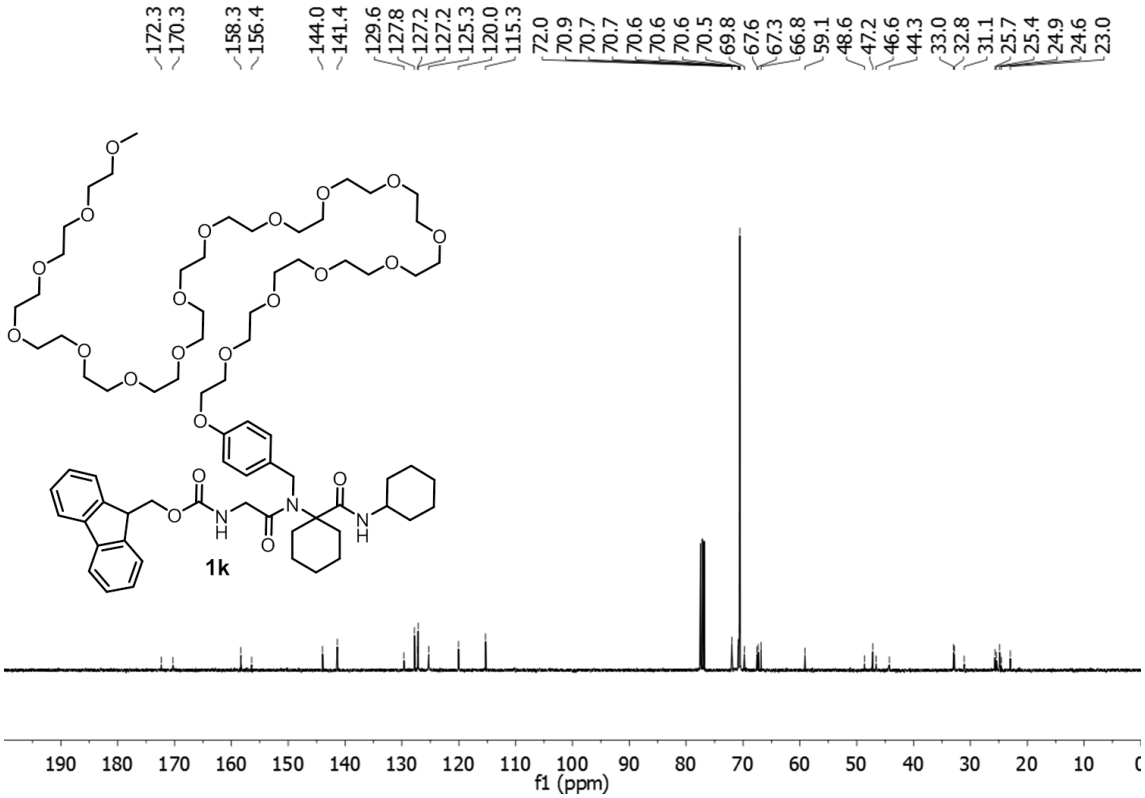


^{1}H NMR (400 MHz, $CDCl_3$): **1k**

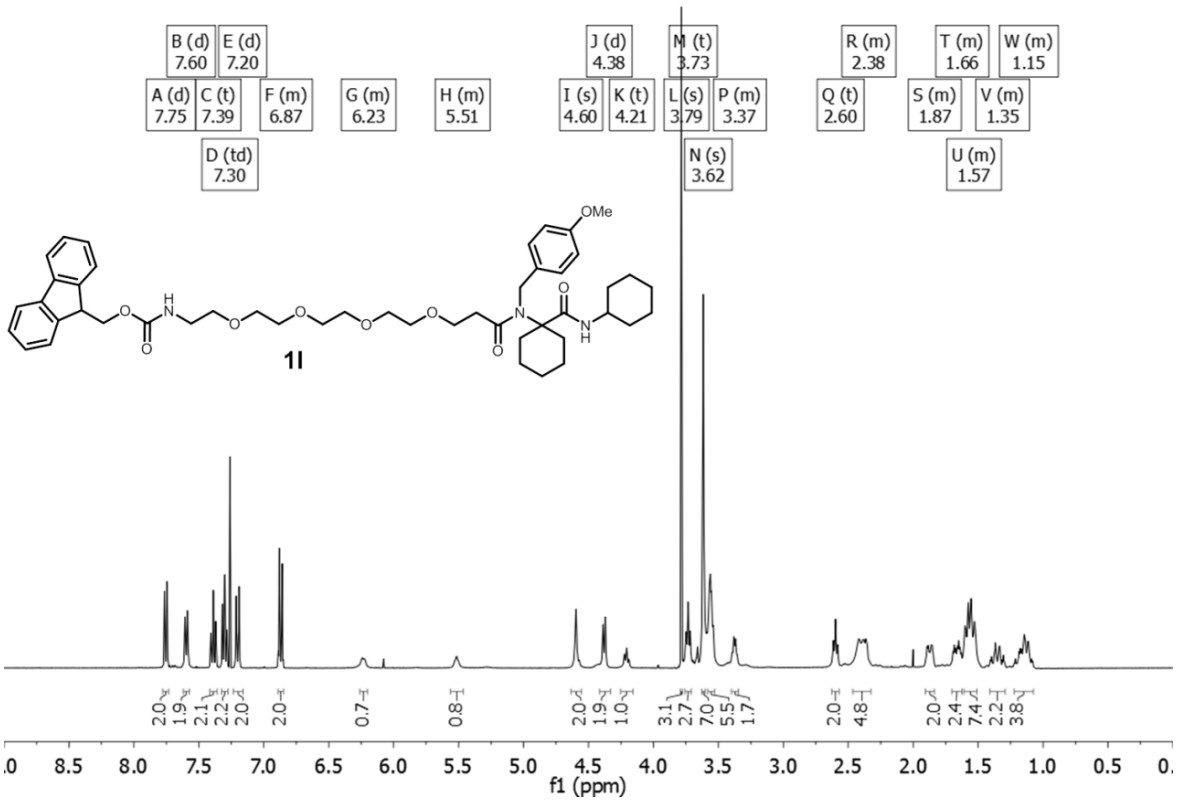


^{13}C NMR (100 MHz, $CDCl_3$): **1k**

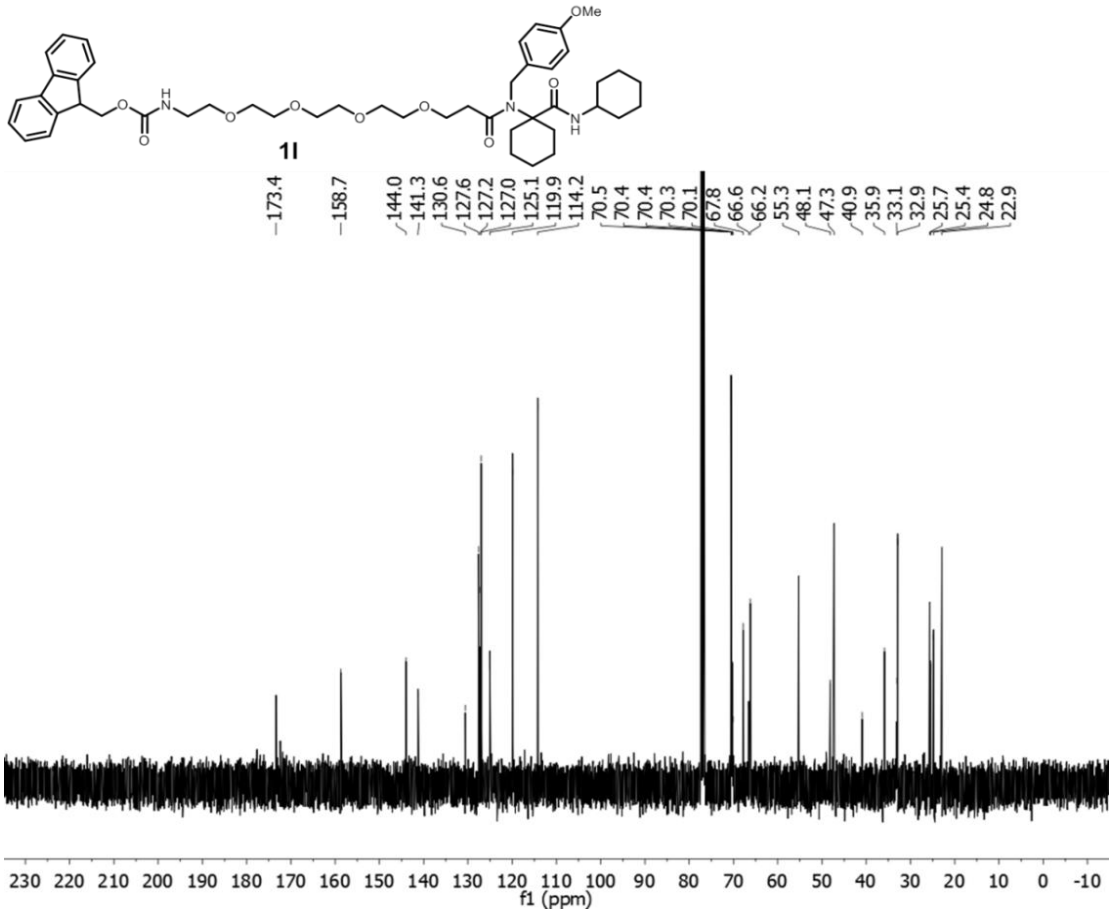


326

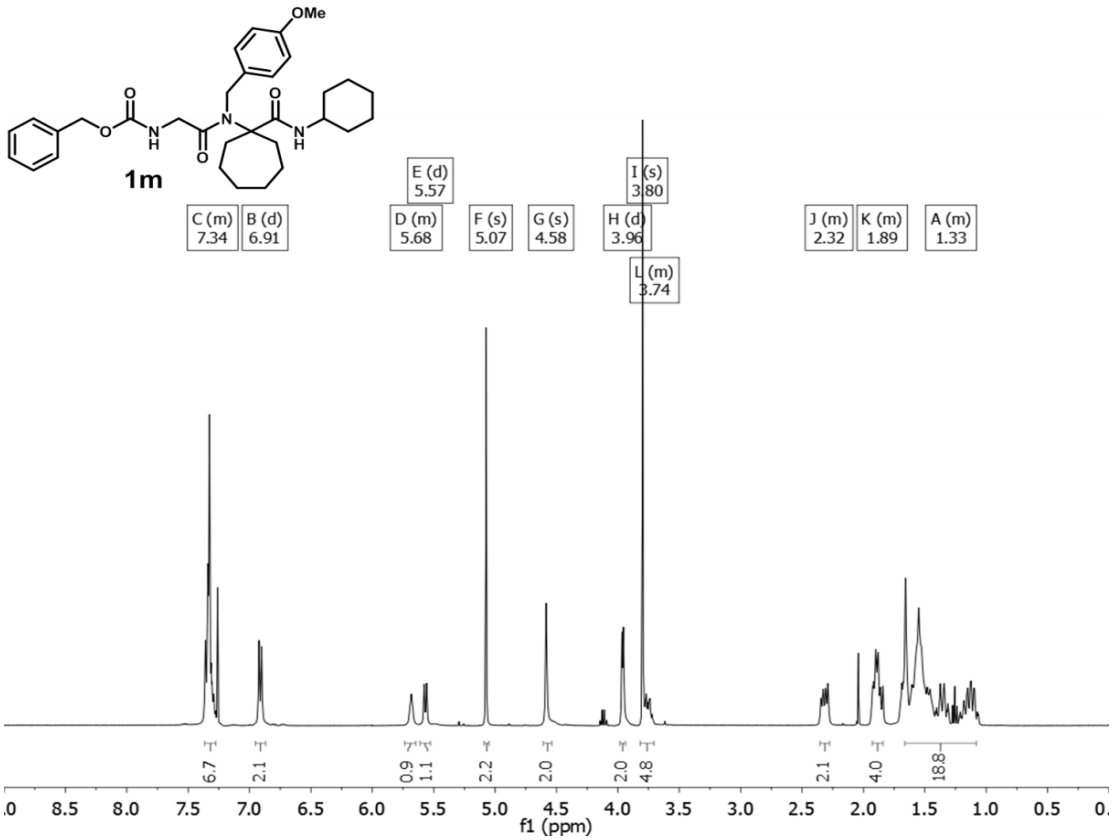
^{1}H NMR (400 MHz, $CDCl_3$): **1l**



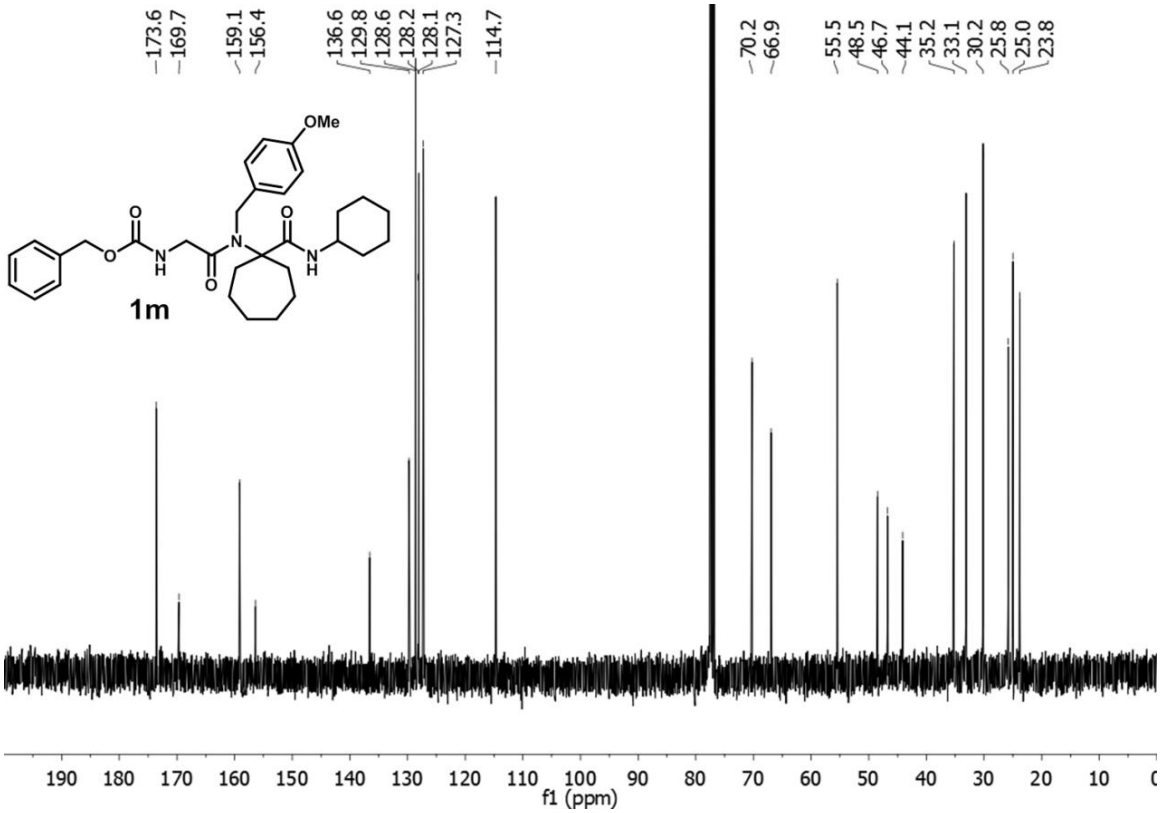
^{13}C NMR (100 MHz, $CDCl_3$): **1l**



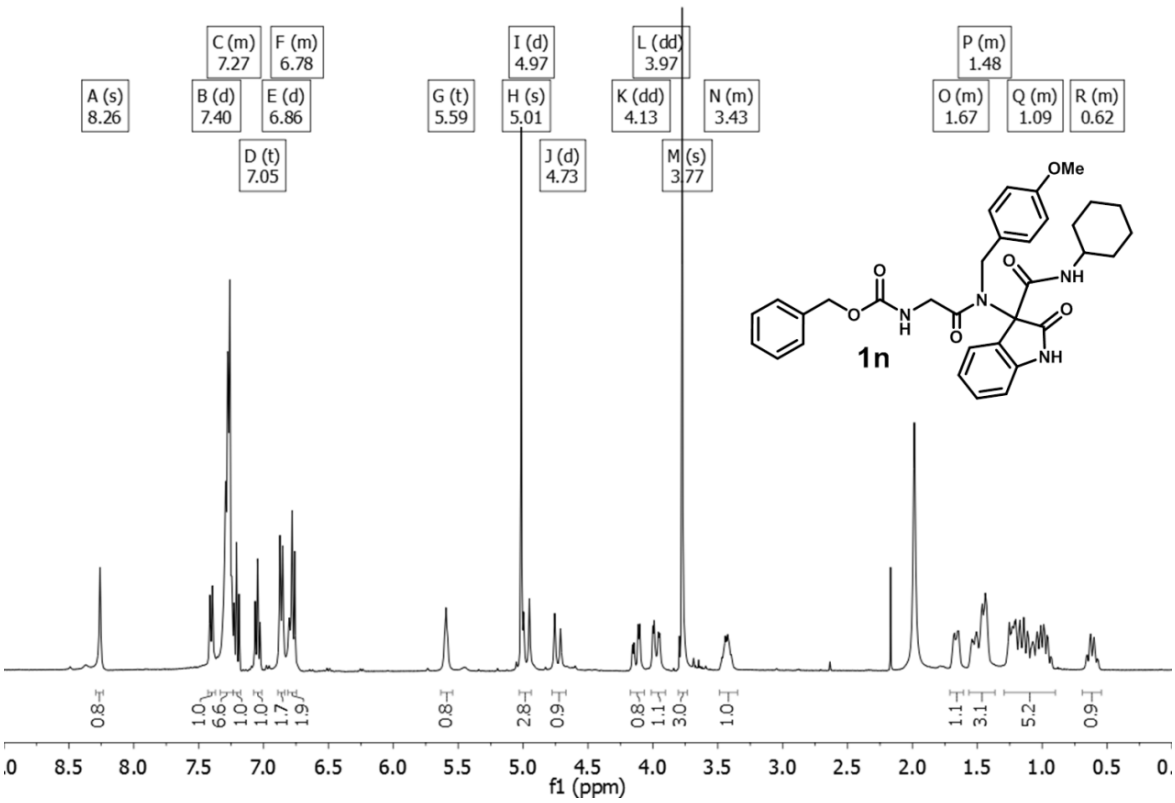
^{1}H NMR (400 MHz, $CDCl_3$): **1m**



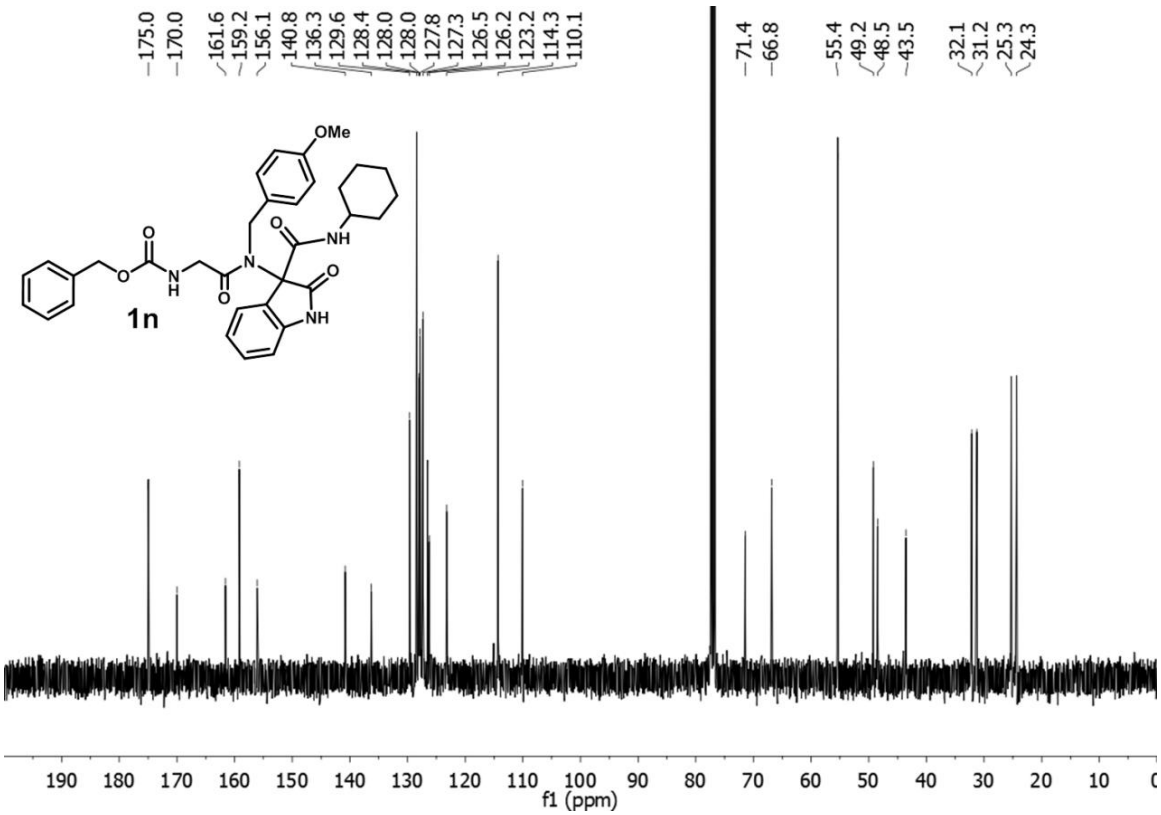
^{13}C NMR (100 MHz, $CDCl_3$): **1m**



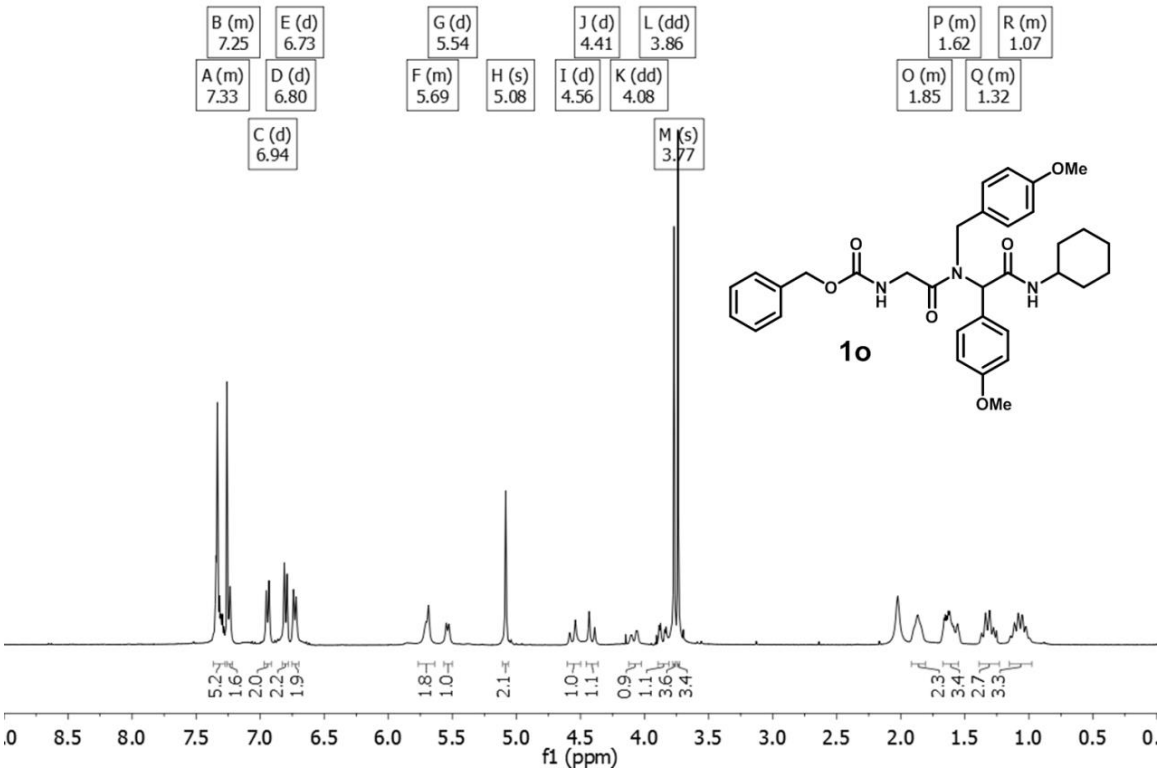
^{1}H NMR (400 MHz, $CDCl_3$): **1n**



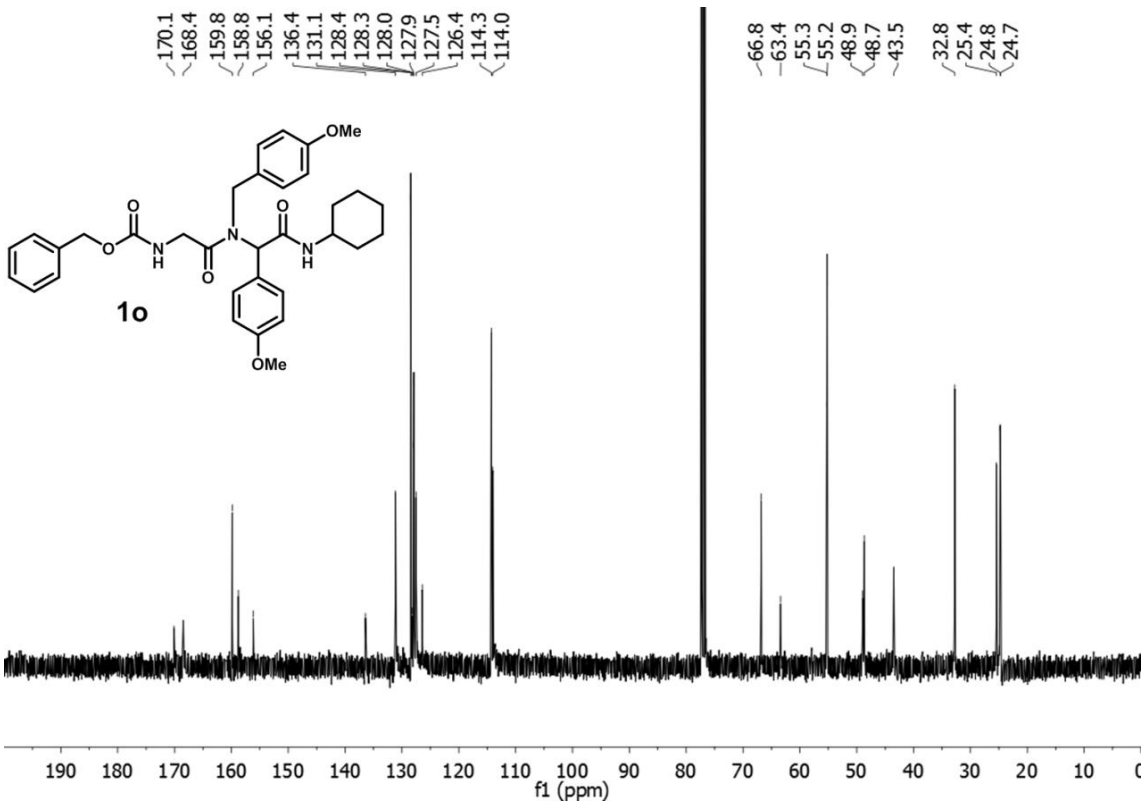
^{13}C NMR (100 MHz, $CDCl_3$): **1n**

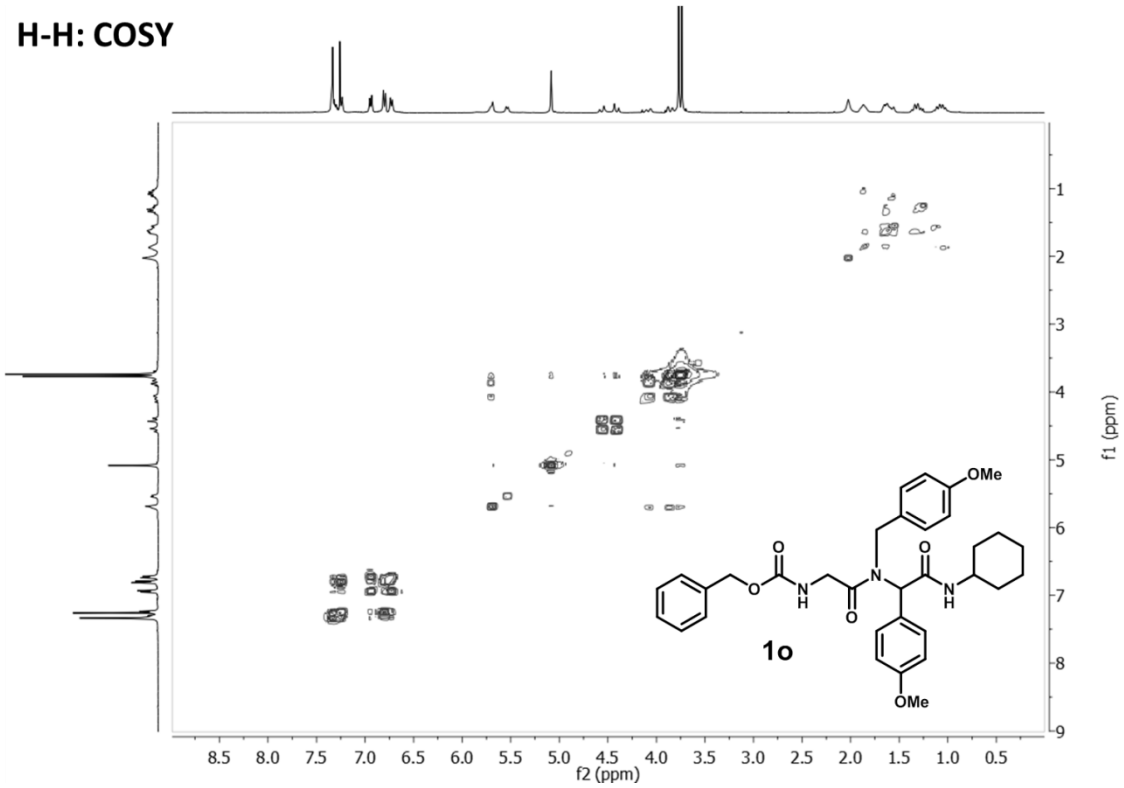


^{1}H NMR (400 MHz, $CDCl_3$): **1o**

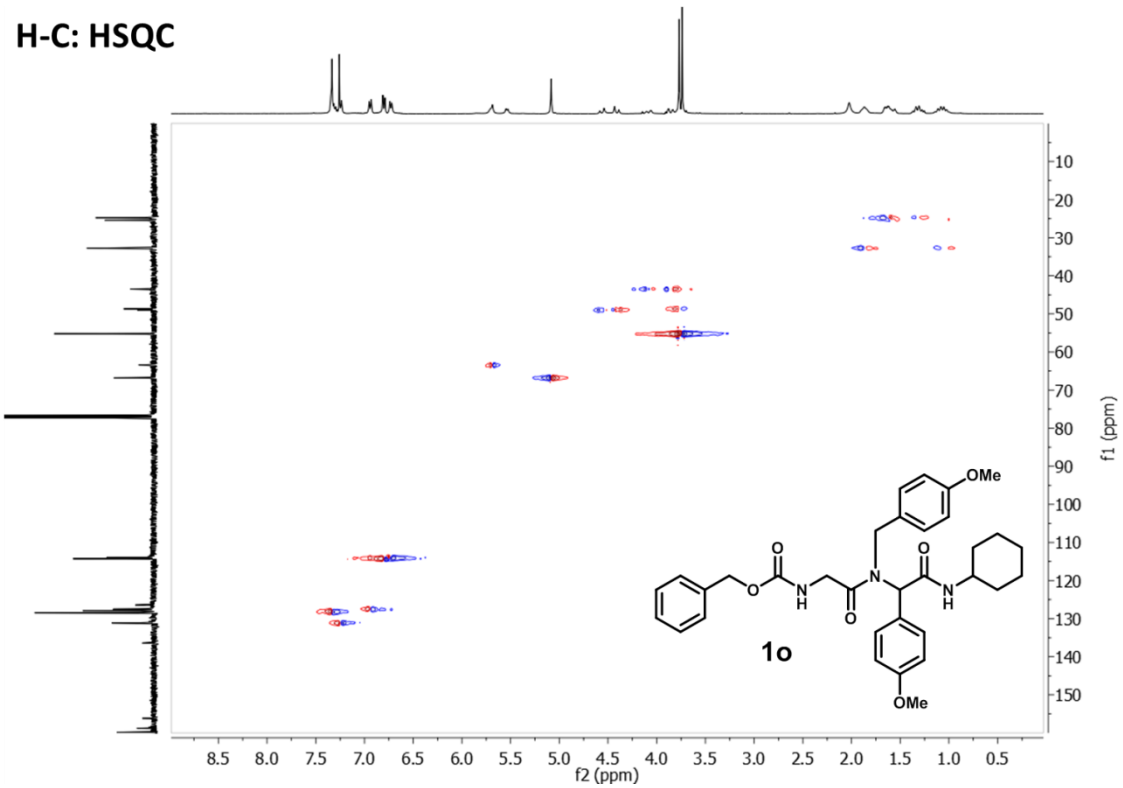


^{13}C NMR (100 MHz, $CDCl_3$): **1o**

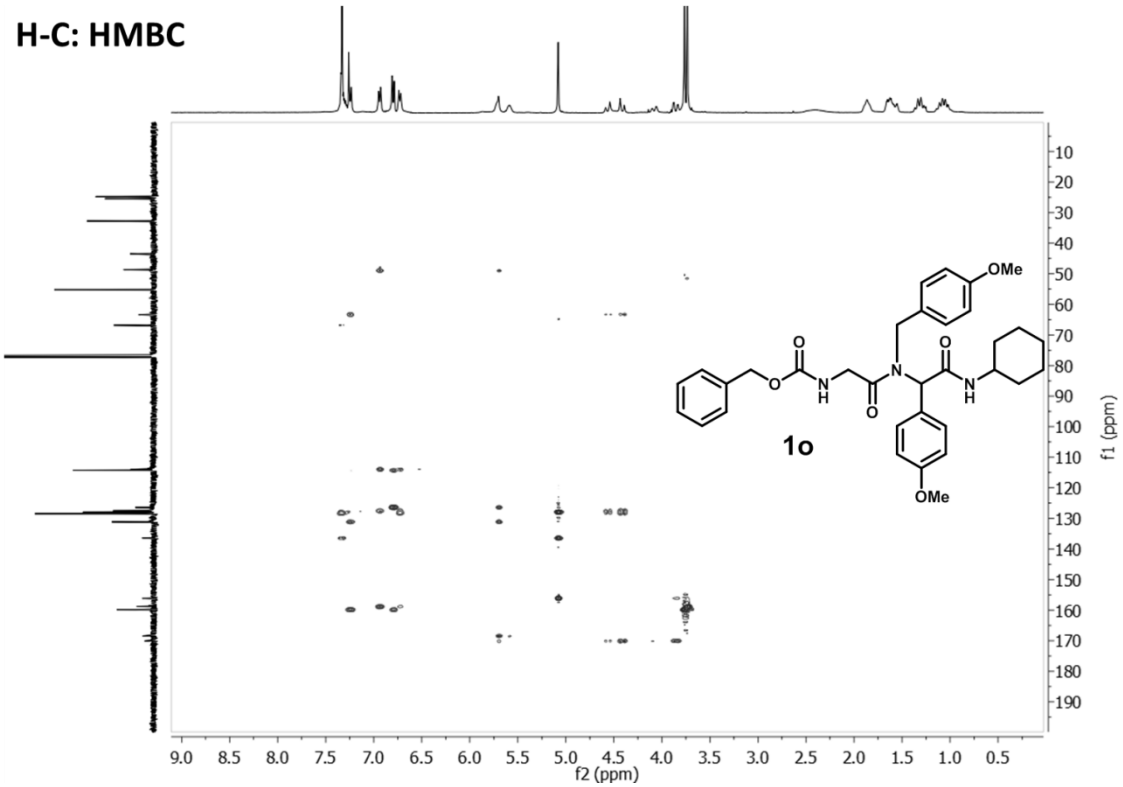




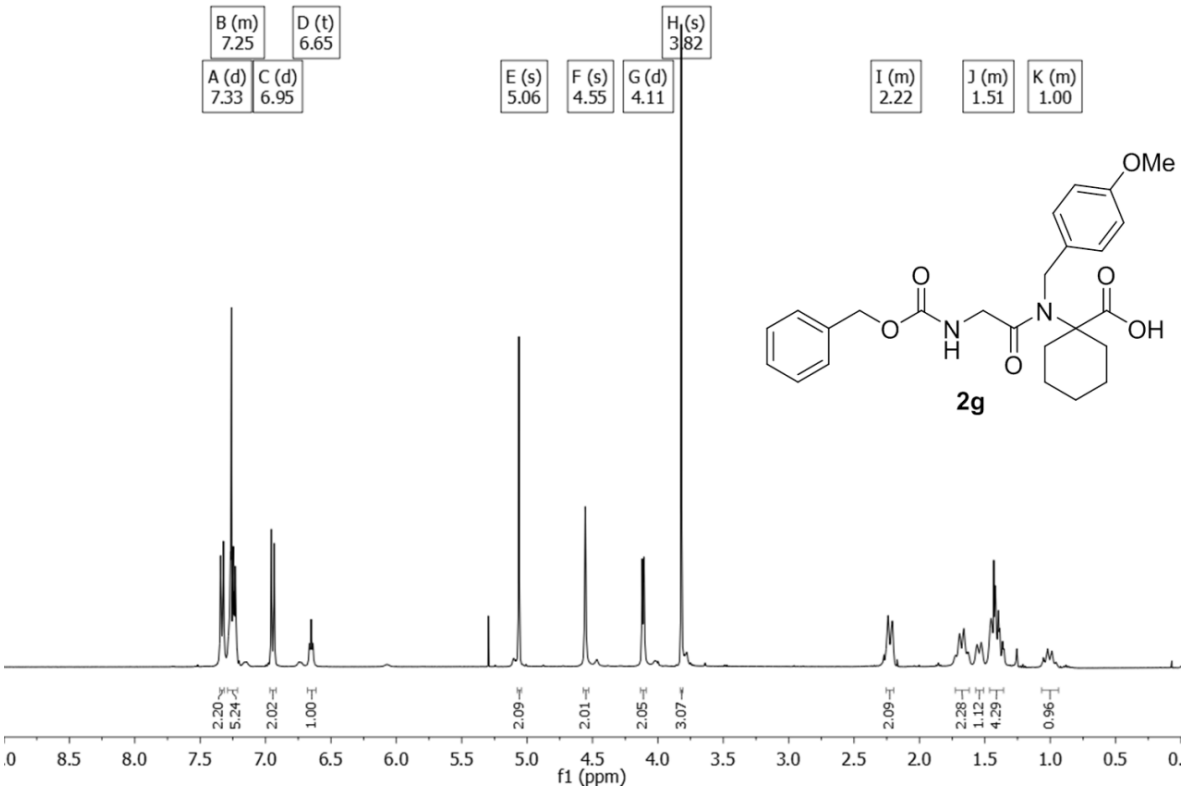
H-C: HSQC



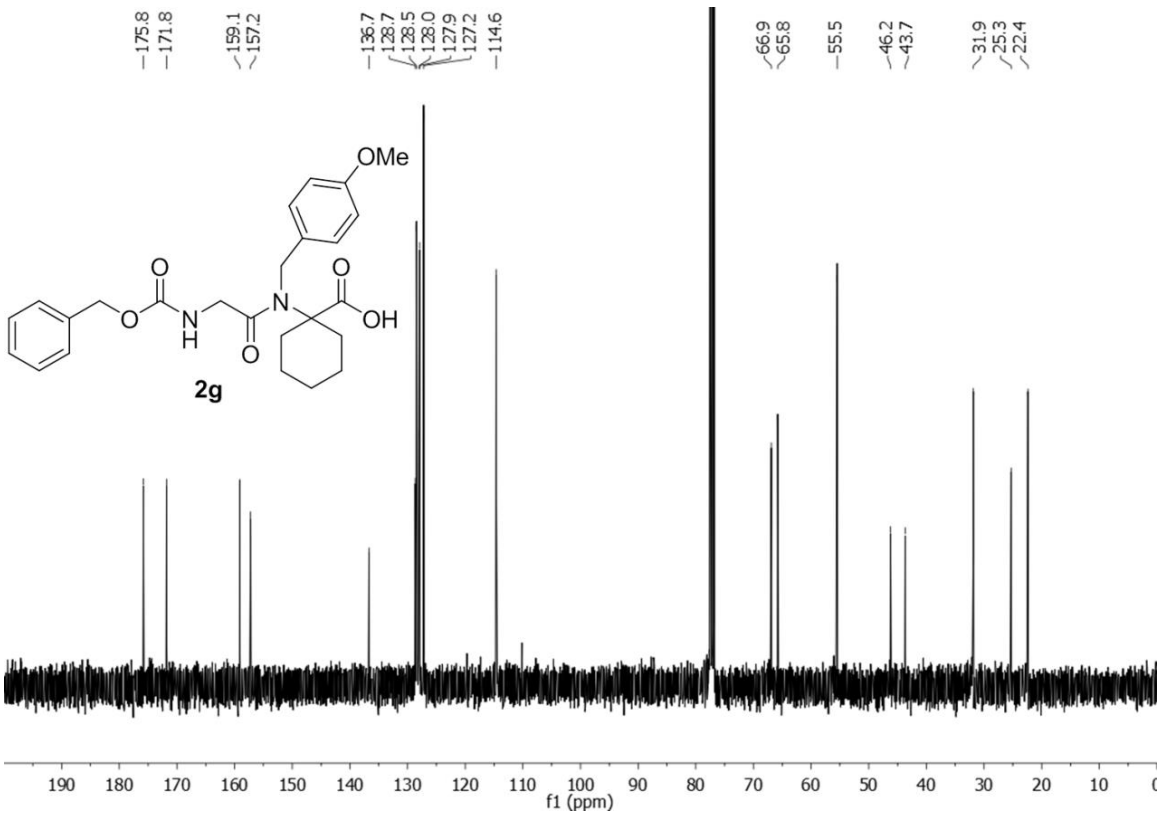
H-C: HMBC



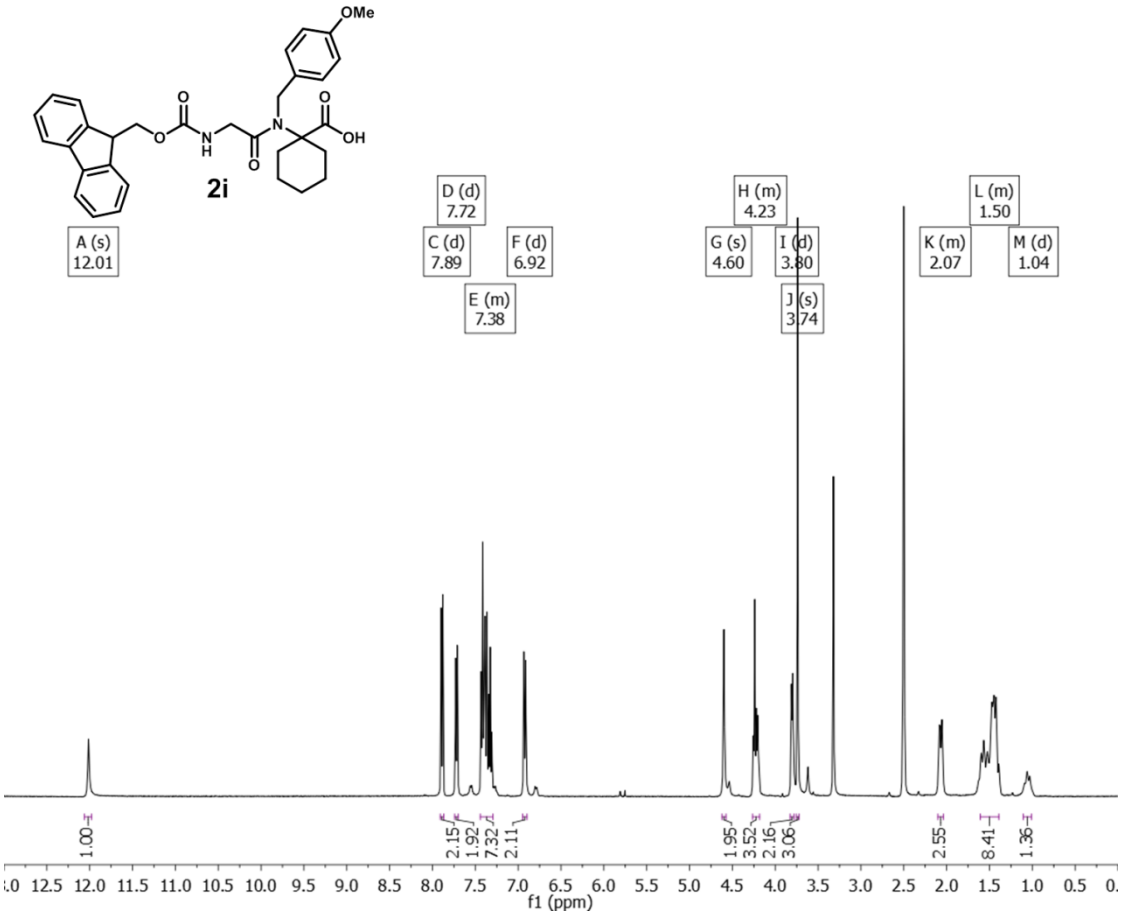
H NMR (400 MHz, $CDCl_3$): **2g**



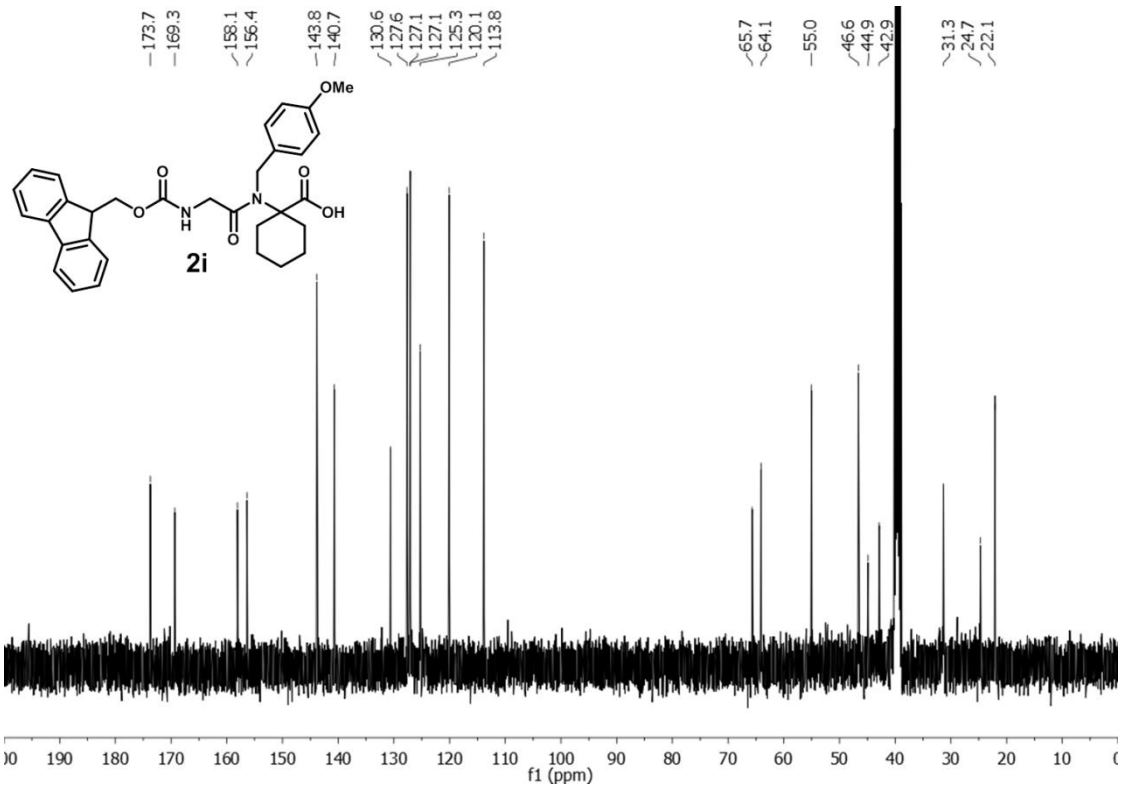
^{13}C NMR (100 MHz, $CDCl_3$): **2g**



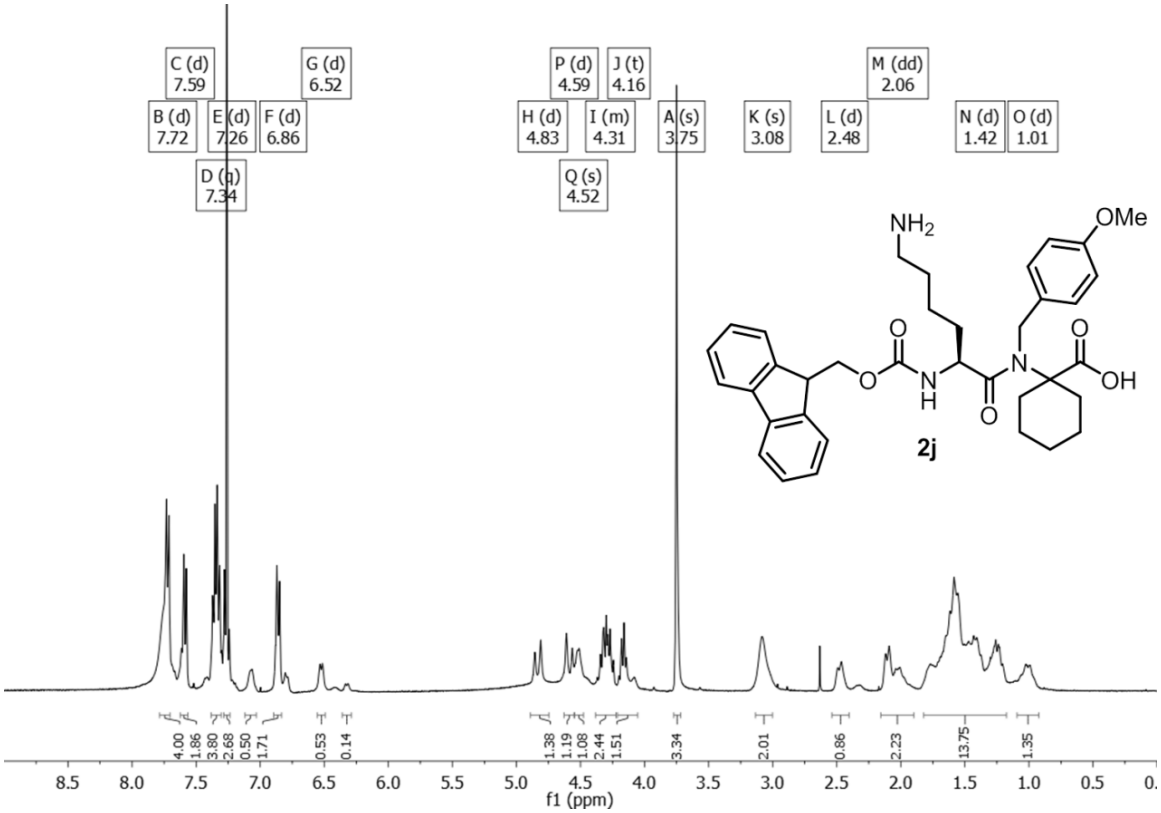
^{1}H NMR (400 MHz, DMSO-d_6): **2i**



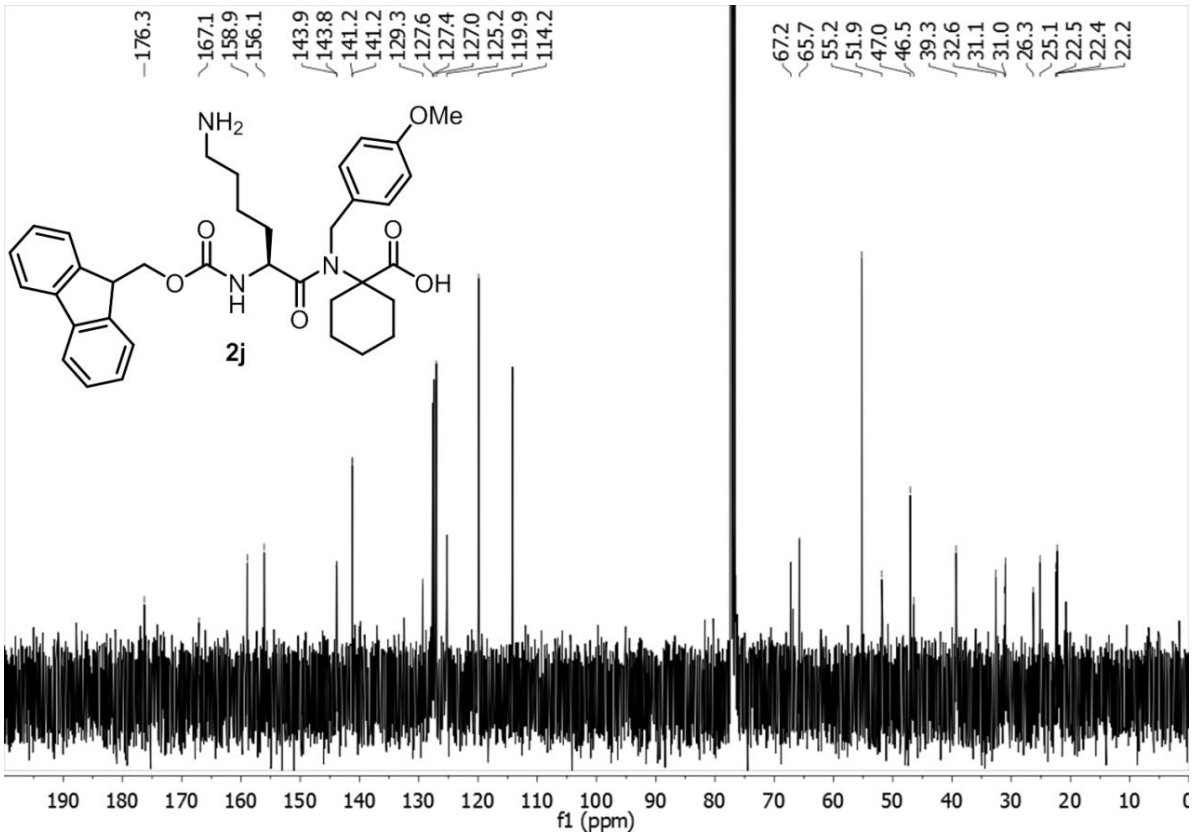
^{13}C NMR (100 MHz, DMSO-d_6): **2i**



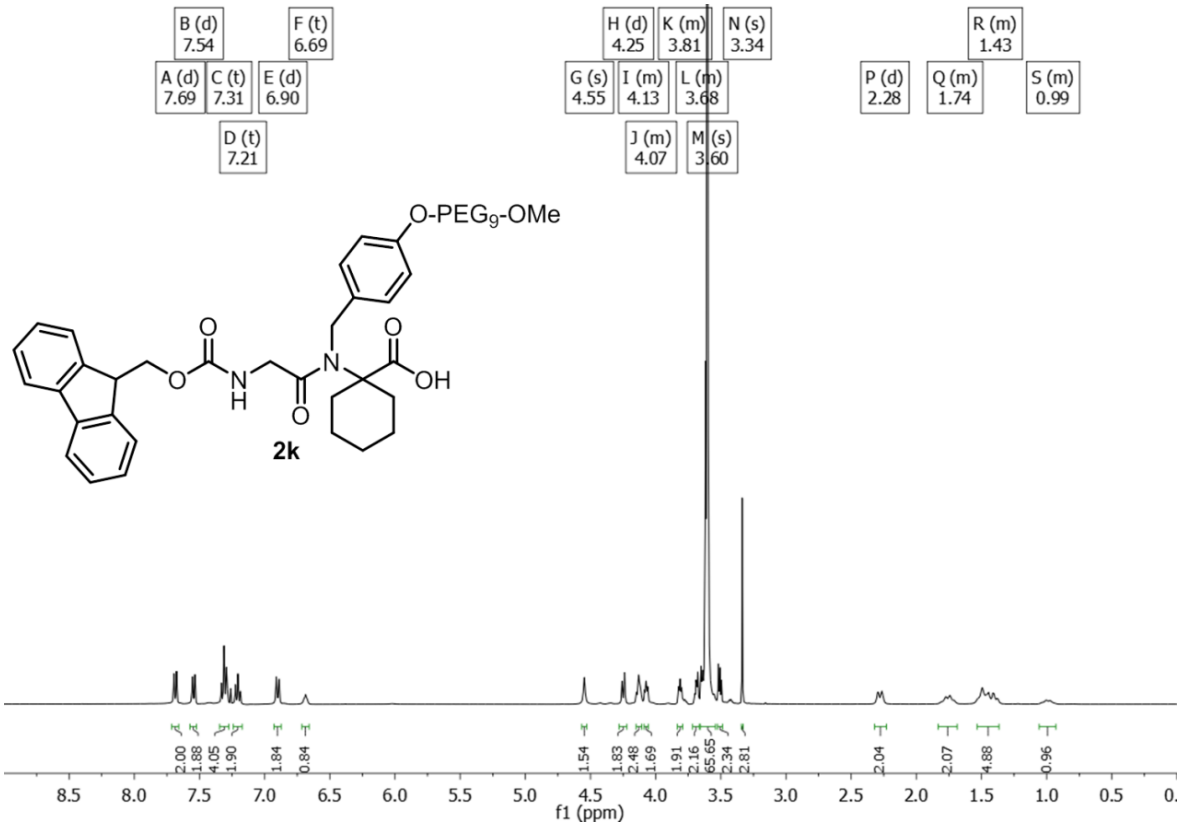
^{1}H NMR (400 MHz, $CDCl_3$): **2j**



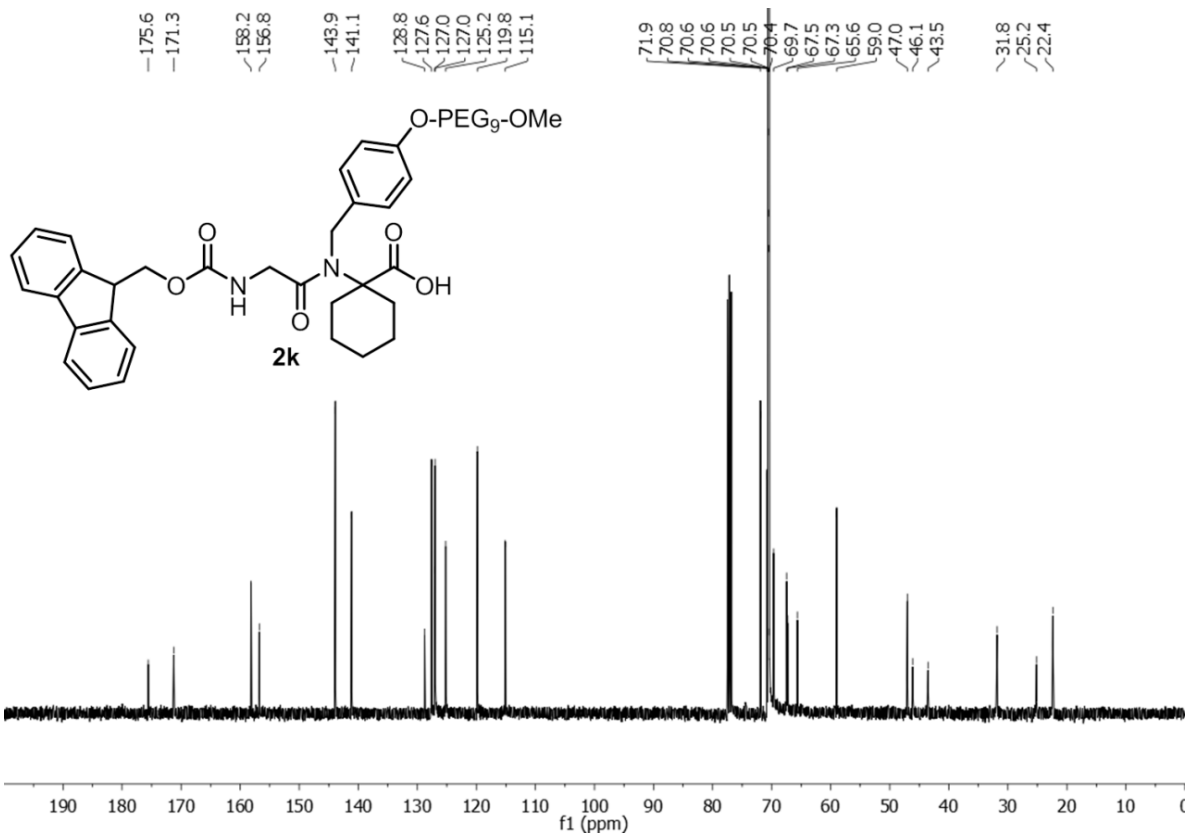
^{13}C NMR (100 MHz, $CDCl_3$): **2j**



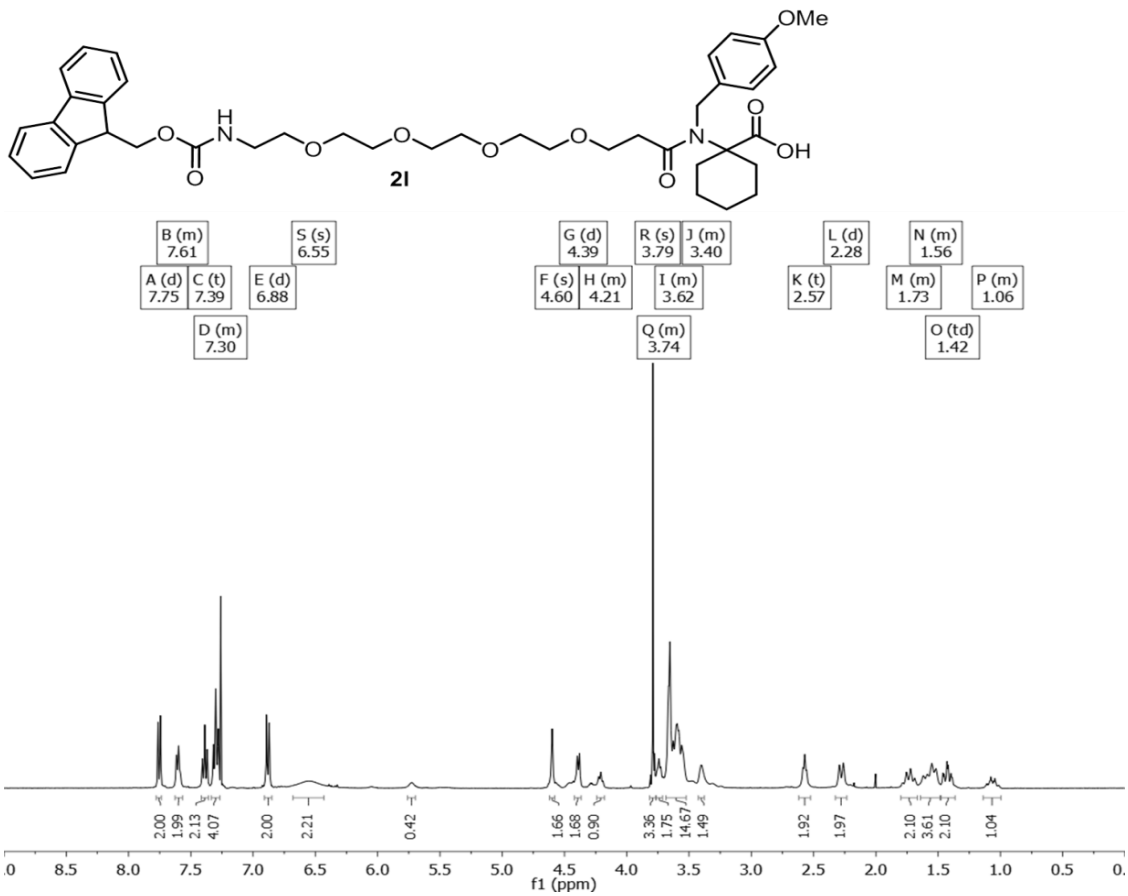
^{1}H NMR (400 MHz, $CDCl_3$): **2k**



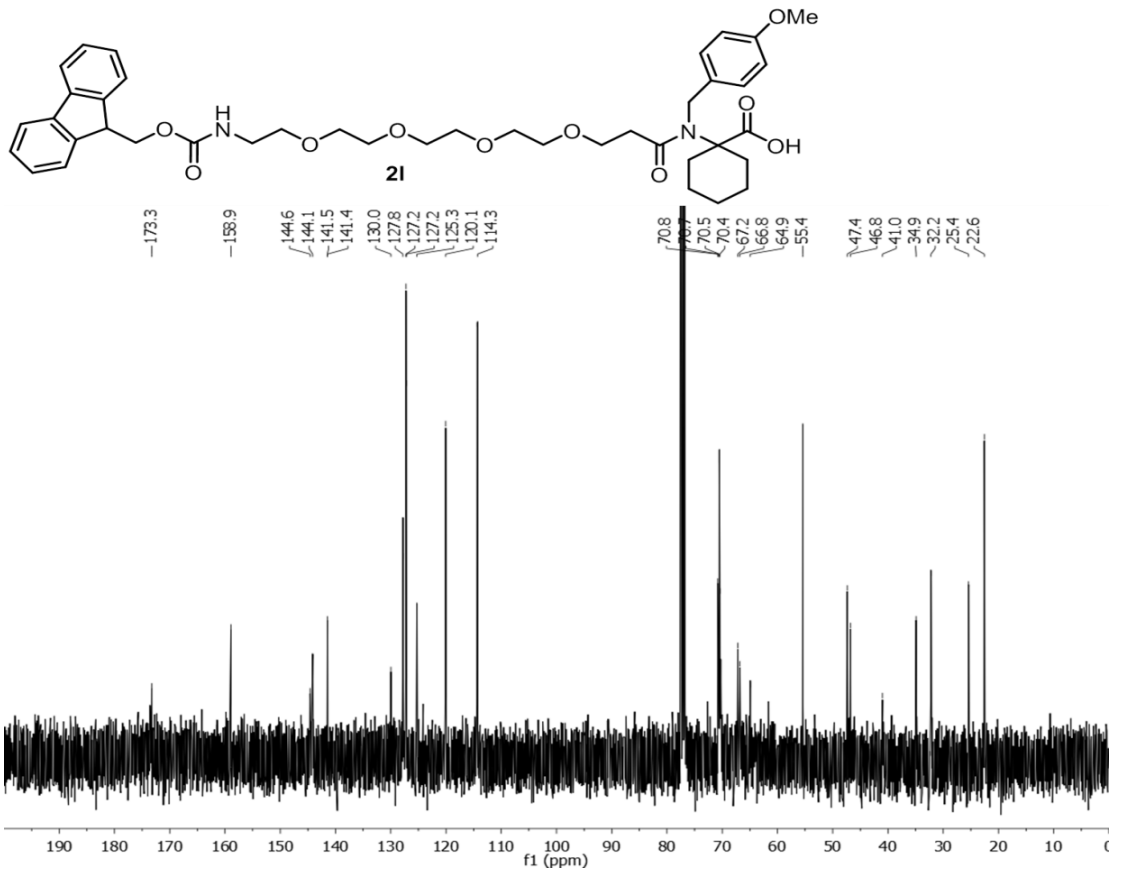
^{13}C NMR (100 MHz, $CDCl_3$): **2k**



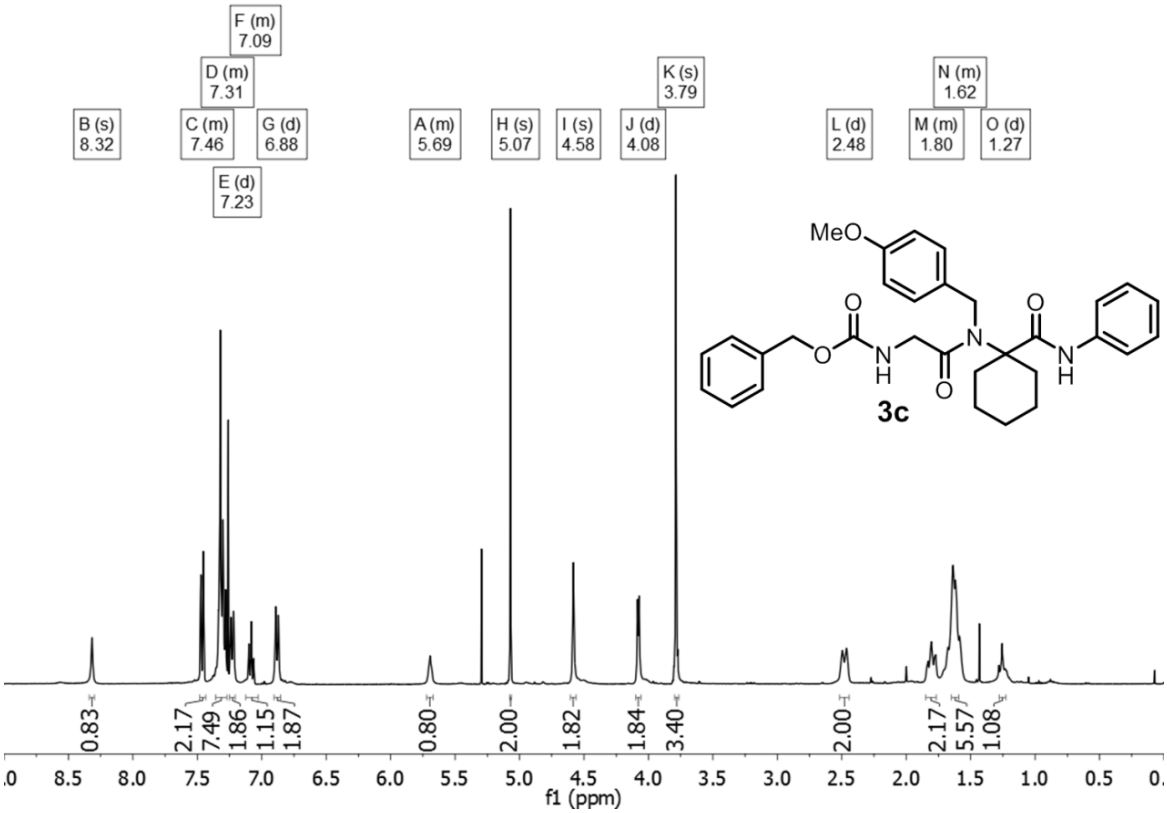
^{1}H NMR (400 MHz, $CDCl_3$): **2l**



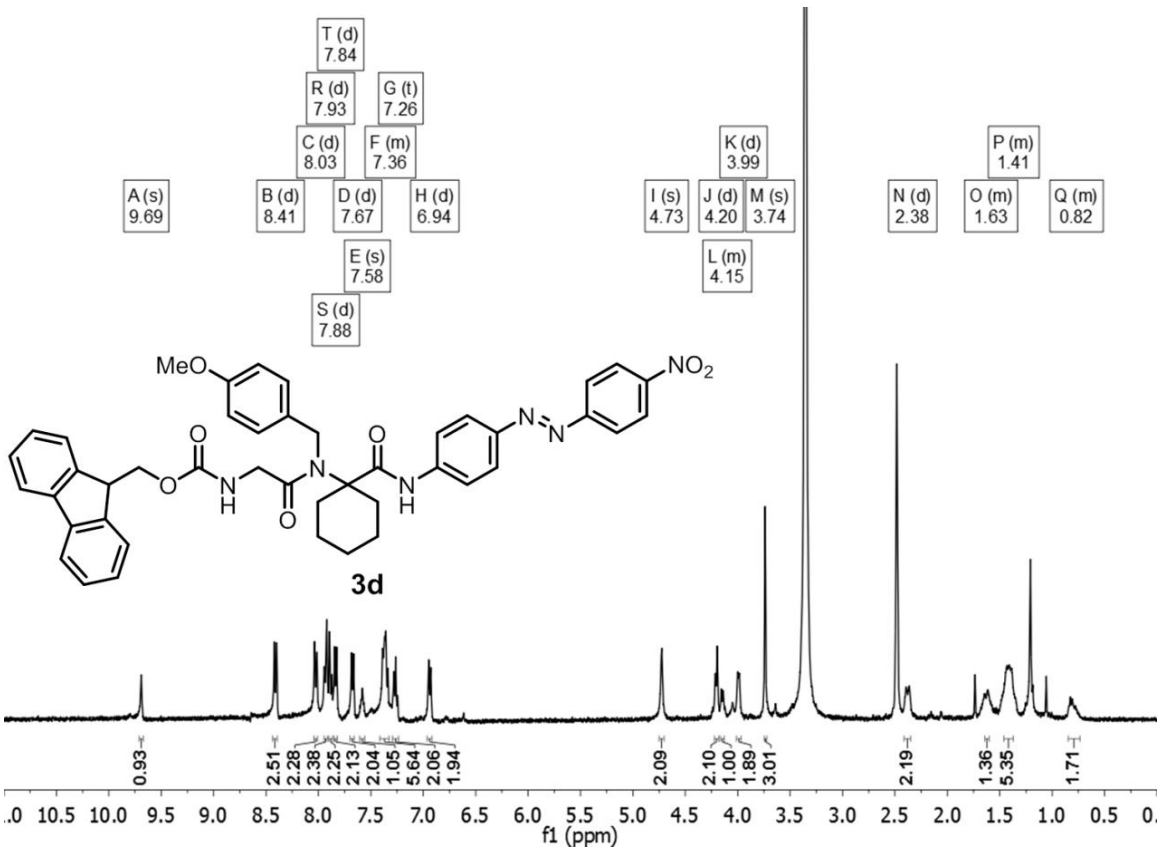
^{13}C NMR (100 MHz, $CDCl_3$): **2l**



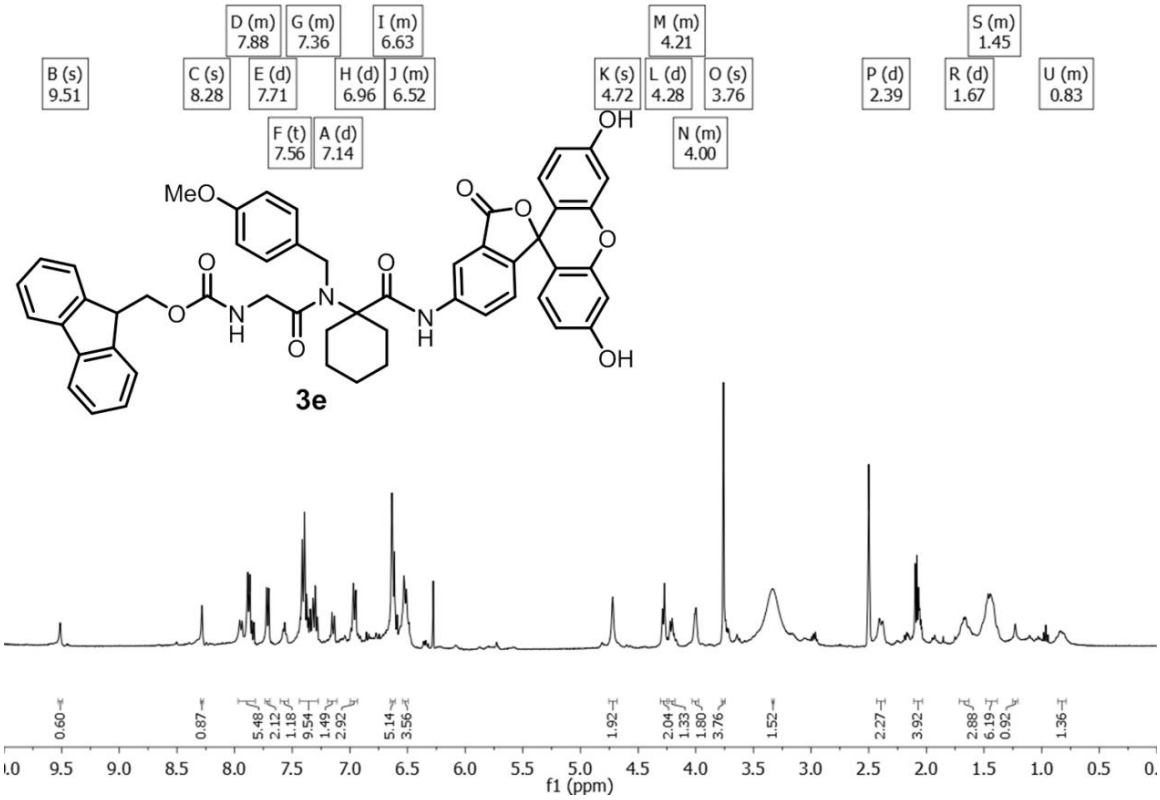
^{1}H NMR (400 MHz, $CDCl_3$): **3c**



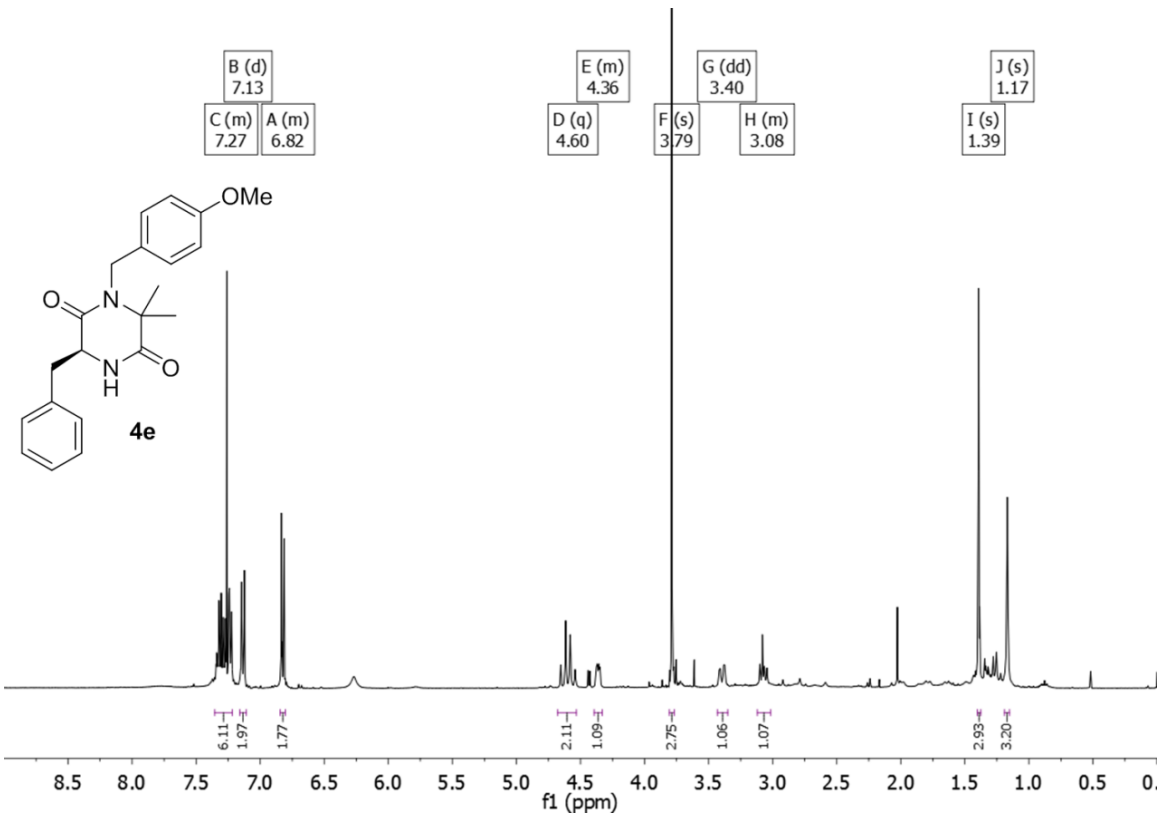
^{1}H NMR (400 MHz, DMSO-d_6): **3d**



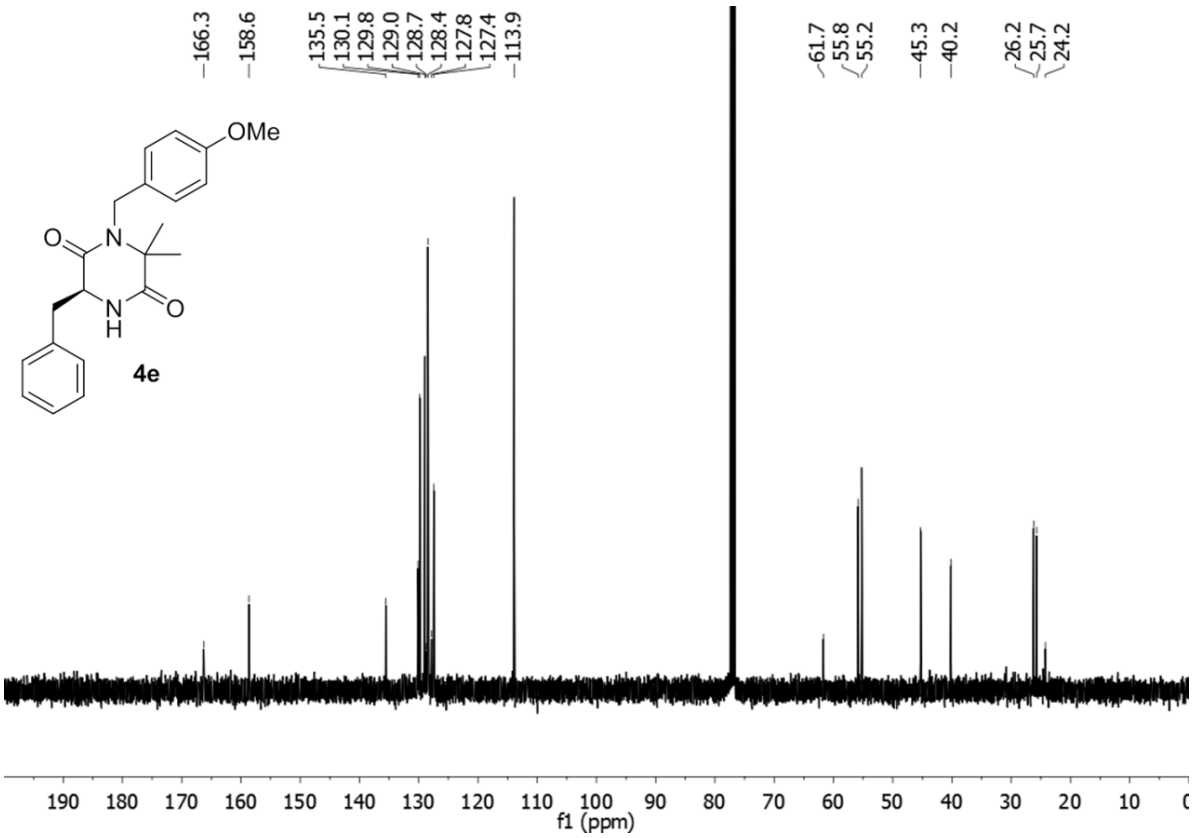
^{1}H NMR (400 MHz, DMSO-d_6): **3e**



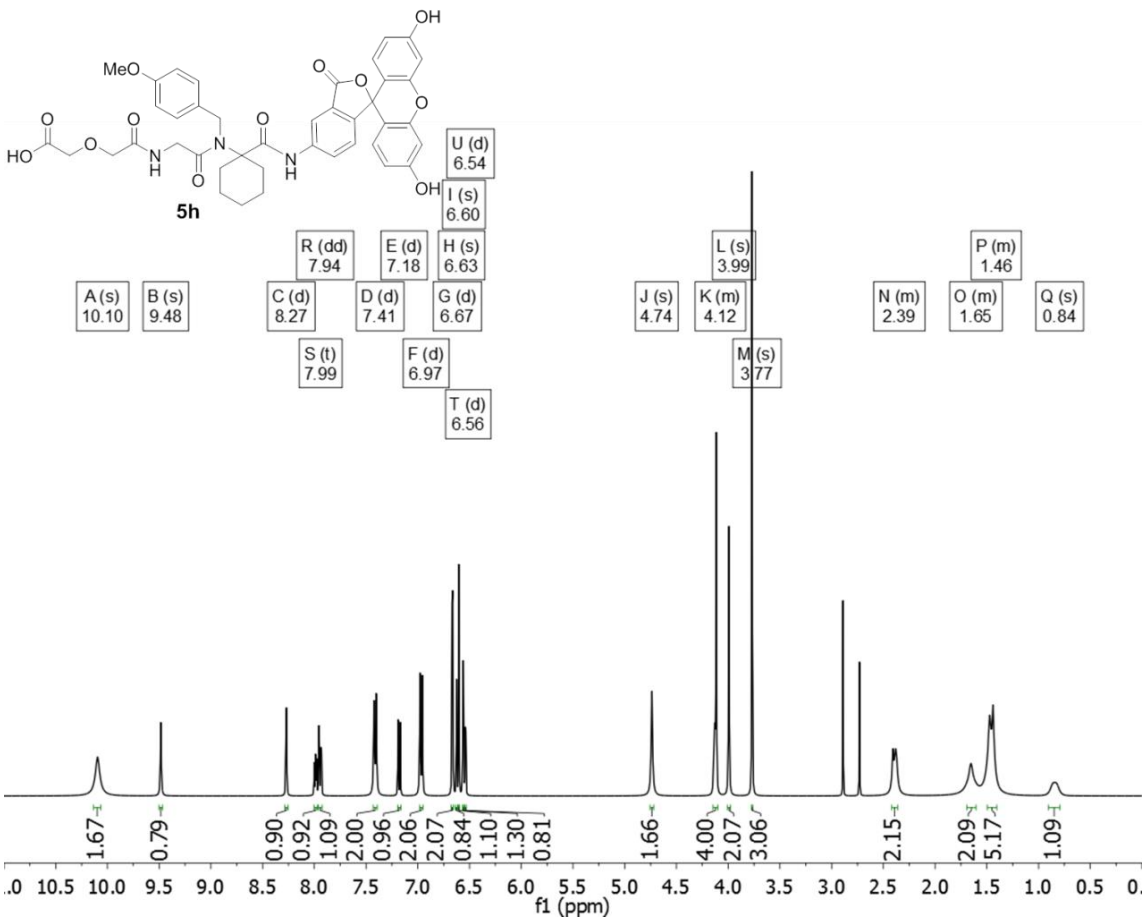
^{1}H NMR (400 MHz, $CDCl_3$): **4c**



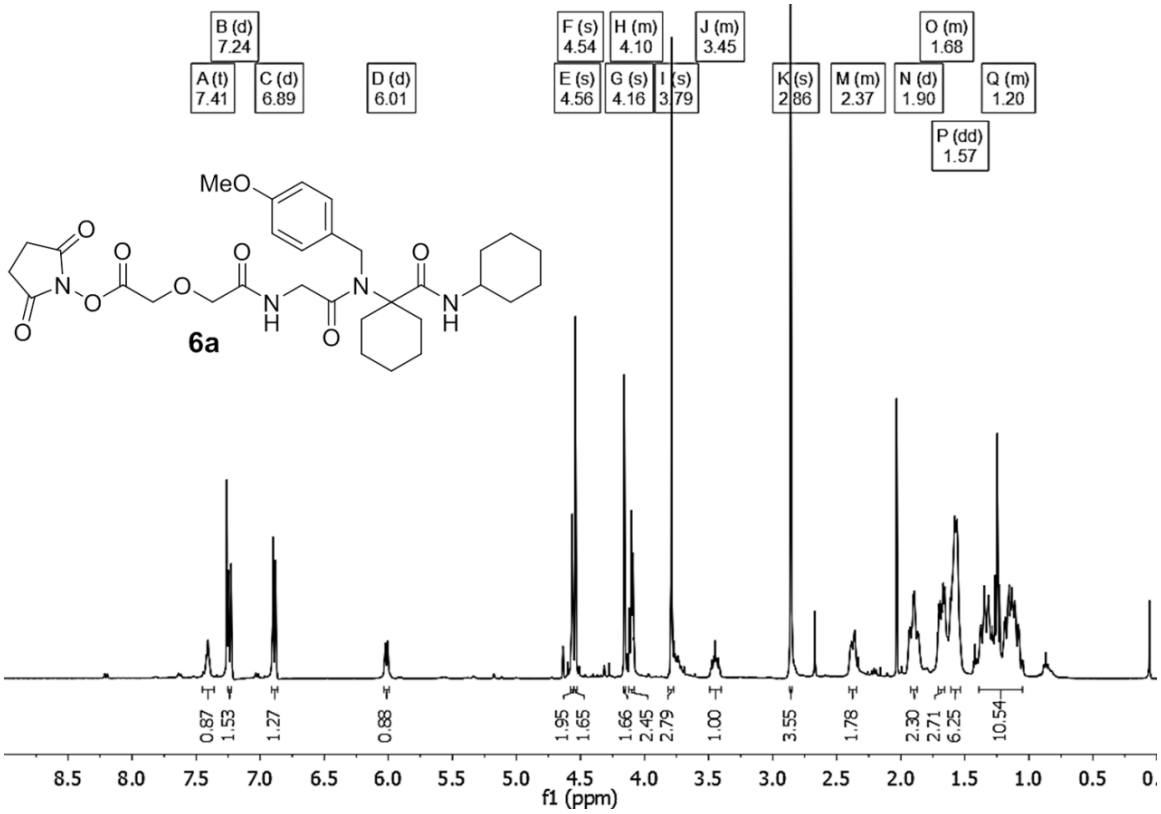
^{13}C NMR (100 MHz, $CDCl_3$): **4c**



^{1}H NMR (400 MHz, DMSO-d_6): **5h**

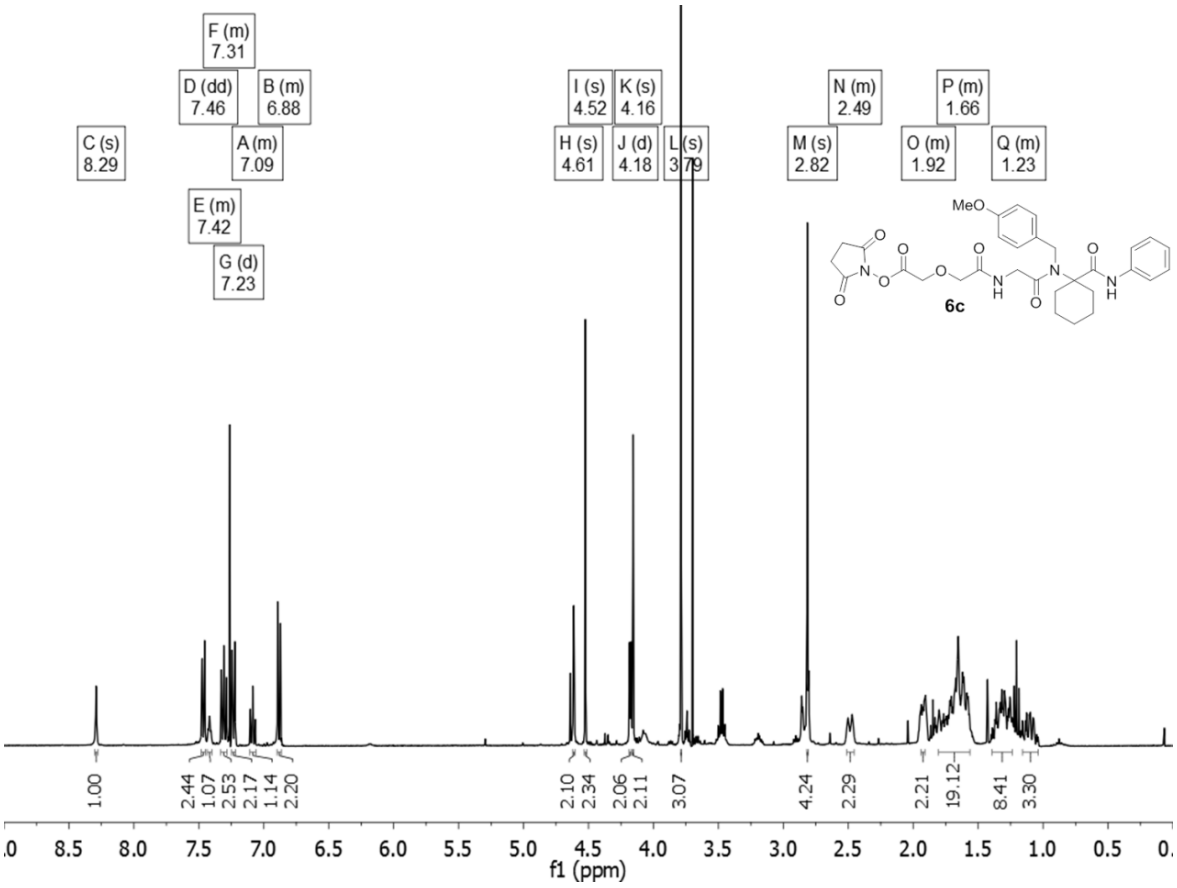


^{1}H NMR (400 MHz, $CDCl_3$): **6a**

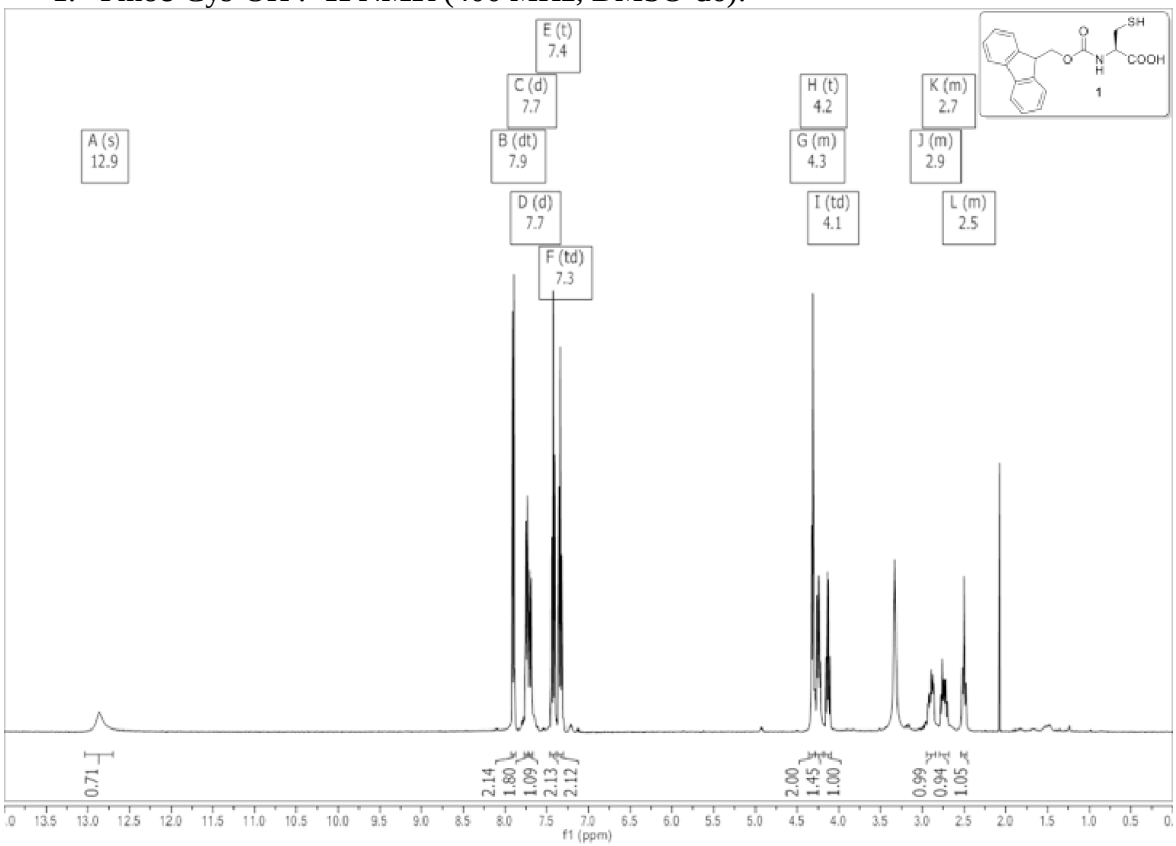


^{1}H NMR (400 MHz, $CDCl_3$): **6c**

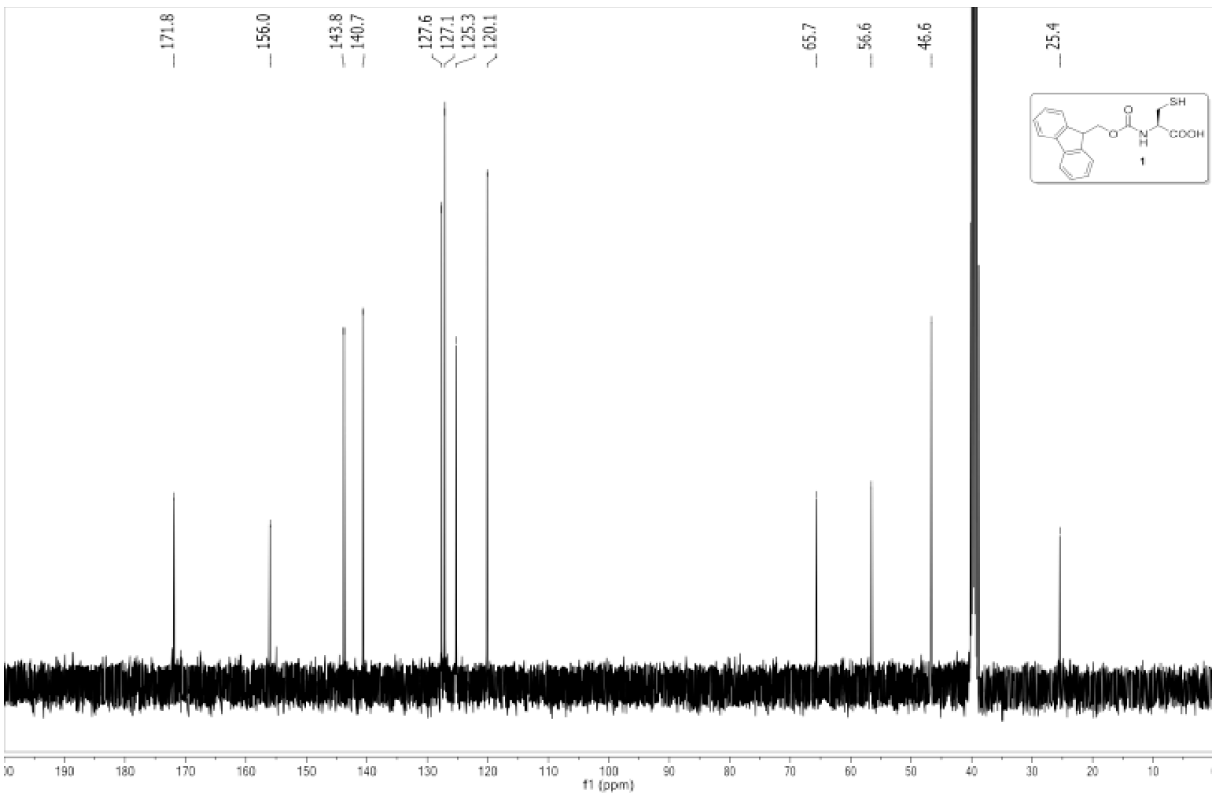
340



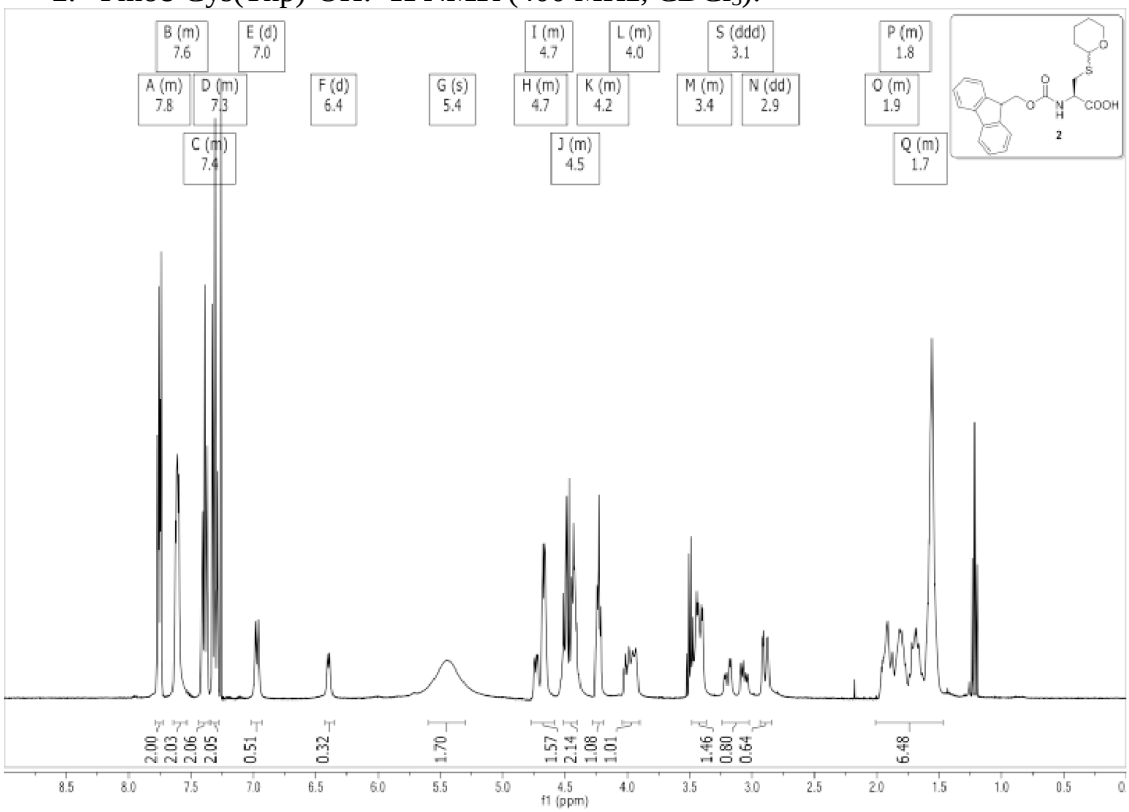
1. Fmoc-Cys-OH : **^{1}H NMR** (400 MHz, DMSO-d6):



^{13}C NMR (100 MHz, DMSO-*d6*):

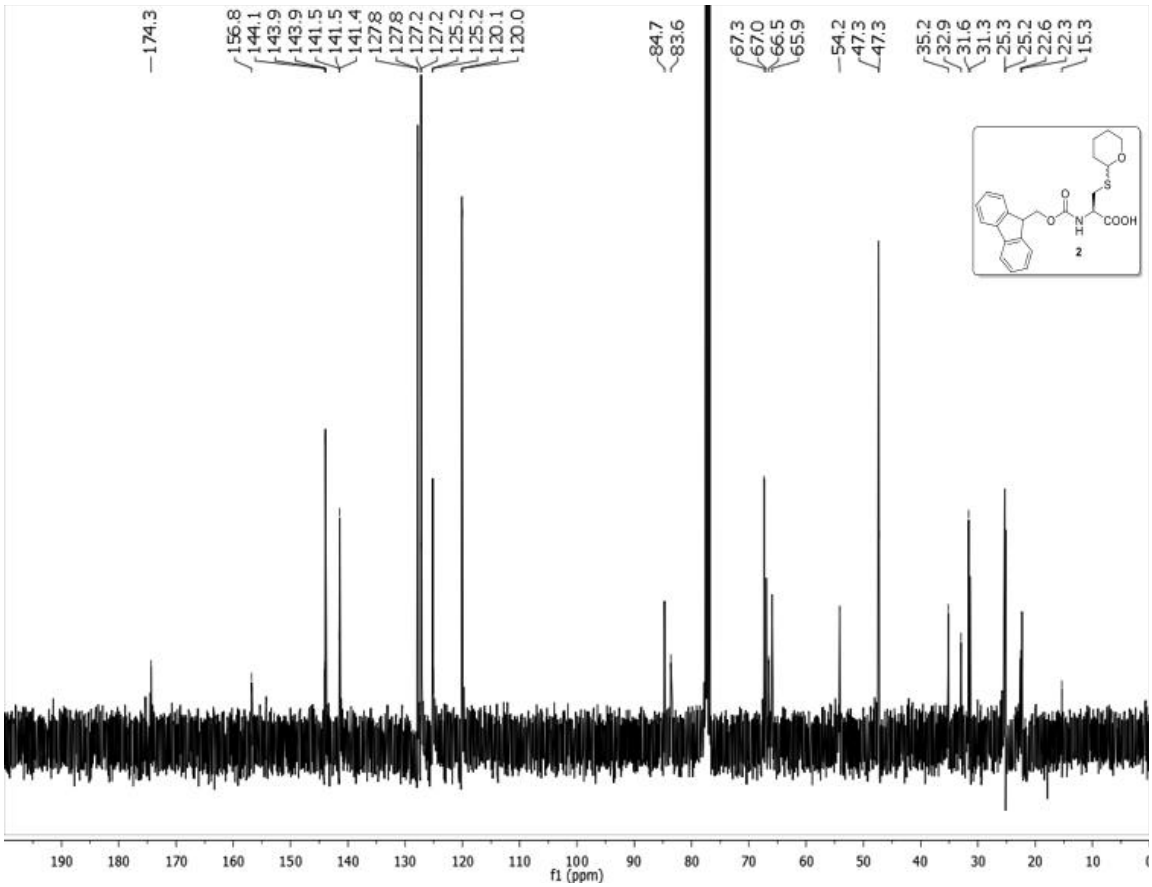


2. Fmoc-Cys(Thp)-OH: **^{1}H NMR** (400 MHz, $CDCl_3$):



^{13}C NMR (100 MHz, $CDCl_3$):

342



^{1}H NMR (400 MHz, Acetone-d_6): **3c**