Development of New Tools for the Synthesis of "Difficult Peptides"

Marta Paradís Bas

Programa de Química Orgànica

Tesi Doctoral

Development of New Tools for the Synthesis of "Difficult Peptides"

Marta Paradís Bas

Dirigida i revisada per:

Dr. Fernando Albericio
(director i tutor)
(Universitat de Barcelona)

Dra. Judit Tulla Puche
(directora)
(Institut de Recerca Biomèdica Barcelona)

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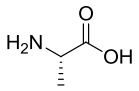
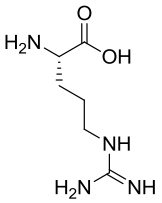
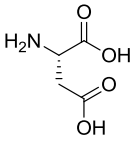
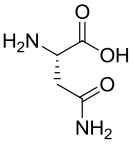
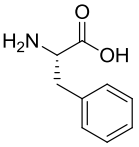
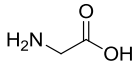
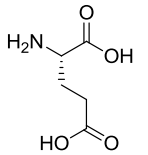
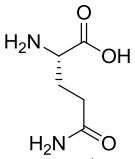
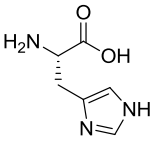
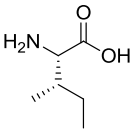
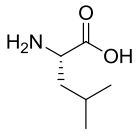
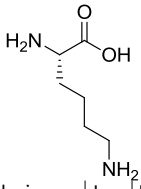
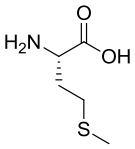
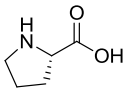
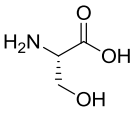
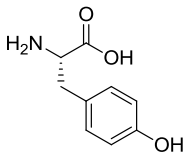
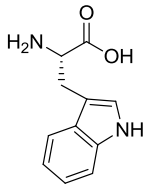
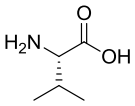
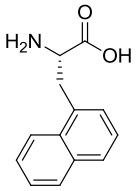
Abbreviations and Acronyms

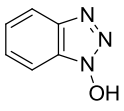
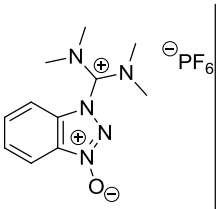
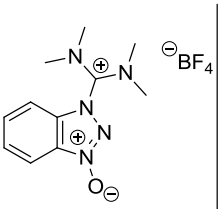
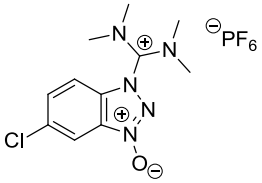
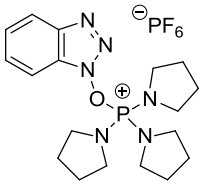
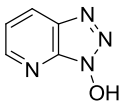
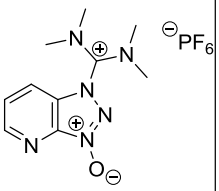
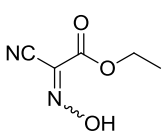
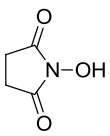
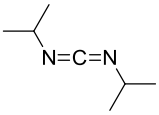
AA	amino acid
Ac	acetyl
ACH	α-cyano-4-hydroxycinnamic acid
ACN	acetonitrile
AM	amino methyl
Boc	*tert*-butoxycarbonyl
BTC	bis(trichloromethyl) carbonate
Bzl	benzyl
CD	circular dichroism
CM	ChemMatrix
CTC	2-chlorotrityl chloride
d	doublet
DCHA	dicyclohexylamine
DCM	dichloromethane
DIEA	*N,N'*-ethyldiisopropylamine
DIPCDI	*N,N'*-diisopropylcarbodiimide
DMAP	4-(dimethylamino)pyridine
DMF	*N,N'*-dimethylformamide
DMSO	dimethyl sulfoxide
EDT	1,2-ethanedithiol
eq	equivalents
ESI	electrospray ionization
ESMS	electrospray mass spectrometry
FDA	food and drug administration
Fmoc	9-fluorenylmethoxycarbonyl
HATU	*N*-[(7-aza-1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
HBTU	*N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
HCTU	*N*-[(6-chloro-1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
HOSu	*N*-hydroxysuccinimide
HPLC	high performance liquid chromatography
HR-MS	high resolution-mass spectrometry

J	coupling constant
m	multiplet
MALDI-TOF	matrix-assisted laser desorption/ionization-time-of-flight
Mmsb	2-methoxy-4-methylsulfinylbenzyl
Mmtb	2-methoxy-4-methylthiobenzyl
MS	mass spectrometry
Mtt	4-methyltrityl
MW	microwave
m/z	mass-charge relation
NMR	nuclear magnetic resonance
OSA	1-octane sulfonic acid
OxymaPure	2-cyano-2-(hydroxyimino)acetate
Pbf	2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl
PEG	polyethylene glycol
PG	protecting group
ppm	parts per million
PyBOP	1-benzotriazole-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate
q	quadruplet
s	singlet
SPPS	solid-phase peptide synthesis
t	triplet
TBTU	*N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide
*t*Bu	*tert*-butyl
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
TIS	triisopropylsilane
TMP	2,4,6-trimethylpyridine
TMS	tetramethylsilane
t_R	retention time
Trt	trityl
UV	ultraviolet
δ	chemical shift

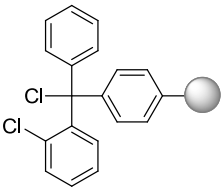
ANNEX I: Amino Acids

 L-alanine | Ala | A	 L-arginine | Arg | R	 L-aspartic acid | Asp | D	 L-asparagine | Asn | N	 L-phenylalanine | Phe | F
 glycine | Gly | G	 L-glutamic acid | Glu | E	 L-glutamine | Gln | Q	 L-histidine | His | H	 L-isoleucine | Ile | I
 L-leucine | Leu | L	 L-lysine | Lys | K	 L-methionine | Met | M	 L-proline | Pro | P	 L-serine | Ser | S
 L-tyrosine | Tyr | Y	 L-triptophan | Trp | W	 L-valine | Val | V	 1-naphthyl-L-alanine | Nal	

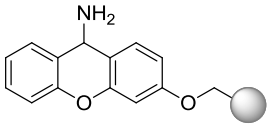
ANNEX II: Coupling Reagents and Additives

 HOBt	 HBTU	 TBTU	 HCTU	 PyBOP
 HOAt	 HATU	 OxymaPure	 HOSu	 DIPCDI

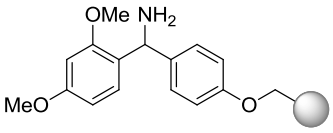
ANNEX III: Resins and Linkers



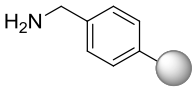
CTC Resin
(2-chlorotrityl chloride)



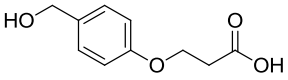
Sieber Resin



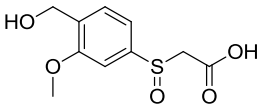
Rink Resin



AM Resin
(aminomethyl)

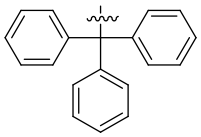


HMPP Linker
[4-(hydroxymethyl)phenoxy-propionic acid]

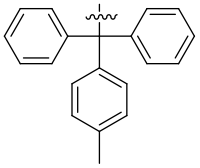


Mmsb-OH Linker
(2-methoxy-4-methylsulfinyl-benzyl alcohol)

ANNEX IV: Protecting Groups

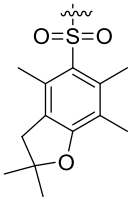


Trt
(trityl)

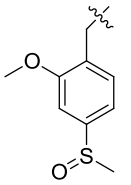


Mtt
(4-methyltrityl)

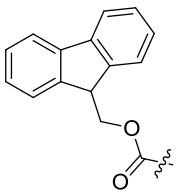
*t*Bu
(*tert*-butyl)



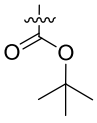
Pbf
(2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl)



Mmsb
(2-methoxy-4-methylsulfinylbenzyl)



Fmoc
(9-fluorenylmethoxycarbonyl)



Boc
(*tert*-butyloxycarbonyl)

Overview of the Thesis

The present thesis is structured as a compendium of publications which have been published in international scientific journals. These reports are organized in three chapters classified depending on the tool evaluated to achieve "difficult peptides" and aggregation prone peptides.

Initially, a **General Introduction** places the readers into the "difficult peptides" field and the peptide aggregation involvement during and after the synthesis. Furthermore, the strategies reported up to date address to overcome these synthetic and purification troubles are extensively described. The strategies comprised herein provide an overview about the two main branches of achieving these peptides by enhancing their solubility: during their synthesis or after their elongation. Moreover, the **Objectives**, according to the main subject of this thesis, address the aims proposed for each chapter.

Chapter 1 is focused on the known "difficult peptide" RADA-16. First, an **Introduction** is included in order to detail the general interest about this peptide and also to explain the basis of the synthetic strategies carried out in this project. Then, the specific research work addressed to obtain this peptide is detailed in the *Results and Discussion* and also the *Experimental Part*, both comprised in the **Publication I** section, as a full paper that includes also its supporting information.

Chapter 2 is based on the strategy which takes advantage of the use of backbone amide protecting groups to synthesize "difficult peptides". The **Introduction** provided is focused on certain concepts that have not been mentioned in the general introduction, but considered relevant to follow the experimental work. Then, in the **Publication II** section, experiments performed about this issue are described in a full paper with its supporting information as the *Results and Discussion* and also the *Experimental Part* of this chapter.

Chapter 3 contains two projects with the principal aim centered on facilitating the post-synthetic manipulation of non-soluble molecules by the short peptide-based solubilizing tag strategy. The two methodologies, the permanent and the temporary linkage have been performed in two differentiated projects. An additional proposal has been included in this chapter, which despite not being based on solubilizing strategies, takes the advantage of the same linker used for the temporary tag strategy. In this new approach, the linker has been explored as a carboxylic acid protecting group, including an application. An initial **Introduction** exposes the state of the strategies addressed to solubilize non-polar sequences by their conjugation to short peptide sequences as

solubilizing tags, in order to expand specific details not included in the general introduction. Moreover, there are mentioned some of the most significant carboxylic acid *C*-terminal protecting groups described in the literature to provide an overview of the importance to contribute to this field. The *Results and Discussion* and also the *Experimental Part* of these projects are exposed in two independent papers, one letter and one communication, and both supporting information are included and presented in **Publication III** and **Publication IV** sections. Publication III, specifically covered a study about short peptide-based solubilizing tag strategies in permanent conjugations; and in the first part of publication IV, the same issue is addressed, although focused on temporary conjugations. The new application on carboxylic acid *C*-terminal protecting groups is found in the second part of the publication IV, where this group is applied in a fragment condensation synthesis.

Finally, the **General Conclusions**, organizes the main final accomplishments after the development of all the projects herein presented. In agreement with the objectives considered in the beginning, and according to the results obtained and presented in the publication sections, this last part shows the contribution of this thesis to the fields herein studied.

GENERAL INTRODUCTION

1. "Difficult Peptides" and Aggregation

The peptide synthesis has become an attainable process since 1963,[1] when Professor Merrifield described the revolutionary concept of constructing these molecules by the solid-phase strategy. This invention was significant enough for him to win the Nobel Prize in Chemistry, at the same time that researchers received an extraordinary tool to facilitate the obtaining of peptides. After the solid-phase peptide synthesis (SPPS) was discovered, synthetic improvements have emerged addressed to the design of orthogonal protecting groups,[2] new functionalized solid supports,[3,4] efficient coupling reagents,[5] or even methodologies to overcome difficulties on the sequence elongation. Nowadays, synthesis of large peptides has become achievable thanks to specific strategies emerged in recent years focusing on development of new synthetic tools. However, in spite of all the efforts invested in these fields, there are certain sequences still correlated with laborious syntheses.

The known as **"difficult peptides"** are an evident example of the need to continue developing strategies to enable their obtaining. This peptide concept was established in the '80s[6] and refers to sequences that show inter- or intra-molecular β-sheet interactions significant enough to form aggregates. These structural associations occur **during the peptide synthesis** in both methodologies, solid-phase and solution-phase (Fig. 1)[7] and they are stabilized and mediated by non-covalent hydrogen bonds, which, depending on the sequence, are favored. Specifically, they arise on the backbone of the peptide, in particular between the hydrogen amides and the carbonyls.[8] The tendency of peptide chains to associate themselves is translated into a list of common behavioral features attributed to "difficult sequences", detailed by Kent[9] and later by Milton.[10] The main relevant synthetic evidences provided by these authors are the following: repetitive incomplete aminoacylations (<15%) despite re-couplings; accentuated difficulties when resin loading is high[11] or when sterically hindered AAs are in the sequence; and slow or incomplete 9-fluorenylmethoxycarbonyl (Fmoc) removal.[12]

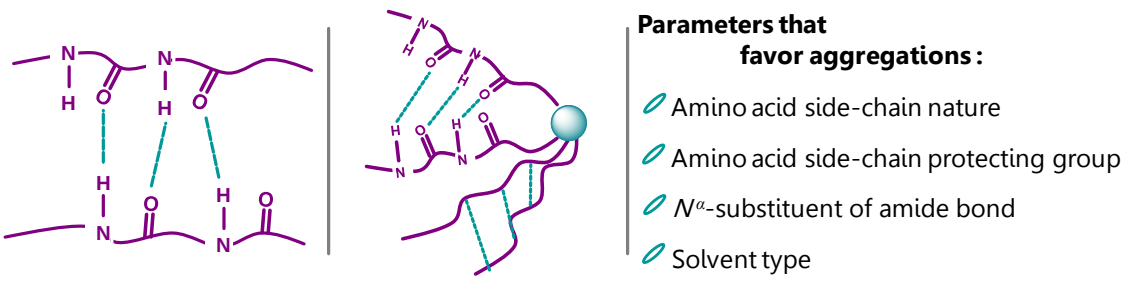


Figure 1. Hydrogen bond inter-chain interactions during peptide synthesis (in solution or on solid-phase) and factors that contribute to their formation.

Some studies have analyzed the influence of each AA on β-sheet interactions, confirming that the capacity to induce intermolecular associations is sequence-dependent.[13] Nevertheless, the prediction of a "difficult peptide" by the AAs present in its sequence, *a priori*, is not evident. Several factors that contribute to increase the propensity to form inter-chain associations are correlated with AA side-chains or even with the protecting group of the AA, with the nature of *N*-substitution of the backbone amide, and the characteristics of the solvent used to synthesize them (Fig. 1).[10,14] Although the literature does not provide a comprehensive list of "difficult sequences", below we refer to some that fit the description and that have been synthesized, or their β-sheet folding mechanisms evaluated.

Sequences	Peptide Type	Ref.
RADA16-I [Ac-(RADARADA)$_4$-NH$_2$] **RADA16-II** [Ac-(RARADADA)$_4$-NH$_2$]	ionic self-assembling (Type I and Type II)	46 56
oligo-Ala, **oligo-Val** **oligo-Gln, oligo-Leu**	homooligo-pept.	17-23
Aβ (1-40) or **Aβ (1-42)**	amyloidogenic	29-33
Amylin	amyloidogenic	34, 35
ACP (65-74) (H-VQAAIDYING-NH$_2$)	-	13, 20 57-60
HIV-1 PR (81-99) (H-PVNIIGRNLLTQIGCTLNF-NH$_2$)	-	20 57-59
PnIA (A10) (H-GCCSLPPCALNNPDYC-NH$_2$)	-	20, 59
Thymosin α1	-	61-64

Table 1. Common examples of sequences that aggregate on solid-phase: "difficult peptides".

On the other hand, there are peptides that form β-sheet associations **in solution** only once their sequences are fully unprotected, not during the elongation. These peptides assemble themselves in the liquid media establishing stable interactions that evolve to **aggregates**. Although that behavior may be considered an appreciated property in certain areas (explained in the following sections), in terms of peptide characterization or purification it supposes a significant drawback. These two post-synthetic steps demand sequences perfectly dissolved to provide an accurate peptide analysis. Again, this phenomenon is not easily predictable by the AAs present in the sequence, as it occurred for "difficult peptides". However, studies based on structural interaction experiments[15] have led to develop computational methods to establish similarities between sequences that anticipate aggregation of peptides.[16]

Sequences	Peptide Type	Ref.
Ac-**V_6D**-NH_2, Ac-**A_6D**-NH_2, Ac-**V_6D_2**-NH_2, Ac-**L_6D_2**-NH_2 Ac-**G_nD**-OH (n=4,8,10)	negatively charged surfactant-like	40-42
Ac-**V_6K_2**-NH_2, Ac-**L_6K_2**-NH_2, Ac-**A_6K**-NH_2, Ac-**KA_6**-NH_2, Ac-**V_6H**-NH_2, Ac-**L_6K**-NH_2, **H_2V_6**-NH_2, **KV_6**-NH_2	positively charged surfactant-like	41, 43
KFE-8 [Ac-$(FKFE)_2$-NH_2] **KLD-12** [Ac-$(KLDL)_3$-NH_2]	ionic self-assembling (Type I)	54 46, 47
DAR16-IV [Ac-(A-$(DA)_4$-$(RA)_3$-R)-NH_2]	ionic self-assembling (Type IV)	56
ELK8-II (Ac-LELELKLK-NH_2)	ionic self-assembling (Type II)	56
EAH16-II [Ac-$(AE)_2$-$(AH)_2$-$(EA)_2$-$(AH)_2$-NH_2] **EAK16-II** [Ac-$(EA)_2$-$(AK)_2$-$(AE)_2$-$(AK)_2$-NH_2]	ionic self-assembling (Type II)	56
RADSC-14 [Ac-$(RADSC)_2$-A_5-C-NH_2] **RADSC-16** [Ac-$(RADSC)_3$-A_3-C-NH_2]	ionic self-assembling (Type I)	55
Q7 (Ac-KFQFQFE-NH_2) **Q11** (Ac-QQKFQFQFEQQ-NH_2)	ionic self-assembling	50-52
DN1 (Ac-QQRFQWQFEQQ-NH_2) **P11-4** (Ac-QQRFEWEFEQQ-NH_2)	ionic self-assembling	26 43, 44
Sup35 (7-13) (H-GNNQQNY-NH_2)	amyloidogenic	36, 37

Table 2. Common examples of sequences that aggregate in solution.

To sum up, aggregation in peptides can be originated in two different moments, during the synthesis or after the global deprotection of sequences. Herein, the peptides have been organized by families which present aggregation in one or both stages. Therefore, all kinds of peptides exposed below have in common the β-sheet association tendency, being highlighted if these interactions are presented **in solution** or during the synthesis (**solid-phase**).

1.1. Homooligo-Peptides

The homooligo-peptides are composed by only one AA type. These peptides are *de novo* synthetic sequences that show high tendency to aggregate, and are composed by hydrophobic AAs such as Ala, Gly, Ile, Leu or Val (Table 1). The aggregation is manifested not only during the synthesis of peptides **on solid-phase**, but also when the sequence is unprotected **in solution**. Those most commonly analyzed are poly-alanine and poly-valine,[17] which show a notable capacity to assemble, in the case of oligo-alanine already starting from the fifth residue. Moreover, other homooligo sequences, such as poly(Gln) have also been explored regarding the aggregating mechanisms,[18,19] or the poly(Leu) to validate new "difficult peptide" synthetic

strategies.[20] The driving force behind the assembly is the hydrogen bond formation between the backbone amides of the peptide chains. The authors who initially studied the behavior of these sequences, mentioned that, due to their characteristics, AA deletion occurred during the synthesis, thus demonstrating the structural conformation that explains this phenomenon.[21–23] Especially, the oligo-alanine has been widely used as a model to study conformational changes after chemical modifications.

1.2. Self-Assembling Peptides

Self-assembling peptides (SAPs), as their name denotes, are another class of sequences characterized by their capacity to self-assemble. The self-assembling tendency appears when the peptide is completely unprotected after the cleavage from the resin, thus precluding its purification once **in solution** phase. These dissolved molecules spontaneously assemble into energetically favored β-structures that subsequently may form fibrils[24] that may evolve to hydrogelation. The SAPs are not necessarily "difficult peptides", although some of them also may form β-sheet interactions during **solid-phase** synthesis and would also fit into this concept. Given their capacity to aggregate in solution, they provide a broad number of applications in biomedicine.[25–27] Although self-assembly has been considered a desired property from a biomaterial perspective, this phenomenon underlies a number of human diseases.[28] In this regard, probably the most known SAP is amyloid-β peptide (Aβ), the primary responsible agent of alzheimer's disease.[29,30] Specifically, the two amyloid sequences involved in this neurodegenerative illness are the 40-mer Aβ (1-40) and the 42-mer Aβ (1-42) (Table 1), which misfold and cause insoluble non-native disordered aggregates, forming oligomers and fibrils.[31–33] Among the amyloidogenic peptides described, the hormone amylin (Table 1), another relevant sequence also present in humans, is highlighted because of its involvement in type 2 diabetes mellitus.[34,35] This hormone is a 37-mer peptide secreted in pancreas, together with insulin, and in a pathological situation, the excess of amylin self-assembles producing deposits in the pancreas and causing non-insulin dependent diabetes. One of the few amyloid-like peptides characterized by x-ray crystallography has been the 7-mer fragment of yeast prion protein Sup35, specifically the region (7-13)[36] (Table 2). The Sup35 protein exists in a non-infectious form but when folded as an infectious variant is called a prion, in which the 7-mer region plays a key role. Moreover, studies on Sup35 heptapeptide fibril formation have been used to understand the aggregation mechanisms.[37] Apart from the aforementioned peptides found in biological systems, there are other *de novo* designed sequences, classified as SAPs, which are not present in nature. These are commonly sub-divided into two families, described below.

1.2.1. Amphiphilic self-assembling peptides

The first family comprises amphiphilic (or amphipathic peptides, also named surfactant-like peptides),[38,39] whose sequences have two differentiated parts, one hydrophobic (more than three non-polar AAs), and one hydrophilic (one or two polar or charged AAs). Depending on the length of the hydrophobic core, the solid-phase peptide synthesis may be hindered as occurs in "difficult peptides", being the largest chain the most difficult to achieve. Nevertheless, **in solution** all these amphiphilic self-assembling peptides tend to assemble by forming nanotubes or nanovesicles.[40] Regarding their applications, it is worth highlighting the membrane protein stabilization allowed by some of these surfactant-like peptides.[41] Sequences such as V_6D, A_6D and V_6K_2 are examples of amphiphilic peptides which exhibit a demonstrated capacity to assemble, at certain concentration, in water solutions[40–43] (Table 2). When the hydrophilic part of these peptides holds one kind of ionic charge, they are simply amphiphilic, whereas when they hold the two charges, they belong to the ionic peptide group.

1.2.2. Ionic self-assembling peptides

The sub-type of ionic self-assembling peptides are characterized by the stabilization of aggregates because of ionic interactions, being this effect caused by the positively and negatively charged AA side-chains.[44,45] The ion-ion associations occurs once sequences are unprotected, thus the stabilization of aggregates in these peptides **in solution** is sever. Although most of the sequences belonging to this classification may be synthesized without difficulties on SPPS, there are some that also present β-sheet interactions during **solid-phase** synthesis, and therefore can also be classified in the group of "difficult peptides". Those *de novo* designs composed by rational arrangement of oppositely charged AAs, have served as self-assembling sequences (Table 1 and Table 2). These structures are sometimes named "peptide lego" and some of them exhibit supra-structures that have reached the market.[46–48] Ionic self-assembling peptides are characterized mainly by the formation of complementary ionic interactions that are electrostatically orientated between two peptide chains facing opposite charges, thus favoring the self-assembling design when this phenomenon is desired. Peptide designs with alternating hydrophobic and hydrophilic AAs were among the initial methods proposed to achieve sequences with β-sheet interactions of interest.[49–53] Later, Zhang, a relevant author in the field of well-defined self-assembling ionic sequences, proposed the division of these ionic self-assembling peptides into modules (I, II, III,...) (Table 1 and Table 2) on the basis of their positive and

negative charge arrangement (type I: + − + − + −; type II: + + − − + +; type III: + + + − − − ; ...).[46,47,54–56]

1.3. Other "Difficult Peptides"

Moreover, there are "difficult peptides" non-classified in a specific sub-type described in the literature. Some examples are the extensively used protein fragment named Acyl Carrier Protein [ACP (65-74)][13,20,57–60] (Table 1) or the fragment of human immunodeficiency virus protease [HIV-1 PR (81-99)][20,57–59] (Table 1), two models employed to evaluate new synthetic methodologies. Researchers specialized in conotoxin peptide synthesis have been used the Ala10→Leu mutant of PnIA [PnIA(A10L)] (Table 1) as a "difficult peptide" to validate synthetic methodologies,[59] and it is also a sequence which has been used for the same proposal by other authors.[20] Another example is the commercially available Thymalfasin (also known as Thymosin α1)[61] (Table 1) described for hepatitis B and C.[62] It is a 28-mer peptide produced by the thymus gland which participates in the mature of the T cells and with a wide range of reported medicinal applications,[63] that have evolved to develop strategies to achieve it.[64]

2. Strategies to Solubilize Peptides

The main obstacle common to all "difficult peptides", and also to those sequences which aggregate in solution, is their insolubility. The non-soluble nature of peptides precludes their synthesis and/or hinders their characterization, and even prevents their purification. The peptide sequence and, most importantly, the AA composition, plays a key role in terms of secondary structure, thereby directly affecting the solubility of the molecules. The SPPS, and also the synthesis of peptides in solution, requires protected sequences to allow the two functional groups to react properly to lead the desired amide bond. The nature of these protecting groups contributes to enhance the hydrophobicity of the sequences and may induce interactions which favor the insolubility of peptides. In general, it is known that peptides that adopt α-helix secondary structures in solution are generally soluble in water, whereas β-sheet conformations are insoluble because of their capacity to interact by hydrophobic interactions which evolve to aggregates.

In order to overcome this drawback, several authors have invested efforts to develop strategies to enhance the solubility of peptides by disrupting the β-sheet interactions. Thus, the low solubility of some peptides is considered not only a barrier, but also a challenge in terms of developing new solubilizing methods. These methodologies can

be classified in two principal groups, those that modulate an *external factor* and those that introduce a *chemical modification* to the peptide sequence.

2.1. External Factors

One of the first aspects to be considered when addressing peptide insolubility is the modification of external parameters. In some cases, external factors are introduced on solid-phase to favor peptide elongation by improving the AAs incorporation, or the protecting groups removal. These parameters may be introduced not only during the SPPS, but also to solubilize sequences in solution. Specifically, the main external parameters evaluated in the literature are the solvent, temperature, pH, the detergents and salts added to the peptide solution.

2.1.1. Solvent selection

Although *N,N*-dimethylformamide (DMF), a dipolar aprotic solvent, is commonly used in solid- and solution-phase peptide synthesis, several studies have explored the capacity of other solvents to substantially disrupt the secondary interactions that make peptides insoluble. In solid-phase the resin plays a key role in preventing intermolecular peptide associations. Consequently, an appropriate solvent to swell the resin may avoid these limitations, as well as the low loading may minimize the intermolecular connection.[65] In some cases, dimethyl sulfoxide (DMSO) achieves solubilization of the hydrophobic sequences because, as a polar aprotic hydrogen bond-accepting solvent, it causes an increase in peptide mobility and consequently favors its solvation.[66] A number of studies have demonstrated the use of DMSO as a solvent for SPPS to perform AA couplings and Fmoc removal treatments, thus enhancing the solubility and purity of the peptides.[7] However, the oxidative properties of DMSO poses limitations for its use in SPPS and in solution when sequences contain AAs susceptible to oxidization, such as Met and Cys.

Another group of solvents that have been studied extensively and used to solubilize peptides in solution are the fluorinated alcohols, which the most widely used have been the 2,2,2-trifluoroethanol (TFE), the 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), and the 1-phenyl-2,2,2-trifluoroethanol (PhTFE),[67] (Fig. 2) among others. In contrast to non-fluorinated alcohols, the main characteristic of these fluorine-containing ones is their strong hydrogen-bond donor capacity, a property that makes them suitable for interfering with the secondary structure of peptides.[67,68] Mixtures of H_2O/TFE in peptide copolymers with alternating neutral and charged AAs favors the α-helix when higher is the TFE proportion,[69] supporting the general idea that TFE stabilizes the α-helix and

only in some cases stabilizes β-helix.[70] Furthermore, for certain peptide sequences there is a reversible α-β transition, that modulates the conformation of the peptide and consequently, its solubility.[71,72] Thanks to the compatibility of fluorinated alcohols with solid-support protocols, HFIP has also been used to enhance solubility during the solid-phase synthesis of "difficult peptides", in those cases in which DMF is not as effective as a solvent. Its use has represented a suitable alternative for acylation, Fmoc removal,[73] and even for peptide cyclization[74]. Other research groups have also explored the use of diverse solvents during SPPS to improve certain couplings.[75,76] These solvents include tetrahydrofuran (THF), acetonitrile, and *N*-methyl-2-pirrolidone (NMP), which affect the swelling of the resin.[65,77]

2.1.2. Temperature increase

The peptide elongation on solid-phase, commonly performed at room temperature, may be carried out also at higher degree Celsius. Particularly, those synthesis performed at high temperatures are known as SPPS-elevated temperature (SPPS-ET). The most crucial aspects that must be considered when SPPS-ET is performed are the side-reactions, such as AA racemization, or the alteration of solvent-swelling on resin, among others. In the '90s, studies based on SPPS-ET developed protocols at 60 °C with several coupling reagents, solvents or resins, which showed efficiency in peptide yields and purities.[78] Furthermore, other reported peptide synthesis that followed this methodology demonstrated minimized racemization at several temperature ranges of 55-75 °C,[79] or 30-80 °C[80] for short peptides even using triethylamine. Moreover, similar protocols at 50 °C may be used for large sequences for certain AA couplings, when under common conditions incorporations are hindered,[65] or to facilitate "difficult peptide" synthesis.[81] On the other hand, in solution peptide manipulation, the increase of temperature produces a disruption of their secondary structure that may enhance their solubility. This involvement on unfolding the peptide depends on the secondary structure transition of each self-assembling type sequence. Thus, modifying the characteristic β-sheet structure to a random coil by temperature is a described possibility to mediate the transition between insoluble into a soluble peptide.[82]

Another way to enhance the temperature in order to solubilize peptides relies on the microwave (MW)-assisted synthesis. Initial studies to perform combinatorial chemistry under MW conditions have been summarized years later,[83] showing the large variety of organic reactions that can be accelerated thanks to these irradiations. Several reports have validated this strategy to enhance solubility which have allowed achieving the synthesis of a wide range of peptides.[84–86]

2.1.3. The pH control

When AAs in the peptide sequence are ionizable, positively or negatively, another external parameter to take into account is the pH, which allows modulation of the neat charge of the sequence in solution, thereby modifying its interactions with solvent and also its solubility.[87,88] The pH value at which a sequence exhibits a conformational α-β transition depends on the pka of the AAs or on their isoelectric point. It is believed that the conformation of an ionizable peptide sequence depends more on external factors than on its intrinsic tendency to form a specific secondary structure. Several authors have taken advantage of ionizable peptides to control peptide interactions. In this regard, they have achieved interesting materials by modifying the pH.[89–91] On SPPS, interfering in the pH of the system to enhance solubility is not common practice because this parameter directly affects the integrity of the protecting groups used in such synthesis and could lead to their removal or even cleavage of the peptide.

2.1.4. Salts addition

Other strategies to enhance the solubility of peptide sequences consist of adding chaotropic salts or denaturant agents (guanidinium chloride, urea or detergents) into the aqueous peptide solution. Regarding chaotropic salts, as their definition indicates, they are added to destabilize hydrophobic interactions and consequently the aggregates. Lithium salts such as LiCl, LiBr or $LiClO_4$[92,93], are the most used for this purpose, together with other salts such as KSCN or $NaClO_4$ (Fig. 2). These salts act by disrupting the intermolecular hydrogen-bonding, first increasing the polarity of the solvent (non-polar, aprotic or non-nucleophilic), and then displacing it by the most favorable ion-dipole interaction between the salt and the peptide. The associations of peptides in solution by ionic groups have been described by a number of researchers,[94,95] and have stimulated other groups to address the addition of these salts to solubilize peptides, confirming it by NMR and circular dichroism. Those studies demonstrated that chaotropic salts can be used during the manipulation of a peptide in solution, and based on the same concept, during solid-phase synthesis to improve peptide synthesis, mainly when THF is used as solvent.[96]

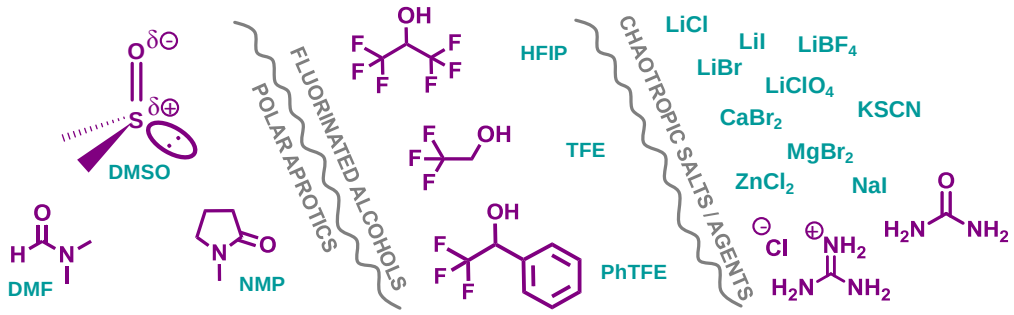


Figure 2. Common solvents and chaotropic agents used to enhance the solubility of peptides in solution and on solid-phase.

The addition, at a certain concentrations, of guanidinium hydrochloride (Gnd·HCl or Gdm·HCl) or urea to a peptide has resulted in increased solubility of the sequence, especially those containing non-polar AAs. The mechanism of action of these two agents suggests that there is a complex formation between the peptide backbone and the denaturant agent through hydrogen-bonds (2 hydrogen donors per agent; and 2 acceptors from carbonyl and amine).[97] The stability of this interaction is stronger than the β-sheet association between peptide chains which form aggregation and this is translated into effective denaturant agents. Principally, guanidinium hydrochloride is used to dissolve peptides in solution or to prevent long peptide folding, by precluding the reaction, in peptide fragment ligations.[98,99] NMR studies have confirmed these denaturant phenomenon based on the changes on chemical shift observed for structured conformations.[100–102] Furthermore, given the effect of guanidinium hydrochloride on solubility, some studies have proposed the introduction of L-Arg in the aqueous solution of proteins to improve their solubility.[103,104] The extension of the arginine application to some non-soluble peptides was used to improve the peptide detection by HPLC. However, this is not an appropriate solubilizing method for the peptide synthesis on solid-phase neither in solution-phase, or even purification, as arginine addition may compete with AA coupling and it implies the presence of an extra impurity, which may hinder purification.

2.1.5. Detergent addition

Detergents are considered another kind of denaturing agent, and are also a useful additive for hydrophobic sequences. Although these agents are extensively used for proteins, the addition of some kinds has also been reported for peptides. The most commonly used detergents are the anionic and non-ionic sub-types, specifically sodium dodecyl sulphate (SDS) as an example of the former, and Triton X100 or Tween 20 as example of the latter (Table 3). They are used to solubilize peptides both in solution and during solid-phase synthesis, thus favoring yields and even peptide

folding mechanisms.[105] Detergents favor micelles formation, thus enhancing the aqueous solubility of peptides; these structural changes have been analyzed to further understand the solubilizing power of detergents.[106,107] Some studies revealed that non-ionic detergents also preserve certain structured sequences, protecting the peptides in well-organized micelles.[108,109] Although detergents have been commonly used for protein solubilization for many years,[110,111] some lines of research recommend that they have to be avoided because may interfere with HPLC chromatography and decrease the signal intensity in mass spectrometry analysis.[112] Recent publications have proposed protocols to improve HPLC compatibility with detergents.[113–115] The most effective way to solubilize hydrophobic peptides or facilitate peptide reactions in solution is probably by combining appropriate solvents with certain detergents, the best known blend is the "magic mixture", which comprises dichloromethane (DCM)/DMF/NMP (1:1:1) containing 1% of Triton X-100 and 2 N of ethylene carbonate.[116–120]

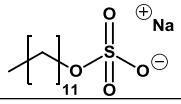
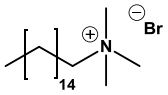
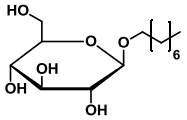
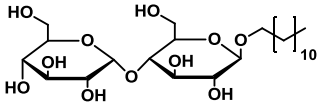
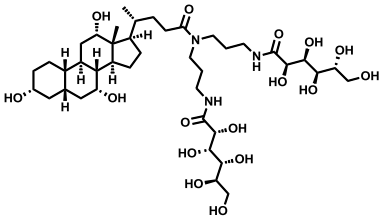
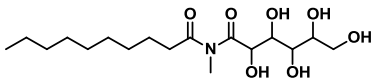
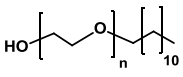
Detergent Name	Detergent Type	Detergent Structure
Sodium dodecyl sulfate (SDS)	anionic	
Hexadecyltrimethyl ammonium bromide (CTAB)	cationic	
Octyl glucopyranoside	non-ionic	
Dodecyl maltopyranoside	non-ionic	
Big CHAP	non-ionic	
N-Decanoyl-*N*-methylglucamine (MEGA-10)	non-ionic	
Genapol X-080	non-ionic	

Table 3. Some detergents used in peptide synthesis to increase sequence solubility.

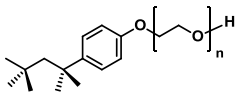
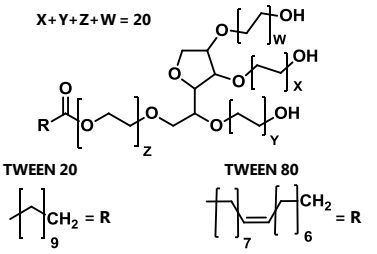
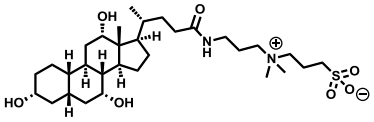
Detergent Name	Detergent Type	Detergent Structure
Triton X-100	non-ionic	
TWEEN 20 TWEEN 80	zwitter-ionic	
CHAPS	zwitter-ionic	

Table 3. (Continued).

2.2. Chemical Modifications on the Peptide Sequence

In order to circumvent the limitations associated with some of the external factors, recent years have witnessed the development of other kinds of strategies. Specifically, more sophisticated tools to improve the solubility of peptides, focused on chemical modifications in the peptide sequences, have emerged. These structural changes modulate the physico-chemical properties of peptides. Chemical modifications introduced into the peptide sequence can be *permanent* as long as the newly introduced moieties do not affect the objective of study of the desired peptide. On the other hand, the incorporated chemical changes can be *temporarily* coupled to a certain functional group. In the latter case, peptides with enhanced solubility may be momentarily manipulated, and the solubilizing tool being removed later to afford the native peptide.

These strategies based on synthetic peptide modifications are comprised in two differentiated groups on the basis of the stage where the insolubility is originated: during the synthesis **on solid-phase**, or after the peptide cleavage, **in solution**. All these approaches are detailed below. Specifically, those tools designed to overcome the insolubility of peptides during solid-phase synthesis are addressed to synthesize "difficult peptides". On the other hand, those methods developed to dissolve sequences in solution are focused to facilitate the peptide characterization and purification.

2.2.1. Solubilization on solid-phase

2.2.1.1. Backbone amide protecting groups

The development of new protecting groups for peptide chemistry has focused mainly on α-amino, α-carboxylic acid, and on the side chain of functionalized AAs. However, orthogonal protection of backbone amides may be necessary in specific cases.[2] In spite of the amides are functional groups commonly non-protected in peptide synthesis, there are a few number of relevant side-reactions where amides are involved in. The diketopiperazine (DKP)[121,122] (Fig. 3b) or aspartimide[123–125] (Fig. 3a) formation, both are initiated by the nitrogen from the amide bond under certain conditions.

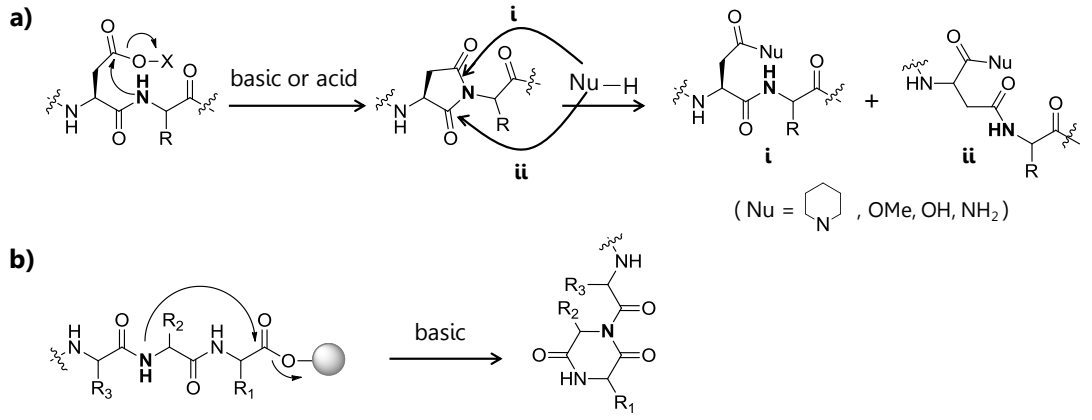


Figure 3. Mechanisms involved in the formation of two side-products: (a) aspartimide; and (b) DKP.

The incorporation of a substituent in the amide position reduces the nucleophilicity of the nitrogen and subsequently precludes these internal cyclizations. Moreover, in addition to contribute to minimize these two by-products, this substitution on the amides becomes mandatory when the aggregation in SPPS needs to be avoided.

The backbone amide functions in peptides may be protected permanent- or temporarily. In the first type, the group remains on the sequence as a non-cleavable substituent (such as methyl groups); and in the second case, the group is named backbone amide protecting group, because it is removable under certain conditions. Numerous efforts have been addressed to design these kind of protecting groups, especially because they enable the synthesis of "difficult peptides".

2.2.1.1.1. Pseudoprolines

Pseudoprolines (**ψpro**), developed by Mutter in 1992,[126,127] were the first backbone amide protecting groups described and supposed a crucial finding for the synthesis of "difficult peptides".[128–130] These building blocks are based on the proline structure, as the name suggests. Previous reports, also by Mutter, proved the evident improvement

of peptide synthesis when some AAs are substituted by prolines.[131–133] These benefits are due to the structural peculiarity of prolines, which has absence of the hydrogen on their α-amino group, which disrupts the continuity of hydrogen bonding on the backbone, a process responsible for the formation of insoluble aggregates. Furthermore, the 100% *cis*-amide conformation induced by the proline-like moiety destabilizes the β-sheet folding of peptides.[133] Following these concepts, and in order to mimic the proline structure, Mutter built these dipeptide derivatives based on thi/oxazolidines moieties. The dipeptides are composed by any AA at the *N*-terminal position cycled by the hydroxyl or thiol groups from the side-chains of Ser, Thr, or Cys, which are placed in the *C*-terminal position (Fig. 4). The most common used pseudoprolines are those that contained dimethylation in the R_1 position, the dimethyloxazolidines $AA_x(\psi^{Me,Me}pro)$ and the dimethylthiazolidines $AA_x(\psi^{Me,Me}cys)$. In fact, pseudoprolines composed by almost all the AAs on its *N*-terminal are commercially available. The incorporation of pseudoprolines into a peptide sequence can be performed on solid-phase under standard coupling conditions. Once the peptide is cleaved from the resin by acydolysis, the pseudoproline is hydrolyzed, providing the two corresponding native AAs. Furthermore, many publications have demonstrated that this strategy greatly contributes to the synthesis of "difficult peptides",[134] improves the cyclization of peptides,[135] achieves large glycopeptides,[136,137] and even facilitates protein synthesis.[138,139] Despite the successful results attributed to the use of pseudoprolines in peptide sequences, their limitation resides in the necessity of having Ser, Thr or Cys in the sequence. This drawback has led to an increased demand to explore new backbone protecting groups.

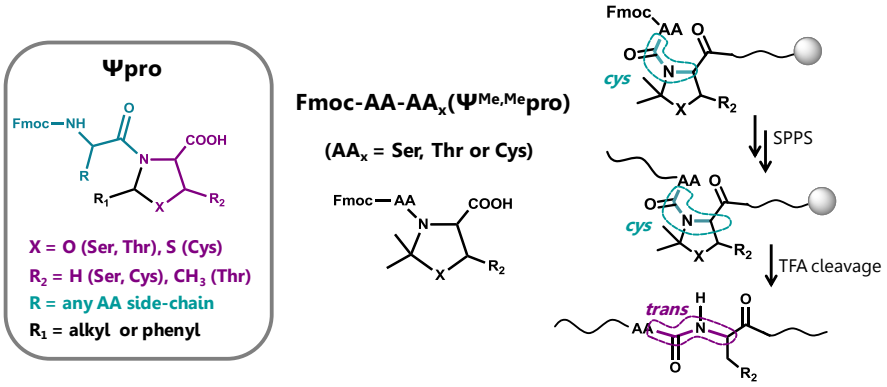


Figure 4. Pseudoproline structure and its incorporation in solid-phase peptide synthesis.

2.2.1.1.2. ortho-Hydroxybenzyl structure-based

Another research line focused on the design of backbone amide protectors was initiated by Sheppard and collaborators, also in the beginning of the '90s (1993), when they proposed the use of the *N*-(2-hydroxy-4-methoxybenzyl) (**Hmb**)[14,140] moiety for Fmoc/*tert*-butyl (*t*Bu) SPPS (see Fig. 5a and Table 4). The *N*-Fmoc-*N*(Hmb)-AAs is previously prepared in solution and then coupled on solid-phase to synthesize the desired peptide (Fig. 5a). After the Hmb-derived AA has been introduced into the sequence, the Fmoc group is removed, and the incoming AA is coupled through a non-standard mechanism (Fig. 5c). Thus, during the introduction of the incoming AA, the proton from the *ortho*-hydroxyl, in one of the tautomers, forms a hydrogen bond with the nitrogen of the secondary amine (α-amino) that favors the initial acylation at the hydroxyl position. Once this ester bond is formed, an intra-molecular *O*→*N*-acyl transfer occurs affording the desired amide bond and leaving the *ortho*-hydroxyl free. The peptide is elongated by standard SPPS conditions and, during the final peptide acidic cleavage from the resin, the Hmb is removed (see Table 4) from the sequence, together with other acid-labile protecting groups, affording the desired peptide target.

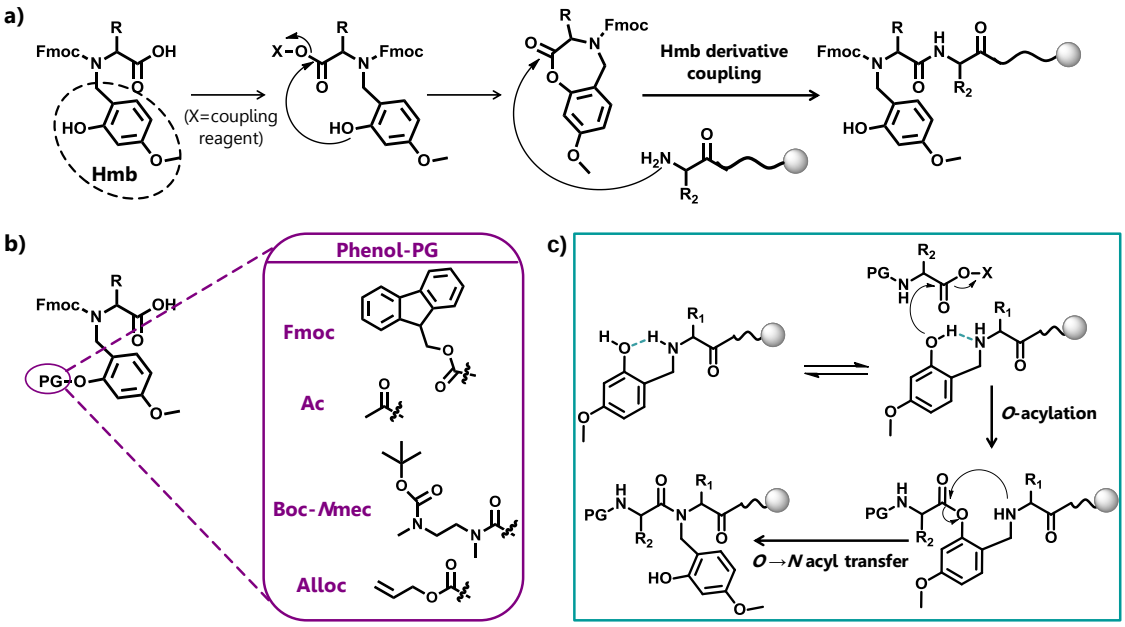


Figure 5. Hmb backbone protecting group: (a) mechanism during the coupling on peptidyl-resin with mono-*N*-Fmoc-*N*(Hmb)-AA derivative; (b) phenol protecting groups; and (c) mechanism of incoming amino acid incorporation on *N*(Hmb)-peptidyl-resin mediated by *O*→*N* acyl transfer.

Although some researchers have reported syntheses of peptides incorporating the building block as mono-*N*-Fmoc-*N*(Hmb) derivative,[141,142] the major syntheses described, including the Sheppard proposal, have put forward the use of *O,N*-bisFmoc-*N*(Hmb) derivative (Fig. 5b).[14,143,144] The main reason for synthesizing and

incorporating Hmb-derivatives bearing extra Fmoc protection on the phenolic hydroxyl group is addressed to prevent the formation of the intermediate isolated and described by Nicolás and collaborators (see Fig. 5a).[142] Thus, the mechanism that takes place during the incorporation of the mono-*N*-Fmoc-*N*(Hmb)AA occurs through an internal cyclization mediated by the nucleophilic attack of the hydroxyl to the activated carboxyl group. The product afforded is the lactone 4,5-dihydro-8-methoxy-1,4-benzoxazepin-2(3H)-one, which undergoes subsequent amino attack on the carbonyl to render the expected amide. This intra-molecular reaction is faster than the inter-molecular amino attack, and it has been demonstrated to be the main cause of the slow incorporation of Hmb-derivatives. In particular, the R substitution in lactone intermediate (see Fig. 5a) supposes a sterical hindrance when the amino group has to reach the carbonyl moiety. That sterical obstacle is the principal cause of the poor reactivity of lactone and consequently slow incorporation of Hmb-derivative. This drawback can be solved with the protection of the phenolic hydroxyl group (Fig. 5b).

When Fmoc is used to protect the *ortho*-hydroxyl from Hmb, the protection is temporary. Commonly, once the Fmoc group is removed in a standard peptide synthesis, the hydroxyl is left free, which does not affect subsequent AA incorporations. However, when certain molecules susceptible to react with this hydroxyl group are incorporated into the sequence, the semipermanent protection of phenol group becomes mandatory, being possible to protect it directly on resin (Fig. 5b). One strategy usually chosen is the acetylation (AcHmb), which is performed on solid-phase by acetic anhydride in presence of DIEA. The AcHmb removal occurred in two steps, first, the hydrazine treatment to afford the Hmb and second, the TFA cleavage conditions to release the unprotected peptide.[145] Some authors have adopted the option of acetylation[145–148] while others have selected alternatives to protect the phenol from Hmb, such as the use of the allyloxycarbonyl group (Alloc)[145] or the *tert*-butoxycarbonyl-*N*′-methyl-*N*-[2-(methylamino)ethyl]carbamoyl (BocNmec)[149] (Fig. 5b). In a similar process as acetylation, Alloc is introduced on solid-phase by diallyl pyrocarbonate ($Alloc_2O$) in presence of DIEA; and the BocNmecHmb is incorporated by activating the phenol with *p*-nitro-phenylchloroformate followed by a treatment with mono-Boc-*N*,*N*-dimethylethylenamine/*N*,*N*′-ethyldiisopropylamine (DIEA) in DCM. Removal of AllocHmb and NmecHmb required also two steps, first, the $Pd(PPh_3)_4$ treatment (for Alloc)[150] or the *N*-methylmorpholine (for Nmec);[151] and second, the standard TFA Hmb removal mentioned before.

On the other hand, the ester bond formation when the AA is being coupled onto the *N*(Hmb)-peptidyl-resin occurs kinetically slower than an standard amide bond, and this

is translated into a prolonged final *O*→*N*-acyl shift. In order to avoid possible side-reactions derived from this slow acylation, it was proposed that the dipeptide Fmoc-AA_2-*N*(Hmb)-AA_1-OH would be pre-synthesized in solution and further introduced into the sequence. Although aspartimide prevention has been reported not only for synthesis which used mono-*N*-Fmoc-*N*(Hmb)-AA,[152] but also when using the dipeptide Fmoc-AA_2-*N*(Hmb)-AA_1-OH,[153] slow incorporations of the bulky dipeptide leading to racemization[153] have been observed.

In spite of these limitations attributed to Hmb backbone amide protector, some dipeptides containing this protecting group are commercially available. Over the years, a number of peptide syntheses[81,152,154,155] have described that Hmb inhibits aspartimide formation and enhances the solubility of peptides facilitating the synthesis of "difficult peptides". Specifically, aggregations are abolished in a peptide when Hmb is introduced into the sequence after the fifth or sixth residue.

Parallel to the use of Hmb for the Fmoc/*t*Bu strategy, an equivalent backbone amide protecting group suitable for Boc/benzyl (Bzl) SPPS strategy was required in order to cover the principal peptide strategies on solid-phase. In this regard, in 1994, two of Sheppard's collaborators for the Hmb proposal, Johnson and Quibell, defined the *N*-(2-hydroxybenzyl) group (**Hbz**)[156] (Table 4). This group shows enhanced acid stability compared with Hmb and is therefore resistant to the continuous TFA treatments required to remove the temporary Boc groups common in Boc/Bzl SPPS strategies. The authors described the preparation of a modified AA protected by Hbz in solution and analogous to the Hmb derivatives, obtaining the *O,N*-bisFmoc-*N*(Hbz)-AA derivative. Although the synthesis was performed using the Fmoc/*t*Bu strategy, they demonstrated the stability of the protecting group to TFA and its final removal by a mixture of trifluoromethanesulfonic acid and TFA (Table 4). The coupling of the incoming AA onto the *N*(Hbz)-peptidyl-resin occurs more slowly than in Hmb-containing peptidyl-resin, probably because of the absence of the electron-withdrawing effect of the methoxy in meta position respect the hydroxyl group.

Other approaches to prevent the poor *O*→*N*-acyl transfer associated with Hmb or Hbz have relied on the development of new backbone protecting groups that modify the benzyl substituent to afford a more efficient transacylation. In 1999, Meutermans and Smythe described a new nitro-activated group, the 6-nitro-2-hydroxybenzyl (**Hnb**)[157–159] (Table 4). The electron-withdrawing nitro group decreases the pK_a of the *ortho* ionizable group, thus favoring *O*-acylation and enhancing the reactivity of the *O*→*N*-acyl transfer. Incorporation of this group was performed in a different manner to that used for the *ortho*-hydroxybenzyl analogs described previously. In this case,

6-nitro-2-hydroxybenzaldehyde is reacted with the α-amino from the peptidyl-resin by a reductive amination. The peculiarity of nitro derivatives is the process by which they are removed, which is mediated by photolysis at a certain wavelength leaving the nitrosobenzaldehyde.[160]

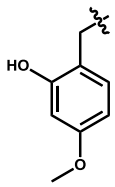
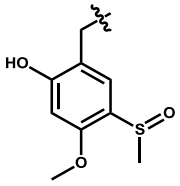
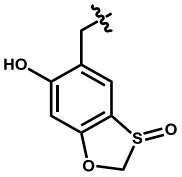
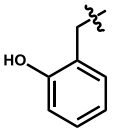
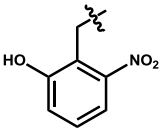
					
	Hmb	**Hmsb**	**1,3-Benzoxathiole-3-oxide derivative**	**Hbz**	**Hnb**
REMOVAL CONDITIONS	TFA/phenol/EDT/TES (92:3:3:2 v/v), 2 h, rt **or** TFA/phenol/TIS (94:3:3 v/v), 2 h, rt	TFA/TMSBr/EDT/thioanisole (89:4.5:2:4.5 v/v), 2 h, rt **or** $SiCl_4$/TFA/EDT/anisole (5.:90:2.5:2.5 v/v), 2 h, rt	NH_4I/Me_2S in TFA (20:20 eq) at 1mg/mL, 2 h, 0 ºC	TFA/EDT/thioanisole/TFMSA (78:4:8:10 v/v), 2 h, rt	λ= 366 nm UV light (1 mM in 1% AcOH/MeOH or 1% AcOH/DMSO 2-3 h, rt
REF.	14, 81, 140-149, 152-155	20, 162, 163	161	156	157-159

Table 4. Backbone amide protecting groups found in the literature based on *o*-hydroxybenzyl structure.

Subsequent to the Hmb group, and based on the *ortho*-hydroxy moiety which enables the *O*-*N* rearrangement, in 1997, Offer together with Quibell and Johnson, proposed the introduction of an electron-withdrawing group in *para* position to the 2-hydroxyl function, specifically the benzoaxazepsin-2(3H)-one moiety. This group, the (6-hydroxy-3-oxido-1,3-benz[d]oxathiol-5-yl)methyl (**1,3-Benzoxathiole-3-oxide derivative**),[161] (Table 4) can be used both in Boc and Fmoc strategies and the advantages associated with the modification on the Hmb protector are similar to those offered by nitro derivatives, since both groups share the same chemical electron properties. These authors proved that acylation occurs at a higher rate in less potent coupling conditions, thus considerably suppressing the epimerization side-reaction promoted by a long acylation. Moreover, the cleavage of the protecting group is completed after a reductive-acidolysis treatment (although it was not designed for this proposal, it may be considered a "safety-catch" protecting group, concept explained in the introduction of chapter 2). Along these lines, some years later, Quibell and Johnson presented the *N*-(3-methylsulfinyl-4-methoxy-6-hydroxybenzyl (SiMB, also named **Hmsb**)[162] (Table 4), a new generation of "safety-catch" protecting groups (see chapter 2) that was easier to synthesize and showed equivalent acylation kinetics to the previous one. Quibell and collaborators have recently described new applications for the Hmsb group that support the efficiency of synthesizing "difficult peptides" when this protecting group is used.[163] More recently, one research group has also focused their attention on the Hmsb amide backbone protection, and have studied specifically the optimization of coupling conditions, as well as its application in some difficult large sequences.[20]

Furthermore, the Hmsb group provides an additional advantage attributed to the fact that since it is acid resistant, it gives the possibility of obtaining the Hmsb-containing sequences after TFA cleavages. This property makes it a "safety-catch" protecting group, a concept that has been further studied in the introduction of chapter 2.

2.2.1.1.3. ortho-Mercaptobenzyl structure-based

Regarding the same *O*→*N*-acyl transfer mechanism associated to Hmb-based protectors to favor the amide bond formation, several researchers described similar protecting groups, but addressed to a different proposal, specifically to assist the obtaining of large peptides/proteins through the chemical ligation of unprotected peptide fragments in solution[164] (Fig. 6). Among all the described auxiliaries for the *N*-terminus, herein, two of them based on a mercaptobenzyl scaffold have been highlighted. In spite of not being designed as a solubilizing tool, we have considered these mercaptobenzyl derivatives as backbone protecting groups because, after the fragment ligation, these groups become amide bond protectors.

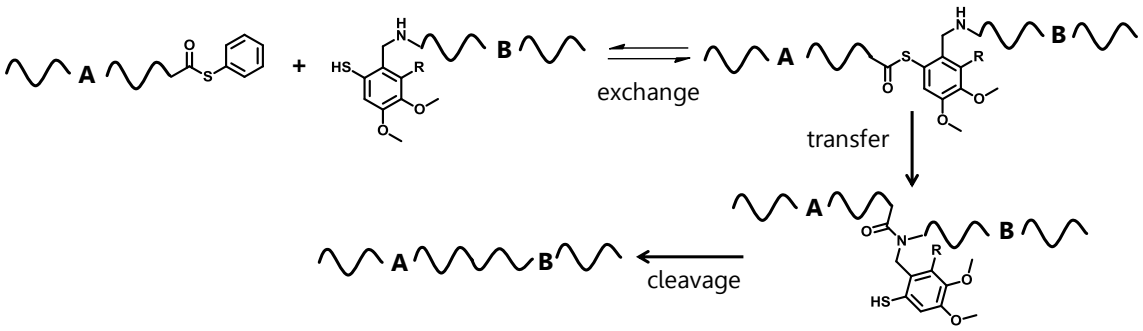
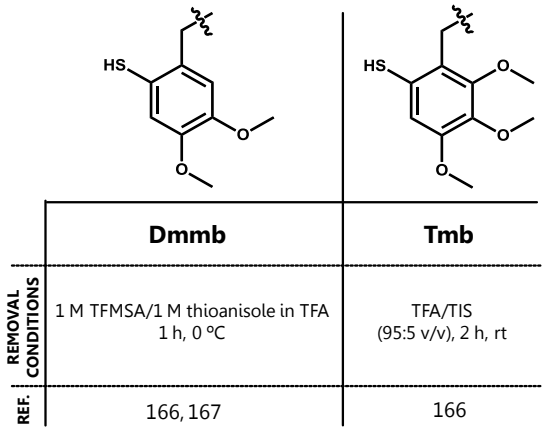


Figure 6. Auxiliary-mediated peptide fragment ligation. Peptides A and B are unprotected sequences and R can be a hydrogen or a methoxy group.

In the earlier 2000s, Dawson[165,166] and Aimoto,[167] in parallel, developed the mercaptobenzyl moiety as an *N*-terminal protecting group. In particular, they defined two analogues, the di-methoxy derivative 4,5-dimethoxy-2-mercaptobenzyl (**Dmmb**,[166,167] also named Dmb, abbreviation that unfortunately was also selected for another protector, see 2.2.1.1.4. section) (Table 5); and the tri-substituted 4,5,6-trimethoxy-2-mercaptobenzyl (**Tmb**,[166] abbreviation that unfortunately was also selected for another protector, see 2.2.1.1.4. section) (Table 5).

The incorporation of the Dmmb group on the peptidyl-resin can be performed by two different methods based on a reductive amination mechanism. In one case, the reaction occurs between the amino group contained in the mercaptobenzyl moiety[167] and the aldehyde present on the *N*-terminal peptidyl-resin. In another case, the incorporation involves an aldehyde group contained in the mercaptobenzyl moiety[165]

and an amino group present on the *N*-terminal peptidyl-resin. On the other hand, the Tmb group is introduced into the sequence by reacting the amino group contained in the mercaptobenzyl moiety through a nucleophilic attack on an acid bromide *N*-terminal peptidyl-resin.[166]



	Dmmb	Tmb
REMOVAL CONDITIONS	1 M TFMSA/1 M thioanisole in TFA 1 h, 0 °C	TFA/TIS (95:5 v/v), 2 h, rt
REF.	166, 167	166

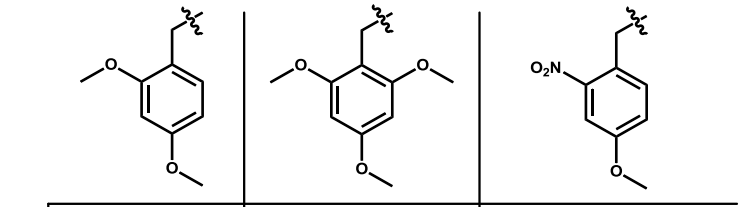
Table 5. Backbone amide protecting groups found in the literature based on *o*-mercaptobenzyl structure.

The incoming AA is coupled onto the *N*(Hmb)-peptidyl-resin through an *S*→*N*-acyl transfer, which occurs in an equivalent manner as the *O*→*N*-acyl shift in *o*-hydroxybenzyl-based protectors. Dawson and collaborators demonstrated that for chemical ligation, the Dmmb group allows the acylation of the incoming AA faster than when Hmb derivative is used.[166] The final Dmmb and Tmb removal, (Table 5) which affords the desired peptide target, is carried out during the peptide acidolytic cleavage from the resin, together with other acid-labile protecting groups. It is worth highlighting that Dmmb removal is slower than that for Tmb, the former requiring strong acids, such as TFMSA or HF.

2.2.1.1.4. para-Methoxybenzyl structure-based

Several backbone amide protectors that are not based on the *ortho*-hydroxyl moiety, are composed by methoxy substituted benzyl structures addressed to enhance the TFA lability to remove them during cleavage of the peptide. The first of those groups proposed was the 2,4-dimethoxybenzyl (Dmob, also named **Dmb**),[168] defined initially as backbone amide protecting group for solution-phase synthesis in 1966 (Table 6). The *ortho* and *para* electron-donating substitution of these protectors allows their fast removal at high concentrations of TFA. It is described that the proton of the amino group forms an internal hydrogen bond with the oxygen from the *o*-methoxy function, equivalently to the *o*-hydroxy moiety in Hmb derivative (see Fig. 5c), which favors the acylation. Nevertheless, the bulkiness of the methoxy group induces severe steric hindrance that, in some cases, may preclude the incorporation of the following AA. In

spite of this limitation, Zahariev and White, independently, demonstrated the efficiency of Dmb in preventing aspartimide side-reactions.[169,170]



	Dmb	Tmob	4-methoxy-2-Nb
REMOVAL CONDITIONS	TFA/scavengers (95:5 v/v), 1 h, rt	TFA/scavengers (95:5 v/v), 1 h, rt	366 nm UV light (in MeCN/H_2O+ 0.1% TFA (pH 2) + 200 eq L-Cys·HCl·H_2O) 1 h, rt
REF.	168-171	140, 143, 172	173

Table 6. Backbone amide protecting groups found in the literature based on *p*-methoxybenzyl structure.

The same authors, in order to minimize the steric hindrance drawback associated to this group, described in their works the introduction in the sequence of the pre-synthesized Fmoc-AA_2-*N*(Dmob)-AA_1-OH building block to allow the synthesis of large peptides.[170,171] However, they specified that this dipeptide method is restricted to the AA-Gly dipeptides because when using other amino acids at the *N*-terminus steric hindrance may preclude their preparation. These dipeptides are commercially available and, as a matter of fact, the Dmb is one of the most used backbone protecting groups, together with pseudoprolines amb Hmb drivatives.

The less studied protecting group with three methoxy units, the 2,4,6-trimethoxybenzyl (Tmb, also named **Tmob**) (Table 6),[172] has also been used as an amide backbone protector for peptides. The incorporation of an extra methoxy group introduces more acid lability to the group compared with its di-substituted analog. Although some comparative studies revealed that Tmob can be removed with only 5% TFA in dichloromethane,[143] standard Tmob removal is performed under the same acidic proportion as common cleavages in Fmoc strategies (Table 6). In spite of preference for the di-substitution over the substituted trimethoxy, Tmob resulted in faster acylation than that achieved with Dmb.[140,143,172] The facilitated acylation observed for this protecting group is due to a similar hydrogen bonding effect as that described for Hmb (Fig. 8c). The greater the number of methoxy groups in *ortho* position, the more favored the acylation, thus influencing more the hydrogen bonding than the steric hindrace of the methoxy group. However, bulkiness is the main reason why sometimes neither Dmb nor Tmob are selected as backbone amide protecting groups, although some Dmb- or Tmob-protected AAs are commercially available.

A family of photolabile backbone protecting groups, that are also based on *para*-methoxybenzyl moieties, has also been explored by Kent and co-workers, namely the 2-nitrobenzyl group (2-Nb) and the two methoxy versions (**4-methoxy-2-Nb** (Table 6) and 4,5-dimethoxy-2-Nb)[173] for use in Boc/Bzl SPPS chemistry. They based their studies on the photolytic nitro properties previously reported[160,174] and also on the photolytic cleavage analysis.[175,176] The introduction of methoxy groups to increase acid lability (prior demonstrated for nitrobenzyl derivative linkers),[177] led Kent to confirm that the incorporation of a second methoxy in *meta* position with respect to the benzyl carbon atom does not favor the photolytic cleavage. It is reasoned because of the extra methoxy position, which represents an electron-withdrawing group with respect to the benzyl carbon atom, and those less electron-rich benzyl carbons lead to decompose less rapid than their more electron-rich analogues. Thereby using the mono-methoxy-substituted nitrobenzyl 4-methoxy-2-Nb analog, they found the most promising backbone amide protecting group. On the other hand, the nitrobenzyl derivatives, besides being cleaved under mild removal conditions, provide the advantage of being acid resistant, allowing to obtain the 4,5-dimethoxy-2-Nb-containing sequences after HF or TFA cleavages. This property enables them to be considered a "safety-catch" protecting group, which has been further studied in the introduction of chapter 2.

2.2.1.1.5. Other structures

Improved alternative protecting groups are the 1-methyl-3-indolylmethyl (**MIM**)[178] or the 3,4-ethylenedioxy-2-thenyl (**EDOTn**)[178] (Table 7), two electron-rich systems, both described by Albericio and collaborators and designed for Fmoc/*t*Bu strategies and maintaining the same range of acid lability as Dmb. The less sterically hindered properties of EDOTn is an advantage, allowing faster acylation than Dmb and thus overcoming the limitations associated with this protector. In 2009, Carpino proposed the dicyclopropylmethyl (**Dcpm**)[179,180] (Table 7) as a backbone protecting group labile to TFA. This group is based on the analog dimethylcyclopropyl (Dmcp)[181], previously described by the same author as an amide side-chain protecting group for Asn and Gln, as well as *C*-terminal amide protector. First, the author described the synthesis of the building block as Fmoc-*N*(Dcpm)-AA-OH for unhindered AAs and later proposed the pre-synthesized dipeptide building block Fmoc-AA_2-*N*(Dcpm)-AA_1-OH to overcome the sterical hindrance,[182] a strategy also proposed and mentioned previously for other backbone amide protectors.

Two other backbone amide protecting groups not based on benzyl structures are the substituted **furfuryl** and **thienylmethyl**,[143] both put forward by Quibell in 1999 (Table 7) and designed for Fmoc/*t*Bu SPPS strategy. These two benzyl structures show greater acid lability than Hmb, and only in the case of 5-methoxythienylmethyl derivative the lability is equivalent to the Tmob group. The incorporation of the substituted furfuryl and thienylmethyl moieties is performed by preparing the Fmoc-*N*(furfuryl derivative)AA-OH or the Fmoc-*N*(thienyl derivative)-AA-OH. Their coupling onto the peptidyl-resin is performed under common solid-phase conditions, however the most significant limitation of those groups lies in the extremely low yields obtained when the AA is coupled onto the *N*-(furfuryl/thienyl)peptidyl-resin.

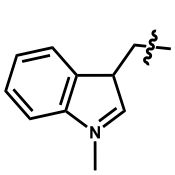
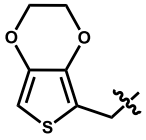
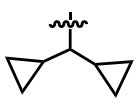
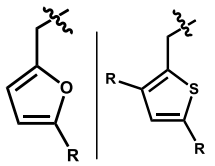
				 R = H, Me or OMe	
	MIM	**EDOTn**	**Dcpm**	derivatives of: **2-furfuryl** | **2-thienylmethyl**	**Etom**
REMOVAL CONDITIONS	TFA/CH_2Cl_2/H_2O (95:2.5:2.5 v/v), 1 h, rt	TFA/CH_2Cl_2/H_2O (95:2.5:2.5 v/v), 1 h, rt	TFA/H_2O/TIS (95:3:2 v/v), 1 h, rt	TFA/H_2O/TIS (95:5 v/v) R = H (2-3 h), rt R = Me or OMe (2 min), rt	TFA/CH_2Cl_2 or H_2O (50:50 v/v), 1 h, rt
REF.	178	178	179, 180, 182	143	183

Table 7. Backbone amide protecting groups found in the literature that are not based on any common structure.

More recently, the amide backbone protecting group *N*-alkoxymethyl-based, the ethyloxymethyl (**Etom**)[183] (Table 7) was proposed by Spengler and Albericio to prove its efficiency on enhancing solubility for the Fmoc/*t*Bu SPPS. This group was incorporated into the sequence under standard conditions as dipeptide Alloc-*N*-(Etom)AA_2-*N*(Etom)-AA_1-OH, previously synthesized. The removal of Etom is carried out under 50% TFA/DCM, however it is also conceivable under mild acidic TFA treatment (5%).

2.2.1.2. Isopeptides

Depsipeptides, also known as *O*-acyl isopeptides or merely isopeptides, are suitable as solubilizing peptide strategy because the temporary ester bond in the sequence disrupts the hydrogen bond continuity of backbone amides to the same extent as backbone protectors, thus preventing β-sheet interactions and consequently aggregations. This strategy is not mediated by the introduction of an extra protecting group. Indeed, the sequence is synthesized by SPPS, and the ester bond is performed through the hydroxyl group of side-chain of Ser or Thr, instead of performing the

common amide through the α-amino. The peptide is elongated and then cleaved from the resin, affording the *O*-acyl isopeptide (Fig. 7).

After the acidic cleavage of the depsipeptide, the unprotected primary amino group of the depsi unit is protonated, thus the intramolecular attack of that amino on the carbonyl of the ester is not produced and it is possible to obtain a stable depsipeptide in solution. In order to achieve the native peptide sequence, once the peptide is released from the resin and unprotected, the last step consists of the *O*→*N*-acyl shift, which is mediated by mild basic conditions in aqueous media. Thus, the *O*→*N* arrangement occurs through a five-member ring intermediate, reaction controlled by the pH (Fig. 7).

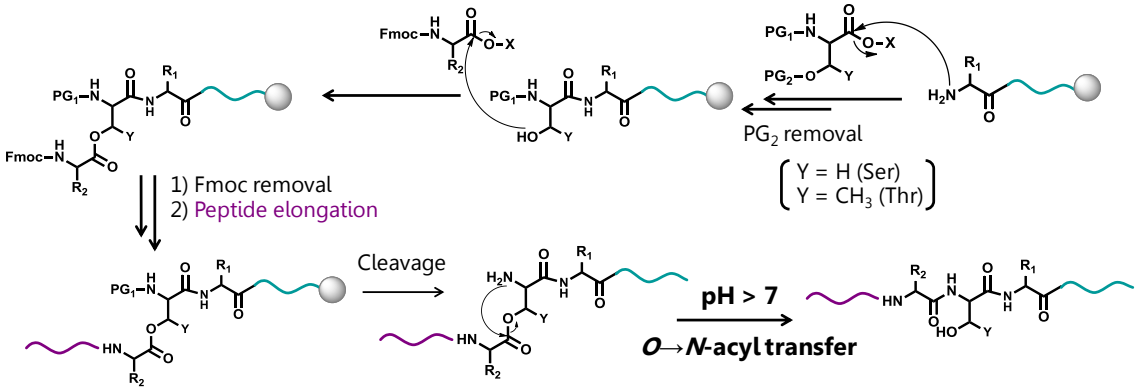


Figure 7. Isopeptide concept: synthesis by SPPS and the *O*→*N* shift in solution to achieve the native sequence.

The first peptides synthesized by SPPS following the *O*→*N*-acyl transfer strategy were published in 1998 by Horikawa and Ohfune[184,185], based on earlier studies with peptides synthesized in solution.[186–189] However, it was in 2004 when the application of isopeptides as a β-sheet disrupting strategy was exploited, in parallel, by Carpino,[190] Kiso,[191,192] Mutter[193] and Aubagnac[194] to synthesize "difficult peptides", where the ester bond was produced through the Ser/Thr-hydroxyl (Fig. 7). Initially, the solid-phase synthesis of these isopeptides was performed in a stepwise manner, including the esterification step; however, some side-reactions were detected after introduction of the depsi unit. These non-desired reactions are associated with racemization during the ester formation or the well-known DKP formation after deprotection of the amino group from the second AA after the depsi unit. Various solutions were proposed in order to minimize or prevent these drawbacks in "difficult peptide" synthesis. Some of them were mainly based on the use of pre-synthesized isodipeptides in solution to prevent racemization;[195,196] and others, on the replacement of the Fmoc of the seond AA after the depsi unit by other amino protecting groups labile to milder basic conditions.[195,197] The same idea of acyl migration has been reported for *O*→*N*- and

S→*N*-acyl isopeptides, depending on the AA involved in the migration step. Therefore, the preparation of new isodipeptides units[198,199] has opened up a diversity of building blocks, in the beginning subjected only to Ser/Thr, but now extended to any AA.[200] During recent decades, several published syntheses have demonstrated the efficiency of depsipeptide strategies.[201,202] In this regard, these strategies favor the cyclization of peptides[203] and also peptide elongations by microwave SPPS.[204]

Although this strategy was merely developed to address the solubility of peptides during the solid-phase elongation, the stability of the isopeptide to cleavage conditions allows to preserve its optimized solubility properties, thus facilitating its manipulation and purification. Other recent variations of the isopeptide method have focused on the *O*→*N*-acyl shift step, proposed by Mutter and named "switch-peptides",[193] or the "click-peptides"[205] proposed by Kiso, where the modulation of the acyl-transfer is mediated by a last selective and controlled reaction, not only by the pH. Thus, in order to favor the stability of isopeptides in solution under neutral conditions, a number of groups have proposed distinct protecting groups for the isopeptide site (PG_1 in Fig. 7), assigned as *switch elements*, that induce the acyl-transfer via a specific trigger. Some examples of proposed switch elements are based on photocleavable protecting groups such as 6-nitroveratryloxycarbonyl (Nvoc), which is removed under photolytic conditions, thus leading to the *O*→*N*-acyl transfer.[205,206] The azide temporary protecting group, one of the most widely used switch elements, is preserved after cleavage of the peptide, and once the azide is reduced to amino, the *O*→*N* arrangement takes place, affording the native sequence.[207,208]

2.2.2. Solubilization in solution

2.2.2.1. "Pegylation" or glycosylation

The two most studied chemical modifications to increase the aqueous solubility of non-polar peptides are "pegylation" and glycosylation. Both strategies are addressed to enhance solubility, thus facilitating not only the peptide manipulation, but also the peptide reactions in solution. “Pegylation” involves the conjugation of a peptide to a polyethylene glycol (PEG) tag; and glycosylation to a sugar moiety. The "**pegylation**" strategy was initially introduced in proteins by Davis and collaborators[209] in the ’70s and this method was subsequently adopted by other authors for smaller molecules or peptides.[210,211] The most significant property associated with the PEG conjugation to a peptide[212] is that it provides a substantial increase in solubility of hydrophobic sequences in water. This mechanism relies on the formation of more hydrogen bonds with water through ethylene oxide units[213,214] Another advantage, apart from the

enhancement of solubility, lies on the synthetic facilities to link them to a peptide sequence.[215] The most frequent active site in peptides for the attachment of PEG moieties are the amino groups of lysines (α or ε) or the *N*-terminus of the peptide,[216] although new strategies have emerged expanding the versatility of "pegylation" (Fig. 8).[217–220] The incorporation of PEG tags into peptide sequences has been extensively studied and some authors have summarized the strategies most used to introduce them in solution and solid-phase peptide synthesis.[215,221]

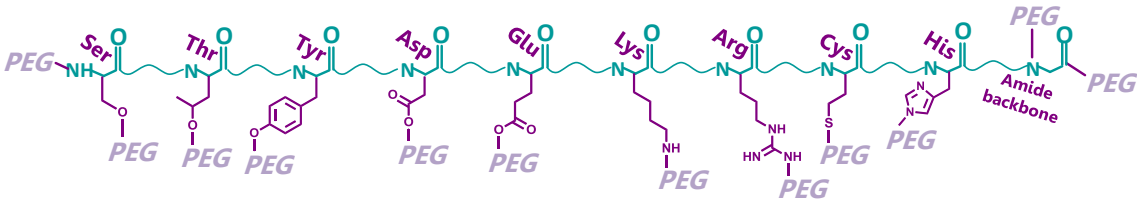


Figure 8. Peptide positions at which it is possible to introduce a polyethylene glycol chain.

Several examples in the literature have confirmed the efficiency of conjugated PEGs to modify both, the properties of peptides and their architectures.[222,223] The PEG moieties most widely used have a molecular weight average of M_w<1000 Da. Moreover, although many kinds of PEG moieties are commercially available, monodisperse PEG units are the most preferred to avoid tough characterizations or even unachievable purifications.[216] Various authors have linked the PEG unit through an orthogonal cleavable linker, whereby the PEG moiety temporarily preserves the solubility of certain sequences, thereby facilitating the characterization and purification of the peptide.[127] The PEG moiety is then detached from the peptide by a final selective reaction that allows to recover the native sequence. In addition to these appreciated properties, the "pegylation" strategy has additional advantages over other strategies, referred to its lack of toxicity, which has brought about approval by the Food and Drug Administration (FDA). Furthermore, conjugation of drugs to a PEG unit enhances the half-life of these therapeutic agents, thus expanding the number of "pegylated" peptide applications.[211,224,225] Recent studies further support the use of "pegylation" strategies as attractive options to modify non-polar peptides in order to increase their solubility.[226,227]

In a parallel manner, **glycosylation**, in which the peptide sequence is attached to sugar units, has also been developed as a strategy to enhance solubility of peptides and reduce aggregations.[228] In contrast to "pegylation", the incorporation of sugar moieties into a sequence is a reaction that occurs in biological systems when glycoproteins are produced by the glycosyltranferase enzymes.[229] Sialic acid from carbohydrate moieties

is responsible for increasing the solubility of the connected hydrophobic molecule, as well as for introducing other therapeutic properties.[230]

Appropriate orthogonal hydroxyl protecting groups are required for the solution and solid-phase synthesis of glycopeptides. In spite of the difficulties encountered to attach a sugar moiety to a sequence, several authors have further addressed this strategy in recent years (Fig. 9a).[231,232] The main linkage of sugar to a peptide sequence is formed through the side-chain of certain AAs, with the modified AA building block generally being previously synthesized and then sequentially introduced into the peptide sequence (Fig. 9b). Depending on the functional group involved in the linkage between the peptide and the saccharide, the sequence afforded is known as *N*-, *O*-, or *C*-glycopeptide. Improved syntheses of glycopeptides both in solution[233–235] and on solid-phase[86,236–238] have been described even for long peptide sequences. In both strategies, peptides containing conjugated PEG moieties or saccharides require the condensation of large molecules, which may lead to difficult reactions. In this case, the combination of various solubilizing strategies is mandatory.

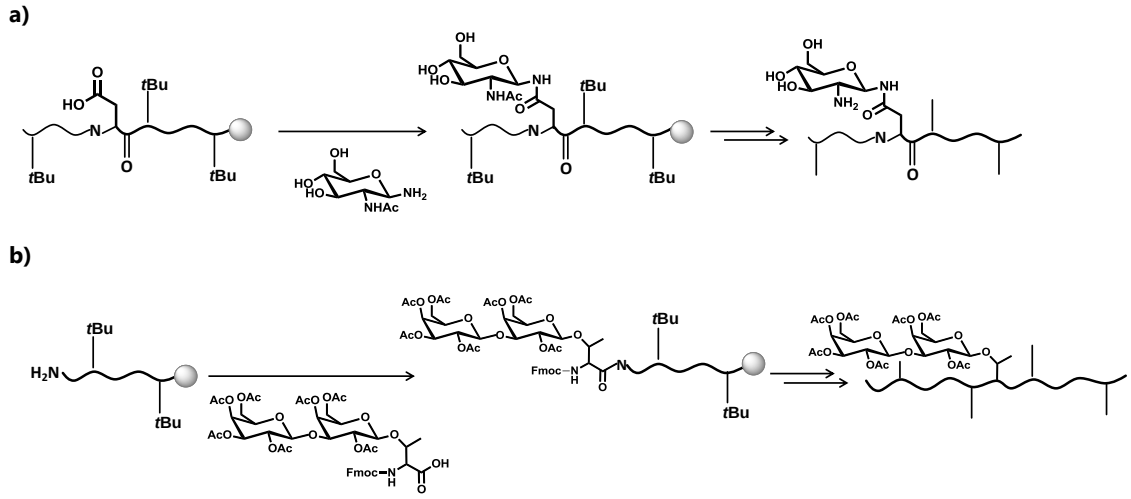


Figure 9. Two main exemplified strategies to introduce sugar moieties on solid-phase to afford glycopeptides by: (a) stepwise incorporation; and (b) pre-synthesized sugar building block incorporation.

2.2.2.2. Solubilizing short peptide-tags

Given the need to understand the interactions or the behavior of biological systems, recent years have witnessed an increase in the demand for the synthesis of proteins with high purity. The field devoted to the design, development, and optimization of methods to achieve the synthesis of proteins is experiencing constant progress. On the other hand, the use of recombinant methods to achieve proteins has significant bottlenecks with regard to obtaining proteins of considerable purity. In order to overcome purification issues, several authors have supported the strategies based on

peptide fusion, developed initially in the '70s and '80s. Nowadays, these strategies are probably among those most widely used to purify proteins obtained by recombinant methods. Fusion between a protein and peptide sequence, named peptide tag, is produced by expression systems that introduce these peptides into the *N*- or *C*-terminus of target proteins.[239] The principal concept underlying such fusion is based on the specific properties conferred by the tag to the large molecule, which allow its purification. After purification of the protein, when necessary removing the previously attached peptide tag the molecules are detached by different mechanisms, for example by the action of specific enzymes.

The most commonly used tag is the poly-histidine-tag [usually $(His)_6$],[240,241] amino acid that shows high affinity to metal ions, thus allowing purification by columns composed of a combination of metals that immobilize proteins at a certain pH.[242] Other peptide tags bind specifically to certain antibodies, such as FLAG-tag.[243] Furthermore, proteins have also been combined with peptides with high affinity to bind streptavidin protein (Strep-tag and SBP-tag),[244] where purification is based on affinities to momentarily immobilize the tagged proteins to be isolated from other non-tagged impurities. One of the shortest peptide-tag sequence used for recombinant protein purification is the poly-arginine-tag [usually $(Arg)_5$],[245,246] thus cationic exchange columns are used to isolate proteins carrying this tag.

In parallel, although less studied than peptide tags for recombinant proteins, some **short peptide tags** have been conjugated to peptide sequences. In this case, the attachment of the tag to a peptide is not addressed to facilitate the peptide separation by column affinity methods, but it is focused on enhancing the solubility of the non-polar peptide targets. Hydrophobic peptide sequences are examples of molecules that would be synthesized following this strategy, especially taking into account the relevance of solubilizing peptides with respect to HPLC characterization or peptide purification. In this group of peptide tags, short sequences are preferred, generally comprising 5-10 residues, rather than small proteins or large peptides, which are extensively selected for protein purification purposes. The short peptide tags used to increase solubility are formed by poly-AAs containing one type of AA (homooligo-peptides), some of these polar and with charged side-chains. Therefore, the basic AAs disposed as poly-arginine and poly-lysine, and the acidic ones sequences poly-glutamic and poly-aspartic are the peptide tags used for this purpose (studied in more detail in the introduction of chapter 3). Synthetic strategies to afford the non-polar peptide connected to the poly-AA tag may be carried out easily on solid-phase because the tag is also formed by AAs. Major examples described in the

literature are those solubilizing tags introduced on the *C*-terminus of a target peptide, although some references also report a straightforward synthesis by linking the tag to an AA side-chain.[247]

The conjugation of a non-polar peptide to a given tag can be achieved in two ways. The two molecules can be linked through a non-hydrolysable bond, such as an amide bond, or through a bifunctional linker that is stable under cleavage conditions but labile to other treatments. Concerning the first method, after the SPPS and the cleavage, the tag persists on the peptide target and it is not possible to detach the two molecules, thus resulting in a permanent conjugation (Fig. 10a). By contrast, in the second approach, the tag is temporarily conjugated to achieve a soluble peptide for further selective release (Fig. 10b). However, after cleavage of the peptide from the resin in both strategies, the peptide and the solubilizing tag remain linked, thus allowing the manipulation of the non-soluble peptide and facilitating the characterization and also the purification. After isolation of the conjugate from any impurities formed during the peptide synthesis, a final step is performed in a temporarily conjugated strategy, whereby the target peptide is released by a specific treatment which removes the linker.

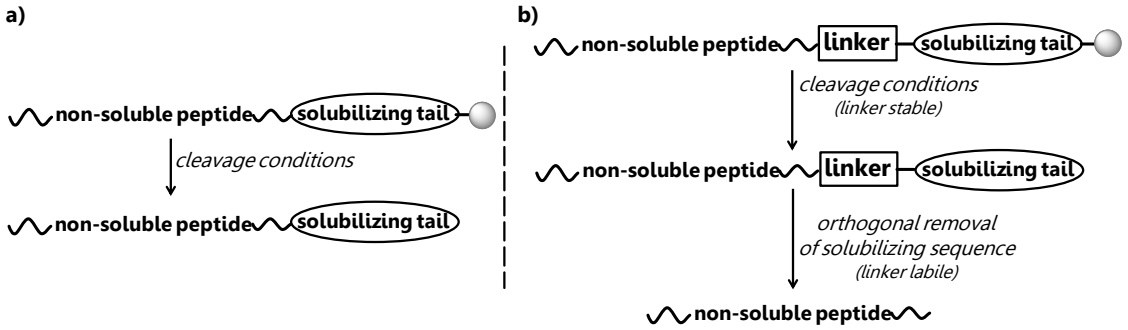


Figure 10. Schematic concept of the "solubilizing tail" strategies by conjugation: (a) permanently; and (b) temporarily.

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OBJECTIVES

The general aim of the present thesis is focused on the development of tools to achieve, by solid-phase, the synthesis of "difficult peptides", as well as the purification of aggregation prone peptide sequences. Evaluation of synthetic strategies and the solubility after the elongation for some selected standard complex sequences will serve of comparison to validate the novel strategies described in this thesis. More specifically, the objectives are the following:

Design and evaluation of several synthetic strategies based on solid-phase to reach one of the well-known "difficult peptides", the RADA-16, as the main aim of **chapter 1**. Analysis by HPLC of some commercialized samples to define the most significant parameters to be considered when characterizing this kind of peptides. Examination of a post-synthetic RADA-16 procedure to provide a purification tool to achieve aggregating peptides.

Synthesis of a new backbone amide protecting group, the 2-Methoxy-4-methylsulfinylbenzyl (Mmsb), to be later incorporated on solid-phase into "difficult peptide" sequences, as the goal of **chapter 2**. Elongation of three complex sequences by using the temporary Mmsb group, as a "safety-catch" moiety to improve their syntheses, as well as allowing their purifications.

Enhancing the solubility of non-polar molecules in solution through the conjugation of a short-peptide tag, as the first objective of **chapter 3**. Evaluation of AA tag type and AA tag spatial disposition influences of linear and branched poly-AA as solubilizing tag sequences. Validation of this strategy by a permanent conjugation of an insoluble drug to a selected solubilizing tag. The second objective relies on studying the efficiency of the Mmsb moiety as a linker to temporarily connect a non-soluble peptide to a poly-AA tag.

New application of Mmsb-OH linker to take part of the orthogonal *C*-terminal protecting groups. In addition, in **chapter 3**, evaluation of the proposed protecting group by synthesizing an example of one well-known peptide segment. Stability and lability studies regarding this protecting group to validate its usefulness in peptide fragment condensation in solution.

CHAPTER 1.

Synthetic Approaches to Reach a "Difficult Peptide"

Introduction

1. The "Difficult Peptide" RADA-16

"Difficult peptides" are characterized by their tendency to form β-sheet inter- or intramolecular interactions during the synthesis, which is considered, at the same time, a challenge. The inaccessibility of functional groups to react affording the amide bond is precluded precisely by those molecular associations. In spite of the synthetic difficulties related to those sequences, their impact in biomedicine has encouraged the researchers to invest great efforts to develop new methodologies to reach them. The appreciated capacity of "difficult peptides" to compose β-sheet secondary structures resides on the sequence transformation from a non-organized (random coil) to a more organized (β-sheet) structure. When this behavior is also produced after the peptide synthesis, thus in solution and under appropriated conditions, these peptides may form aggregates or fibrils, supramolecular stable architectures with interesting properties in the nanomaterials field.

Specifically, those peptide sequences with self-assembling capacity and amphiphilic character are the most valued because the assembly occurs spontaneously in solution and may be modulated by external factors. The facility to manipulate the peptide behavior in amphiphilic peptides has given the possibility to get in aqueous solution initially aggregates, later fibers and subsequently, as a last stage of their organization, hydrogel formations.[1] Hydrogels are supramolecular organizations stable enough to be applied in biology, for example as regenerative tissues, drug delivery systems, nanotubular materials, among others.[2,3] The wide range of applications in medicinal engineering of amphiphilic peptides have attracted the interest in designing new peptide sequences, not necessarily extracted from natural structures,[4] mainly addressed to force its self-assembly.[5] Principal designs are focused merely on the synthesis of the subtype of these peptides known as ionic self-assembling peptides, mentioned in the general introduction. In addition to the hydrogen bonds characteristic of the peptide backbone amides, the ionic surfactant-like peptides present the positive-negative ion-ion interactions which are orders of magnitude stronger than the hydrogen bond. Those associations allow the peptides to aggregate in appropriated pH conditions and this is the reason which those ionic sequences form extremely stable hydrogels.

One known example of ionic self-assembling peptide is the called RADA-16, also named RADA-16-I because of belonging to type I (+ − + − + −,...), referring to one-to-one positive-negative alternation within the subtype of ionic self-assembling peptides. The primary structure of RADA-16 is composed by a total of 16 residues with the peculiarity of being composed of repeating the Arg-Ala-Asp-Ala (R-A-D-A)

sequence four times (Fig. 1a). It belongs to a group of ionic self-assembling structures in which the first described member of those peptides was the EAK-16,[6,7] which is a segment of a yeast protein with binding Z-DNA properties. On the contrary, RADA-16 is an artificial *de novo* designed structure, being all the group of those peptides designed by Zhang and collaborators.[8,9] Precisely the alternating and complementary charges that this kind of sequences exhibited under an appropriated pH, is the reason that lead them to adopt a β-sheet conformation in which two faces are defined: the hydrophobic part (Ala residues) and the positive/negative charged core (Arg/Asp residues) (Fig. 1b). Different structural analyses of RADA-16 have been performed to understand the nanofiber formation and its tridimensional organization.[10–12] In the most recent published report,[13] the RADA-16 has been described as parallel β-sheet strands secondary structure, which are prolonged in the third dimension. These strands are stabilized in one direction by hydrophobic interactions from alanines, and on the other direction, by cation-anion interactions from arginines and aspartates from consecutive peptide chains in parallel position (Fig. 1c).

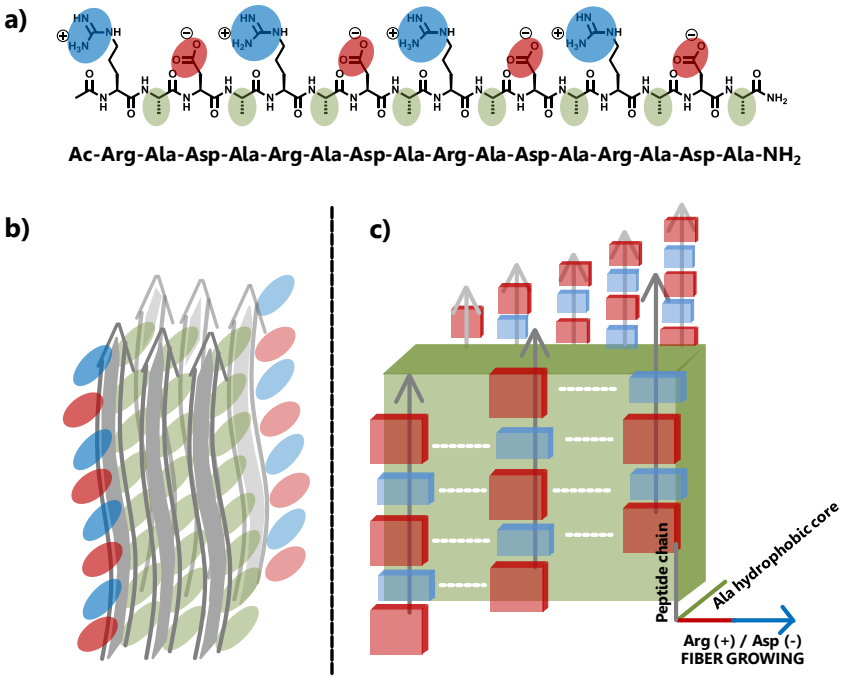

Figure 1. RADA-16 structure according to Cormier analysis.[13] (a) The AA sequence with the corresponding charged side-chains; (b) the tridimensional schematic representation of the disposition of peptide chains when aggregation occurs; and (c) the tridimensional schematic representation of peptide chains orientation when fiber formation occurs. Alanines (in green); arginines (in red); aspartates (in blue); peptide chain direction (in grey); and ionic interactions (in white dots).

In order to obtain the hydrogel material composed by RADA-16 it has been reported that it is required, once the peptide is resuspended in water, the addition of cations or,

the merely introduction of the peptide into the physiological media, with certain salt concentration to stimulate the peptide to form fibers. Salt addition to induce the self-assembly has been studied and, depending on the sequence, ions from salt could affect the secondary structure or most importantly, favor the peptide-peptide interactions. For example, positively charged face composed of a peptide chain which presents exclusively lysine residues would be filled of the complementary negative ion from the salt composition, being possible the approximation of another positively charged face from other equivalent peptide chains. An analogous effect would occur for negatively charged residues. Thereby, RADA-16, which is formulated in approximately 99.5% of water, has to be administrated *in vitro* or *in vivo* by addition of salt solution either some monovalent cations. That peptide was launched in 2002 by the north-American company BD Bioscience as a bioactive hydrogel named PuraMatrix®,[14] later commercialized with a different name by other companies and other ranges of purity. In spite of being a peptide not considered a drug because it is not related with a specific interaction in the biological machinery, their intrinsic properties are enough attractive to sell it as a material to be employed in biological systems, even in animals or who knows if will be feasible for humans in a near future. The principal application of RADA-16 is associated to its capacity of formulating this hydrogel to promote the attachment of cells, as it was demonstrated with different mammalian cells. Furthermore, the absence of immune response when RADA-16 is delivered to an animal model, in addition to studies of toxicity and biocompatibility, have opened the application window associated to that peptide to the biological systems. Thus, in recent years, publications related to RADA-16 have appeared for different purposes, specifically focused on the regenerative medicine, which are directly associated with its capacity to induce cell adhesion and migration. It has been proved *in vitro*, and also in animal models, in systems such as bones, nervous, cardiac or blood (Table 1).[8,15–24] In some studies, the RADA-16 hydrogel is applied combined with certain motifs responsible for certain biological activities, thus the self-assembling peptide in that case enhances those bioactivities.[25,26] These bioconjugates are specified in table 1 and named as functionalized RADA.

Application	Related Field	Ref.
cell adhesion	tissue regeneration	13, 14
brain issue ingeneering (functionalized RADA)	tissue regeneration	15, 16
bone tissue regeneration (functionalized RADA)	tissue regeneration	17, 18
promote angiogenesis (functionalized RADA)	tissue regeneration	19
neurite outgrowth and active synapse formation	tissue regeneration	20, 21
inhibition on growth of human leukemia cells and also in nude mices *in vivo*	cancer treatment	22
inhibition on growth of breast cancer cells *in vitro* (functionalized RADA)	cancer treatment	23

Table 1. Some significant published medicinal applications of RADA-16.

The extended list of published reports about RADA-16 applications highlights the importance of obtaining this kind of peptides in the laboratory by optimized protocols. However, from the synthetic point of view, in some cases an ionic self-assembling peptide may, at the same time, belong to the "difficult peptide" group, which is characterized by presenting the aggregation behavior during the synthesis. In particular, RADA-16 is one of those peptides that aggregate in both stages, during and after the synthesis. Therefore, based on Boc/Bzl or Fmoc/*t*Bu SPPS, several approaches have to be considered to reach this kind of sequences. Although the first attempt to synthesize peptides on solid-phase is stepwise, in which AAs are sequentially introduced on the solid support, when the sequences are considered "difficult peptides", standard methodologies are not suitable to reach those peptides with high purities. Other more elaborated strategies have to be addressed to overcome synthetic troubles. When it is not desired to introduce modifications on the sequence or extra protecting groups to overcome the limitations associated with stepwise approaches, an alternative strategy which combines the synthesis on solid-phase and liquid-phase was described some years ago.[27] This methodology is known as convergent strategy because the native sequence is rationally and retro-synthetically detached in segments, which in a synthetic flow would be connected in the appropriate manner. Two general and classical convergent methodologies have been described as suitable strategies for synthesis of large sequences or even "difficult peptides": *solid-phase fragment*

condensation[28,29] and *fragment condensation in solution*.[30] Both of them share the requirement that the condensation of two fragments occurs mandatorily between fully protected sequences. Although several synthetic processes require the evaluation and the synthesis by using both kinds of condensations to get the peptide target,[31] herein are detailed the most relevant aspects of the two methodologies which would serve as a guide to an appropriated choice depending on the peptide characteristics.

2. Solid-Phase Fragment Condensation Approach

The convergent strategy based on solid-phase fragment condensation consists on performing the ligation between two peptide segments on the solid support, as its name suggests. In this strategy, the initial rational detachment of the whole peptide in fragments is crucial, thereby SPPS is the tool used to sequentially reach the peptide chains of each fragments, separately. Once a "difficult peptide" or a large sequence is decided to be synthesized by this strategy, firstly it must be divided, considering that the AA that will be in the *C*-terminal free carboxylic acid of the new fragment would be selected preferably from those not susceptible to racemization, such as Pro or Gly. Racemization is more favored in this strategy as compared to the stepwise approach, because this side-reaction occurs first when the α-hydrogen is abstracted and then the oxazolone intermediate is formed, and is thus reduced or destabilized when Fmoc or Boc are protecting the amino in a single AA sequential coupling. On the contrary, if a peptide fragment is being coupled, the absence of carbamate group close to the activated carboxylic acid and the presence of an acyl group there, promotes the oxazolone formation and subsequently the racemization. Another aspect to have in consideration about the AAs involved in the condensation is related to their hindrance: when no β-branched AAs or when no bulky side-chain protecting groups are selected, the fragment condensation occurs faster and with higher yields, besides side-reactions are precluded (see other requirements in Table 2).

In this strategy, detailed in figure 2, a principal solid support is selected, on which the corresponding *C-terminal fragment* (whose α-amino is going to react during the condensation) is synthesized stepwise on a solid support which allows to obtain the desired *C*-terminal functional group. The other peptide segment, the *N-terminal fragment* (whose carboxylic acid is going to react during the condensation) is synthesized on a certain resin which allows to reach, after the cleavage, a carboxylic acid free in its *C*-terminus and the functionalized side-chains properly protected. The *N*-terminal fragment is coupled onto the peptidyl-resin, where the *C*-terminal fragment had been previously elongated, by fragment condensation under standard conditions

using the common excess of this fragment and coupling reagents used in stepwise SPPS (1.5-3 eq.). When more fragments have to be coupled onto that peptidyl-resin to complete the target sequence, they are prepared analogously to the first *N*-terminal fragment, and after removal of the α-amino protecting group from the peptidyl-resin, this new *N*-terminal fragment is coupled, and so on, and so forth. After all these solid-phase ligations, the last α-amino protector is removed on solid-phase and the final peptide sequence is cleaved from the resin with concomitant removal of the side-chain protecting groups. Only in those cases where only one fragment condensation is required to get the desired target, the last α-amino protector removal could be performed in conjunction with the cleavage step, whenever that group would be labile to cleavage conditions. In contrast, when more fragment condensations are required, the protecting group must be orthogonal, thus being labile to different conditions as compared with the final cleavage.

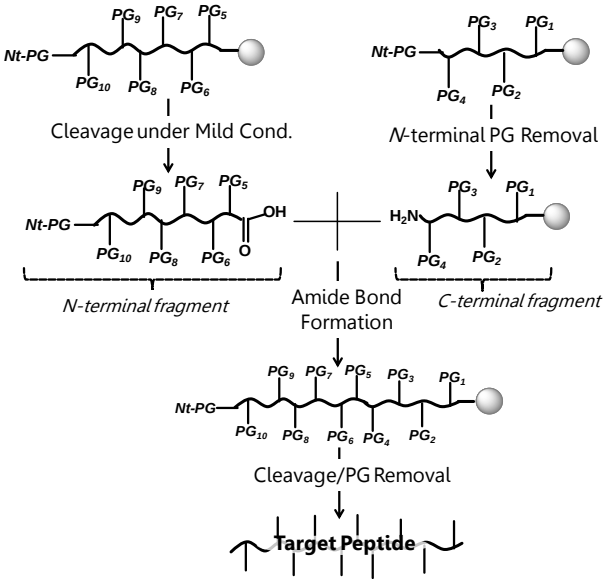

Figure 2. General scheme of the convergent strategy: solid-phase fragment condensation.

The first proposed peptide synthesis by solid-phase fragment condensation was performed in the early '80s and several advantages were attributed to the use of this methodology compared with the classical stepwise strategy.[28] In subsequent years this approach was supported by different authors[29,32,33] as the most appropriated methodology to synthesize certain peptide sequences by both, the Boc/Bzl or the Fmoc/*t*Bu solid-phase strategies. A reported sequence characteristic where fragment condensation by solid-phase would be profitable, relies on the presence of some repeating AAs fragment along the sequence. This fragment, thereby, would be prepared in large quantity on the same resin and subsequently being introduced on

the peptidyl-resin, the number of times necessary to reach the final peptide.[34] One example of these kind of peptides is the previously mentioned RADA-16. In the literature, several sequences have been synthesized by solid-phase fragment condensation and most importantly, recent published reports[35–37] validate this strategy, which remains a reasonable option to be considered when conventional strategies are not able to reach certain peptides.

3. Fragment Condensation in Solution Approach

Parallel to the solid-phase convergent strategy, the fragment condensation in solution was established as an advantageous alternative to build large or "difficult peptide" sequences when limitations associated to firstly described fragment condensation have to be overcome.[38,39] These principal obstacles that may be encountered in the solid-phase ligations are related to: (a) equivalent excesses required of the protected *N-terminal fragment* to carry out the fragment condensation (Fig. 2); and (b) the hindrance of the protected *N-terminal fragment* (Fig. 2). The first issue involves the necessity to synthesize more quantity of *N*-terminal fragment, assuming the loss of peptide material. The second limitation becomes severe when fragment condensation is performed on solid-phase instead of in solution phase, thus the solid support nature would not allow the access of bulky molecules. These two aspects are considered reasons that would lead researchers to prefer fragment condensation in solution instead of the solid-phase alternative. However, other essential requirements must be considered when this kind of fragment coupling becomes the strategy of choice (Table 2). Racemization, as well as the hindrance are some of the parameters that must be taken in consideration before deciding the detachment point in the retro-synthetic analysis, in a similar criteria as the solid-phase alternative condensation.[40,41]

In this strategy, detailed in figure 3, in parallel, two solid supports are properly selected to synthesize independently the *N*- and *C*-terminal segments, which subsequently will be coupled. The *N-terminal fragment* is elongated on a solid support, which, after cleavage, allows to afford a sequence with a carboxylic acid free in its *C*-terminus, but with a protected *N*-terminus (semi-permanent protecting group, *Nt-PG*). The *C-terminal fragment* is synthesized on a solid support, which, after cleavage, allows to render a sequence with a non-reactive *C*-terminus (amide or semi-permanent protecting group, *Ct-PG*), but with an amino free in its *N*-terminus.

In both peptides, *N*- and *C*-terminal fragments, the cleavage conditions to release the sequences before fragment condensation allow all the protecting groups to remain on the side-chains of the AAs, thus only two functional groups (the carboxylic acid and the

amino), one for each fragment, rest unprotected. Fragment condensation is carried out in a suitable solvent which allow both fragments to be dissolved completely (Table 2). Contrary to solid-phase couplings, a wide range of solvents are available to perform the amide bond formation in solution. Equimolar quantities of the two peptides would be enough to be successful on the ligation step by using standard coupling reagents, or by stronger conditions depending on the particular difficulties encountered.

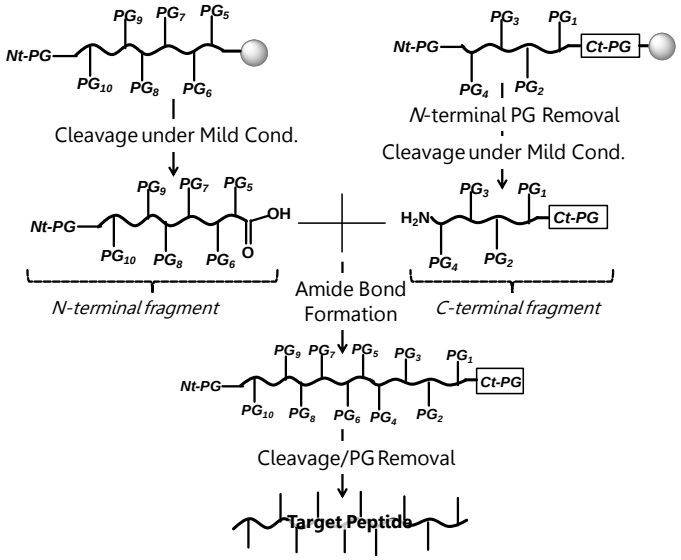

Figure 3. General scheme of the convergent strategy: fragment condensation in solution.

Large peptide sequences, even proteins or "difficult peptides", nowadays are mostly synthesized by following the fragment condensation in solution approach, where the solid-phase syntheses of fragments are separately performed by Boc/Bzl or Fmoc/*t*Bu strategies. Therefore, not only in the past certain complicated sequences were synthesized by this kind of fragment condensation method,[42] but also nowadays there are authors which carry on selecting this strategy to reach peptides.[43,44] Other peptide fragment condensation strategies in solution have emerged in the recent years,[45] such as the well-known native chemical ligation strategy,[46] which allows the condensation between unprotected peptide segments enabling the obtaining of complex peptides.[47,48]

Requirements for Solid-Phase Fragment Condensation	Requirements for Fragment Condensation in Solution
N-terminal fragment synthesized on resin which allows keeping protected side-chains	*N*-terminal and *C*-terminal fragments synthesized on resin which allows keeping protected side-chains
low resin loading preferably in *C*-terminal fragment synthesis	high solubility of *N*-terminal and *C*-terminal fragments in the same solvent during fragment condensation
high solubility in standard SPPS of *N*-terminal fragment	the amino acid on the *C*-terminus from the *N*-terminal fragment should not be susceptible to racemization
the amino acid on the *C*-terminus from the *N*-terminal fragment should not be susceptible to racemization	avoid bulky amino acids when choosing the separation point
avoid bulky amino acids when choosing the separation point	coupling conditions in fragment condensation should avoid the removal of any PG
coupling conditions in fragment condensation should avoid the removal of any PG	

Table 2. Synthetic requirements to perform the fragment condensation strategy on solid-phase[31] or in solution.[37]

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Publication I

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FULL PAPER

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RADA-16: A Tough Peptide – Strategies for Synthesis and Purification

Marta Paradís-Bas,[a,b] Judit Tulla-Puche,[a,b] Aikaterini A. Zompra,*[a,b] and Fernando Albericio*[a,b,c,d]

Keywords: Solid-phase synthesis / Peptides / Self-assembly / Synthetic methods / Nanostructures

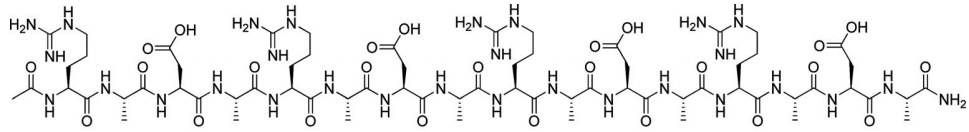
The self-assembling capacity of certain molecules can be exploited for a diverse range of biomedical applications. The ionic complementary peptide RADA-16 is well-known for its propensity to self-assemble, which derives from its architectural arrangement. Herein, we describe rational synthetic strategies of synthesis of RADA-16 based on fragment condensation, and its subsequent purification through optimized methods. Our methodology should prove suitable for the preparation of other peptides prone to self-assembly.

Introduction

Over the past few years nanobiotechnology and biomaterials based on peptide and protein self-assembly systems have garnered great attention.[1,2] The interest lies chiefly in the possibility of manufacturing new nanostructured scaffolds with customizable mechanical properties and biological functions.[3,4] Moreover, peptides and proteins are excellent model systems for studying biological self-assembly because they are highly biocompatible and their sequences can be modified to obtain defined molecular properties. Several types of self-assembling peptides have been systematically studied.[5] This class of biological materials has countless biomedical applications, including tissue regeneration,[6] drug delivery,[7] protein crystallization,[8] and cellular internalization.[9]

In this study, we chose the well-known self-assembling peptide Ac-$(RADA)_4$-NH_2 (RADA-16, Figure 1), a 16 amino acid peptide belonging to the family of ionic complementary peptides. It comprises repeated segments of hydrophobic (Ala) and hydrophilic (Arg and Asp) groups with alternating positive and negative charged amino acid residues. This structure enables RADA-16 to undergo ordered self-assembly in solution to form nanofibers.

RADA-16 is highly prone to self-assemble into very stable and highly organized β-sheet structures that tend to form hydrogels. It has been extensively used for three-dimensional cell culture scaffolds as well as for drug delivery and regenerative medicine.[10–12] Recently, several functional motifs have been used to generate RADA-16 analogues that promote cell adhesion, migration, neurite outgrowth, and cell differentiation.[13]



Ac-Arg^1-Ala^2-Asp^3-Ala^4-Arg^5-Ala^6-Asp^7-Ala^8-Arg^9-Ala^{10}-Asp^{11}-Ala^{12}-Arg^{13}-Ala^{14}-Asp^{15}-Ala^{16}-NH_2

Figure 1. Structure of RADA-16.

[a] Institute for Research in Biomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain
E-mail: azompra@upatras.gr
fernando.albericio@irbbarcelona.org
Homepage: http://www.irbbarcelona.org
[b] CIBER-BBN, Networking Center on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, 08028 Barcelona, Spain
[c] Department of Organic Chemistry, University of Barcelona, Marti i Franquès 11, 08028 Barcelona, Spain
[d] School of Chemistry and Physics, University of KwaZulu-Natal, 4001 Durban, South Africa

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The self-assembling capacity of RADA-16, which results in a huge range of nanobiotechnology and biomedical engineering applications,[14–17] is not only its beauty, but also the cause of difficulty in both its synthesis in decent yield and its purification. Although RADA-16 is commercially available and has been synthesized by research groups, the absence of a detailed full chromatographic characterization[18] encouraged us to explore combined optimum synthetic and purification strategies as well as a convenient HPLC method for its characterization.[19]

Prompted by the demand for RADA-16 in sufficiently high purity for clinical use, we sought to explore synthetic and chromatographic (HPLC) strategies to obtain this product in high purity. We rationalized that the peculiarity of repeated segments in the RADA-16 sequence would make this peptide an excellent model for developing different peptide synthetic strategies to obtain the desired target with maximum possible purity.

In this work, rationalized synthetic strategies to obtain RADA-16 have been proposed and carried out to establish the most suitable approach to the synthesis of this peptide with acceptable levels of purity. Stepwise solid-phase synthesis, in manually and automatic modes, were first attempted, since these are the most common and straightforward ways to synthesize peptides. Two more sophisticated strategies based on fragment condensation approaches ("solid-phase fragment condensation" and "fragment condensation in solution") were performed to evaluate which was the most suitable strategy to reach the peptide target.

Approaches outlined herein should prove valuable for other peptide targets that contain repetitive sequences and that undergo self-assembly into other structures.

Results and Discussion

Prior to the development of synthetic strategies to obtain RADA-16, an HPLC study of this peptide [in this case, Ac-$(RADA)_4$-NH_2 provided by different companies] was required to optimize the best analytical procedures with which to characterize the self-assembling peptide. Several HPLC factors were analyzed and optimized,[19] as an example, one of the analytical parameters that seriously affects the chromatographic analysis of RADA-16 was the concentration of peptide injected onto the HPLC column. This factor, which is not typically significant when other peptides are analyzed, is crucial when RADA-16 or other self-assembled peptides are studied. The appreciable differences in chromatographic profiles of RADA-16 dissolved at concentrations of 0.5, 1 and 2 mg/mL show the importance of this parameter (Figure 2). This study allowed us to conclude that the optimum concentration of HPLC injection for Ac-$(RADA)_4$-NH_2 was ca. 0.5 mg/mL, which reduces aggregation during application on the HPLC column while still allowing peptide purity evaluation.

The initial strategy described herein to obtain RADA-16 was based on stepwise solid-phase syntheses of RADA-16 in manual and automatic modes, using solid-phase peptide synthesis according to Fmoc/*t*Bu chemistry on ChemMatrix resin.[20–23] This resin, which is considered the best suited for this class of peptides, enabled us to overcome the challenge associated with strongly hydrophobic synthetic targets, including problems attributed to peptide aggregation or to poor resin solvation. Although after several recouplings, our manual synthesis ultimately afforded the desired peptide, however, final characterization by HPLC and MALDI-TOF MS revealed a rather poor final purity of the

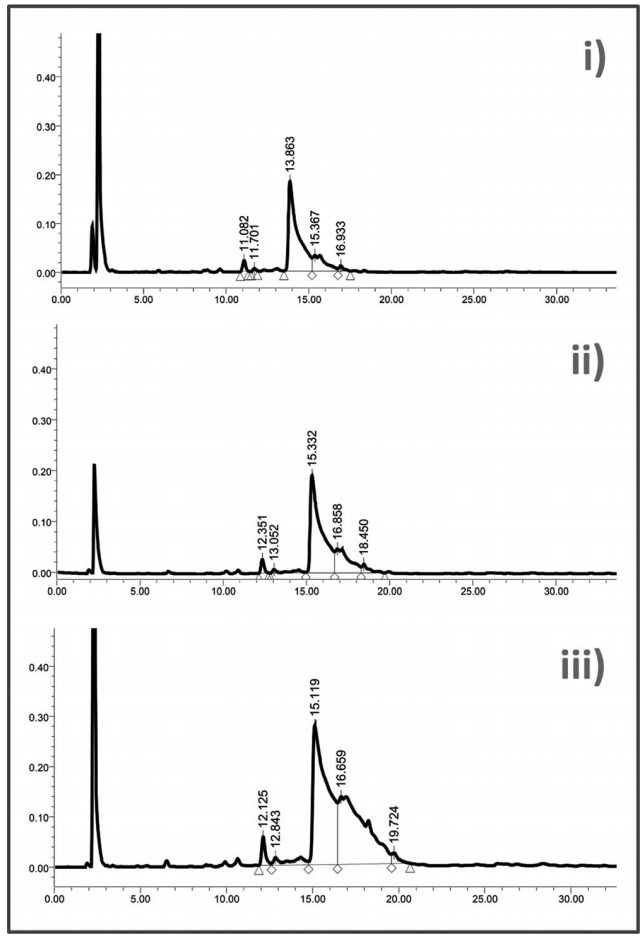


Figure 2. Comparison of HPLC profiles [conditions: column I heated at 50 °C, eluent based on TFA system, gradient (%B): 0–30] of Ac-$(RADA)_4$-NH_2 material provided by one chemical supplier dissolved with H_2O/0.1 % TFA and analyzed at different concentrations: (i) 0.5, (ii) 1, and (iii) 2 mg/mL.

crude product. Most importantly, impurities associated with this strategy eluted close to the desired product, thus complicating the purification, which was already problematic due to the poor solubility of the peptide (Figure 3, A). In fact, one of the principal impurities was derived from deletion of the three amino acids Arg, Ala, and Asp, which are side-products that cannot be easily removed, especially when the stepwise approach is hampered by β-sheet formation. A similar HPLC profile was observed when automated synthesis was performed (Figure 3, B).

Since neither manual nor automated stepwise synthesis gave sufficiently pure peptide, we investigated the solid-phase fragment condensation strategy, whereby presynthesized, protected fragments were sequentially coupled onto the resin, enabling the peptide sequence to be completed on solid phase. By following this strategy, side products formed from incomplete couplings are more easily removed because they differ from the peptide by the length of one fragment, rather than by only one amino acid.[24–26] Taking into account that RADA-16 contains the tetrapeptide sequence RADA (Arg-Ala-Asp-Ala) in quadruplicate, we decided to

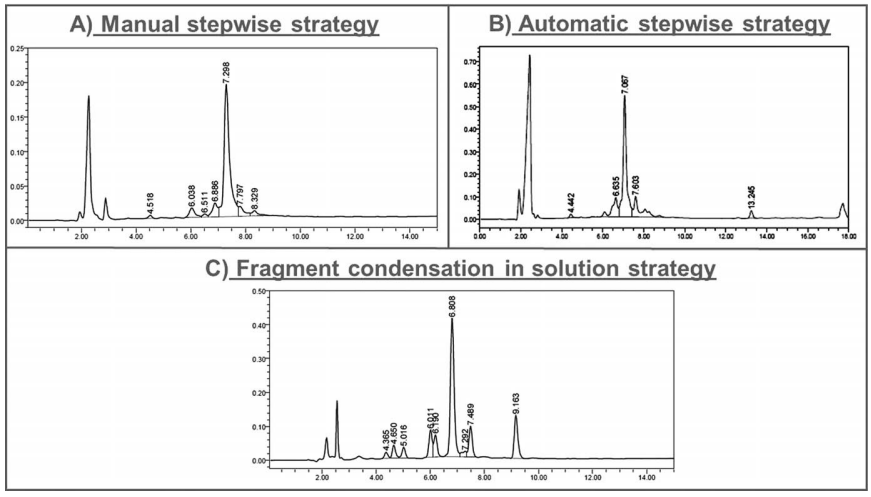
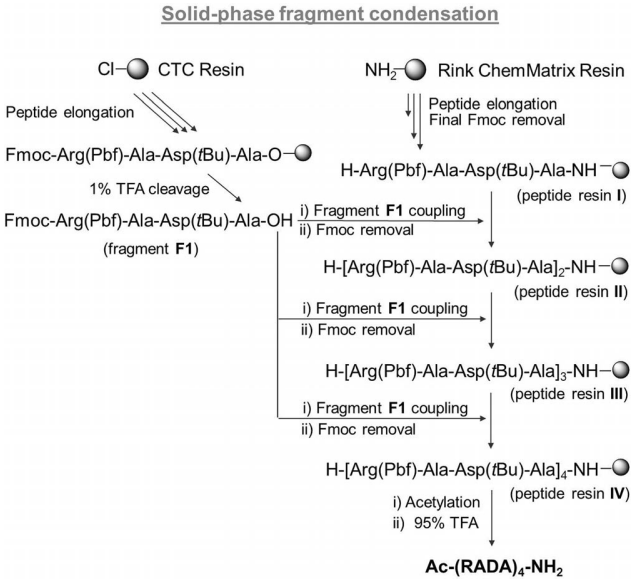


Figure 3. HPLC profiles of the crude peptide obtained in three different syntheses of RADA-16: (A) manual stepwise solid-phase, (B) automatic stepwise solid-phase, and (C) fragment condensation in solution.

prepare the Fmoc-protected tetrapeptide Fmoc-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-OH (Fragment **F1**) on solid-phase, and then link, consecutively, **F1** until the desired sequence of RADA-16 was complete (Scheme 1).

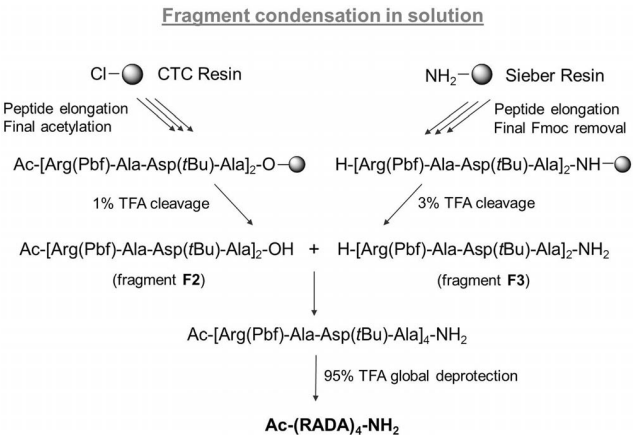


Scheme 1. Synthesis of Ac-$(RADA)_4$-NH_2 (RADA-16) by solid-phase fragment condensation.

To synthesize the protected fragment **F1**, with a C-terminal carboxyl moiety, we accomplished peptide elongation on CTC resin.[27] Cleavage of the peptide from the resin under mild acidic conditions provided **F1** in high purity (99%), thus obviating the need for further purification. Although the solubility of the obtained product – a requisite for successful couplings on resin – was unpredictable,[28] fortunately, the target protected tetrapeptide was completely soluble in *N*,*N*-dimethylformamide (DMF), which is an appropriate solvent for solid-phase coupling. To incorporate the synthesized protected fragment **F1** onto the resin, careful choice of resin,[29] peptide loading,[30] coupling reagents,[31,32] and reaction conditions[33] is essential. Again, the ChemMatrix support was chosen for this strategy. At this point, we first tried to incorporate fragment **F1** directly onto the polymer support by using a (4 + 4 + 4 + 4 + resin) strategy. However, this approach was unsuccessful and was abandoned. We then turned to the solid-phase fragment condensation (4 + 4 + 4 + RADA-resin) strategy (Scheme 1); thus, first four amino acids (RADA) were coupled stepwise conferring "peptide resin **I**". Subsequently, the first fragment **F1** was incorporated onto peptide resin **I** through a double-coupling protocol to afford the resin-bound octapeptide (peptide resin **II**).

The best way to monitor each coupling of **F1** onto the resin was to cleave an aliquot of peptide–resin and analyze it by reverse-phase HPLC, which, although more tedious, was essential because standard tests used in stepwise synthesis, such as the Kaiser test and the TNBS-test only give a first indication. After Fmoc-removal, the **F1** fragment was incorporated (three times) using relatively harsh coupling reagents to afford the dodecapeptide on resin (peptide resin **III**). HPLC analysis revealed a mixture of products, although the desired peptide was obtained in 46% purity. After the last fragment **F1** incorporation, the Fmoc group was removed and the resulting deprotected peptide was acetylated and then cleaved from the resin. The main advantage of this strategy was that it minimized formation of side products that differ from the desired product by only one or two residues, as indicated in narrower peaks observed by reverse-phase HPLC (Figure 3, C). Although for peptides that are not prone to self-assembly, purification of the crude material obtained from the fragment coupling strategy was more convenient than from crude material obtained from a stepwise strategy, in our case, the poor solubility of the crude material precluded that.

Finally, seeking to establish an optimized strategy for RADA-16 synthesis that would ultimately enable synthesis on a larger scale, we explored fragment condensation in solution.[34] Numerous examples of this type of synthesis, which combines the respective advantages of solid-phase and solution phase synthesis, have been reported. We chose the 8 + 8 strategy (Scheme 2), which required two protected fragments: the N-terminal fragment (Ac-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-OH; Fragment **F2**) and the C-terminal fragment (H-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-NH_2; Fragment **F3**). The success of fragment condensation depends on various factors: (1) the solubility and final purity of the protected fragments, (2) the scale of synthesis, (3) the coupling reagents and conditions used, and most importantly (4) the final isolation of the product with concomitant removal of any remaining reagents. This strategy has proved to be the strategy of choice for industrial synthesis of several difficult peptide targets[35,36] because it can produce sufficiently pure final product for clinical use.



Scheme 2. Synthesis of Ac-$(RADA)_4$-NH_2 (RADA-16) by fragment condensation in solution.

In this work, we synthesized the two protected fragments by solid-phase, manually, on medium scale (0.8 mmol) with two different resins. In each case, the peptide was cleaved from the resin without affecting the protecting groups. Because the two fragments were obtained in high purity, no further purification was required. The key point of this strategy was that both fragments were completely soluble in DMF, because, as previously mentioned, although the solubility of fragments in DMF was mandatory for coupling, the solubility of any designed protected fragment is unpredictable. The choice of coupling reagents PyBOP/DIEA with HOAt as additive, and the use of equimolar amounts of protected fragments, was found to be suitable for this strategy. Phosphonium salts are better than aminium salts as activating agents for reactions in which the carboxylic component was not in excess (e.g., fragment condensation or cyclization), because they do not react irreversibly with the amino function, as aminium salts tend to do.[37,38] A clear advantage of fragment condensation in solution over fragment condensation on solid-phase is the quality of the crude peptide obtained.

Chromatographic methods are generally unsuitable for purification of peptides such as RADA-16, which are typically insoluble in standard chromatography solvents. Thus, the relatively pure crude material with impurities that are chemically differentiated from the expected product ob-

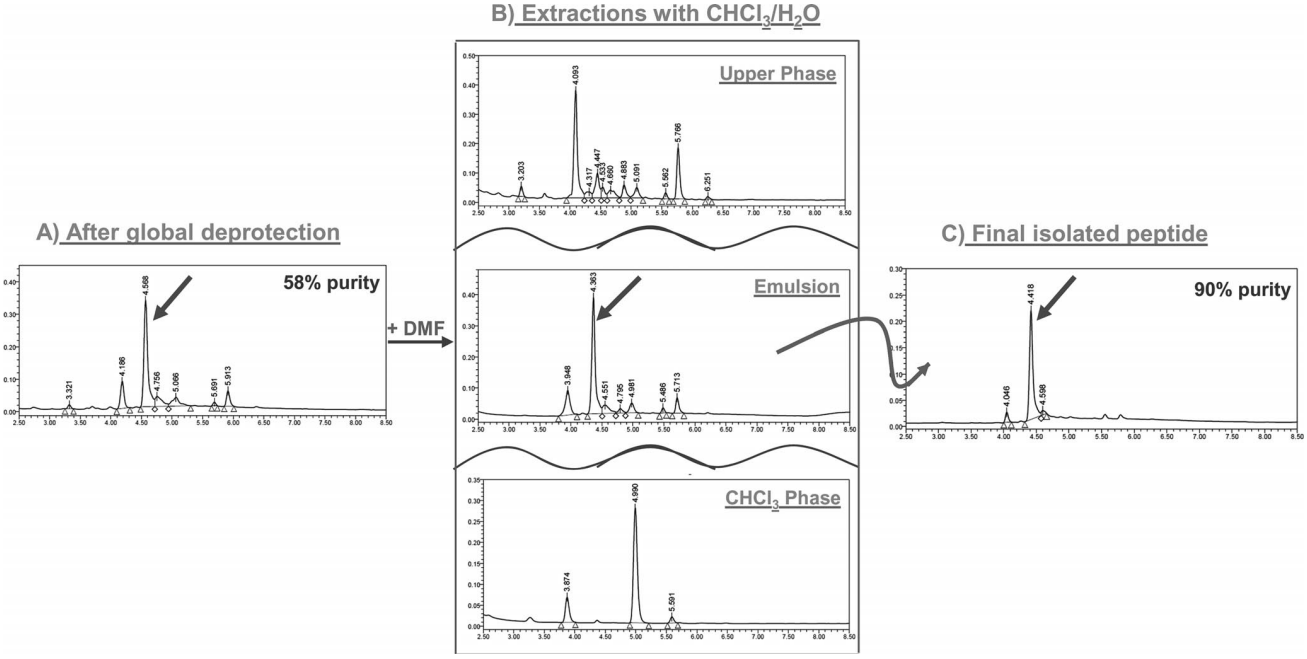


Figure 4. HPLC profiles obtained (A) before, (B) during, and (C) after $CHCl_3/H_2O$ extractions performed in the synthesis of RADA-16 by fragment condensation in solution.

tained from fragment condensation in solution should be purified by washing and precipitation. Washing with *tert*-butyl methyl ether helped to remove HOAt, which very often remains on the peptide. However, although this step can be very efficient in small-scale syntheses, as was the case in our attempts, for a larger scale a deeper work-up was needed (Figure 4). During this procedure, several impurities were removed (Figure 4, B); one of the principal impurities that entered the organic phase had the UV profile of HOAt (as indicated by photodiode array detector from HPLC analysis). The expected product was mainly detected in the emulsion phase and was isolated as described in detail in the experimental part and presented in (Figure 4). This purification procedure gave RADA-16 in higher purity (90%; see Figure 4, C) than did either of the stepwise syntheses or the solid-phase fragment condensation synthesis.

Conclusions

It is clear that Ac-$(RADA)_4$-NH_2 is a tough peptide to synthesize and purify. The strategy outlined herein, which combines the advantages of solid-phase methodology and solution chemistry, has allowed the preparation of the target peptide with sufficient purity for biological purposes. The strategy described herein is based on the solid-phase preparation of two protected fragments with sufficient purity that they do not require purification. Their coupling in solution gives the target product in a purity that can be resolved further by standard laboratory operations. In addition, a wide range of chromatographic parameters to establish the optimum HPLC conditions to characterize the RADA-16 were analyzed.

This work has shown that, despite the sophistication of recently developed synthetic strategies for the preparation of peptides (solid supports, handles, coupling reagents, etc.), the synthesis of self-assembled peptides remains a major challenge. The methodology developed herein should be of broad applicability to similar peptide targets.

Experimental Section

General: Analytical HPLC was carried out with an instrument comprising two solvent delivery pumps, an automatic injector, and a variable wavelength detector (photodiode array). UV detection was performed at 215 and 220 nm. Two HPLC columns were used: column I [C18 column (XTerra, Waters: 4.6 × 150 mm, 5 μm)] or column II (SunFire, Waters: C18, 3.5 μm 4.6 × 100 mm). All peptide fragments analyzed from different synthetic strategies of RADA-16 were run using linear gradients of two eluents at room temperature (25 °C) and a flow rate of 1.0 mL/min: eluent A (H_2O + 0.045% TFA) and eluent B (MeCN + 0.036% TFA). HPLC/ES-MS was performed on a reverse-phase C18 column (3.9 × 150 mm, 5 μm) using aqueous (+ 0.1% formic acid) and MeCN (+ 0.07% formic acid) as eluents. MALDI-TOF MS was carried out with a Voyager-DETMRP Biosystem, using α-cyano-4-hydroxycinnamic acid (ACH) as matrix.

RADA-16 Syntheses

Stepwise strategies: We explored manual and automatic stepwise solid-phase syntheses of RADA-16, with the double objective of optimizing the preparation and also to obtain sufficient amount of peptide for use as a reference material.

Manual Synthesis: RADA-16 was synthesized manually by solid-phase peptide synthesis by using 9-fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl (*t*Bu) chemistry on an aminomethyl-ChemMatrix resin[39] (Matrix Innovation, scale 0.1 mmol, loading: 0.62 mmol/g, 35–100 mesh). Peptide elongation was performed in polypropylene syringes fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. The linker Rink-Amide (3 equiv., Iris Biotech) was anchored to the resin with *N*,*N*′-diisopropylcarbodiimide (DIPCDI)/1-hydroxybenzotriazole (HOBt) (3 equiv. each) in DMF for 12 h. At this point, a positive ninhydrin test indicated free amino groups, so the linker reaction was repeated, this time for 2 h. The Fmoc groups were removed by treatment with piperidine/DMF (1:4) in the presence of 0.1 M HOBt (1 × 2 min, 2 × 10 min), which was used to minimize aspartimide formation.[40] The following Fmoc-L-protected amino acid derivatives were used: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Asp(*t*Bu)-OH. Chain extension was carried out by using 5 equiv. of the amino acid, at each coupling step, in the presence of 2-(6-chloro-1-*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) (5 equiv.) and diisopropylethylamine (DIEA) (10 equiv.) for 1 h. In case of a positive ninhydrin test, a recoupling was performed, using a fivefold molar excess of the Fmoc-amino acid, activated essentially in situ by DIPCDI/7-aza-1-hydroxybenzotriazole (HOAt) in DMF for 3 h. Incorporation of the following residues required multiple recouplings: Ala^2, Asp^3, Ala^4, Arg^5, Ala^6, Arg^9, Ala^{10}, Asp^{11}, and Ala^{12}. After Fmoc removal of the last Arg residue, acetylation was performed by treatment with Ac_2O (10 equiv.) and DIEA (10 equiv.) for 30 min. All washings of the peptidyl-resin between Fmoc removal, amino acid coupling and the final acetylation were done with DMF. Once the fully protected peptide had been synthesized, it was subject to global deprotection (removal of the side-chain protecting groups and cleavage from the resin) by treatment with a mixture of TFA/triisopropylsilane (TIS)/H_2O (95:2.5:2.5) for 4 h. The peptide was isolated by precipitation with cold diethyl ether, centrifuged, dissolved in H_2O/MeCN/TFA (7:2.8:0.2, starting with neat TFA), and sequentially lyophilized. It was then characterized by HPLC [Column I; t_R = 7.29 min, 75% purity, gradient (%B): 5–25 in 15 min] and MALDI-TOF MS showed the mass of the desired peptide {*m*/*z* calcd. for Ac-$(RADA)_4$-NH_2 ($C_{66}H_{113}N_{29}O_{25}$) 1711.8; found 1712.8 $[M + H]^+$}. Other impurities were also detected from the HPLC analysis and characterized by MALDI-TOF MS: the peak that eluted at t_R = 6.88 min (peak area: 6.84% purity) corresponded to a byproduct that lacks the tripeptide RDA {*m*/*z* calcd. for Ac-$(RADA)_4$-NH_2 – RAD ($C_{53}H_{91}N_{23}O_{20}$) 1369.6; found 1370.7 $[M + H]^+$}.

Automated Synthesis: The automated synthesis was conducted on the same resin used for the manual synthesis (aminomethyl-ChemMatrix), with an ABI 433A synthesizer (Applied Biosystems, Foster City), on 0.1 mmol (peptide) scale, following standard Fmoc chemistry and using a 10-fold excess of Fmoc-protected L-amino acids and *N*-[(1*H*-benzotriazol-1-yl)-dimethylamino-methylene]-*N*-methylmethanaminium tetrafluoro-borate *N*-oxide (TBTU, 0.45 M) in the presence of HOBt as coupling reagents in DMF. Each deprotection step was carried out in 15 min and each coupling step in 35 min. Acetylation, cleavage and isolation of the completed peptide were done as described for the manual synthesis. The product

was characterized by HPLC [Column I, t_R = 7.06 min, 60% purity, gradient (%B): 5–25 in 15 min] and by MALDI-TOF MS, which showed the mass of the desired peptide {*m*/*z* calcd. for Ac-(RADA)$_4$-NH$_2$ ($C_{66}H_{113}N_{29}O_{25}$) 1711.8; found 1712.6 [M + H]$^+$}.

Solid-Phase Fragment Condensation Strategy (4 + 4 + 4 + RADA-resin)

Fragment F1: Fmoc-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-OH was synthesized manually by using standard Fmoc protocols on 2-chlorotrityl chloride (CTC) resin (1.55 mmol/g, 10 g). The first Fmoc-Ala-OH (1.00 mmol/g resin, scale of synthesis 10 mmol) was incorporated in the presence of DIEA (10 equiv.), which was added in two portions: first, 1/3 of the volume and, after 10 min, the remaining 2/3. The mixture was allowed to react for 1 h. Next, a capping step with MeOH (0.4 mL/g) was done. In each deprotection step, removal of the Fmoc group was performed with piperidine/DMF (1:4) (2 × 2 min, 2 × 10 min). The remaining couplings were done using threefold molar excess of the corresponding Fmoc-amino acid activated essentially in situ by DIPCDI/HOBt in DMF for 3 h. Between coupling and subsequent deprotection steps, the resin was washed with DMF (3 × 3 min), CH_2Cl_2 (1 × 5 min), DMF (1 × 3 min), and CH_2Cl_2 (3 × 3 min), using 10 mL of solvent/g of resin per wash. The complete protected peptide was cleaved from the resin by using TFA/CH_2Cl_2 (1:99) (6 × 3 min), and then collected in H_2O to avoid side-chain deprotection. After evaporation of CH_2Cl_2 and lyophilization, the peptide was characterized by HPLC [Column I, t_R = 9.14 min, 99% purity, gradient (%B): 40–100 in 15 min] and by HPLC/ES-MS, which confirmed the desired product Fragment **F1** {*m*/*z* calcd. for Fmoc-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-OH ($C_{48}H_{63}N_7O_{12}S$) 961.4; found 962.6 [M + H]$^+$}. This product was sufficiently pure that it did not require any further purification.

Peptide Resin I (RADA-CM Resin): Synthesized on aminomethyl-ChemMatrix resin (0.062 mmol scale) following the same procedure used for the manual synthesis of RADA-16. At the end, an aliquot was taken and treated with TFA/TIS/H_2O (95:2.5:2.5) for 2 h. HPLC/ES-MS confirmed the desired (H-Arg-Ala-Asp-Ala-NH_2) product (*m*/*z* calcd. for $C_{16}H_{30}N_8O_6$ 430.2; found 431 [M + H]$^+$).

Peptide Resin II ([RADA]$_2$-CM resin): Fragment **F1** (3 equiv.), which was completely soluble in DMF, was coupled onto peptide resin **I** in the presence of 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium hexafluorophosphate 3-oxide (HATU) (3 equiv.) and DIEA (6 equiv.) for 1 h. Due to a positive ninhydrin test, a recoupling was performed using fragment **F1** (3 equiv.) and equimolar amounts of DIPCDI/HOAt for 3 h. Complete coupling was confirmed by a negative ninhydrin test. An aliquot was taken and treated with TFA/TIS/H_2O (95:2.5:2.5) for 2 h. HPLC [Column I; t_R = 5.88 min, 89% purity, gradient (%B): 20–80 in 15 min] and HPLC/ES-MS confirmed the desired product (*m*/*z* calcd. for $C_{47}H_{67}N_{15}O_4$ 1065.5; found 1066.1 [M + H]$^+$).

Peptide Resin III ([RADA]$_3$-CM resin): The Fmoc group of peptide resin **II** was removed by treatment with piperidine/DMF (1:4) in the presence of 0.1 M HOBt. Three couplings were required until a negative ninhydrin test was obtained. Fragment **F1** (1.5 equiv.) was incorporated onto peptide resin **II** in three steps: first, using HCTU (1.5 equiv.)/DIEA (3 equiv.) for 1 h; second, with DIPCDI (1.5 equiv.)/HOAt (1.5 equiv.) for 3 h; and finally, with HCTU (1.5 equiv.)/DIEA (3 equiv.) for 1 h. An aliquot was taken and treated with TFA/TIS/H_2O (95:2.5:2.5) for 3 h. HPLC [Column II; t_R = 3.89 min, 98% purity, gradient (%B): 5–100 in 8 min] and MALDI-TOF MS indicated the desired peptide {*m*/*z* calcd. for Fmoc-(RADA)$_3$-NH$_2$ ($C_{63}H_{94}N_{22}O_{20}$) 1478.7; found 1479.8 [M + H]$^+$}.

Ac-(RADA)$_4$-NH$_2$: Fragment **F1** (1.5 equiv.) was coupled onto peptide resin **III** in two steps: first, with HCTU (1.5 equiv.)/DIEA (3 equiv.) for 1 h, which gave a positive ninhydrin test; and then, using DIPCDI/HOAt (1.5 equiv. of each) for 3 h. After Fmoc group removal, the acetylation step was performed by using Ac_2O (10 equiv.)/DIEA (10 equiv.) for 30 min. An aliquot of peptide resin was taken and then treated with TFA/TIS/H_2O (95:2.5:2.5) for 4 h. The HPLC revealed a mixture of products [Column II; main peak t_R = 4.24 min, 41% purity, gradient (%B): 5–25 in 8 min], including the desired peptide, RADA-16. MALDI-TOF MS showed the desired product [*m*/*z* calcd. for Ac-(RADA)$_4$-NH$_2$ ($C_{66}H_{113}N_{29}O_{25}$) 1711.8; found 1712.6 (M + H)$^+$], as well as two incorrect mass signals: one corresponding to Ac-(RADA)$_3$-NH$_2$ (*m*/*z* calcd. for $C_{50}H_{86}N_{22}O_{19}$ 1298.6; found 1299.8 [M + H]$^+$) and another impurity (*m*/*z* found 1671.9 [M + H]$^+$) that corresponds to the nonacetylated peptide [H-(RADA)$_4$-NH$_2$ (*m*/*z* calcd. 1669.8)]. Based on these results, the acetylation treatment was repeated. The peptide-resin was cleaved using TFA/TIS/H_2O (95:2.5:2.5) for 4 h. HPLC results still revealed a mixture of products, but the peak at t_R = 4.94 min had disappeared and the purity of the desired peptide was superior to that previously seen in the aliquot [Column II; main peak t_R = 4.26 min, 46% purity, gradient (%B): 5–25 in 8 min].

Fragment Condensation in Solution (8 + 8): Two protected octapeptides Ac-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-OH (Fragment **F2**) and H-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-NH_2 (Fragment **F3**) were synthesized separately on different resins, and then cleaved from the resin with full preservation of protecting groups.

Fragment F2 [Ac-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-OH]: Synthesized manually by using standard Fmoc protocols on CTC resin (1.55 mmol/g, 10 g). The first Fmoc-Ala-OH (0.8 mmol/g resin, scale of synthesis 8 mmol) was incorporated using DIEA (10 equiv.) added in two portions: first, 1/3 of the volume and, after 10 min, the remaining 2/3. The mixture was allowed to react for a total of 1 h. Next, a capping step with MeOH (0.4 mL/g) was performed. In all deprotection steps, removal of the Fmoc group was carried out with piperidine/DMF (1:4) in the presence of HOBt 0.1 M (1 × 2 min, 2 × 10 min). The remaining couplings were accomplished by using threefold molar excess of each Fmoc-amino acid activated in situ by DIPCDI/HOBt in DMF for 2 h. Washings between coupling and subsequent deprotection steps were performed with DMF (3 × 3 min), CH_2Cl_2 (1 × 3 min), DMF (1 × 3 min) and CH_2Cl_2 (3 × 3 min), using 10 mL of solvent/g of resin per wash. No positive ninhydrin tests were observed at any point during the synthesis; therefore, no recouplings were performed. Acetylation was accomplished with Ac_2O (10 equiv.)/DIEA (10 equiv.) for 30 min. The protected peptide was cleaved from the resin by using TFA/CH_2Cl_2 (1:99) (8 × 3 min), and then collected in H_2O to avoid side-chain deprotection. After evaporation of CH_2Cl_2, MeCN was added to increase solubility. The product was then lyophilized and characterized by HPLC [Column II; t_R = 5.50 min, 98% purity, gradient (%B): 40–100 in 8 min] and by HPLC/ES-MS, which confirmed the desired product, fragment **F2** (*m*/*z* calcd. for $C_{68}H_{106}N_{14}O_{20}S_2$ 1502.7; found 1504.0 [M + H]$^+$). Owing to the high purity (98%) of the final product, no further purification was performed.

Fragment F3 [H-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-NH$_2$]: Synthesized on Sieber amide resin[41] (loading: 0.53 mmol/g, 15 g), which allows cleavage of protected peptides from the resin using 3% TFA. In each deprotection step, removal

of the Fmoc group was accomplished with piperidine/DMF (1:4) in the presence of HOBt 0.1 M (1 × 2 min, 2 × 10 min). The couplings were done using a threefold molar excess of each Fmoc-amino acid activated by DIPCDI/HOBt (3 equiv. each) in DMF for 2 h. Only one recoupling was required: Arg[1] (recoupled using the same coupling reagents in half-molar quantity for 2 h). The protected peptide was cleaved from the resin by using TFA/CH_2Cl_2 (3:97) (3 × 3 min), and then H_2O was added. After evaporation of CH_2Cl_2, MeCN was added to increase the solubility of the protected peptide. The product was lyophilized and then characterized by HPLC [Column II; t_R = 3.21 min, 97% purity, gradient (%B): 40–100 in 8 min] and by HPLC/ES-MS, which confirmed the desired structure fragment **F3** (*m*/*z* calcd. for $C_{66}H_{105}N_{15}O_{18}S_2$ 1459.7; found 1462 $[M + H]^+$, 731 $[M + 2H]^+/2$). Due to the excellent purity of the product, no further purification was required.

Fragment Condensation in Solution: The coupling was carried out on 0.2 mmol scale. Thus, Fragment **F2** (0.2 mmol) was dissolved in DMF (30 mL). To the solution were added DIEA (2 mmol), then, added dropwise over 30 min, a solution of 1-benzotriazole-1-yloxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP)/HOAt (0.2 mmol each) in DMF (43 mL). Fragment **F3** (0.2 mmol) was dissolved in DMF (30 mL) and added slowly to the solution. The reaction mixture was left at room temperature for 16 h and monitored by HPLC. The DMF was then evaporated in the presence of toluene and the product was precipitated by adding cold *tert*-butyl methyl ether (3 times). The precipitate was dissolved in MeCN/H_2O (1:1), with sonication (3 min), and then lyophilized. This process (from the addition of cold *tert*-butyl methyl ether up to the lyophilization) was then repeated twice. The side-chain protecting groups were removed by treatment with TFA/TIS/H_2O (95:2.5:2.5) for 4 h. The peptide was precipitated by adding cold diethyl ether, centrifuged, washed with diethyl ether, dissolved in MeCN/H_2O (1:1), and then lyophilized. The product was characterized by HPLC, which exhibited one main peak (Column II; t_R = 4.56 min, 58% purity, gradient (%B): 5–25 in 8 min), and by MALDI-TOF MS, which confirmed the desired product, RADA-16.

To isolate the desired product and remove any remaining coupling reagents or by-products, the following work-up was performed. Water (0.75 mL) was added to a part of the reaction mixture (200 mg) in DMF (1.5 mL), and a precipitate appeared. The upper phase was extracted three times with $CHCl_3$, and the combined upper phases together with the pellet/emulsion were centrifuged (4000 rpm for 8 min at 4 °C) to isolate the solid and to remove the upper phase. The solid material was washed with cold diethyl ether, and then centrifuged twice. The precipitate was then dissolved in MeCN/H_2O (1:1) and lyophilized. It was then characterized by HPLC, which exhibited a main peak (Column II; t_R = 4.41 min, 90% purity, gradient (%B): 5–25 in 8 min). Aliquots from different phases during the work-up were taken ($CHCl_3$ phase, emulsion, upper phase), and then characterized by HPLC.

Supporting Information (see footnote on the first page of this article): An accurate HPLC analysis of six RADA-16 materials provided by different chemical suppliers has been performed.

Acknowledgments

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SUPPORTING INFORMATION

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Title: RADA-16: A Tough Peptide – Strategies for Synthesis and Purification
Author(s): Marta Paradís-Bas, Judit Tulla-Puche, Aikaterini A. Zompra,* Fernando Albericio*

HPLC ANALYSIS OPTIMIZATION

An accurate HPLC analysis of six RADA-16 materials provided by different chemical suppliers has been performed:

Materials and Methods

Analytical HPLC was carried out on an instrument comprising two solvent delivery pumps, automatic injector and a variable wavelength detector (photodiode array). UV detection was at 215 nm and 220 nm. HPLC columns: Column I (C18 column (XTerra, Waters: 4.6 x 150 mm, 5 μm) or Column II (SunFire, Waters: C18, 3.5μm 4.6 x 100mm) were used at a flow rate of 1.0 mL/min. Liquid chromatography studies of RADA-16 provided by different chemical suppliers were run at linear gradients in three different pairs of eluents A and B (specified its content in every case) at flow rate of 1.0 mL/min: a) TFA based system: eluent A (H_2O-0.45% TFA) and eluent B (MeCN-0.036% TFA); b) OSA based system: eluent A [H_2O-0.1% 1-Octane Sulfonic Acid Sodium Salt (OSA)] and eluent B (MeCN); c) Perchlorate based system: eluent A (H_2O-0.1% (85% H_3PO_4 aquose)-0.13% sodium perchlorate) and eluent B (MeCN-40% H_2O-0.1% (85% H_3PO_4 aquose)-0.13% sodium perchlorate).

Evaluation of different HPLC conditions to characterize RADA-16

Six different RADA-16 peptides provided by different chemical suppliers were used to optimize the appropriated HPLC conditions to evaluate the quality of this self-assembling peptide. Several chromatographic parameters were tested, and detailed below, in order to provide a wide range of liquid chromatography conditions.

Peptide sample preparation. A RADA-16 sequence (H-$(RADA)_4$-OH) at 1 mg/mL concentration was dissolved in two different conditions [H_2O-MeCN (1:1) and H_2O-0.1% TFA]. The same linear gradient in column I heated at 50-60ºC was used to evaluate the proper solvent for peptide RADA-16.

Effect of sample concentration. In a 50-60ºC heated column I at the linear gradient and eluent system (1-octane sulfonic acid salt based system), the Ac-$(RADA)_4$-NH_2 material was analyzed at different concentrations. Peptide samples at 0.5 mg/mL, 1 mg/mL and 2 mg/mL were injected separately in HPLC with the other chromatographic parameters fixed.

Linear gradient optimization. The RADA-16 sequence H-$(RADA)_4$-OH, was chosen to evaluate one of the most important HPLC factors, the mobile phase gradient. When the purity of a crude peptide is reported, a previous accurate study of optimum gradient is forced to be done. An eluent system based on TFA at different polar linear gradients run at 30 min, was proposed and tested using column I.

Effect of temperature in the HPLC column. When a sample tends to self-assemble or forms aggregations then one of the appropriate approaches to analyze it by HPLC can be to apply temperature to the column (column I). Chromatographic analysis of the same peptide material at 25ºC and in a hot column conditions (50-60ºC) were performed to compare results.

Mobile phase (eluents) selection. Three different eluent systems are proposed in this work to analyze some RADA-16 sequences. The first method is based on TFA in aqueous and MeCN media (system a, detailed above), the second one based on 1-octane sulfonic acid sodium salt in aqueous and MeCN media (system b) and the third one based in sodium perchlorate in aqueous and MeCN media.

Results-Discussion

A wide range of HPLC conditions have been studied in this work, evaluating the quality of synthetic RADA-16, taking into account the eluent system chosen and the linear gradient as well. Although there are many potential buffers for chromatography, three of them based on TFA, OSA and perchlorate systems were selected in this research. These three buffers differ significantly in ionic strength content and this affects especially the interaction of the peptide with the HPLC column and can explain the differences between profiles. The most appropriate for this self-assembling peptide were those eluents which contain some TFA in H_2O and MeCN media [chromatographic profiles show clearly the differences when the eluent parameter is changed (Figure S1).

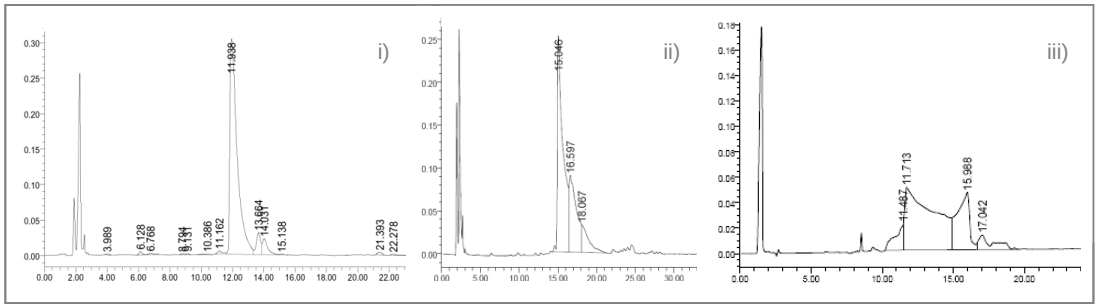

Figure S1. Evaluation of eluent effect by HPLC analysis of Ac-$(RADA)_4$-NH_2 material provided by one chemical supplier dissolved with H_2O-0.1% TFA and analyzed at the same HPLC conditions (column I heated at 60ºC, gradient (%B):5-35) but in 3 different eluent systems: H_2O and MeCN with TFA (i); H_2O and MeCN with perchlorate sodium salt (ii); H_2O and MeCN with 1-octane sulfonic acid sodium salt (iii).

Besides, TFA (1%) helps the RADA-16 sample to be dissolved in H_2O, thus in this work, that preparation sample protocol was applied to analyze all peptide fragments. HPLC analysis of the same peptide sample when different solvent to solubilize it were tested, reveal that this factor is very important (Figure S2).

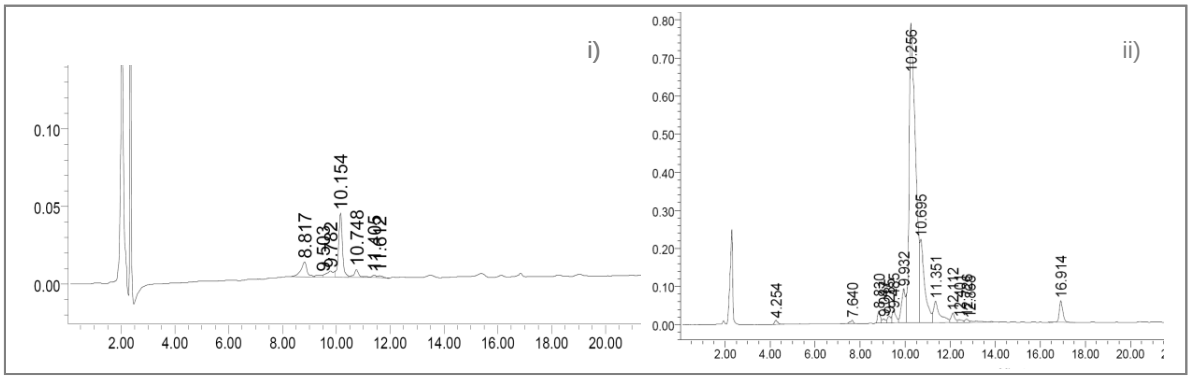

Figure S2. Evaluation of RADA-16 peptide sample preparation: HPLC chromatograms (conditions: Column I heated at 50ºC, eluents based on TFA system, gradient (%B): 0-30) of H-$(RADA)_4$-OH material provided by one chemical supplier, dissolved in two different conditions: H_2O-MeCN (1:1) (i) and H_2O-0.1% TFA (ii).

Characterization by liquid chromatography requires an optimization of the linear gradient in order to distinguish the impurities from the expected material. In this work, a wide range of linear gradient combinations based on TFA eluent system

allow us to find the appropriated one to analyze RADA-16, which consist on gradient (%B): 5-10 in 30 min. The heating of column at 50-60ºC was another factor should be taken into account, since the higher temperature achieved, less aggregating components of RADA-16 are formed during the analysis, facilitating the identification of some impurities present in the solid material.

HPLC AND ES-MS ANALYSIS OF SYNTHESIZED RADA-16

The RADA-16 synthesized by fragment condensation in solution and obtained after work-up was characterized by HPLC and HPLC-ES-MS:

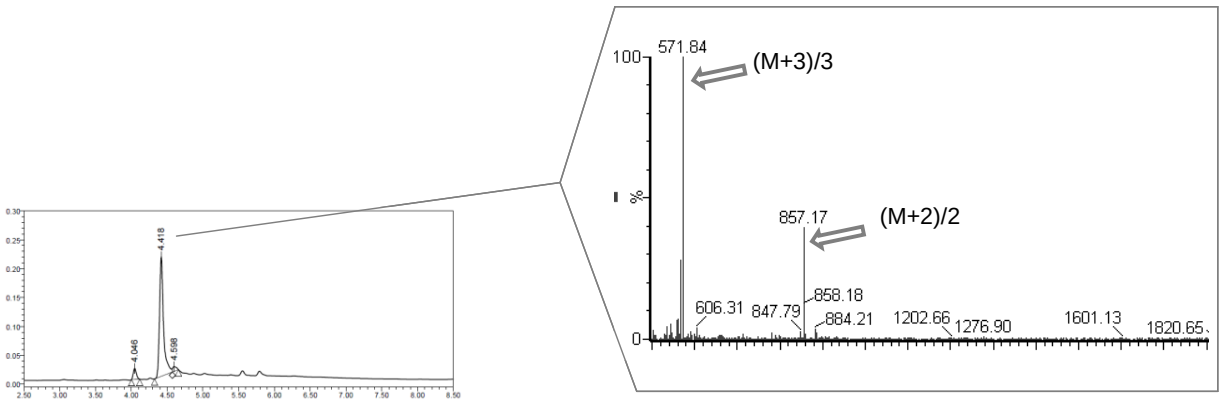

Figure S3. HPLC profile and ES-Mass Spectra of RADA-16 synthesized by fragment condensation in solution by HPLC (on the left) and HPLC-ES-MS (on the right). The M corresponds to Ac-$(RADA)_4$-NH_2 expected mass.

CHAPTER 2.

New Backbone Amide Protecting Group

Introduction

1. Safety-Catch Structure-Based

1.1. Safety-Catch Concept

In 1971 professor Kenner and collaborators were the first who defined the safety-catch term, as a principle applicable to the synthesis of peptides on solid-phase.[1] The same concept was also mentioned by Merrifield and Barany in a book section.[2] Specifically, the safety-catch idea described by Kenner establishes that it may be assigned to those molecules that meet the situation where a "...stable bond is eventually labilised at the appropriate moment by a specific chemical modification". Therefore, various kind of structures may be suitable for this description, being the unique requirement that the removal of this linked molecule occurs after a chemoselective transformation.

1.2. Safety-Catch Linkers

The first safety-catch structures for SPPS were proposed as linkers already anchored on the resin, whose advantage lies in the fact that they afford peptide sequences fully protected (the *N*-, *C*-terminus and side-chains). These linkers are introduced by reacting the polystyrene resin in order to functionalize it as **sulfonamides** (Fig. 1a), and are also known, related to its inventor, as *Kenner's safety-catch linkers*. The peculiarity of the acylsulfonamide peptidyl-resin lies in its stability to nucleophiles, acids or bases. Thus, under these conditions, the linker does not allow the release of the peptide. Particularly, in alkaline media, and due to the pka of the NH group (around 2.5), the deprotonation of the group is more favored than the desired nucleophilic attack. On the contrary, after a chemoselective transformation, such as the alkylation of this NH group, the acylsulfonamide results activated towards nucleophiles because of the enhanced electrophilicity introduced to the carbonyl (Fig. 1a). Specifically, Kenner proposed, for the cleavage, some nucleophiles, such as alkaline hydroxides, the ammonia or the hydrazine to render, respectively, the acid, the amide or the hydrazide *C*-terminal sequences (Fig. 1a). Although this author developed this novel protocol to render protected peptides containing different *C*-terminal modifications, one limitation in the Kenner's original idea resides in the last reaction, when poor nucleophiles are not able to cleave the peptide; or when insufficient resin loadings are obtained that favor side-reactions such as isomerization in aspartic or glutamic AAs.

Some years later, Ellman[3] and Kiessling,[4] independently, proposed improvements based on increasing the electrophilicity of the amide carbonyl towards nucleophiles, by introducing an electron-withdrawing group instead of the methyl moiety (Fig. 1b). In 1996, Ellman and co-workers described an optimized incorporation of the first

carboxylic group onto the linker to reach higher resin loadings, and also a new alkylating agent which produced cyanomethylation. Both modifications provided significant progress when several substituted amines were used as nucleophiles, even the anilines. In the same study, a new safety-catch functionalized resin (the **aliphatic sulfonamide**, Fig. 2)[5] was presented to facilitate the incorporation of the first carboxylic group onto the resin for those molecules which contained some α-electronegative substituents (such as the *N*-Fmoc, -Boc AAs), thus situations where the original Kenner's linker rendered poor resin loadings.

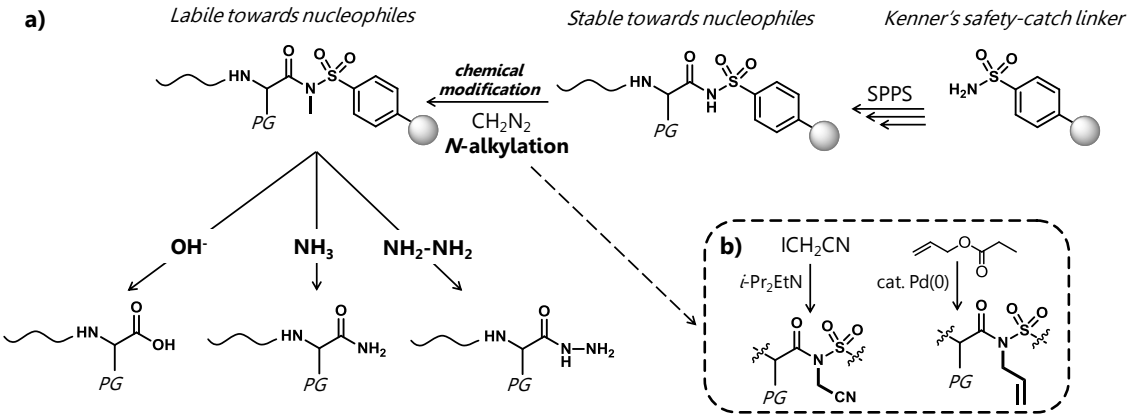

Figure 1. SPPS onto safety-catch sulfonamide linker. (a) Kenner's description which afforded *C*-terminal peptide derivatives depending on the nucleophile; and (b) two described alternatives to the *N*-alkylation.

On the other hand, in 2006, Kiessling focused his research on overcoming the troubles of the cyanomethylation reaction, regarding the low yields obtained in the *N*-alkylation step, as well as the side-reactions associated to the basic conditions required for this reaction. He proposed the *N*-allylation reaction (instead of *N*-methylation), thus the allyl substitution may be incorporated by neutral palladium-catalyzed conditions. These mild conditions precludes the non-desired side-products; and again, the intermediate formed is more activated than in the case of the Kenner's linker. Regarding the Ellman and Kiessling optimized acylsulfonamides, several works, most of them published recently, have addressed the efforts to facilitate various difficult, but demanding reactions, mainly peptide head-to-tail cyclizations,[6–9] but also to produce *C*-terminal thioesters,[10,11] or even to reach glycopeptides.[12]

Other examples of activating agents were also developed to improve the yields of the nucleophilic attack and the activation of the carbonyl.[13] These mentioned contributions using sulfonamides as safety-catch linkers, together with other kinds of sulfonamide variations, were summarized by Link and Heidler,[14] and their versatility has been confirmed for a wide range of organic molecules synthesized,[14–17] apart from peptides.

The stability of those molecules attached to sulfonamide linkers towards the specific alkylation, and also to the cleavage conditions, are two requirements for the use of Kenner-type linkers. In addition to the opportunity to obtain fully protected peptides, these linkers also contribute to expand the number of those already existing handles for Boc/Bzl strategy, which in the beginning were necessarily subjected to arduously HF cleavages. More importantly, the orthogonality of these linkers offer an evident advantage that lies in the wide range of basic, acidic or nucleophilic conditions that may be applied to those molecules connected to these linkers, without causing cleavage.

Apart from the Kenner-type linkers, in 1998[18] first, and later in 2009,[19] Pátek and Lebl summed up the broad diversity of safety-catch linkers described up to date. We have addressed our attention on highlighting those non-based Kenner's linkers which bear the sulfide function, due to the contribution to this field presented in this chapter. Therefore, two types of **sulfoxide-based safety-catch linkers** may be distinguished: (i) linkers based on the sulfide derivatives studied by Marshall[20] (***p*-mercaptophenol**, Fig. 2) and (ii) linkers based on the sulfoxide derivatives proposed by Lebl and co-workers[21,22] (the safety-catch amide linker, **SCAL**, Fig. 2), also studied by Kiso[23] (the 4-(2,5-dimethyl-4-methylsulfinylphenyl)-4-hydroxybutanoic acid, **DSB**, Fig. 2) and, as well as by Undén[24] (the 3-(4-Hydroxymethylphenylsulfanyl)propanoic acid, **HMPPA**, Fig. 2).

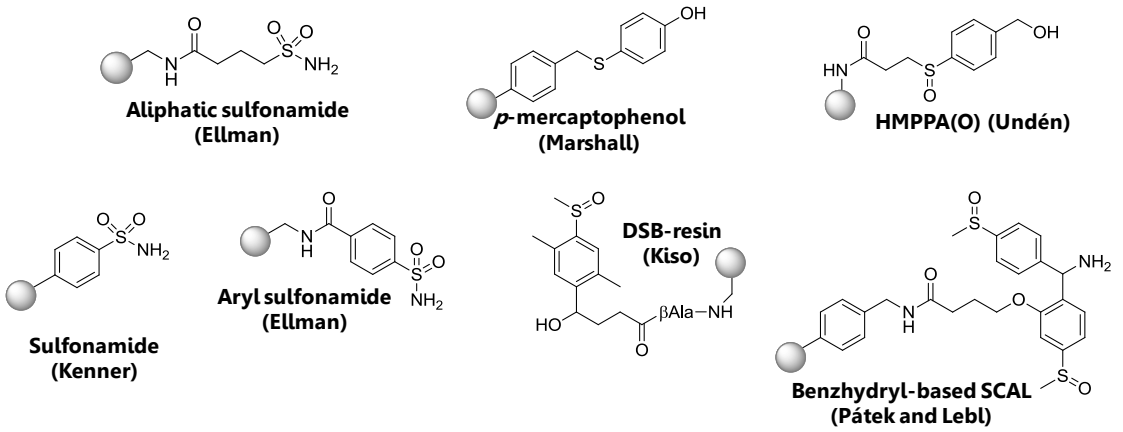

Figure 2. Some sulfonamide and sulfoxide-based safety-catch linkers described in the literature.

The first type of sulfide-containing linkers (for example, the ***p*-mercaptophenol**, Fig. 2) were aimed to reach the synthesis of fully protected peptide sequences. The procedure consists of three steps: the sequence elongation onto the linker, a chemical modification through the oxidation of the sulfide to sulfone, and the cleavage of the peptide by nucleophiles. Thereby, the sulfide function, which preserves the nucleophilic

attack on the ester moiety, becomes activated towards nucleophiles after being oxidized to the sulfone function, allowing the hydrolysis of the ester. Specifically, Marshall used the glycine sodium salt as nucleophile to obtain a Gly in the *C*-terminus. The limitation encountered for these linkers resides on the oxidative step, which was not recommended when the sequence contains AAs susceptible to be oxidized, such as Met, Trp, Cys or its cystine derivative. In spite of this drawback, some syntheses of non-peptide-like molecules have been proposed by using the Marshall type linker.[25]

The second kind of sulfide-containing linkers (for example, the **SCAL**, the **DSB**, and the **HMPPA(O)**, Fig. 2) introduces an opposite effect, as they contain the sulfide oxidized to the sulfoxide form. It is worth highlighting that although the HMPPA is introduced onto the resin with the sulfide non-oxidized, this linker is immediately oxidized to sulfoxide after its incorporation on the resin, and before the peptide elongation, being named HMPPA(O) (Fig. 2). The procedure consists of three steps: the sequence elongation onto the linker, a chemical modification through the reduction of the sulfoxide to sulfide, and the cleavage of the peptide by acidolysis. Herein, the linker provides the singularity that the electron-withdrawing effect of the sulfoxide does not allow the peptide being cleaved under acidic conditions. Thereby, this type of safety-catch linkers are stable to hydrolysis under: strong acids, such as the TFA used in Boc/Bzl chemistry, they are also resistant to nucleophiles, or even to those conditions used to remove the *N*-terminal protector in solid-phase chemical ligations.[26] This behavior was not only advantageous for peptides, but also profitable for the obtaining of a large diversity of synthetic organic molecules.[27,28] The reduction of the sulfoxide imparts an electron-donating effect that allow the subsequent release of the sequence in acidic media. The peptide cleavage conditions reported by Pátek or Kiso consist of $SiCl_4$/anisole/TFA or $(CH_3)_3SiBr$ (TMSBr)/thioanisole/TFA cocktails, being the reducing agent and the acid in the same mixture, thus the reduction and the cleavage occur concomitantly (known as reductive acidolysis). On the other hand, some studies, inspired by the previously published reduction of methionines by NH_4I[29] or by TMSBr,[30] have reported successful results on elongations of peptides which contain disulfide bridges that have been synthesized onto SCAL and that have been cleaved by the NH_4I/$(CH_3)_2S$/TFA cocktail.[28,31]

1.3. Safety-Catch *C*-Terminal and Amino Acid Side-Chain Protecting Groups

Although the main research developed on the safety-catch idea was focused on linkers, by exploiting its singular advantage of the stability/lability, several authors have applied this concept also to protecting groups for **amino acid side-chains**. In this section, only those based on the sulfoxide function have been highlighted. Parallel to

those explained in the 1.2. section, the removal of the sulfoxide derivative protecting groups contained on the AA side-chain is performed by a reductive acidolysis mediated by the sulfoxide→sulfide chemical modification. One of the most simple protecting group which is suitable for these criteria is the *p*-(Methylsulfinyl)benzyl (**Msib**, also named **Msob**) (Fig. 3b), described in 1988 by Samanen and co-workers[32] and based on previous studies based on another application.[33] Firstly, Samanen proposed the Msib to protect carboxylic acids, specifically for the *C*-terminus, and also envisioning its use for Asp and Glu side-chains (Fig. 3a); this last AA protector (**OMsob**) was proved later by Kiso.[34]

Professor Kiso also decided to open the applicability of the Msib moiety by incorporating it as an hydroxyl protecting group for Thr or Ser.[35] The same author developed a similar protector, modifying it as a carbamate group, the 4-methylsulphinylbenzyloxycarbonyl (**Msz**) (Fig. 3a). It was conceived for amino groups, not only as *N*-terminal protector, but also for the ε-amino from Lys side-chain;[34] as well as for the protection of the phenol function for Tyr.[36] Removal of the Msz, either positioned in the amino or the hydroxyl group, is carried out under the same aforementioned reductive acidolytic conditions, but also under basic treatments (1 M NaOH in MeOH), thus fully orthogonal to Msob removal. More recently, Alewood and co-workers[37] have developed the 4,4-dimethylsulfinylbenzhydryl (**Msbh**) (Fig. 3a) protecting group for Cys protection to form disulfide bonds by a selective protocol, since it is compatible with other cysteine protecting groups.

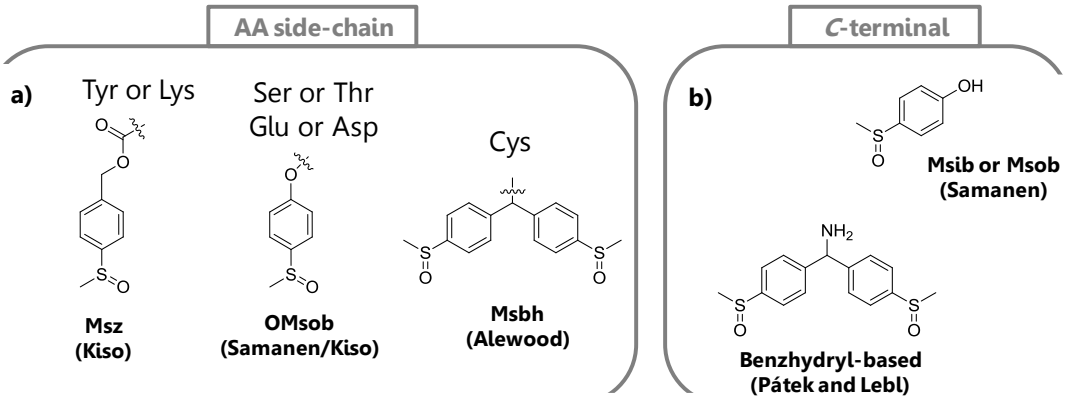

Figure 3. Some sulfoxide-based safety-catch protectors for: (a) the AA side-chains; and (b) the *C*-terminus.

In the '90s, Professor Lebl and collaborators, followed the same research line as Samanen and designed another sulfoxide-based protector for carboxylic acids (*para*-substituted benzhydryl-based, Fig. 3b) at the ***C*-terminal position.**[21] This protecting group was defined as a fruitful method to perform peptide fragment condensation in solution, because the removal of the *C*-terminal protecting group is carried out after

the condensation, and those conditions do not affect the peptide integrity, thus being removed concomitantly with other side-chain protectors.

1.4. Safety-Catch Backbone Amide Protecting Groups

The last significant protecting groups based on the safety-catch concept were designed specifically for the backbone amide group in peptides. As it was aforementioned in the general introduction section, the incorporation of these protecting groups provide the advantage of disrupting the β-sheet interactions by enhancing the solubility of peptides during solid-phase, thus allowing the synthesis of "difficult peptides". When the backbone amide protecting group belongs to those defined as safety-catch protectors, another benefit is afforded, which lies in the expanded possibility to treat the sequence under a wide range of conditions, as occur for linkers or AA protectors. The safety-catch backbone amide protecting groups described in the literature up to date, have been mentioned in the general introduction section, and herein are collected in figure 4.

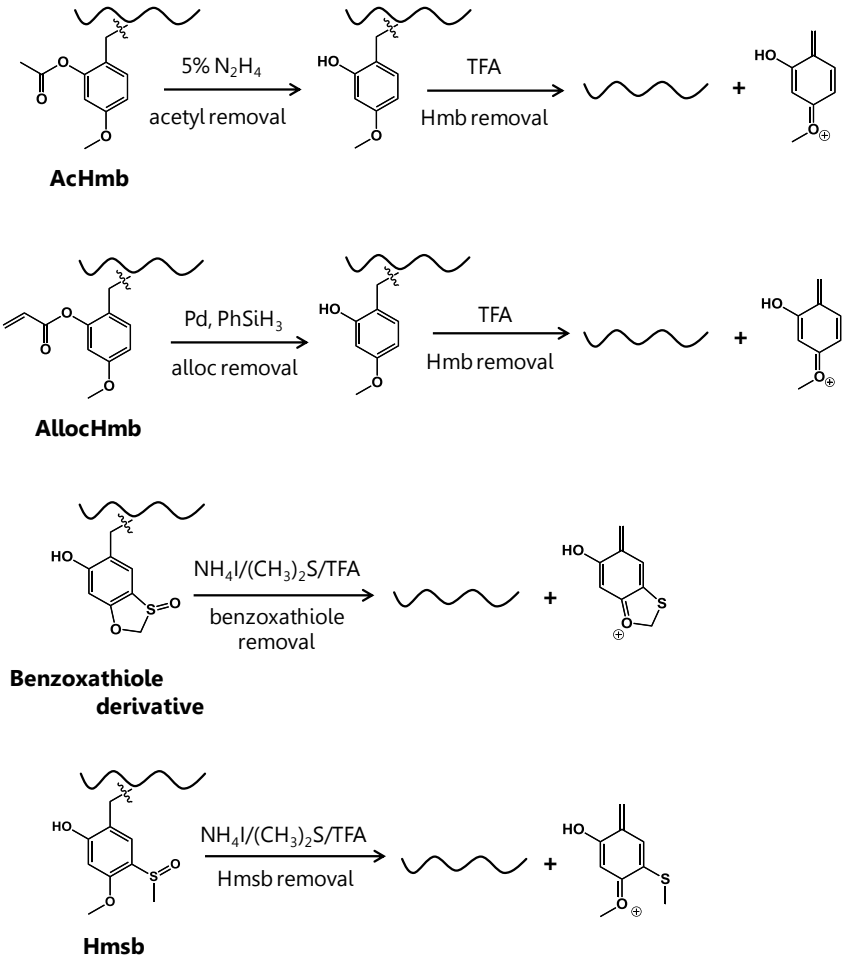

Figure 4. Safety-catch backbone amide protecting groups and their removal conditions.

The two first Hmb derivatives (Fig. 4), although they have not been described specifically as safety-catch protectors, they may be included in this concept because the Ac or the Alloc incorporated onto the phenol provides to the Hmb stability under a wide range of conditions during the SPPS. They are suitable for the safety-catch description because only after a certain chemical modification (in these protectors the removal of the Ac or the Alloc group), the Hmb protecting group becomes acid-labile. On the other hand, the last two backbone protectors (Fig. 4) were based on the already mentioned sulfoxide strategy, both groups presents stability in acidic media and when the sulfoxide is reduced to the thioether, subsequently the resulting group becomes cleavable under TFA.

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Publication II

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Solid-Phase Synthesis

2-Methoxy-4-methylsulfinylbenzyl: A Backbone Amide Safety-Catch Protecting Group for the Synthesis and Purification of Difficult Peptide Sequences

Marta Paradís-Bas,[a, b] Judit Tulla-Puche,*[a, b] and Fernando Albericio*[a, b, c, d]

Abstract: The use of 2-methoxy-4-methylsulfinylbenzyl (Mmsb) as a new backbone amide-protecting group that acts as a safety-catch structure is proposed. Mmsb, which is stable during the elongation of the sequence and trifluoroacetic acid-mediated cleavage from the resin, improves the synthetic process as well as the properties of the quasi-unprotected peptide. Mmsb offers the possibility of purifying and characterizing complex peptide sequences, and renders the target peptide after NH_4I/TFA treatment and subsequent ether precipitation to remove the cleaved Mmsb moiety. First, the "difficult peptide" sequence H-$(Ala)_{10}$-NH_2 was selected as a model to optimize the new protecting group strategy. Second, the complex, bioactive Ac-$(RADA)_4$-NH_2 sequence was chosen to validate this methodology. The improvements in solid-phase peptide synthesis combined with the enhanced solubility of the quasi-unprotected peptides, as compared with standard sequences, made it possible to obtain purified Ac-$(RADA)_4$-NH_2. To extend the scope of the approach, the challenging Aβ(1-42) peptide was synthesized and purified in a similar manner. The proposed Mmsb strategy opens up the possibility of synthesizing other challenging small proteins.

Introduction

Solid-phase peptide synthesis (SPPS) is nowadays the most common strategy used to synthesize biopolymers in high yield and purity. This approach has been made possible thanks to great efforts of the scientific community to develop a wide range of functionalized resins,[1] coupling reagents,[2–5] and orthogonal protecting groups.[6,7] After extensive fine-tuning over the last 50 years, one would assume that chemists would have all the tools necessary to prepare a short sequence of any nature. However, this is not the case. The so-called "difficult sequences", which are short sequences that are apparently unreachable targets,[8–11] continue to pose a challenge.

These kinds of peptides are characterized by incomplete removal of the amino protecting group and poor incorporation of the protected amino acids at some stage of the synthesis, which prevents peptide elongation even after repeating reaction treatment. It is accepted that these difficulties are due to the formation of peptide secondary structure, such as β-sheets.[12,13] During the synthesis, these intra- or intermolecular interactions, which are usually sequence-dependent, spontaneously form aggregations. These assemblies are attributed to the formation of the backbone amide hydrogen bond with carbonyl groups of peptide chains also bound to the resin.

Self-interactions require the presence of the NH of the peptide bond, therefore, Pro-containing peptide sequences are relatively free of this problem. In this regard, Mutter described a revolutionary concept in protecting group strategy: the pseudoproline. This concept converts Ser, Thr, and Cys into their five-membered ring dimethyl derivatives, thi/oxazolidines, which, during the final global deprotection, render the native residues.[14,15] Following the same idea of masking the presence of the NH moiety, Kiso, Carpino, and Beyermann independently proposed the synthesis of Ser and Thr depsipeptides, which are more manageable (soluble building blocks) and can be converted into the corresponding peptides in basic medium.[16–19]

Parallel to the work of Mutter, Sheppard and co-workers put forward the use of an amide-protecting group, *N*-(2-hydroxy-4-methoxybenzyl) (Hmb).[20,21] However, during activation, the building block N^{α}-Fmoc-N^{α}-(Hmb)amino acid forms a 4,5-dihydro-8-methoxy-1,4-benzoxapin-2(3*H*)-one, which shows poor reactivity.[22] Thus, these residues should be incorporated in the

[a] *M. Paradís-Bas, Dr. J. Tulla-Puche, Prof. F. Albericio*
Institute for Research in Biomedicine (IRB Barcelona)
Baldiri Reixac 10, Barcelona, 08028 (Spain)
Fax: (+34) 93-4037126
E-mail: judit.tulla@irbbarcelona.org
albericio@irbbarcelona.org

[b] *M. Paradís-Bas, Dr. J. Tulla-Puche, Prof. F. Albericio*
CIBER-BBN, Networking Centre on Bioengineering
Biomaterials and Nanomedicine, Barcelona Science Park
Baldiri Reixac 10, Barcelona, 08028 (Spain)

[c] *Prof. F. Albericio*
Department of Organic Chemistry, University of Barcelona
Martí i Franquès 1-11, Barcelona, 08028 (Spain)

[d] *Prof. F. Albericio*
School of Chemistry & Physics, University of Kwazulu-Natal
University Road, Westville, X54001, Durban, 4001 (South Africa)

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form of *O*,*N*-bis(Fmoc) derivatives. Once these building blocks have been introduced, and after removal of both Fmoc groups, the coupling of the incoming amino acid is facilitated through a seven-membered ring O→N transacylation mechanism. In addition, the same authors proposed the use of a so-called "reversible backbone amide" protecting group, *N*-(3-methylsulfinyl-4-methoxy-6-hydroxybenzyl) (SiMB). In addition to the free hydroxyl group, SiMB contains an electron-withdrawing sulfoxide substituent *para* to the OH, with the unique purpose of enhancing the acylation step.[23] Global deprotection is carried out through a reductive acidolysis to convert the sulfoxide into the thioether and then allow the amide protecting group to be cleaved by trifluoroacetic acid (TFA). Similarly, the so-called "reversible" backbone protecting groups based on the nitro group, which is cleavable under photolysis, were proposed by Kent and co-workers.[24]

During recent years, our group has focused on the synthesis of these "difficult sequences". In this regard, we have used dimethoxybenzyl (Dmb) and developed 1-methyl-3-indolylmethyl (MIM) and 3,4-ethylenedioxy-2-thenyl (EDOTn)[25] to reduce intra- and inter-chain interaction, as well as prevent side reactions associated with the presence of the NH group, such as aspartimides[26–28] and internal diketopiperazine formation.[29,30]

The design of the last two protecting groups mentioned has shown some advantages over benzyl derivatives. However, we have focused our efforts on a more global solution for the synthesis and on the characterization and purification of complex sequences that is not limited to Ser/Thr/Cys but valid for a large number of residues. Here we describe a "safety-catch" backbone amide-protecting group[31] that is compatible not only with the 9-fluorenylmethoxycarbonyl (Fmoc) strategy, but also with TFA treatment, with the latter being used to detach the peptide from the resin and remove the classical side-chain protecting groups. The synthesis of a quasi-protected peptide, which still bears the safety-catch protecting group, facilitates the characterization and purification of the peptide, and allows the target free-peptide to be rendered after a final and clean chemical treatment.

The Fmoc SPPS strategy is based on the use of a temporary protecting group that is removed by a base (piperidine) and cleavage upon global deprotection (by TFA), therefore, we chose not introduce any extra chemical reaction. We envisaged that the pair alkylsulfoxide/thioether would serve our requirements. Thus, the electron-withdrawing sulfoxide group introduces stability to a benzyl moiety, and it can be easily reduced to the corresponding electron-donating thioether, which confers acid lability to the same benzyl group. Specifically, 2-methoxy-4-methylsulfinylbenzyl (Mmsb), which is stable to TFA, was reduced to 2-methoxy-4-methylthiobenzyl (Mmtb), which is labile to TFA, thus perfectly matching our requirements (Figure 1).

The Mmsb moiety has been used for the preparation of the corresponding carbamate-based amine protection and integrated as a linker for the preparation of peptide acids.[32] The demethoxy (*p*-methylsulfinylbenzyl) version was also used as a carboxyl protecting group[33] and as a linker,[34] and its corresponding carbamate-based amine protection.[35] More

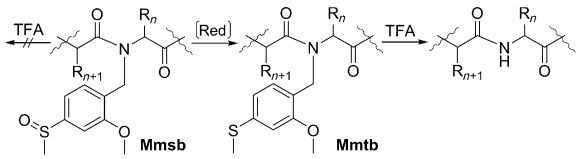

Figure 1. The concept of the new backbone amide-protecting group (Mmsb).

sophisticated linkers based on the same principle have also been used for SPPS.[36]

Here, we used the Mmsb group to prepare three "difficult sequences". After TFA global deprotection, the Mmsb-containing peptides were characterized and purified, when necessary, before performing a reductive TFA treatment, which reduces the sulfoxide to thioether and concomitantly removes it in a one-pot reaction.

First, the H-$(Ala)_{10}$-NH_2 sequence was selected as a model to study and optimize the new synthetic methodology proposed herein. Second, two interesting bioactive peptides, Ac-$(RADA)_4$-NH_2 (RADA-16) and Aβ(1-42), both with a high tendency to self-assemble, were synthesized for the validation of the Mmsb strategy, established in the first model. The first sequence is a challenging peptide for SPPS because of its hydrophobicity and its high tendency to form secondary structures that jeopardize the deprotection of the amino function and also acylation.[37] This peptide has been widely used to assay novel synthetic approaches.[38,39] The second peptide, RADA-16, is of interest because of its biomedical applications,[40,41] but it is again associated with a complex synthesis.[42] In this case, aggregation is related to its high content of alternating positively and negatively charged amino acids. Finally, we addressed the synthesis of the molecule considered to be responsible for amyloid formation in Alzheimer's disease, the extensively studied Aβ(1-42).[43,44] The synthesis of this peptide is challenging[18,45,46] because this molecule shows a strong tendency to form aggregates, which directly affects the elongation sequence and, at the same time, its solubility, thus complicating its purification.

Results and Discussion

To introduce the new Mmsb backbone amide-protecting group into the peptide sequence, the corresponding Fmoc derivative (Fmoc-*N*(Mmsb)-Ala-OH in our case) was prepared. The synthesis of this building block was accomplished in five steps (Scheme 1, see the Supporting Information for details).[47] Commercially available 3-methoxythiophenol (**1**) was methylated in practically quantitative yield with iodomethane (MeI) by careful dropwise addition of triethylamine to prevent dialkylation. Next, alkylated product **2** was formylated by reaction with freshly prepared Vilsmeier reagent,[48] which rendered the two possible isomers. 2-Methoxy-4-methylthiobenzaldehyde (**3**) was isolated (48% yield) after purification by reverse-phase chromatography on a C18 column. The reductive amination of aldehyde **3** with the amine of the unprotected alanine was reached in a one-step reaction with $NaBH_3CN$ in dioxane/H_2O (1:1).[25] *N*(Mmtb)-Ala-OH (**4**) was protected with the Fmoc

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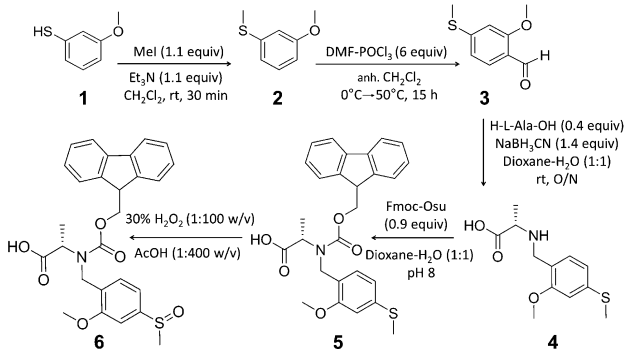

Scheme 1. Synthesis of Fmoc-*N*(Mmsb)-Ala-OH. O/N = overnight.

group by using a slight excess of Fmoc-OSu in basic media to afford Fmoc-*N*(Mmtb)-Ala-OH (**5**), which was easily purified by reverse-phase chromatography on a C18 column to remove the excess of unreacted alanine during the reductive amination step. Finally, oxidation of **5** with H_2O_2 rendered Fmoc-*N*(Mmsb)-Ala-OH (**6**) in excellent purity (98.0%) and without further purification; this building block was used directly for SPPS. The overall yield to convert **1** into **6** was 19%.

First, we tested our synthetic strategy on H-$(Ala)_{10}$-NH_2. As a control, the standard peptide was also synthesized with only the use of Fmoc-Ala-OH (**7**; Figure 2). Two modified H-$(Ala)_{10}$-NH_2 sequences were synthesized with one Fmoc-*N*(Mmsb)-Ala-OH at positions 6 (**8**) and 8 (**9**), respectively.

The three peptides were synthesized on Rink amide-polystyrene (PS) as a solid support and *N*,*N*′-diisopropylcarbodiimide (DIPCDI)/ethyl 2-cyano-2-(hydroxyimino)acetate (OxymaPure)[49] as coupling reagent. In this regard, Fmoc-*N*(Mmsb)-Ala-OH was introduced with only 1.5 equiv (AA/coupling reagent), and the best conditions for the key incorporation of the incoming residue consisted of three couplings with Fmoc-Ala-OH (10 equiv), DIPCDI (10 equiv), and OxymaPure (10 equiv) at 45 °C, with MeCN/*N*,*N*-dimethylformamide (DMF) (3:1) as solvent. Once again, we observed that MeCN is a suitable solvent for incorporations onto hindered amino acids.[50]

Except for the coupling of the incoming Ala on the *N*-Mmsb residue, no double couplings were performed. However, the three peptide elongations were checked by the Kaiser test[51] after each coupling. The tests revealed that synthesis of the standard peptide took place with incomplete incorporations of the last four Ala residues.

The two modified Mmsb-containing peptides were cleaved from the resin by using TFA/CH_2Cl_2 (19:1), rendering both sequences with similar purity (Figures S3 and S4 in the Supporting Information), and the total stability of the Mmsb group was confirmed by MS analysis (*m*/*z* calcd for $C_{39}H_{63}N_{11}O_{12}S$: 909.44; found: 932.60 $[M+Na]^+$ (**8**) and 932.45 $[M+Na]^+$ (**9**)).

Finally, we used MS analysis to study the reductive acidolysis of the Mmsb-containing peptides caused by treatment with NH_4I/TFA (Figure 3 and Figure S12 in the Supporting Information). It was found that the Mmsb group was completely removed after 30 min in the case of **8** and after 2 h in the case of **9**. This deprotection was accomplished without the need for scavengers (some articles report the use of Me_2S)[23,32].

One proposed solution to prevent the main impurity detected in both modified Mmsb-containing peptide syntheses (9% in **8** and 6% in **9**) associated with the truncated peptide after coupling of the *N*-Mmsb residue involved preparing the dipeptide (in this case Fmoc-Ala-*N*(Mmsb)-Ala-OH) in solution (see the Supporting Information for details). This strategy was used

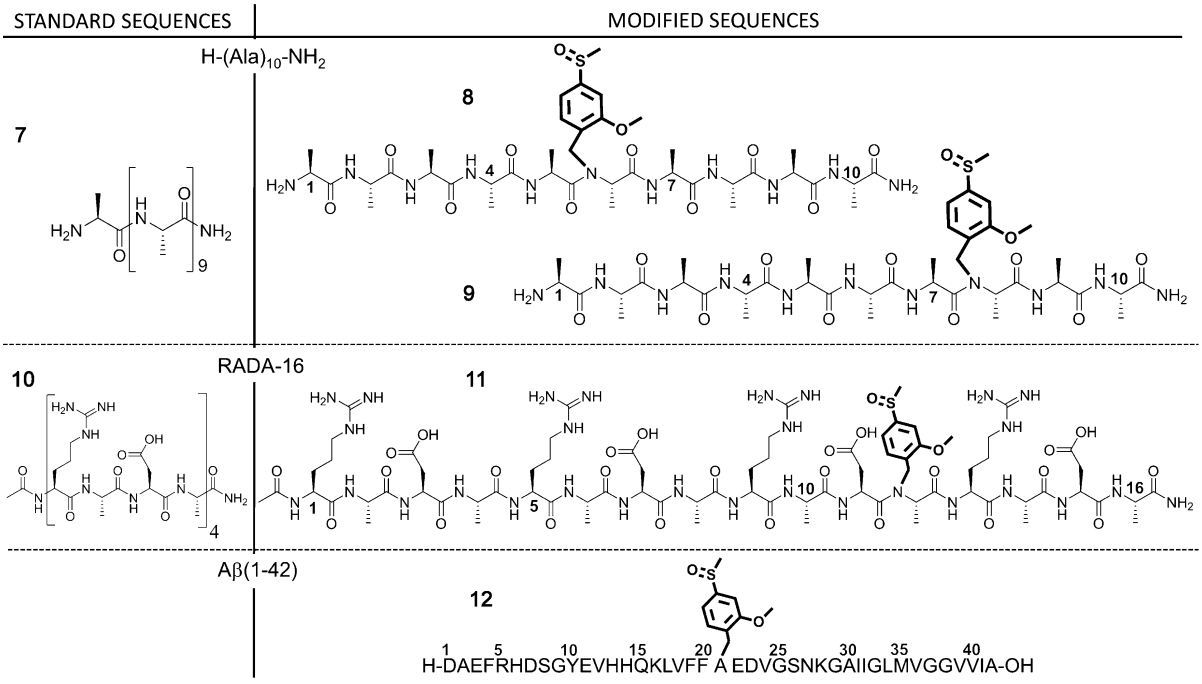

Figure 2. Structures of the synthesized peptides.

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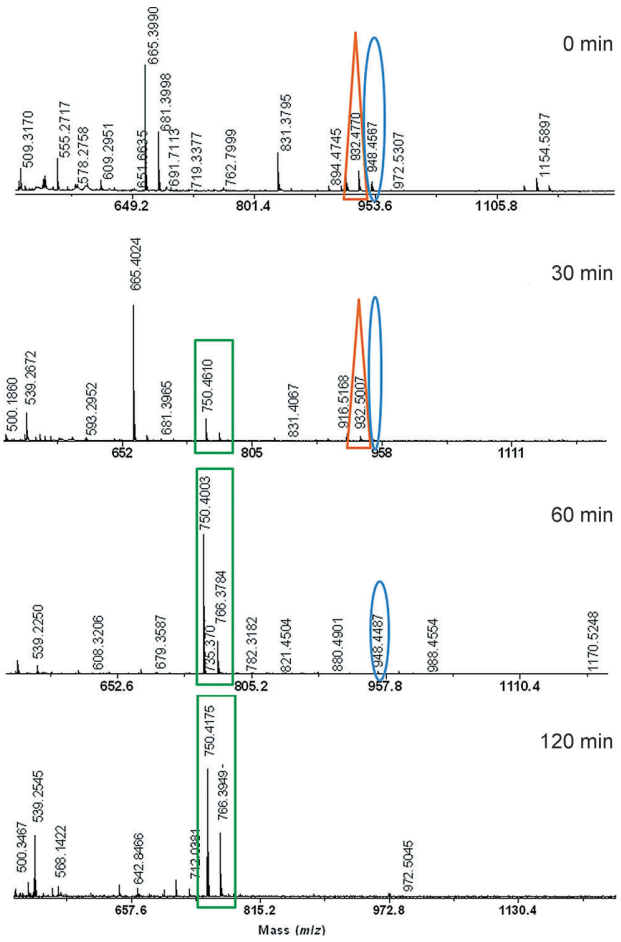

Figure 3. Monitoring of the Mmsb group removal by MALDI-TOF MS spectra. Starting material (Mmsb-protected H-(Ala)$_{10}$-NH$_2$) **8** is highlighted in blue, whereas the reaction intermediate (Mmtb-protected H-(Ala)$_{10}$-NH$_2$) and final product (unprotected H-(Ala)$_{10}$-NH$_2$) are shown in orange and green, respectively.

for the synthesis of modified H-(Ala)$_{10}$-NH$_2$ **8**, which showed an increased purity and the absence of the mentioned impurity (Figure S8 in the Supporting Information), thus confirming an approach to overcome the steric hindrance associated with the *N*(Mmsb) residue. The main conclusion drawn from these analyses is that the influence of the backbone protection has an effect that is apparent even six amino acids further down the backbone. Furthermore, in these two syntheses (**8** and **9**), no peptides lacking Ala residues were detected (Figure 4b and Figure S4b in the Supporting Information). The standard peptide synthesized with common Ala (**7**) showed four sequences lacking Ala residues, as shown by MS (Figure 4a); therefore, these results are consistent with those obtained from the qualitative Kaiser test. In addition, the increase in solubility of the modified sequences in common peptide solvents (H_2O or H_2O/MeCN, 1:1) was extraordinary (Figure 4b).

In addition to HPLC and MS analysis of standard and modified H-(Ala)$_{10}$-NH$_2$, and by means of circular dichroism (CD), we explored the effect on secondary structure when one Mmsb unit was introduced into the peptide sequence (Figure 5). The CD spectrum of the standard H-(Ala)$_{10}$-NH$_2$ peptide **7** showed

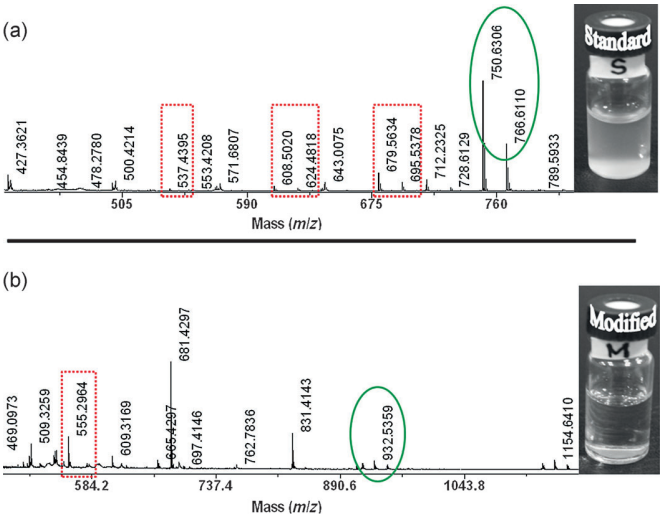

Figure 4. MALDI-TOF MS and solubility assays of H-(Ala)$_{10}$-NH$_2$ crude peptides synthesized: a) by using the common Ala, **7**, and b) by using the modified Fmoc-*N*(Mmsb)-Ala-OH, **8**. Concentration: 1 mg mL^{-1} in H_2O. Mass impurities assigned (red label), mass expected peptide (green label).

a β-sheet structure profile (minimum at 215 nm), characteristic of self-assembling sequences, whereas the two modified peptides (**8** and **9**) showed a random coil structure (minimum at 200 nm). These data suggested the involvement of the Mmsb in the secondary structure of this peptide, possibly explaining the synthetic improvements observed in modified H-(Ala)$_{10}$-NH$_2$.

Encouraged by the results obtained in this peptide model using the new method, we explored the application of Mmsb to other "difficult peptides".

We recently reported that an acceptable quality of RADA-16 can be obtained only by coupling two protected octapeptides in solution.[42] In that study, we showed that neither the protected nor unprotected RADA-16 can be purified by chromatography. We learned that the difficulty arises after the incorporation of 4–5 amino acids. Thus, two syntheses, a standard

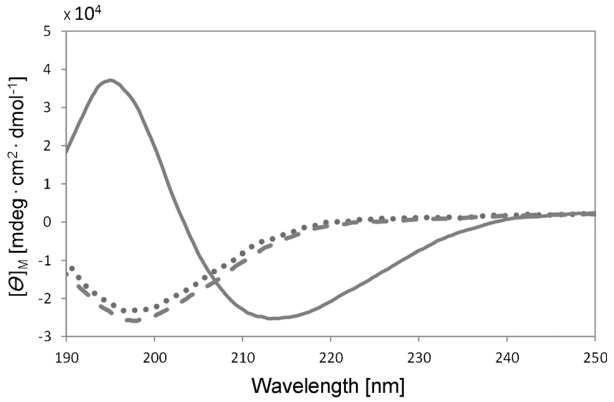

Figure 5. CD spectrum of a 25 μM solution of synthesized H-(Ala)$_{10}$-NH$_2$ in H_2O/2,2,2-trifluoroethanol (TFE) (9:1) at pH 5.2 of standard sequence **7** (solid curve) and modified sequences **8** and **9** (dotted and dashed curves, respectively).

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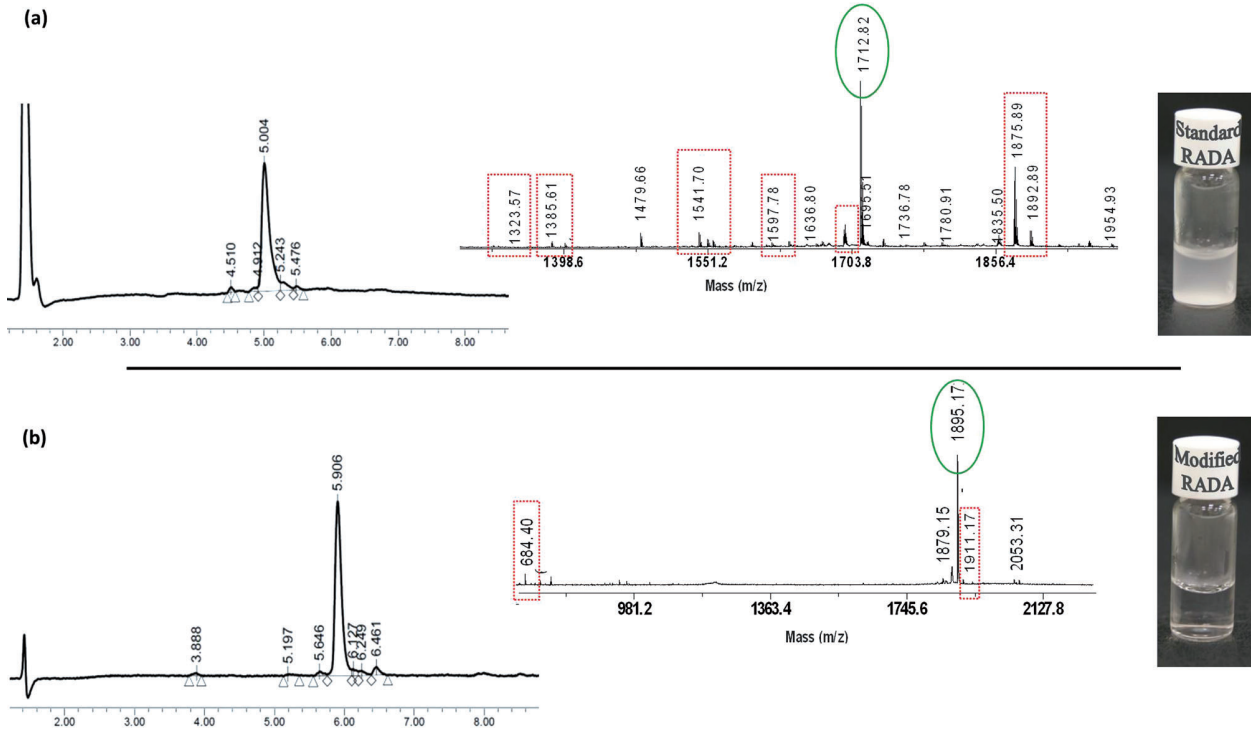

Figure 6. HPLC/MALDI-TOF MS/solubility assays of crude RADA-16 peptides synthesized: a) by using the common amino acids, and b) by using the modified Fmoc-*N*(Mmsb)-Ala-OH in position 12. Concentration: 1 mg mL^{-1} in H_2O. Mass impurities assigned (red label); mass expected peptide (green label).

RADA-16 (**10**) and a modified version (**11**), were carried out on Rink amide-ChemMatrix resin,[52] by using DIPCDI/OxymaPure as coupling reagent. On the modified peptide, Fmoc-*N*(Mmsb)-Ala-OH was introduced in position 12 (**11**). Peptide elongations were again checked qualitatively by the Kaiser test. Although no double couplings were performed, the synthesis of the standard RADA-16 showed, by Kaiser test, incomplete couplings in the last four residues. Fmoc-*N*(Mmsb)-Ala-OH was introduced as in the modified H-(Ala)$_{10}$-NH$_2$ examples. The coupling of the incoming Fmoc-Asp(*t*Bu)-OH was studied (Table 1 in the Supporting Information), concluding that the confluence of a MeCN/DMF (3:1) solvent mixture and three couplings at 45 °C using DIPCDI and OxymaPure, was once again crucial for satisfactory introduction of the incoming residue.

The final global deprotection/cleavage required prolonged acid treatment because of the high content of Pbf side-chain protecting groups of Arg [4 h with TFA/triisopropylsilane (TIS)/H_2O (38:1:1)]. Under these more demanding conditions, the acid-stability of the Mmsb group was confirmed. Although no significant differences between the two RADA-16 peptides were detected by HPLC (Figure 6), the MALDI-TOF MS analysis of these compounds revealed that standard RADA-16 contained several deletions (Figure 6a and Figure S2 in the Supporint Information; already anticipated by the Kaiser test results during synthesis), whereas the modified RADA-16 had none. Careful study of the HPLC and MS results concluded that the modified RADA-16 showed less than 1% of the impurity of

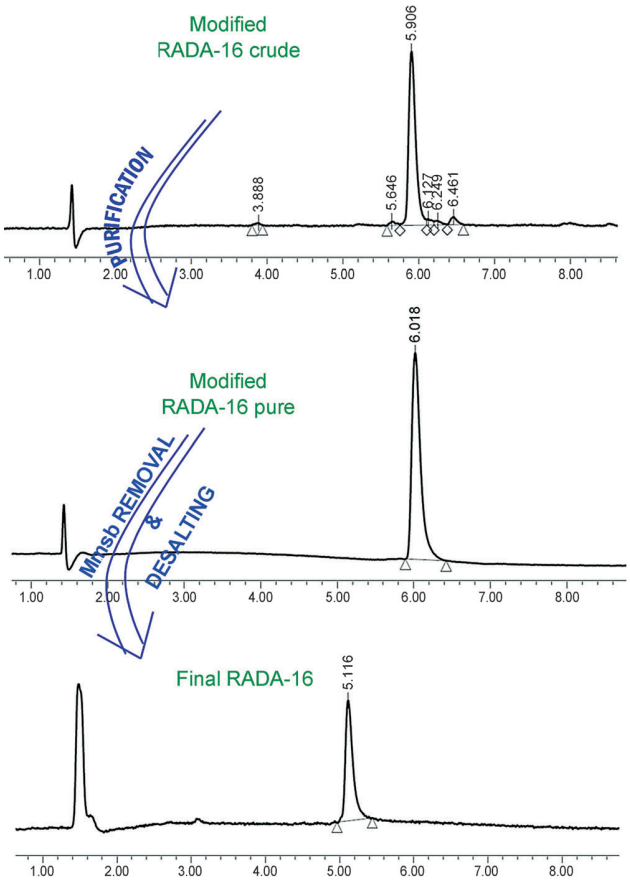

Figure 7. HPLC chromatograms of modified RADA-16 peptide: top) after peptide synthesis, middle) after purification, and bottom) final peptide after removing Mmsb group followed by desalting.

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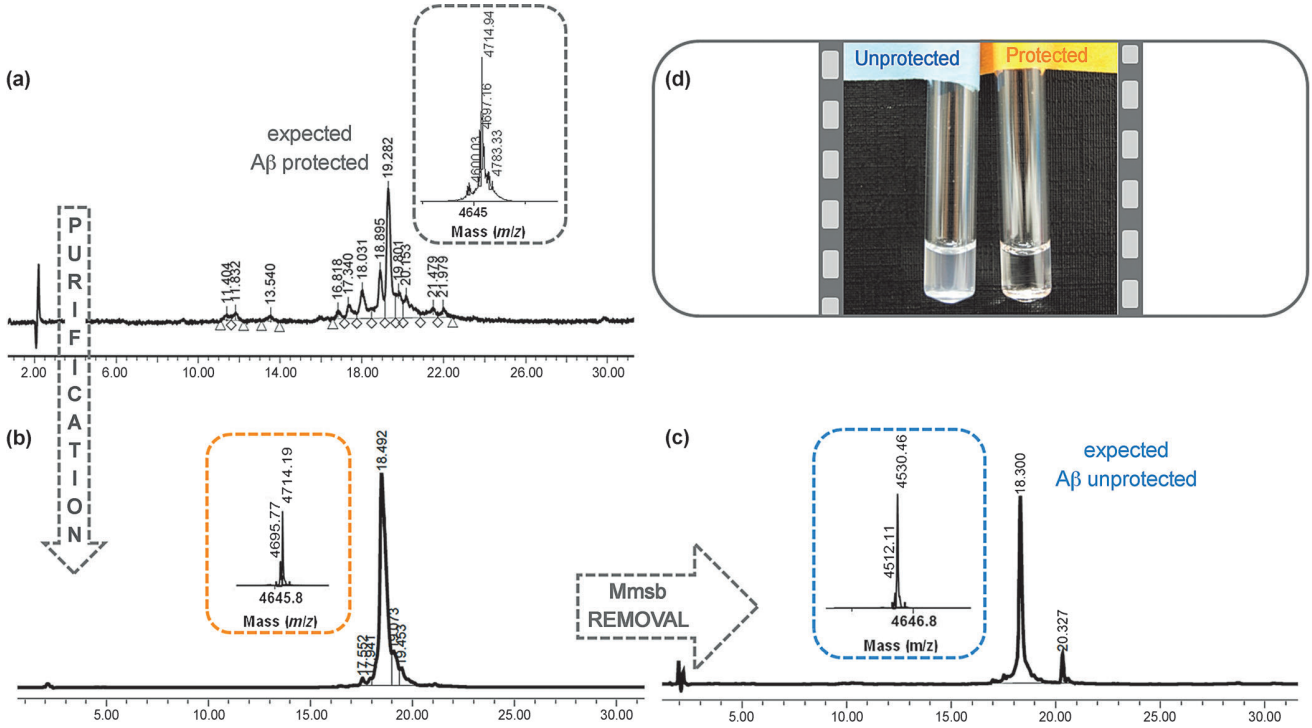

Figure 8. Analysis and characterization of synthesized modified Aβ(1-42): HPLC and MALDI-TOF MS of a) crude Mmsb-protected peptide after synthesis, b) purified Mmsb-protected peptide, c) unprotected peptide after Mmsb removal, and d) solubility of Aβ(1-42) at 1 mg mL^{-1} in H_2O of the Mmsb-protected analogue (orange label) and final unprotected Aβ(1-42) (blue label).

truncated peptide (5-mer, uncompleted incorporation of Asp after coupling of modified *N*(Mmsb) residue; Figure 6b and Figure S5 in the Supporting Information).[53] Most importantly, the solubility of the modified RADA-16 crude peptide in water was significantly higher than that of the standard peptide (Figure 6).

The lower solubility of the standard RADA-16, combined with the poor HPLC resolution of the expected peptide, for which the impurities eluted close to the desired product, prevented its purification. In contrast, the greater solubility of Mmsb-containing RADA-16 allowed its isolation. The final modified peptide was purified by HPLC analysis on a reverse-phase C18 column, and the Mmsb-protected peptide was isolated in excellent purity (99.9%) (*m/z* calcd for $C_{75}H_{123}N_{29}O_{27}S$: 1893.89, found: 1894.69 $[M+H]^+$; Figure 7 and Figure S9 in the Supporting Information). To remove the Mmsb group, the purified peptide was treated with a concentration of 1 mg mL^{-1} NH_4I/TFA and the backbone protecting group was completely removed after 45 min, with only a final desalting step (see the Supporting Information for details) required to reach the pure target RADA-16 peptide (Figure 7 and Figure S14 in the Supporting Information).

Inspired by the possibility of purifying RADA-16, we moved onto a second level, extending the application of the Mmsb backbone-protecting group to the Aβ(1-42) sequence. This amyloidogenic peptide was produced on an aminomethyl resin with a previous incorporation of 3-(4-hydroxymethylphenoxy)propionic acid as a linker in an automatic synthesizer with non-optimized conditions. In the middle of the sequence assembly (**12**, ^{21}AA), we manually inserted Fmoc-*N*(Mmsb)-Ala-OH, followed by the incoming Fmoc-Phe-OH. Both amino acids were coupled by following the same conditions described previously for other modified peptides. Once the elongation of the peptide was complete, the peptidyl resin was treated with TFA/TIS/H_2O (38:1:1) for 2 h, again confirming the acid-stability of Mmsb. Although the crude peptide obtained showed poor purity (35%; Figure 8a and Figures S6 and S7 in the Supporting Information) by HPLC analysis on a C4 column, our principal aim was to enhance the solubility of Aβ(1-42) and facilitate its purification.

The Mmsb-containing Aβ(1-42) was isolated by HPLC using the analogous semi-preparative C4 column. The enhanced solubility of the modified peptide (Figure 8d) allowed its purification (Figure 8b, and Figures S10 and S11 in the Supporting Information). Mmsb removal was completed after 2 h, checked by HPLC and MS (MALDI-TOF and HRMS-ESI), with both techniques confirming the synthesis of the natural Aβ(1-42) in 90% purity and with the expected mass (HRMS-ESI *m/z* calcd for $C_{212}H_{321}N_{55}O_{62}S_2$: 4693.3098, found: 1129.3273 $[M+4H]^+/4$, 903.6643 $[M+5H]^+/5$ and 753.3872 $[M+6H]^+/6$; Figure 8c and Figures S15 and S16 in the Supporting Information).

Conclusion

Mmsb is a new backbone amide-protecting group that is used in a safety-catch strategy. This new group is stable during global deprotection conditions. Mmsb represents a new concept for the synthesis and purification of "difficult peptides". Its

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presence in a peptide confers several advantages over a standard synthesis. Thus, the elongation of the peptide sequence takes place smoothly and with better yields because of the disruption of the secondary structure. Furthermore, its use could prevent side-reactions, such as aspartimide and diketopiperazine formation, while facilitating others, such as cyclization. We have demonstrated that the presence of only one Mmsb molecule favors the incorporation of at least 10 amino acids in the case of H-$(Ala)_{10}$-NH_2 and RADA-16. In addition, the Mmsb-protected peptides are much more soluble than standard peptides, thus allowing better HPLC resolution and, most importantly, their purification. The improved solubility could be explained by the absence of secondary structure and also by the polarity of the sulfoxide. The strategy described here could be applied to any amino acid other than Ala. Preparation in solution of dipeptides (equivalent to pseudoprolines or depsipeptides) is advisable when using Mmsb-hindered amino acids, so as to facilitate incorporation in the peptide sequence. The removal of Mmsb takes place under conditions that do not damage the peptide and allows the desired peptide to be obtained after only one filtration. Notably, our results on the synthesis of Aβ(1-42), for which only one Mmsb group was introduced within 42 AA residues, support the proposed synthetic approach. The results show that this novel synthetic technique enhances the manipulation of the peptide, especially with regard to its solubility and purification. We envisage that this technology will have broad use not only for the synthesis of "difficult peptides", but also for the purification of a large range of other peptides.

Experimental Section

Synthesis of standard peptide sequences

H-$(Ala)_{10}$-NH_2 (**7**) and RADA-16 (**10**) peptides were synthesized on Rink-Amide AM Polystyrene resin (250 mg, 0.12 mmol, 0.48 mmol g^{-1}) and Rink-Amide AM ChemMatrix resin (100 mg, 0.053 mmol, 0.53 mmol g^{-1}), respectively. Polystyrene resin was conditioned by washing with DMF (3×1 min) and CH_2Cl_2 (3×1 min), and ChemMatrix resin was conditioned by initial washing with TFA/CH_2Cl_2 (1:99) (5×1 min), CH_2Cl_2 (5×1 min), DIEA/CH_2Cl_2 (1:19) (5×1 min) and CH_2Cl_2 (5×1 min). Commercial amino acids were coupled in both synthesis as follow: Fmoc-L-AA(PG)-OH (3 equiv), DIPCDI (3 equiv), and OxymaPure (3 equiv) in DMF, with a 5 min preactivation and with a total coupling time of 1 h. After every coupling, the resin was washed with DMF (3×1 min) and CH_2Cl_2 (3×1 min). Then, a Kaiser test was carried out to check which amino acid was not coupled completely [4 AA: Ala^1-Ala^4 in the case of H-$(Ala)_{10}$-NH_2 (**7**); and 4 AA: Ala^9, Ala^6, Ala^4, Arg^1 in the case of RADA-16 (**10**)]. No double couplings were performed independently of the test results. Next, the Fmoc removal was performed with piperidine/DMF (1:4) (25 mL g^{-1} resin, 1×1 min, 2×5 min), followed by resin washings with DMF (3×1 min), CH_2Cl_2 (3×1 min), and DMF (3×1 min). The cycle of AA coupling/Fmoc removal was performed until complete elongation of peptides, including the last Fmoc removal. In the case of RADA-16 (**10**), a final step of acetylation was accomplished by Ac_2O (10 equiv) with DIEA (10 equiv) in DMF for 30 min. Final resin washings were carried out with DMF (3×1 min) and CH_2Cl_2 (3×1 min). The peptides were subjected to cleavage treatment depending on the amino acid side chain-protecting groups: TFA/CH_2Cl_2 (19:1) for 1 h (H-$(Ala)_{10}$-NH_2, **7**) and TFA/TIS/H_2O (38:1:1) for 4 h (RADA-16, **10**). The mixture was partially evaporated under reduced pressure and the peptides were precipitated with cold diethyl ether. The liquid layer was removed by centrifugation and the solid was washed with cold diethyl ether to give white solids that were dissolved in H_2O/MeCN (1:1) and lyophilized. The crude peptides were analyzed by HPLC and MALDI-TOF MS (see Figures S1 and S2 in the Supporting Information).

Synthesis of modified peptide sequences

The modified peptide sequences [two analogues modified H-$(Ala)_{10}$-NH_2: one in position 6 (**8**), the second in position 8 (**9**); and the modified Ac-$(RADA)_4$-NH_2 in position 12 (**11**)] were synthesized on the same resins described in case of each standard peptide. The resin washings, commercial amino acid couplings, and Fmoc removal cycles were performed by following the same protocol described for the standard sequences synthetic methodologies. The Kaiser test was used to detect which amino acid was not completely coupled (neither in **8**, **9**, nor **11** were uncompleted couplings detected). No double couplings were performed except in the case of the incoming amino acid to H-*N*(Mmsb)-Ala-OH. The synthesis of modified Aβ(1–42) (**12**) was carried out by an automatic microwave peptide synthesizer (Discover, CEM corporation). The synthesis was performed with an Aminomethyl ChemMatrix resin (160 mg, 0.1 mmol, 0.62 mmol g^{-1}) with a previous incorporation of 3-(4-hydroxymethylphenoxy)-propionic acid as a linker, coupled with the same system described in standard sequences. Commercial and common Fmoc-L-AA(PG)-OH amino acids were used. The coupling system consisted of *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene] *N*-methylmethanaminium hexafluorophosphate (HBTU) with *N*,*N*-diisopropylethylamine (DIEA) and DMF as a solvent and the Fmoc removal was performed with piperidine/DMF (1:4).

Incorporation of the synthesized building block Fmoc-*N*(Mmsb)-Ala-OH

After Fmoc removal of the previous amino acid, the Fmoc-*N*(Mmsb)-Ala-OH (1.5 equiv) was coupled by using DIPCDI (1.5 equiv) and OxymaPure (1.5 equiv) in DMF for 1 h. Then, washings with DMF (3×1 min) and CH_2Cl_2 (3×1 min) were performed and Kaiser test confirmed the complete incorporation of the AA.

Incorporation of the incoming Fmoc-AA-OH to H-*N*(Mmsb)-Ala-OH

Modified RADA-16 (**11**) was selected to find the best coupling conditions to introduce the incoming amino acid (see Table S1 in the Supporting Information) and the extension of this reaction was evaluated by HPLC analysis of the crude peptide after an aliquot of peptidyl-resin was treated with a cleavage cocktail. The solvent was then evaporated and the peptide was precipitated with cold diethyl ether. Amino acid coupling consecutive to H-*N*(Mmsb)-Ala-OH was accomplished by following the same conditions used in all modified peptide sequences, at 45 °C for 2 h in DMF/MeCN (1:2), with the coupling reagent system DIPCDI (10 equiv) and OxymaPure (10 equiv) for Fmoc-Phe-OH (10 equiv). A total of three consecutive couplings (using the same coupling conditions) were performed.

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Cleavage/global deprotection of side-chain protecting groups of modified peptides

After all the peptide elongation, cleavage, and post-cleavage treatments were performed by following specified conditions depending on the amino acid side chain-protecting groups nature, the peptides were subjected to cleavage treatment TFA/CH_2Cl_2 (19:1) for 1 h (**8** and **9**), TFA/TIS/H_2O (95:2.5:2.5) for 4 h (**11**) and TFA/TIS/H_2O (95:2.5:2.5) for 2 h (**12**). The mixture was partially evaporated under reduced pressure and the peptides were precipitated with cold diethyl ether. The liquid layer was removed by centrifugation and the solid was washed with cold diethyl ether to give white solids that were dissolved in H_2O/MeCN (1:1) and lyophilized to afford the modified sequences. The crude peptides were analyzed by HPLC and MALDI-TOF MS. Peptides were characterized by HPLC and MALDI-TOF MS (see Figures S3–S7 in the Supporting Information).

Removal of the Mmsb amide protecting group

Lyophilized modified crude peptides **8**, **9**, **11**, and **12** (1–5 mg) were dissolved in neat TFA (concentration 1 mg mL^{-1}) in a round-bottomed flask with constant stirring at RT. Ammonium iodine (30 equiv) was then added to the reaction mixture and the reaction was monitored by HPLC (an aliquot was taken from the reaction mixture, the TFA was removed by N_2 (g), and the peptide was precipitated in diethyl ether and redissolved in H_2O/MeCN (1:1)). When MALDI-TOF MS analysis revealed the completion of the reaction, removal of the iodine byproduct was carried out by filtration of the reaction mixture. The majority of volatiles were removed under reduced pressure and the precipitation of the peptide was performed by transferring the peptide solution dropwise to cold diethyl ether. After three times centrifugation and diethyl ether washings, the solid was lyophilized and the peptides were analyzed by MALDI-TOF MS (see Figures S12–16 in the Supporting Information).

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Keywords: peptides · protecting groups · solid-phase synthesis · synthesis design · synthetic methods

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Supporting Information

2-Methoxy-4-methylsulfinylbenzyl: A Backbone Amide Safety-Catch Protecting Group for the Synthesis and Purification of Difficult Peptide Sequences

Marta Paradís-Bas,[a, b] Judit Tulla-Puche,*[a, b] and Fernando Albericio*[a, b, c, d]

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Supporting Information

2-Methoxy-4-methylsulfinylbenzyl, a backbone amide safety-catch protecting group for synthesis and purification of difficult peptide sequences

Marta Paradís-Bas,[a, b] Judit Tulla-Puche*[a, b] and Fernando Albericio*[a, b, c, d]

[a]Institute for Research in Biomedicine (IRB Barcelona) Barcelona, Baldiri Reixac 10, 08028 Barcelona, Spain; Fax: (+34)93-4037126; E-mail: judit.tulla@irbbarcelona.org, albericio@irbbarcelona.org.
[b]CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain
[c]Department of Organic Chemistry, University of Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain
[d]School of Chemistry & Physics, University of Kwazulu-Natal, Durban 4001, South Africa

Materials and General Methods

Amino acids used for peptide syntheses Fmoc-L-AA(PG)-OH were commercially available containing the common side chain protecting groups for Fmoc/*t*Bu strategy. The solid supports (polystyrene and ChemMatrix), as well as the coupling reagents (DIPCDI, OxymaPure or HBTU), to synthesize the peptides were supplied from commercial vendors. Manual SPPS were carried out in polypropylene syringes fitted with a polyethylene porous disc; all synthetic cycles at room temperature, following the specified conditions described below. Solvents (DMF and CH_2Cl_2) and soluble reagents were removed by suction. The automatic SPPS was done in a Microwave Peptide Synthesizer (Discover, CEM Corporation) with a software system Liberty and in a 0.1 mmol scale. The dipeptide amide formation to be used as building block was performed in a 10 mL sealed glass tube in a focused monomode microwave oven (Discover, CEM Corporation) featured with a surface sensor for internal temperature determination. Cooling was provided by compressed air, ventilating the microwave chamber during the reaction.

The HPLC characterizations were performed on a Waters instrument comprising a separation module (Waters 2695) and a photodiode array detector (Waters 2998) with a software system controller Empower. The analytical column used to characterize the synthesized building blocks and the major peptide sequences was a reverse-phase C18 column (XBridge^{TM} BEH130, 4.6 x 100 mm, 3.5 µm); except for Aβ(1-42) sequences that were analyzed in a reverse-phase C4 column (Symmetry300^{TM}, 4.6 x 150 mm, 5 µm). UV detection was measured at 220 nm. Flow rate was at 1 mL/min. The eluent system used in all HPLC conditions was based in linear gradients of eluent B (MeCN + 0.036% TFA) into eluent A (H_2O + 0.045% TFA). The HPLC gradients on a reverse-phase C18 column were performed over 8 min at 25 ºC; and on a reverse-phase C4 column were performed at 60ºC, over 60 min or 30 min, which is specified in every experiment.

The HPLC purifications were carried out in two different Waters equipments: in the case of RADA-16, this peptide was purified with a semi-prep HPLC-MS (ESI) Waters 2545 binary gradient system comprising a sample manager (Waters 2767) and a UV detector (Waters 2489) with a dual absorbance selected at 254 and 220 nm. The column used was a reverse-phase C18 XBridge^{TM} BEH130, 419 x 100 mm, 5 µm OBD^{TM}) where the conditions were based in linear gradients of MeCN (+ 0.1% TFA) into H_2O (+ 0.1% TFA). The MS module was a Micromass ZQ. The software used was a MassLynx version. In the case of Aβ(1-42) and also the dipeptide building block (Fmoc-Ala-*N*(Mmsb)-Ala-OH), a 600 Delta system comprising a sample manager (Waters 2747) and a UV detector (Waters 2487) with a dual absorbance selected at 254 and 220 nm was used. The software used was a MassLynx version. The columns used were specified in every case and the conditions were based in linear gradients of MeCN (+ 0.1% TFA) into H_2O (+ 0.1% TFA). The CombiFlash Rf 200 purification of certain intermediates of building block syntheses was performed in a Teledyne Isco system on a 50 g C18 Redi*Sep* Rf GOLD column. UV detector was measured at 254 nm with gradients of eluent B (MeCN + 0.036% TFA) into eluent A (H_2O + 0.045% TFA).

Circular Dichroism measurements were carried out in a Jasco *J*-815 CD spectrometer; at 25 ºC, and recorded from 250 to 190 nm at a scanning speed of 50 nm/min and a time response of 4 s. Samples were dissolved in H_2O/2,2,2-trifluoroethanol (TFE) (9:1) at 0.25 µM and deposited in a quartz cell of 1 mm path length. Each CD measurement was represented as an average of 4 repeated scans in steps of 0.2 nm. Data were processed with Jasco's Spectra Manager Software and the measurements were converted from mdeg to molar ellipticities [θ] expressed in mdeg · cm^2 · dmol^{-1}.

Mass analyses were performed by different systems: the reverse phase-high performance liquid chromatography-electrospray mass spectrometry (RP-HPLC-ESI MS) analysis to characterize the building blocks was performed on a Waters Micromass ZQ spectrometer comprising a separation module (Waters 2695), an automatic injector (Waters 717 autosampler) and photodiode array

detector (Waters 2998) with a software system controller MassLynx v. 4.1. UV detector was acquired at 220 nm and linear gradients of MeCN (+ 0.07% formic acid) into H_2O (+ 0.1% formic acid) at a flow rate of 0.3 mL/min over 8 min. The Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight mass spectrometry (MALDI-TOF MS) peptide characterizations were performed on an Applied Biosystems Voyager-DE RP instrument, using α-cyano-4-hydroxycinamic acid as a matrix. The characterization of impurity in unprotected Aβ(1-42) peptide was performed in a LC/MSD-TOF (Agilent Technologies) associated to a electrospray mass spectrometer (ESI-MS) and the sample was dissolved in H_2O/MeCN (1:1) containing 0.1% of formic acid. The HR-MS (ESI) spectrometry characterization of Aβ(1-42) peptide sequences were performed on a LTQ-FT Ultra (Thermo Scientific) spectrometer. The ionization was performed by a nanoESI (Advion BioSciences, Ithaca, NY, USA) with direct infusion of sample. The sample Mmsb protected Aβ(1-42) and the unprotected Aβ(1-42) were dissolved in H_2O/MeCN (1:1) with 0.5% of formic acid and were directly infused to MS analysis. Data were processed with Xcalibur software vs.2.0SR2 (ThermoScientific).

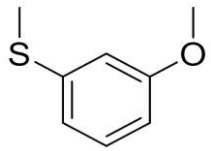
Desalting of the peptide was performed on a prepacked disposable PD MidiTrap™ column containing 5.3 ml of Sephadex™ G-10. These columns allow removing the salts because of different sizes compared with the sample (peptide) molecules larger are excluded from the Sephadex porous and eluted before the salts. The buffer chose to elute the peptide depends on the solubility of them. In the present work the RADA-16 peptide was eluted with 0.045% TFA in H_2O. The collected tubes were analyzed by HPLC and the fractions which contained the peptide sample were combined. Peptide sample eluted first and second the salts. Lyophilization gave the expected peptide without salts.

^{1}H and ^{13}C NMR spectra were recorded on a Varian MERCURY 400 (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR) spectrometer. Chemical shifts (δ) are expressed in parts per million downfield from tetramethylsilyl chloride. Coupling constants are expressed in Hertz.

Building Block synthesis

A) Fmoc-*N*(Mmsb)-Ala-OH

3-methoxymethylthiobenzene (**2**)



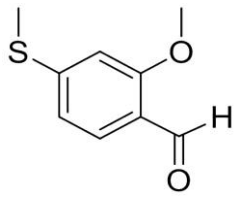
In a round-bottomed flask, 3-methoxythiophenol (**1**) (2.50 mL, 20.2 mmol) was dissolved in CH_2Cl_2 (15.6 mL) and cooled in an ice-water bath and MeI (1.38 mL, 22.2 mmol) was then added. Then, triethylamine (3.09 mL, 22.2 mmol) was added dropwise and the mixture was warmed to room temperature and stirred for 30min. The course of the reaction was followed by HPLC. After completion of the reaction, the organic phase was washed with H_2O (3 x 10 mL) and Brine (3 x 10 mL). The organic fractions were dried ($MgSO_4$) and the solvent was removed *in vacuo* to afford the title compound **2** as a yellow oil, which was used in the next reaction without further purification (3.07 g, 99%).

HPLC analysis (t_R = 4.09 min; gradient from 50% to 60% MeCN over 8 min). The main product corresponds to the expected title compound **2**.

^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si): δ 7.20 (t, J = 8.0 Hz, 1H), 6.85 (ddd, J = 7.8, 1.7, 0.8 Hz, 1H), 6.82 – 6.80 (m, 1H), 6.68 (ddd, J = 8.2, 2.5, 0.8 Hz, 1H), 3.80 (s, 3H, OCH_3), 2.48 (s, 3H, SCH_3).

^{13}C NMR (100 MHz, $CDCl_3$): δ 160.0 (C), 139.9 (C), 129.7 (CH), 118.9 (CH), 112.2 (CH), 110.7 (CH), 55.3 (OCH_3), 15.8 (SCH_3).

2-methoxy-4-methylthiobenzaldehyde (**3**)



A freshly Vilsmeier reagent (6 equiv) was prepared as follows: in a 250 mL round-bottomed flask, phosphorous oxychloride (10.68 mL, 116.7 mmol) was added followed by dry CH_2Cl_2 (25 mL) under an argon atmosphere. The solution was cooled to 10 ºC and a solution of dry DMF (6.65 mL, 85.6 mmol) in dry CH_2Cl_2 (25 mL) was added dropwise. After the Vilsmeier reagent was prepared, a solution of 3-methoxymethylthiobenzene (**2**) (3 g, 19.5 mmol) in dry CH_2Cl_2 (6 mL) was added slowly. The ice bath was removed and the solution was heated under reflux to 50 ºC and stirred for 15 h. After the reaction mixture had cooled

to room temperature, the mixture was poured carefully into crushed ice and the product was extracted with CH_2Cl_2 (3 x 25 mL). The organic layers were combined, dried ($MgSO_4$) and then, the solvent was evaporated under vacuum. Residual DMF was removed by co-evaporation with toluene and CH_2Cl_2. The resulting pale brown solid contains as a major isomer the expected product in 55.8 %. This crude was subjected to purification by CombiFlash Rf 200 system (reverse phase C18 silica, linear gradient from (B/A) 5:95 to 30:70 over 40 min, then isocratic 30:70 over 40 min) to yield the title compound **3**, after lyophilization, as a white solid (1.63 g, non optimized 48% yield).

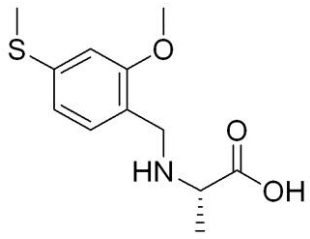
HPLC analysis (t_R = 2.80 min; gradient from 50% to 60% MeCN over 8 min). The expected product title compound **3** shows an HPLC-MS (ESI): *m/z* calcd for $C_9H_{10}O_2S$: 182.04, found: 183.21 $[M + H]^+$. The main impurity detected (HPLC t_R = 2.49 min; gradient from 50% to 60% MeCN over 8 min) corresponds to the other isomer (4-methoxy-2-methylthiobenzaldehyde), HPLC-MS (ESI): *m/z* calcd for $C_9H_{10}O_2S$: 182.04, found: 183.19 $[M + H]^+$.

^1H NMR (400 MHz; $CDCl_3$; Me_4Si): δ 10.33 (d, *J* = 0.6 Hz, 1H, CHO), 7.74 (d, *J* = 8.2 Hz, 1H), 6.83 (dd, *J* = 8.2, 1.1 Hz, 1H), 6.78 (d, *J* = 1.6 Hz, 1H), 3.91 (s, 3H, OCH_3), 2.52 (s, 3H, SCH_3).

^{13}C NMR (100 MHz, $CDCl_3$): δ 188.9 (CHO), 161.8 (C), 149.6 (C), 129.0 (CH), 121.9 (C), 117.1 (CH), 108.1 (CH), 55.8 (OCH_3), 14.9 (SCH_3).

(*S*)-2-((2-methoxy-4-(methylthio)benzyl)amino)propanoic acid

[***N*(Mmtb)-Ala-OH**] (**4**)

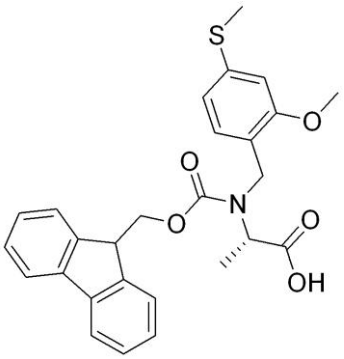


$NaBH_3CN$ (0.20 g, 3.1 mmol) was added to a suspension of 2-methoxy-4-methylthiobenzaldehyde (**3**) (0.50 g, 2.7 mmol,) and H-L-Ala-OH (0.19 g, 2.2 mmol) in H_2O/dioxane (1:1) (6.6 mL). The pH was adjusted to 5-6 with 1 *N* HCl and the resulting solution was stirred overnight. The solution was washed with CH_2Cl_2 (3 x 4 mL) and the aqueous layer was lyophilized to give the title crude **4** as a white solid (0.66 g), which was used in the next reaction without further purification.

HPLC analysis (t_R = 4.18 min; gradient from 5% to 100% MeCN over 8 min). The main product corresponds to the expected title compound **4**, HPLC-MS (ESI): *m/z* calcd for $C_{12}H_{17}NO_3S$: 255.09, found: 256.00 $[M + H]^+$.

(*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)(2-methoxy-4-(methylthio)benzyl)amino)propanoic acid

[**Fmoc-N(Mmtb)-Ala-OH**] (**5**)



The lyophilized crude 2-methoxy-4-methylthiobenzyl alanine (**4**) was suspended in a mixture of 1% aqueous Na_2CO_3/dioxane (1:1, *v/v*) (14 mL) and cooled with an ice bath. A mixture of Fmoc-OSu (0.75 g, 2.2 mmol) in dioxane (2.3 mL) was slowly added keeping the pH at 9 with 10 % aq. Na_2CO_3. The reaction mixture was stirred overnight and the conversion was checked by HPLC. Extra Fmoc-OSu (0.33 g) was required to complete the reaction. H_2O (30 mL) was added to the reaction mixture, the pH was adjusted to 8 with 1 *N* HCl and washings with MTBE (3 x 20 mL) were performed. The aqueous phase was acidified to pH 1 with HCl/H_2O (1:2) and the product was extracted with EtOAc (3 x 30 mL). The organic fractions were dried with $MgSO_4$, concentrated under *vacuo* and the residue was purified using a CombiFlash Rf 200 system (reverse phase C18 silica, linear gradient from (B/A) from 0:100 to 50:50 over 50 min, then isocratic 50:50 over 10 min) to yield, after lyophilization, compound **5** as a white solid (0.43 g, 41% overall yield for two steps).

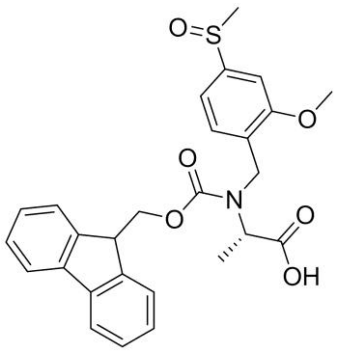
HPLC analysis (t_R = 6.49 min; gradient from 50% to 80% MeCN for 8 min). The main product corresponds to the expected title compound **5**, HPLC-MS (ESI): *m/z* calcd for $C_{27}H_{27}NO_5S$: 477.16, found: 478.02 $[M + H]^+$. The main impurity detected (HPLC t_R = 2.86 min; gradient from 50% to 80% MeCN over 8 min) corresponds to Fmoc-L-Ala-OH, HPLC-MS (ESI): *m/z* calcd for $C_{18}H_{17}NO_4$: 311.11, found: 312.02 $[M + H]^+$.

^{1}H NMR (400 MHz, DMSO-d_6; Me_4Si) (mixture of rotamers): δ 7.92 – 7.63 (m, 3H), 7.47 – 7.30 (m, 4H), 7.20 (dt, *J* = 19.4, 7.4 Hz, 1H), 6.97 – 6.70 (m, 3H), 4.47 – 4.12 (m, 6H), 3.80 and 3.79 (s, 3H, OCH_3), 2.49 and 2.48 (s, 3H, SCH_3), 1.23 and 1.13 (d, *J* = 7.2 Hz, 3H).

^{13}C NMR (100 MHz, DMSO-d_6) (mixture of rotamers): δ 172.94 (COOH), 156.51 (CO from Fmoc), 155.45 (C), 143.70 and 143.61 (C), 140.67 (C), 137.75 and 137.59 (C), 127.88 (CH), 127.52 (CH), 126.95 and 126.91 (CH), 124.95 and 124.90 (CH), 123.14 (CH), 120.03 (C), 117.64 (CH), 108.38 (CH), 66.86 (CH_2 from Fmoc), 55.59 (CH), 55.43 (CH_3), 46.54 (CH), 44.74 (CH_2), 14.88 (CH_3), 14.81 (CH_3).

(2*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)(2-methoxy-4-(methylsulfinyl)benzyl)amino)propanoic acid

[**Fmoc-N(Mmsb)-Ala-OH**] (**6**)



Acetic acid (12 mL) was added to a round bottomed-flask which contained 2-methoxy-4-methylthiobenzyl Fmoc-alanine (**5**) (0.38 g, 0.79 mmol). A solution of aqueous hydrogen peroxide (30%) (2.58 mL, 86.19 mmol) was slowly added to the reaction mixture while stirring, until total conversion was confirmed by HPLC. The mixture was poured onto crushed ice and the product was extracted with CH_2Cl_2 (3 x 15 mL). The organic layers were combined and dried ($MgSO_4$). The organic solvent was evaporated *in vacuo*. Acetic acid was removed by co-evaporation with toluene, dichloromethane and diethyl ether to afford the compound Fmoc-*N*(Mmsb)-Ala-OH (**6**) as a white solid (0.43 g, 98.2% yield). This product was pure enough (98%) to be used on solid-phase peptide synthesis without further purification.

HPLC analysis (t_R = 2.47 min; gradient from 50% to 80% MeCN for 8 min). The main product corresponds to the expected title compound **6**, HPLC-MS (ESI): *m/z* calcd for $C_{27}H_{27}NO_6S$: 449.17, found: 449.07 [M].

^{1}H NMR (400 MHz, DMSO-d_6; Me_4Si) (mixture of rotamers): δ 7.94 – 7.63 (m, 3H), 7.39 (m, 4H), 7.27 (dd, J = 11.0, 4.7 Hz, 1H), 7.23 – 7.09 (m, 3H), 4.52 – 4.10 (m, 6H), 3.88 and 3.87 (s, 3H, OCH_3), 2.76 (s, 3H, $SOCH_3$), 1.26 and 1.15 (d, J = 7.2 Hz, 3H).

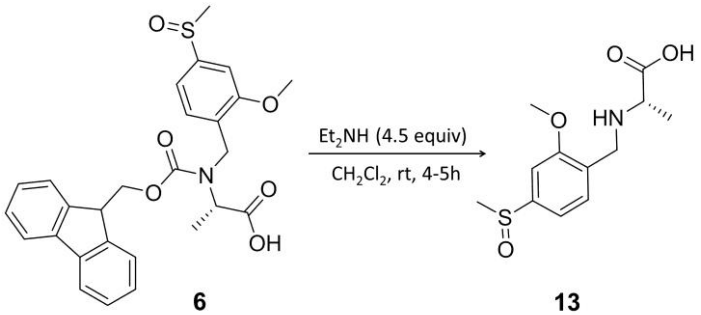
^{13}C NMR (100 MHz, DMSO-d_6) (mixture of rotamers): δ 172.97 (COOH), 156.66 (CO from Fmoc), 155.53 (C), 145.93 (C), 143.64 and 143.58 (C), 140.67 (C),

129.27 (CH), 127.65 (CH), 127.49 (CH), 126.90 and 126.85 (C), 124.82 and 124.75 (CH), 120.06 and 120.00 (CH), 115.27 (CH), 105.13 (CH), 66.84 (CH_2 from Fmoc), 55.70 (CH_3), 46.91 (CH), 46.54 (CH), 44.87 (CH_2), 43.26 and 43.21 (CH_3), 15.59 and 14.85 (CH_3).

B) Fmoc-Ala-*N*(Mmsb)-Ala-OH

(2S)-2-((2-methoxy-4-(methylsulfinyl)benzyl)amino)propanoic acid

[**H-N(Mmsb)-Ala-OH**] (**13**)



Fmoc-*N*(Mmsb)-Ala-OH **6** (0.15 g, 0.3 mmol) was dissolved in CH_2Cl_2 (0.8 mL), and diethylamine (0.14 mL, 1.4 mmol) was added dropwise into the mixture with stirring. The reaction was monitored by HPLC until the reaction was completed (4-5 h). The solvent was removed *in vacuo* and the diethylamine was co-evaporated with toluene. H_2O was added and the dibenzofulvene adduct precipitated. The solid was removed by centrifugation and the aqueous phase was lyophilized to afford the compound H-*N*(Mmsb)-Ala-OH (**13**) as a white solid (0.07 g, 91.5%).

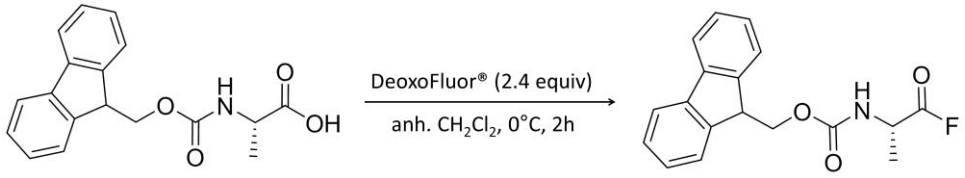
HPLC analysis (t_R = 3.00 min; gradient from 5% to 50% MeCN for 8 min). The main product corresponds to the expected title compound **13**, HPLC-MS (ESI): *m/z* calcd for $C_{12}H_{17}NO_4S$: 271.09, found: 272.08 $[M + H]^+$.

^{1}H NMR (400 MHz, CD_3OD; Me_4Si): δ 7.61 (d, J = 7.8 Hz, 1H), 7.44 (d, J = 1.4 Hz, 1H), 7.32 (dd, J = 7.8, 1.5 Hz, 1H), 4.28 (d, J = 3.0 Hz, 2H), 4.02 (s, 3H, OCH_3), 3.59 (q, J = 7.2 Hz, 1H), 2.82 (s, 3H, $SOCH_3$), 1.53 (d, J = 7.2 Hz, 3H).

^{13}C NMR (100 MHz, CD_3OD): δ 160.33 (COOH), 149.68 (C), 133.69 (C), 117.06 (CH), 117.04 (C), 107.04 (CH), 107.03 (CH), 56.73 (CH and CH_3), 46.40 (CH_2), 43.67 (CH_3), 16.14 (CH_3).

(S)-(9H-fluoren-9-yl)methyl (1-fluoro-1-oxopropan-2-yl)carbamate

[**Fmoc-L-Ala-F**] (**14**)



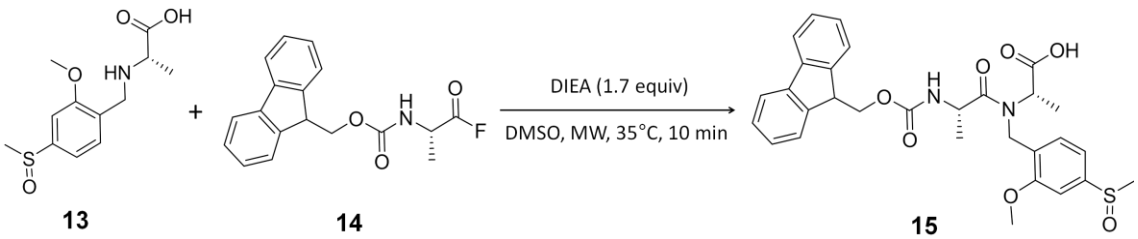
14

In a round-bottomed flask, commercial Fmoc-L-Ala-OH (0.20 g, 0.6 mmol) was dissolved in anhydrous CH_2Cl_2 (25 mL). The reaction mixture was cooled to 0ºC and stirred. The Bis(2-methoxyethyl)aminosulfur trifluoride (DeoxoFluor®) (0.17 mL, 0.8 mmol) was added dropwise into the mixture. After 45 min, extra DeoxoFluor® equivalents (0.17 mL, 0.8 mmol) were added to the reaction to complete the conversion. After a total of 1.75 h the reaction was stopped. The organic layer was washed once with H_2O (50 mL) and once with brine (50 mL). The organic phase was dried ($MgSO_4$) and the solvent was evaporated *in vacuo* to afford the compound Fmoc-L-Ala-F (**14**) as a brown solid (0.15 g, quantitative yield). The product was used in the next reaction without further purification.

HPLC analysis (t_R = 4.61 min; gradient from 50% to 70% MeCN for 8 min). The main product corresponds to the expected title compound **14**.

(2*S*)-2-((2*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-*N*-(2-methoxy-4-(methylsulfinyl)benzyl)propanamido)propanoic acid

[**Fmoc-Ala-*N*(Mmsb)-Ala-OH**] (**15**)



13 **14** **15**

Amide formation was carried out under MW conditions, in batches. H-*N*(Mmsb)-Ala-OH (0.03 g, 0.1 mmol) and Fmoc-L-Ala-F (0.02 g, 0.06 mmol) were placed in a microwave

reactor vessel with dimethyl sulfoxide (2 mL) and *N*,*N*-diisopropylethylamine (DIEA) (0.01 mL, 0.06mmol). The mixture was heated under microwave irradiation at 35 ºC for 10 min. A total of 4 consecutive MW cycles were performed by adding Fmoc-L-Ala-F (0.02 g, 0.06 mmol) in every cycle. H_2O (4 mL) was added to the reaction mixture and the product was extracted with CH_2Cl_2 (3 x 4 mL). The organic layers were combined, dried ($MgSO_4$) and the solvent was removed under reduced pressure. The compound Fmoc-Ala-*N*(Mmsb)-Ala-OH was obtained as an oil and subsequently purified [by reverse phase on a semi-prep HPLC with a XBridge™ Prep C18 OBD™ (19 x 100, 5 μm) column; the elution system was the following: B (MeCN + 0.1% TFA) into A (H_2O + 0.1% TFA); linear gradient (B/A) at a flow rate of 16 mL/min: 40:60 isocratic over 20 min, then from 40:60 to 45:55 over 10 min and finally from 45:55 to 50:50 over 10 min. The product afforded was Fmoc-Ala-*N*(Mmsb)-Ala-OH (**15**) (0.012 g, 7.5%, overall yield for two steps). This purified product (94%, HPLC purity) was used directly in SPPS to synthesize the H-$(Ala)_{10}$-NH_2 (**8**) by the dipeptide strategy.

HPLC analysis (t_R = 3.27 min; gradient from 45% to 55% MeCN for 8 min). The expected product title compound **15** (24.8%, HPLC purity) was detected by HPLC-MS (ESI): *m/z* calcd for $C_{30}H_{32}N_2O_7S$: 564.19, found: 565.22 $[M + H]^+$.

^{1}H NMR (400 MHz; DMSO-d_6; Me_4Si) (mixture of rotamers): δ 7.89 (d, *J* = 7.5 Hz, 1H), 7.76 – 7.55 (m, 2H), 7.41 (t, *J* = 7.4 Hz, 1H), 7.35-7.14 (m, 3H), 4.93 – 4.62 (m, 1H), 4.60-4.50 (m, 2H), 4.33 – 4.16 (m, 4H), 3.89 and 3.88 (s, 3H, OCH_3), 2.73 and 2.71 (s, 3H, $SOCH_3$), 1.21 (d, *J* = 7.0 Hz, 3H), 1.15 (d, *J* = 8.9 Hz, 3H).

^{13}C NMR (100 MHz, DMSO-d_6) (mixture of rotamers): δ 173.69 (COOH), 173.16 (CON), 157.27 (C), 155.92 (CO from Fmoc), 146.91 (C), 144.40 and 144.31 and 144.18 (C), 141.13 (C), 128.81 and 128.75 (CH), 128.06 (CH), 127.50 (CH), 125.83 (CH), 125.78 (C), 120.51 (CH), 115.75 (CH), 105.67 and 105.62 (CH), 66.08 (CH_2 from Fmoc), 56.26 (CH_3), 47.53 (CH), 47.07 (CH), 47.02 (CH), 43.68 (CH_3), 43.53 (CH_2), 18.18 and 18.16 (CH_3), 14.72 (CH_3).

Solid-Phase Peptide Synthesis

Synthesis of Modified Peptide Sequences

a) Synthesis of Modified RADA-16

Incorporation of the incoming Fmoc-AA-OH onto H-N(Mmsb)-Ala-OH

In the synthesis of modified RADA-16 (**11**), an accurate coupling optimization of the incoming amino acid [Fmoc-Asp(*t*Bu)-OH] was performed and summarized in a table (Table S1). The best coupling conditions found were: Fmoc-Asp(*t*Bu)-OH (10 equiv) with the coupling reagent system DIPCDI (10 equiv) and OxymaPure (10 equiv) at 45ºC for 2 h. A total of 3 consecutive couplings (using the same coupling conditions) were carried out (Table S1, entry 10). The extension of this reaction was evaluated by HPLC analysis of the peptide crude after an aliquot of peptidyl resin was treated with TFA/TIS/H_2O (95:2.5:2.5) for 1 h. The solvent was evaporated and the peptide was precipitated with cold diethyl ether.

entry	temperature (ºC)	solvent	coupling system (equiv)	time (min)	conversion (%)[a]
1	rt	DMF	AA (10), DIPCDI (10), Oxy[b] (10)	60	70
2	rt	DMF	AA (10), HATU (10), DIEA (10)	60	25
3	rt	THF	AA (5), BTC[c](1.65), TMP[d] (14)	60	25
4	35, MW	CH_2Cl_2	AA (5), DIPCDI (5), Oxy (5)	5	23
5	35, MW	DMF	AA (5), DIPCDI (5), Oxy (5)	5	19
6	35 + 45, MW[e]	CH_2Cl_2	[AA (5), DIPCDI (5), Oxy (5)] x 2	30 each	37
7	45	DMF	[AA (10), DIPCDI (10), Oxy (10)	120	83
8	45	DMF	[AA (10), DIPCDI (10), Oxy (10)] x 3	120 each	96
9	45	MeCN/DMF (3:1)	AA (10), DIPCDI (10), Oxy (10)	120	94
10	45	MeCN/DMF (3:1)	[AA (10), DIPCDI (10), Oxy (10)] x 3	120 each	99

[a] HPLC method, analyzed after cleavage of the peptide from the resin. [b] OxymaPure: Ethyl 2-cyano-2-(hydroxyimino)acetate. [c] Bis(trichloromethyl) carbonate. [d] *2*,4,6-trimethylpyridine. [e] Two consecutive cycles.

Table S1. Coupling conditions to introduce the incoming amino acid (Fmoc-L-Asp(*t*Bu)-OH) to H-*N*(Mmsb)-Ala-Arg-Ala-Asp-Ala-Rink CM resin in the synthesis of modified RADA-16 (**11**).

In the syntheses of modified H-$(Ala)_{10}$-NH_2 (**8** and **9**), the conditions used to couple the incoming amino acid (Fmoc-L-Ala-OH) were the described in entry 10 (Table S1).

b) Synthesis of Modified H-$(Ala)_{10}$-NH_2 (8) by Dipeptide Strategy

The sequence was synthesized on Rink-Amide AM Polystyrene resin (25 mg, 0.01 mmol, 0.45 mmol/g). The resin was conditioned by washings with DMF (3 x 1 min) and CH_2Cl_2 (3 x 1 min). Commercial Fmoc-L-Ala-OH was coupled as follows: Fmoc-L-Ala-OH (3 equiv), DIPCDI (3 equiv) and OxymaPure (3 equiv) in DMF, with a 5-min preactivation and a total coupling time of 1 h. After every coupling the resin was washed with DMF (3 x 1 min) and CH_2Cl_2 (3 x 1 min). Then, a Kaiser test was carried out to check which amino acid was not completely coupled (in this case no uncompleted couplings were detected). No double couplings were carried out.

*Incorporation of the building block Fmoc-*Ala-*N(Mmsb)-Ala-OH*

After Fmoc removal of the previous amino acid, the purified Fmoc-Ala-*N*(Mmsb)-Ala-OH was coupled as follows: Fmoc-Ala-*N*(Mmsb)-Ala-OH (1.5 equiv), benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) (1.5 equiv) and DIEA (3 equiv) in DMF for 1.5 h. Then, washings with DMF and CH_2Cl_2 were performed and a Kaiser test confirmed the complete incorporation of the AA.

Cleavage/Global Deprotection of Side Chain Protecting Groups

After the peptide elongation of the modified sequence, the cleavage and post-cleavage treatments were performed following the same conditions described as for the corresponding standard and the two modified H-$(Ala)_{10}$-NH_2 sequences. The peptide crude was analyzed by HPLC and MALDI-TOF MS.

Analysis of Peptide Crudes

A) Analysis of Standard Peptide Sequences

a) H-(Ala)$_{10}$-NH$_2$ Standard (**7**): Sample preparation: 1 mg of peptide crude H-(Ala)$_{10}$-NH$_2$ (**7**) was completely dissolved in 10µL of TFA after sonication. Then, 1 mL of H$_2$O/MeCN (1:1) was added, although no completed dissolved peptide crude was obtained. HPLC analysis (Figure S1a) (t_R = 3.72 min; gradient from 5% to 40% MeCN over 8 min). The main product (66%, HPLC purity) corresponds to the expected standard H-(Ala)$_{10}$-NH$_2$ (**7**), MS (MALDI-TOF) (Figure S1b): *m/z* calcd for $C_{30}H_{53}N_{11}O_{10}$: 727.40, found: 728.60 $[M + H]^+$, 750.50 $[M + Na]^+$ and 766.60 $[M + K]^+$. Impurities associated with Ala deletions: H-(Ala)$_9$-NH$_2$ *m/z* Calcd for $C_{27}H_{48}N_{10}O_9$: 656.36, found: 657.60 $[M + H]^+$ and 679.60 $[M + Na]^+$; H-(Ala)$_8$-NH$_2$ *m/z* Calcd for $C_{24}H_{43}N_9O_8$: 585.32, found: 586.50 $[M + H]^+$ and 608.50 $[M + Na]^+$; H-(Ala)$_7$-NH$_2$ *m/z* Calcd for $C_{21}H_{38}N_8O_7$: 514.29, found: 515.40 $[M + H]^+$ and 537.4 $[M + Na]^+$; H-(Ala)$_6$-NH$_2$ *m/z* Calcd for $C_{18}H_{33}N_7O_6$: 443.25, found: 444.40 $[M + H]^+$ and 466.40 $[M + Na]^+$).

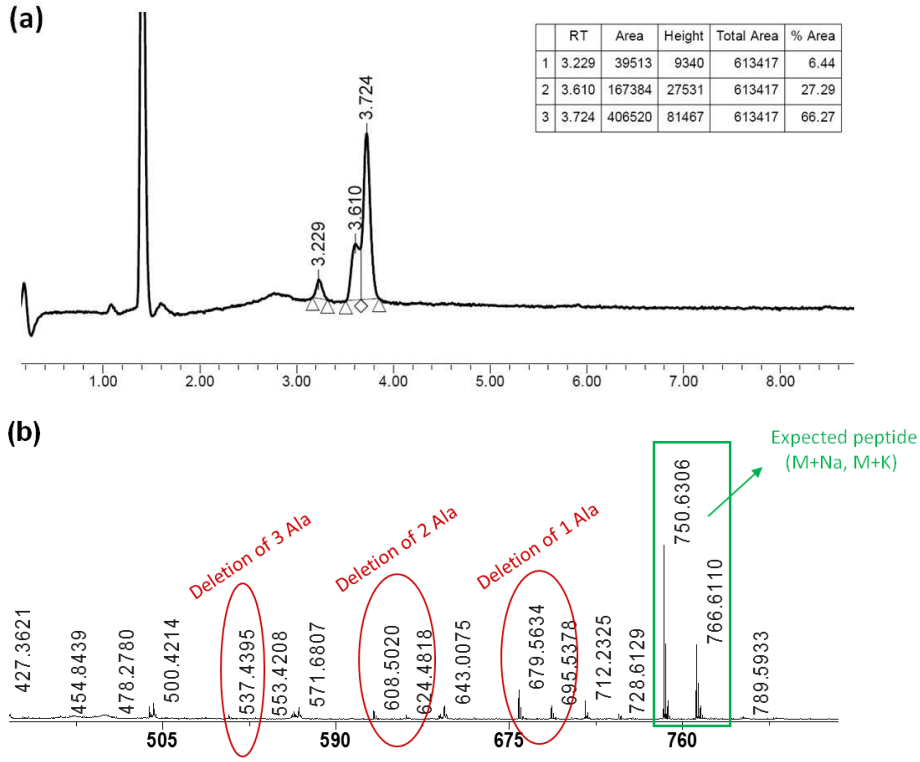


Figure S1. Synthesized Standard H-(Ala)$_{10}$-NH$_2$ (**7**): HPLC chromatogram (a); and MALDI-TOF MS spectrum (b).

b) RADA-16 Standard (**10**): Sample preparation: 1 mg of peptide crude RADA-16 (**10**) was completely dissolved in 10µL of TFA after sonication. Then, 1 mL of H_2O/MeCN (1:1) was added, being these the best conditions to dissolve the RADA-16. HPLC analysis (t_R = 5.00 min; gradient from 5% to 25% MeCN for 8 min). The main product (88%, HPLC purity) corresponds to the expected standard RADA-16 (**10**), MS (MALDI-TOF) (Figure S2): *m/z* calcd for $C_{66}H_{113}N_{29}O_{25}$: 1711.85, found: 1712.90 $[M + H]^+$. Impurities were also detected, such as the peptide sequence with one Ala deletion *m/z* calcd for $C_{63}H_{108}N_{28}O_{24}$: 1640.81, found: 1663.2 $[M + Na]^+$; the peptide sequence with one Arg deletion *m/z* calcd for $C_{60}H_{101}N_{25}O_{24}$: 1555.75, found: 1556.9 $[M + H]^+$; the peptide sequence with two Ala deletions *m/z* calcd for $C_{58}H_{101}N_{27}O_{22}$: 1527.76, found: 1550.7 $[M + Na]^+$; Ac-$(RADA)_3$-NH_2 *m/z* calcd for $C_{50}H_{86}N_{22}O_{19}$: 1298.64, found: 1299.7 $[M + H]^+$; H-$(RADA)_3$-NH_2 *m/z* calcd for $C_{48}H_{84}N_{22}O_{18}$: 1256.63, found: 1257.7 $[M + H]^+$ and the 13-mer peptide *m/z* calcd for $C_{51}H_{89}N_{23}O_{19}$: 1327.67, found: 1328.7 $[M + H]^+$.

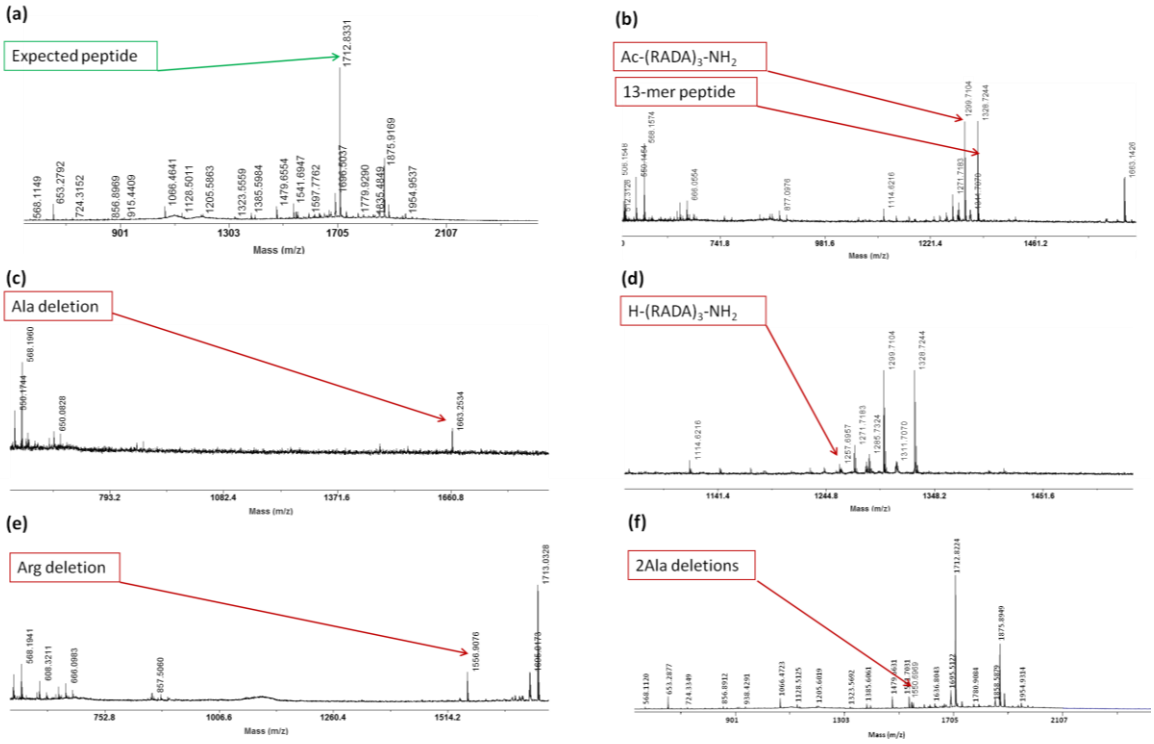


Figure S2. MALDI-TOF MS spectra of fractions collected from HPLC analysis of Standard RADA-16 (**10**).

B) Analysis of Modified Peptide Sequences

a) Modified H-$(Ala)_{10}$-NH_2 in position 6 (**8**): Sample preparation: 1 mg of peptide crude (**8**) was completely dissolved in 1 mL of H_2O/MeCN (1:1). HPLC analysis (Figure S3) (t_R = 4.00 min; gradient from 5% to 60% MeCN over 8 min). The main product (74%, HPLC purity) corresponds to the expected modified H-$(Ala)_{10}$-NH_2 (**8**), MS (MALDI-TOF): *m/z* calcd for $C_{39}H_{63}N_{11}O_{12}S$: 909.44, found: 932.60 $[M + Na]^+$. The product that eluted at t_R = 4.89 min (5%, HPLC purity) corresponds to the expected sequence but with the sulfoxide oxidized to sulfone MS (MALDI-TOF): *m/z* calcd for $C_{39}H_{63}N_{11}O_{13}S$: 925.43, found: 948.6 $[M + Na]^+$ and the byproduct that eluted at t_R = 3.18 min (9%, HPLC purity) corresponds to the truncated sequence 5-mer, MS (MALDI-TOF): *m/z* calcd for $C_{24}H_{38}N_6O_7S$: 554.25, found: 555.29 $[M + H]^+$.

	RT	Area	Height	Total Area	% Area
1	3.181	265611	53141	3003127	8.84
2	3.587	45912	10113	3003127	1.53
3	3.860	37296	10619	3003127	1.24
4	4.003	2217056	445549	3003127	73.82
5	4.128	70741	17056	3003127	2.36
6	4.351	64300	11464	3003127	2.14
7	4.583	14360	3658	3003127	0.48
8	4.899	138574	29951	3003127	4.61
9	7.564	149278	32540	3003127	4.97

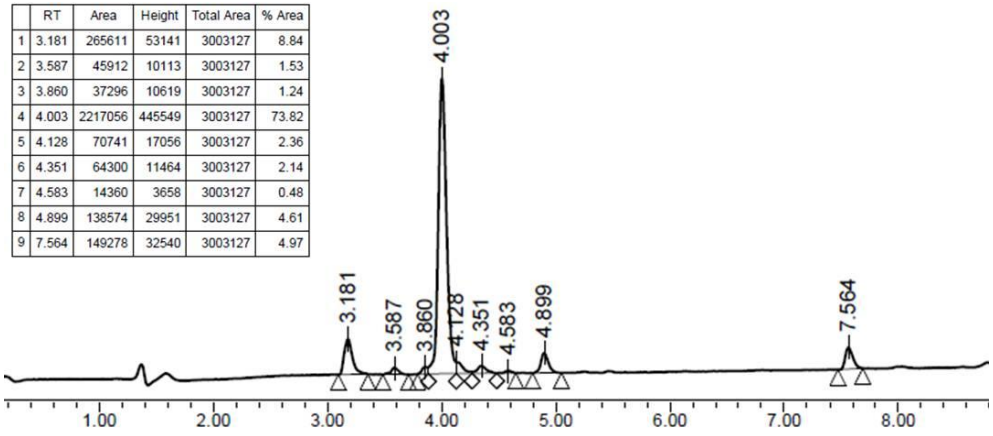


Figure S3. HPLC chromatogram of the modified H-$(Ala)_{10}$-NH_2 crude peptide (**8**).

b) Modified H-$(Ala)_{10}$-NH_2 in position 8 (**9**): Sample preparation: 1 mg of peptide crude (**9**) was completely dissolved in 1 mL of H_2O/MeCN (1:1). HPLC analysis (Figure S4) (t_R = 3.96 min; gradient from 5% to 60% MeCN over 8 min). The main product (63%, HPLC purity) corresponds to the expected modified H-$(Ala)_{10}$-NH_2 (**9**), MS (MALDI-TOF): *m/z* calcd for $C_{39}H_{63}N_{11}O_{12}S$: 909.44, found: 932.45 $[M + Na]^+$. The peak that eluted at t_R = 5.01 min (2%, HPLC purity) corresponds to the expected sequence with the sulfoxide oxidized

to sulfone, MS (MALDI-TOF): *m/z* calcd for $C_{39}H_{63}N_{11}O_{13}S$: 925.43, found: 948.50 $[M + Na]^+$ and the byproduct that eluted at t_R = 3.06 min (6%, HPLC purity) corresponds to the truncated sequence 3-mer, MS (MALDI-TOF): *m/z* calcd for $C_{18}H_{28}N_4O_5S$: 412.18, found: 413.30 $[M + H]^+$.

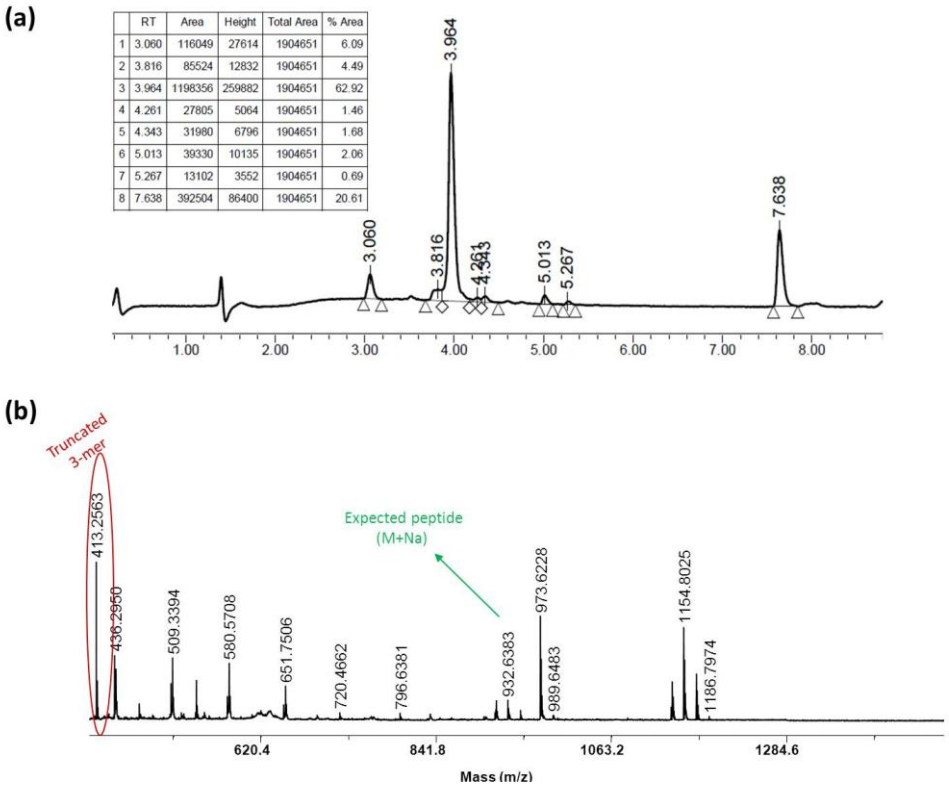


Figure S4. Modified H-$(Ala)_{10}$-NH_2 (**9**) crude analysis by: HPLC (a) and MALDI-TOF MS (b).

c) Modified RADA-16 (**11**): Sample preparation: 1 mg of peptide crude (**11**) was completely dissolved in 1 mL of H_2O/MeCN (1:1). HPLC analysis (Figure S5a) (t_R = 5.91 min; gradient from 5% to 25% MeCN over 8 min). The main product (89%, HPLC purity) corresponds to the expected modified RADA-16 (**11**), MS (MALDI-TOF) (Figure S5b): *m/z* calcd for $C_{75}H_{123}N_{29}O_{27}S$: 1893.89, found: 1895.17 $[M + H]^+$. The peak that eluted at t_R = 6.46 min (3.6%, HPLC purity) corresponds to the expected sequence with the sulfoxide oxidized to sulfone, MS (MALDI-TOF): *m/z* calcd for $C_{75}H_{123}N_{29}O_{28}S$: 1909.88, found 1911.1 $[M + H]^+$ and the byproduct that eluted at t_R = 3.88 min (0.7%, HPLC

purity) corresponds to the truncated sequence **5-mer**, MS (MALDI-TOF): *m/z* calcd for $C_{28}H_{45}N_9O_9S$: 683.31, found: 684.4 $[M + H]^+$.

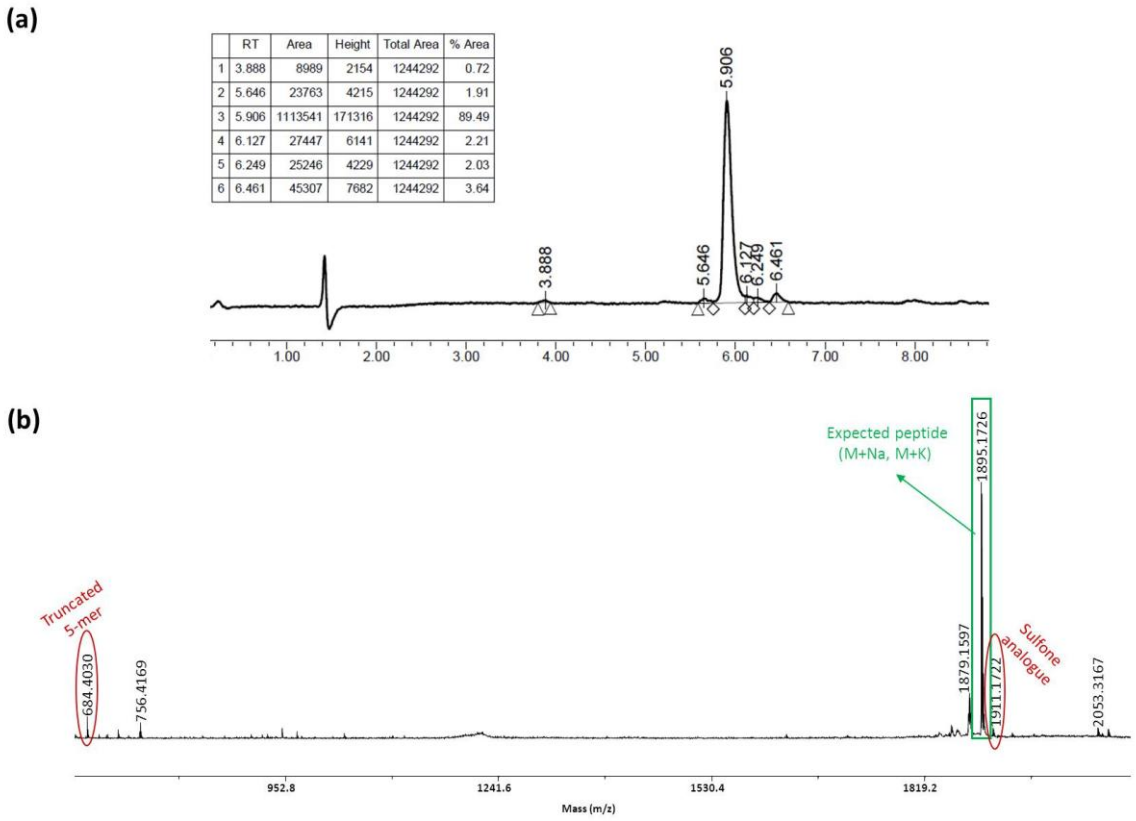


Figure S5. Modified RADA-16 (**11**) crude analysis by: HPLC (a) and MALDI-TOF MS (b).

d) Modified Aβ(1-42) (**12**): Sample preparation: 1 mg of crude peptide (**12**) was completely dissolved in 1 mL of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). HPLC analysis (t_R = 19.28 min; C4 column at 60 ºC, gradient from 10% to 50% MeCN for 30 min). The main product (89%, HPLC purity) corresponds to the expected modified Aβ(1-42) (**12**), MS (MALDI-TOF) (Figure S6): *m/z* calcd for $C_{212}H_{321}N_{55}O_{62}S_2$: 4693.31, found: 4714.94 $[M + Na]^+$. In order to obtain more exact measurements by mass spectrometry, an accurate HR-MS (ESI) (Figure S7) analysis was performed, confirming the detection of the expected peptide: *m/z* calcd for $C_{212}H_{321}N_{55}O_{62}S_2$: 4693.3098, found: 1129.3273 $[M + 4H]^+/4$, 903.6643 $[M + 5H]^+/5$ and 753.3872 $[M + 6H]^+/6$.

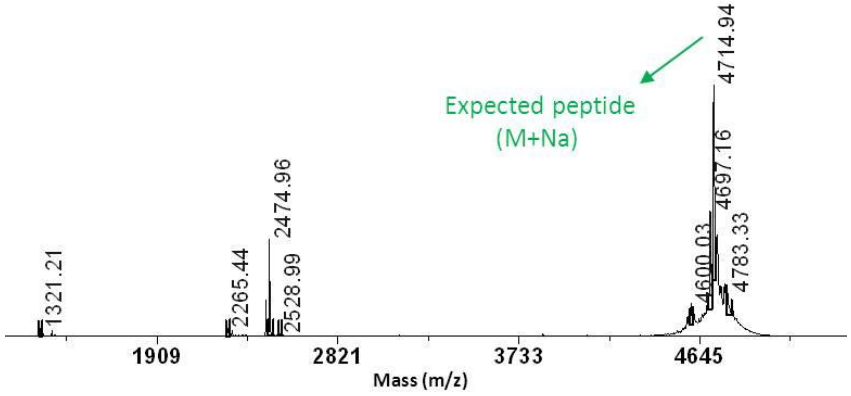


Figure S6. MALDI-TOF MS spectrum of modified Aβ(1-42) (**12**).

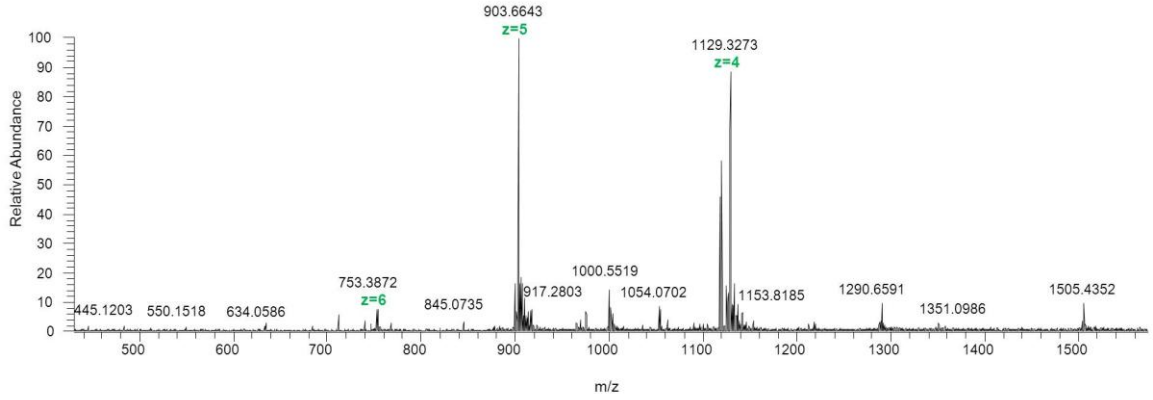


Figure S7. HR-MS (ESI) spectrum of modified Aβ(1-42) (**12**).

e) Modified H-(Ala)$_{10}$-NH$_2$ (**8**, by **dipeptide strategy**): Sample preparation: 1 mg of peptide crude was completely dissolved in 1 mL H_2O/MeCN (1:1). HPLC analysis (Figure S8) (t_R = 3.81 min; gradient from 10% to 40% MeCN for 8 min). The main product (97%, HPLC purity) corresponds to the expected modified H-(Ala)$_{10}$-NH$_2$ (**8**), MS (MALDI-TOF): *m/z* calcd for $C_{39}H_{63}N_{11}O_{12}S$: 909.44, found: 932.45 $[M + Na]^+$. The peak corresponding to the truncated peptide (5 mer) was not detected.

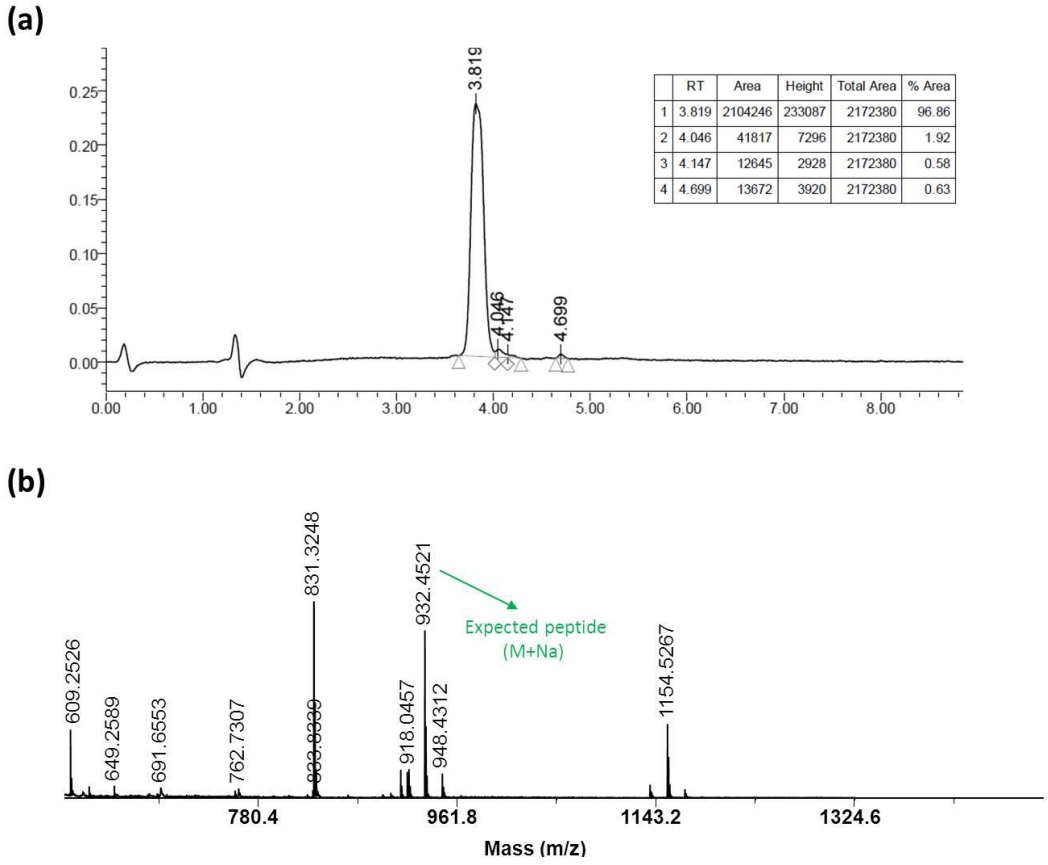


Figure S8. Analysis of H-(Ala)$_{10}$-NH$_2$ **8** by Dipeptide strategy by: HPLC (a) and MALDI-TOF MS (b).

Purification of Modified Peptides

A) Modified RADA-16 (**11**): 65 mg of modified RADA-16 crude were divided in 4 batches (approx. 15 mg each). Every portion was dissolved in H_2O/MeCN (1:1) (0.9 mL) and injected into a semi-preparative HPLC provided with a reverse-phase C18 column (XBridge^{TM} Prep BEH130, Waters: 19 x 100 mm, 5

μm). A linear gradient of (%B) 5-10% over 1 min followed by 10-20% over 7 min was followed, at a flow rate of 16 mL/min: The elution system was the following: B (MeCN + 0.1% TFA) into A (H_2O + 0.1% TFA). After purification and lyophilization, 27.1 mg of pure modified RADA-16 were obtained with a 41.7% purification yield, and excellent purity (99.9%). HPLC analysis of purified product (t_R = 5.116 min; gradient from 5% to 25% MeCN for 8 min) and the MS (MALDI-TOF) confirmed the mass of the expected product (Figure S9): *m/z* calcd for $C_{75}H_{123}N_{29}O_{27}S$: 1893.89, found: 1894.69 $[M + H]^+$.

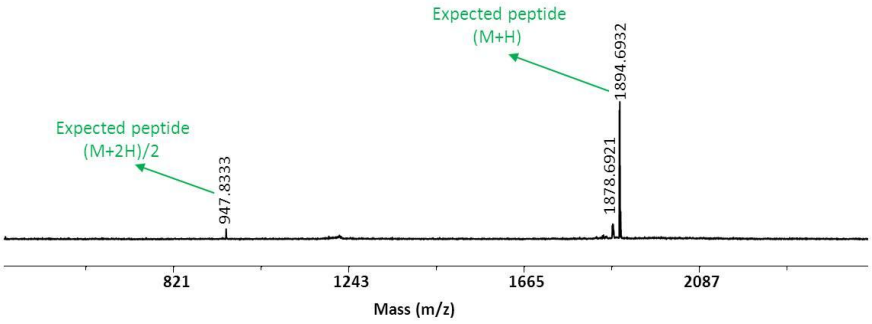


Figure S9. MALDI-TOF MS spectrum of pure modified RADA-16 (**11**).

B) Modified Aβ(1-42) (**12**): Some portion of Aβ(1-42) was dissolved in HFIP (0.8 mL) and injected in a semi-preparative HPLC provided with a reverse-phase C4 column (Jupiter, Phenomenex: 21 x 150, 10μm). A linear gradient of two eluents and flow rate of 20 mL/min (%B): 0-10% in 5 min followed by 10-50% in 60 min was followed. The elution system followed: B (MeCN + 0.1% TFA) into A (H_2O + 0.1% TFA). After purification and lyophilization, modified Aβ(1-42) was obtained with 90% of purity (measured by HPLC). HPLC analysis of purified product (t_R = 18.492 min; C4 column at 60ºC, gradient from 10% to 50% MeCN for 30 min) and the MS (MALDI-TOF) confirmed the mass of the expected product: *m/z* calcd for $C_{212}H_{321}N_{55}O_{62}S_2$: 4693.31, found: 4714.19 $[M + Na]^+$ and 2360.98 $[M + 2H]^+/2$. In order to obtain more exacted measurements by mass spectrometry, an accurate HR-MS (ESI) analysis was performed, confirming the detection of the expected peptide: *m/z* calcd for

$C_{212}H_{321}N_{55}O_{62}S_2$: 4693.3098, found: 1174.8373 $[M + 4H]^+/4$ and 940.0719 $[M + 5H]^+/5$.

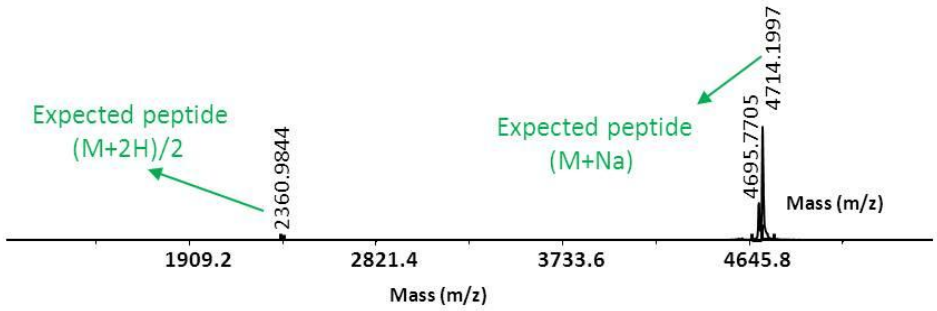


Figure S10. MALDI-TOF MS spectrum of pure modified Aβ(1-42) (**12**).

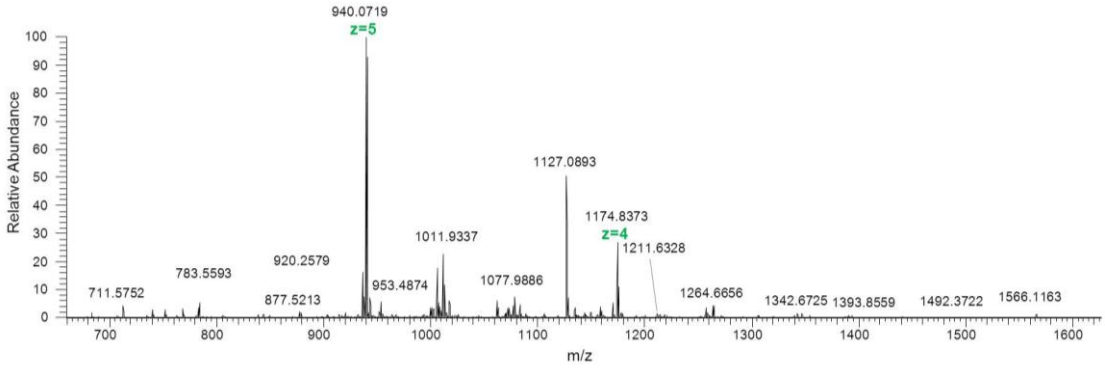


Figure S11. HR-MS (ESI) spectrum of pure modified Aβ(1-42) (**12**).

Removal of the Mmsb Amide Protecting Group

A) Modified H-$(Ala)_{10}$-NH_2 at Position 6 (**8**)

2 mg of pure modified H-$(Ala)_{10}$-NH_2 (**8**) was dissolved in neat TFA (2 mL), and ammonium iodine (3.6 mg) was added to the mixture. The reaction was monitored by MALDI-TOF MS and was stopped after 2 h. The work-up was accomplished by removing the solvent by evaporation and further precipitation and washings of the peptide with cold diethyl ether to afford the expected unprotected product as a white solid peptide. Analysis by MS (MALDI-TOF) revealed the completed reaction: *m/z* calcd for $C_{30}H_{53}N_{11}O_{10}$: 727.40, found: 750.42 $[M + Na]^+$ and 766.39 $[M + K]^+$. The starting material [modified H-$(Ala)_{10}$-NH_2 (**8**)] was not detected.

B) Modified H-(Ala)$_{10}$-NH$_2$ at Position 8 (**9**)

2 mg of pure modified H-(Ala)$_{10}$-NH$_2$ (**9**) was dissolved in neat TFA (2 mL), ammonium iodine (3.6 mg) was added to the mixture. The reaction was monitored by MALDI-TOF MS (Figure S12) and it was stopped after 2 h. The work-up was accomplished by removing the solvent by evaporation and precipitation and washings with cold diethyl ether to afford the expected unprotected product as a white solid peptide. MS (MALDI-TOF) analysis revealed the completed reaction: *m/z* calcd for $C_{30}H_{53}N_{11}O_{10}$: 727.40, found: 750.43 $[M + Na]^+$ and 766.41 $[M + K]^+$. The starting material [modified H-(Ala)$_{10}$-NH$_2$ (**9**)] was not detected.

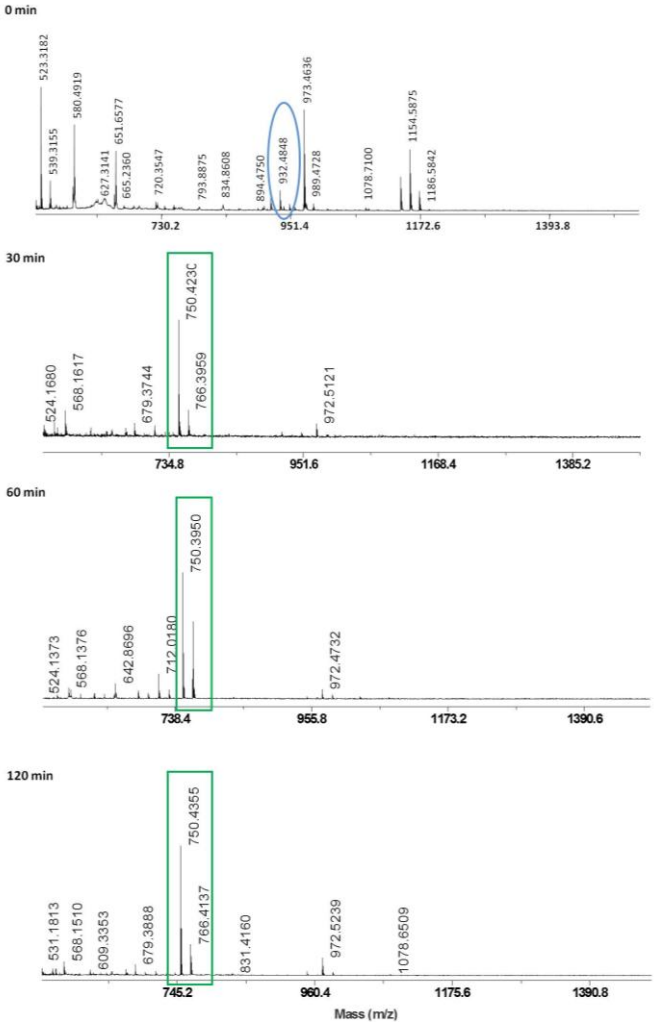


Figure S12. MALDI-TOF MS spectra of monitoring Mmsb removal of modified H-(Ala)$_{10}$-NH$_2$ at position 8 (**9**). Starting material (Mmsb protected H-(Ala)$_{10}$-NH$_2$ (**9**)) (blue). Final product (unprotected H-(Ala)$_{10}$-NH$_2$) (green).

C) Modified RADA-16 (**11**)

5 mg of pure modified RADA-16 (**11**) were dissolved in neat TFA (5 mL), and ammonium iodine (11.5 mg) was added to the mixture. The reaction was monitored by MALDI-TOF MS (Figure S13) and it was stopped after 45 min. The work-up was accomplished by removing the solvent by evaporation, precipitation and washings with cold diethyl ether to afford the expected unprotected product as a white solid peptide. MS (MALDI-TOF) analysis revealed the completed reaction (Figure S14): *m/z* calcd for $C_{66}H_{113}N_{29}O_{25}$: 1711.85, found: 1713.00 $[M + H]^+$ and 856.97 $[M + 2H]^+/2$. The starting material [RADA-16 (**11**) was not detected.

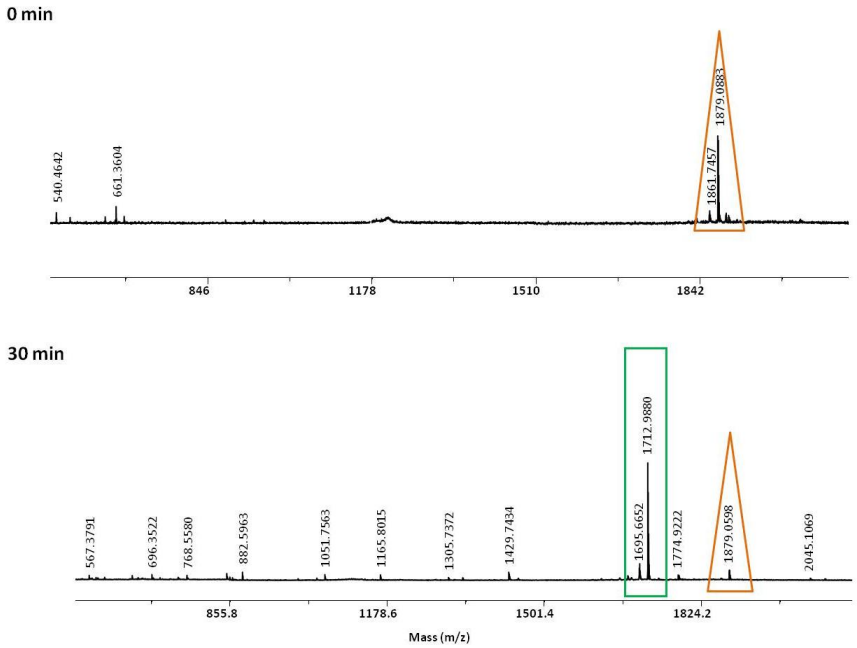


Figure S13. MALDI-TOF MS spectra monitoring of Mmsb removal from modified RADA-16 (**11**). Intermediate of the reaction (Mmsb protected RADA-16) (orange). Final product (unprotected RADA-16) (green).

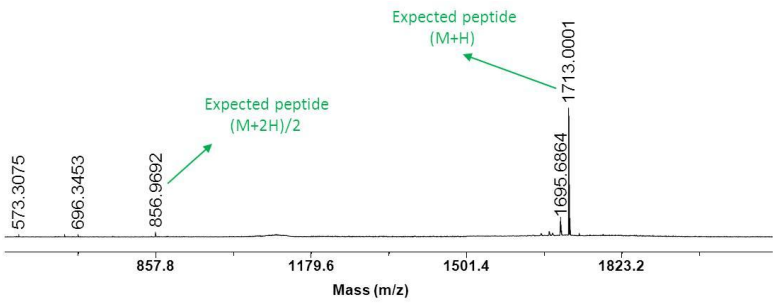


Figure S14. MALDI-TOF MS spectrum of final lyophilized unprotected RADA-16 after Mmsb removal.

D) Modified Aβ(1-42) (**12**)

0.8 mg of pure modified Aβ(1-42) were dissolved in neat TFA (800 μL), and ammonium iodine (1 mg) was added to the mixture. The reaction was stopped after 2 h. The work-up was accomplished removing the solvent by evaporation, precipitation and washings of the peptide with cold diethyl ether and the characterization showed the expected unprotected product as a white solid in 90% purity. MS (MALDI-TOF) analysis revealed the completed reaction (Figure S15): *m/z* calcd for $C_{203}H_{311}N_{55}O_{60}S$: 4511.27, found: 4714.20 $[M + Na]^+$ and 2360.98 $[M + 2H]^+/2$. The starting material [modified Aβ(1-42) (**12**)] was not detected. In order to obtain more exact measurements by mass spectrometry, an accurate HR-MS (ESI) analysis (Figure S16) was performed, confirming the detection of the expected peptide: *m/z* calcd for $C_{203}H_{311}N_{55}O_{60}S$: 4511.2696, found: 1129.3273 $[M + 4H]^+/4$, 903.6643 $[M + 5H]^+/5$ and 753.3872 $[M + 6H]^+/6$.

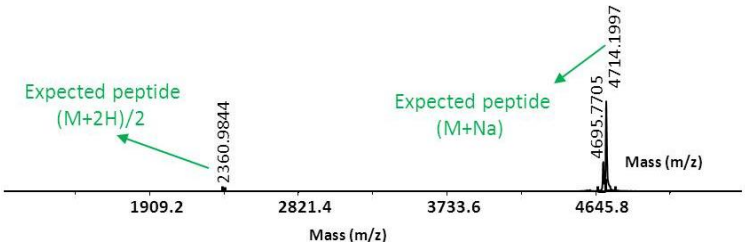


Figure S15. MALDI-TOF MS spectrum of final lyophilized unprotected Aβ(1-42) after Mmsb removal.

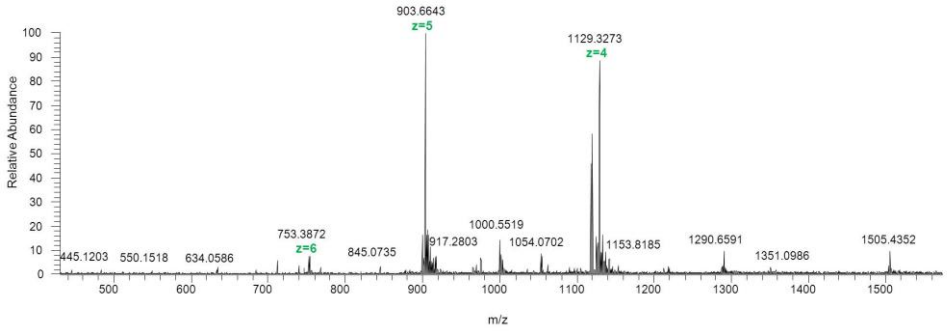


Figure S16. HR-MS (ESI) spectrum of final lyophilized unprotected Aβ(1-42) after Mmsb removal.

The 10% impurity detected by HPLC (t_R = 20.3 min; C4 column at 60ºC, gradient from 10% to 50% MeCN for 30 min) was isolated and characterized by mass spectrometry ESI-MS. The UV profile (Figure S17) indicates the presence of the synthesized protecting group and the mass value confirms that the impurity corresponds to the truncated peptide protected in *N*-terminal with the sulfoxide reduced (Mmtb-^{21}Ala-^{1}Ala-OH, Figure S18).

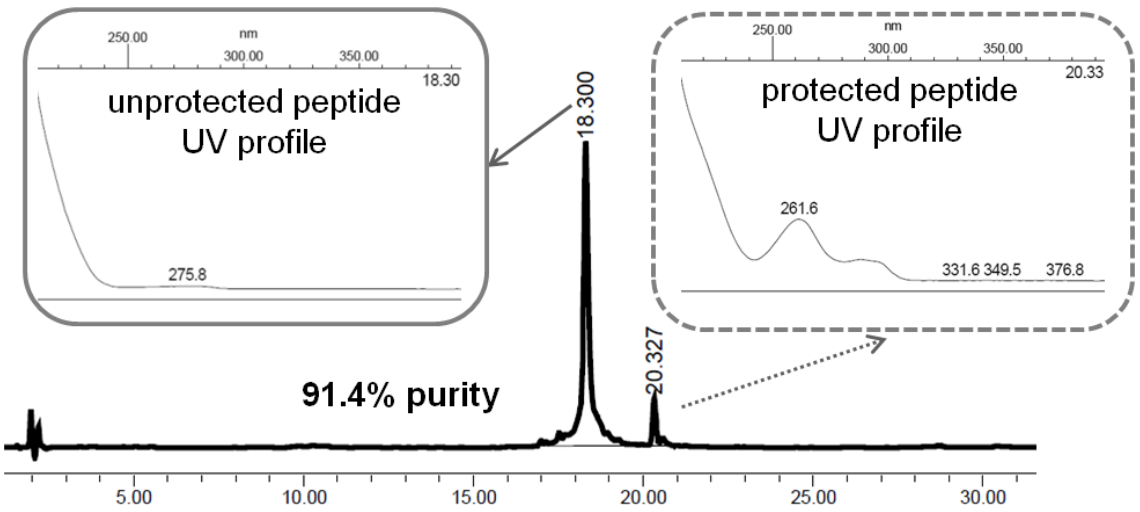


Figure S17. HPLC photodiode array analysis of final lyophilized unprotected Aβ(1-42) after Mmsb removal including the UV profile of every product detected.

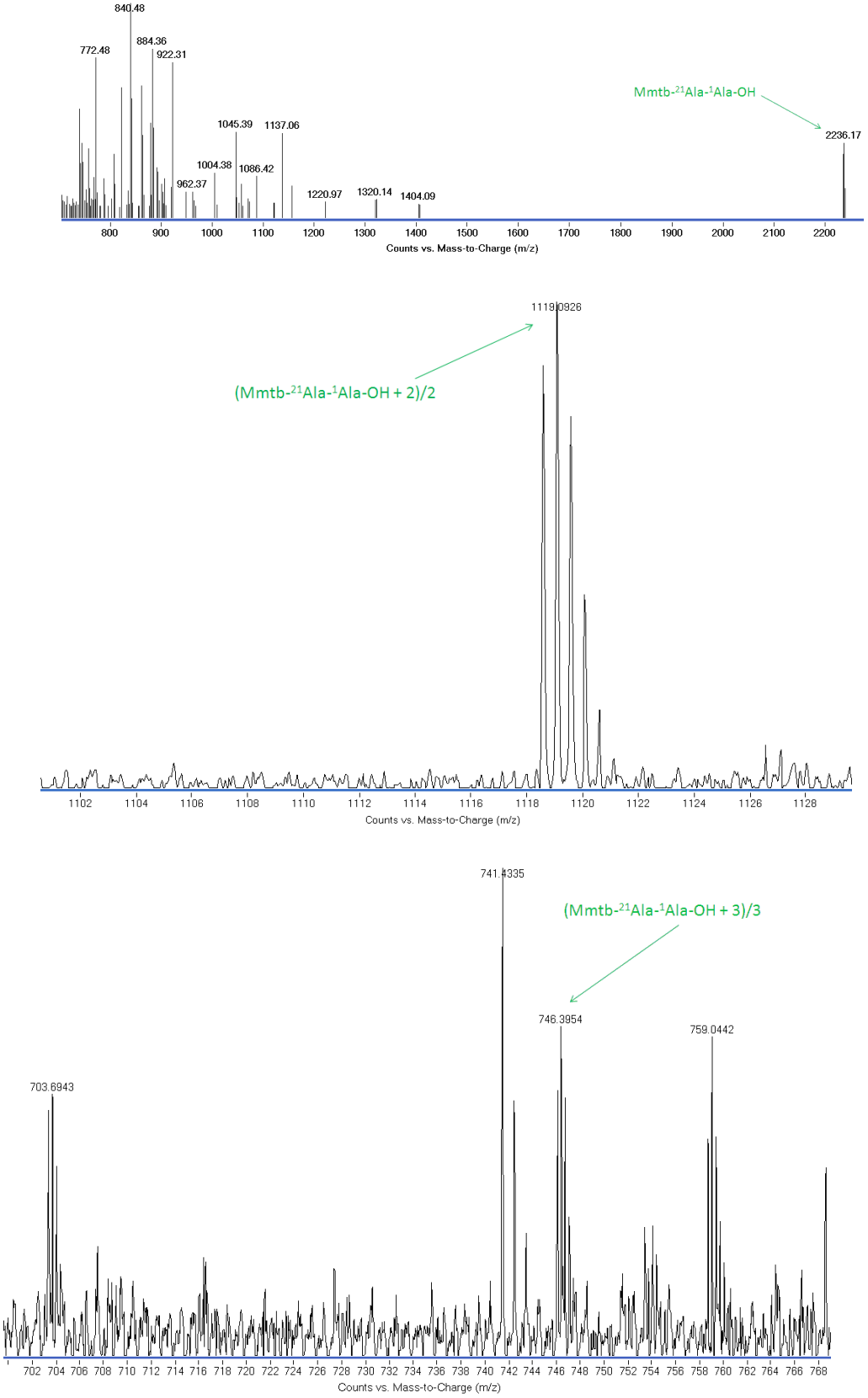


Figure S18. ESI-MS spectra of isolated impurity from lyophilized unprotected Aβ(1-42) after Mmsb removal (t_R = 20.3 min, Figure S17).

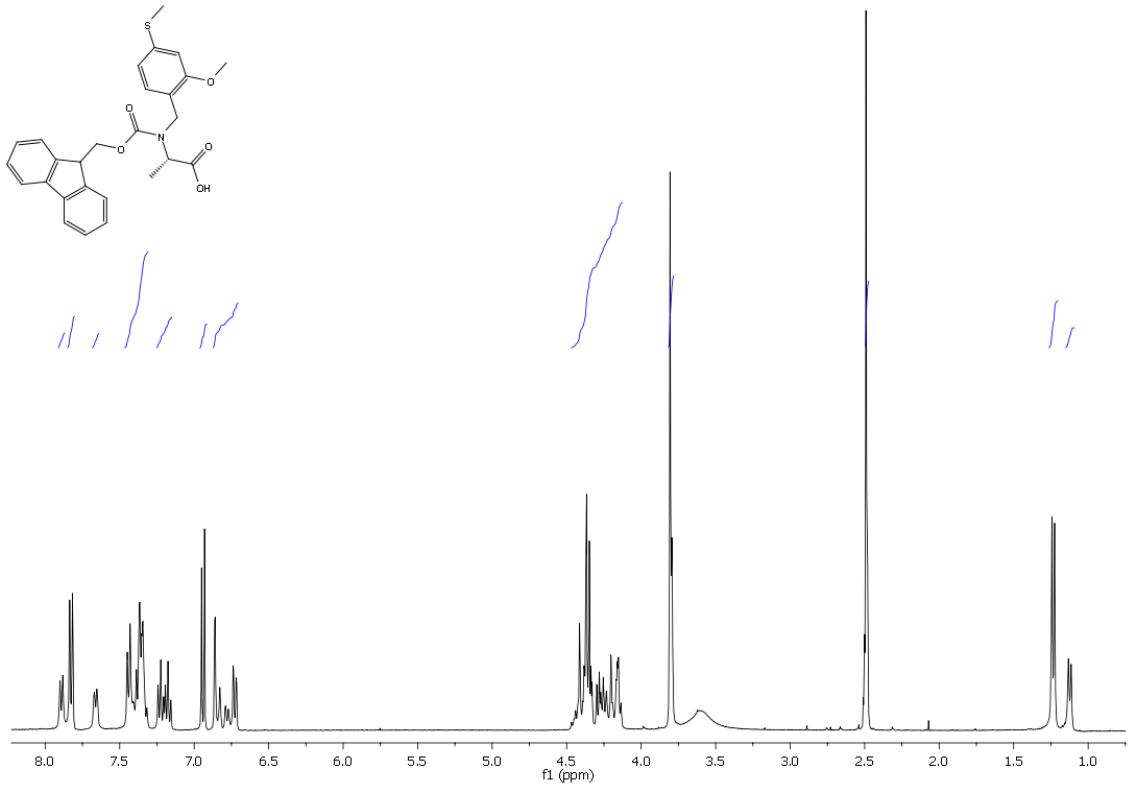
Desalting of Peptides

Modified RADA-16 (**11**): The RADA-16 crude obtained after Mmsb-removal (1.6 mg) was dissolved in 1% TFA and H_2O/MeCN (1:1) (1.6 mL) and the solution was passed through a Sephadex G-10 column. The buffer chosen for the elution of the peptide was 0.045% TFA in H_2O. After filling up the column with a total of 2000 μL of buffer fractions were checked by HPLC, and those containing peptide were lyophilized to remove the liquid to afford the desalted peptide.

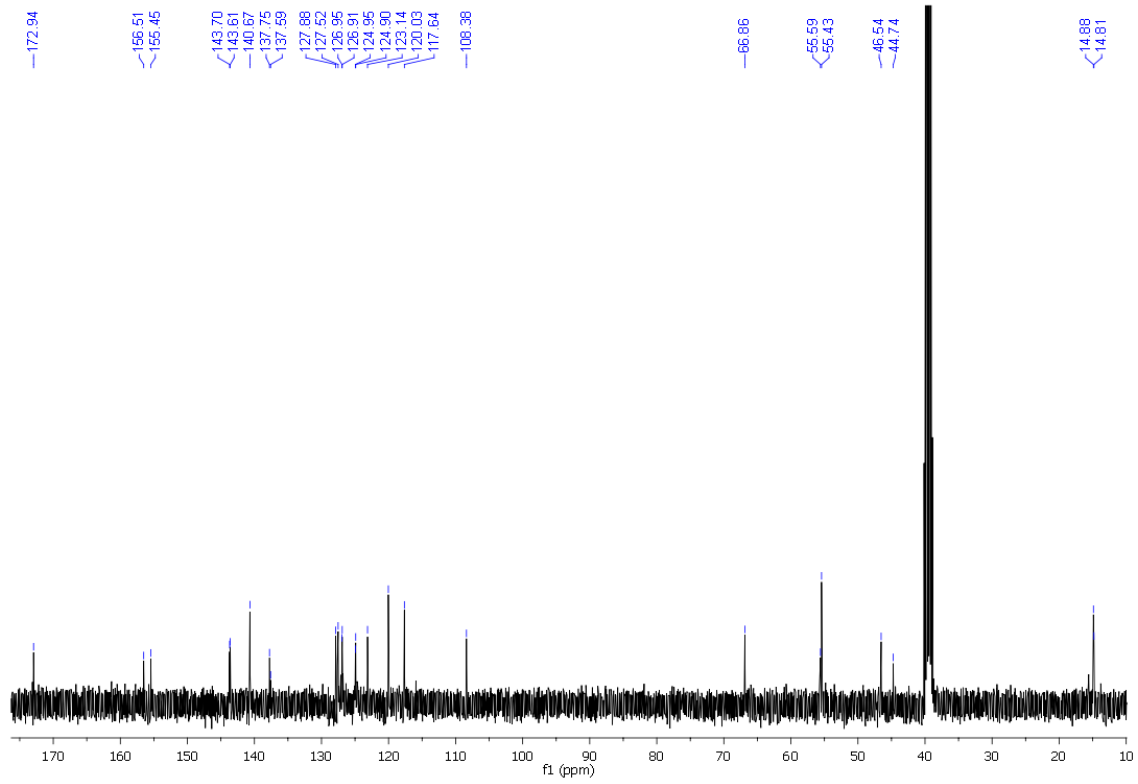
NMR spectra of Building Blocks

[**Fmoc-N(Mmtb)-Ala-OH**] (**5**)

^{1}H NMR (400 MHz, DMSO-d_6; Me_4Si)

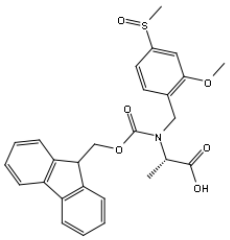


^{13}C NMR (100 MHz, DMSO-d_6)

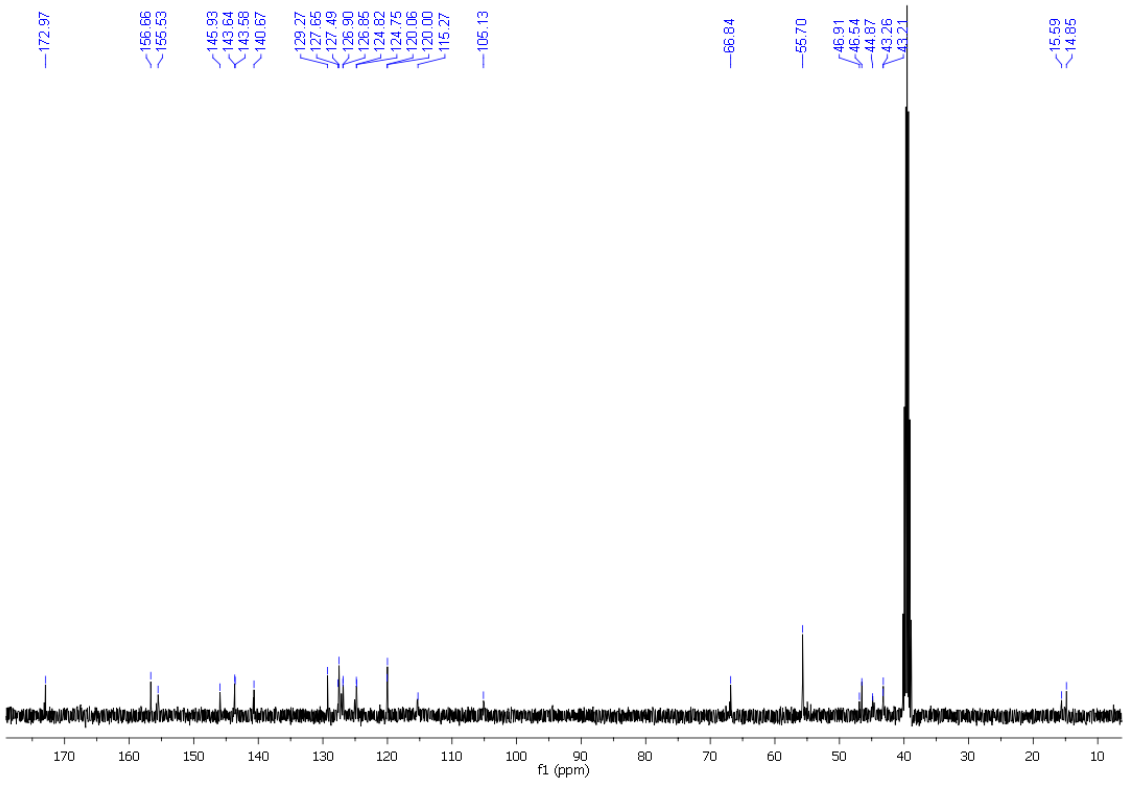


[**Fmoc-N(Mmsb)-Ala-OH**] (**6**)

^{1}H NMR (400 MHz, DMSO-d_6; Me_4Si)

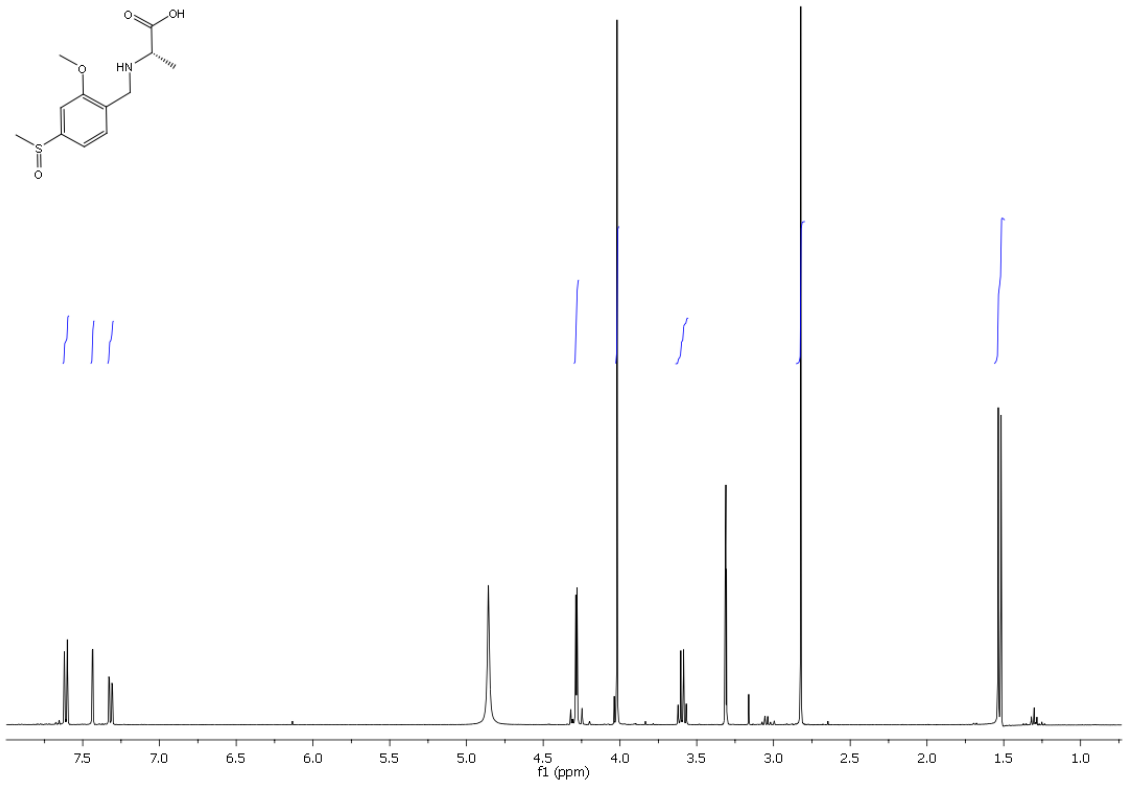


^{13}C NMR (100 MHz, DMSO-d_6)

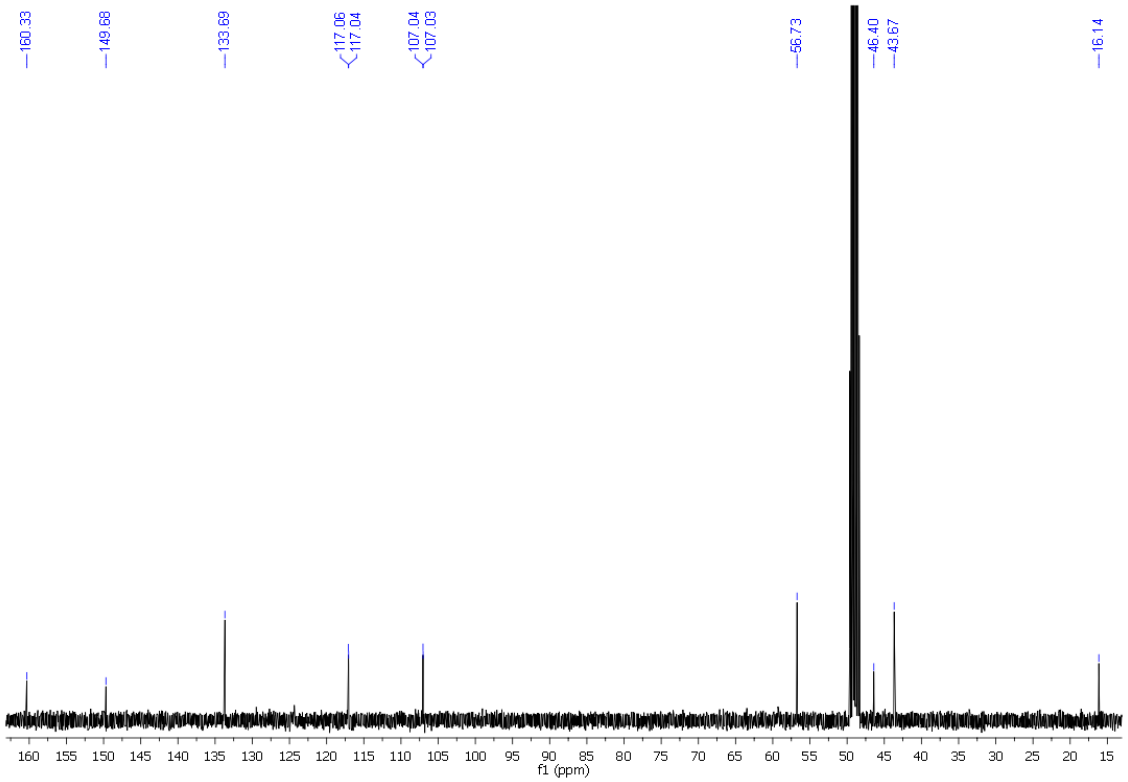


[**H-N(Mmsb)-Ala-OH**] (**13**)

^{1}H NMR (400 MHz, CD_3OD; Me_4Si)



^{13}C NMR (100 MHz, CD_3OD)



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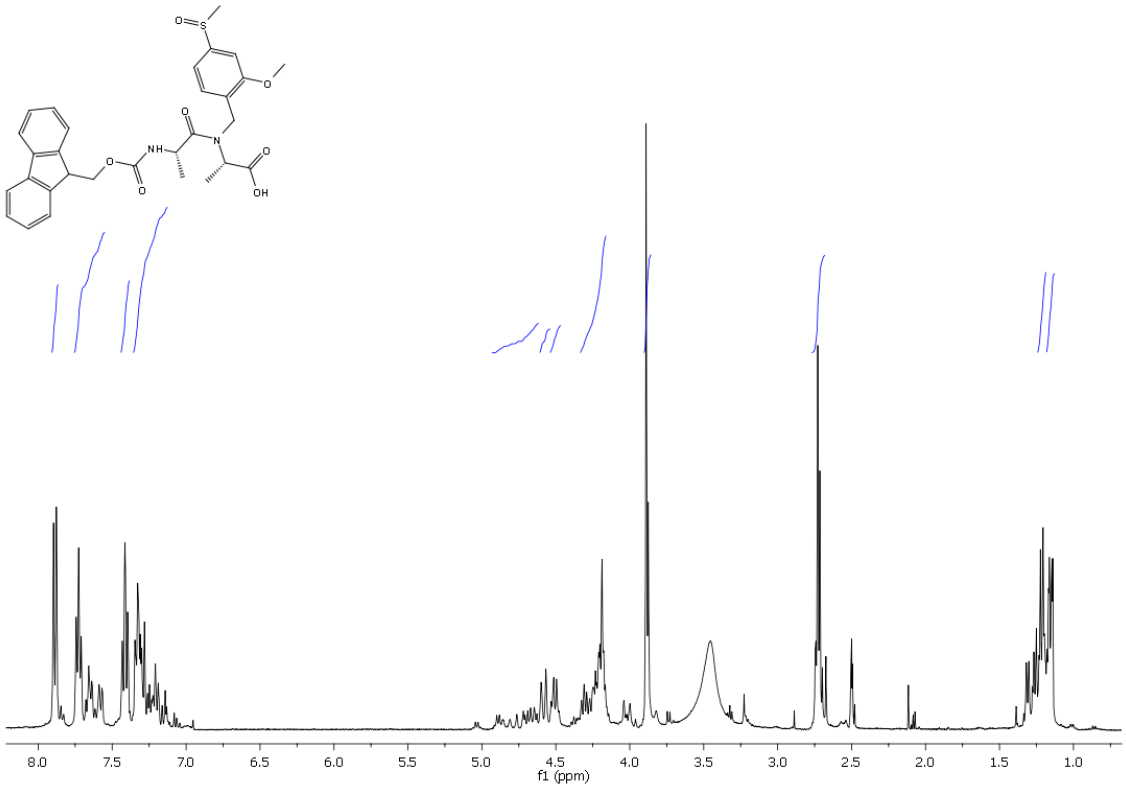
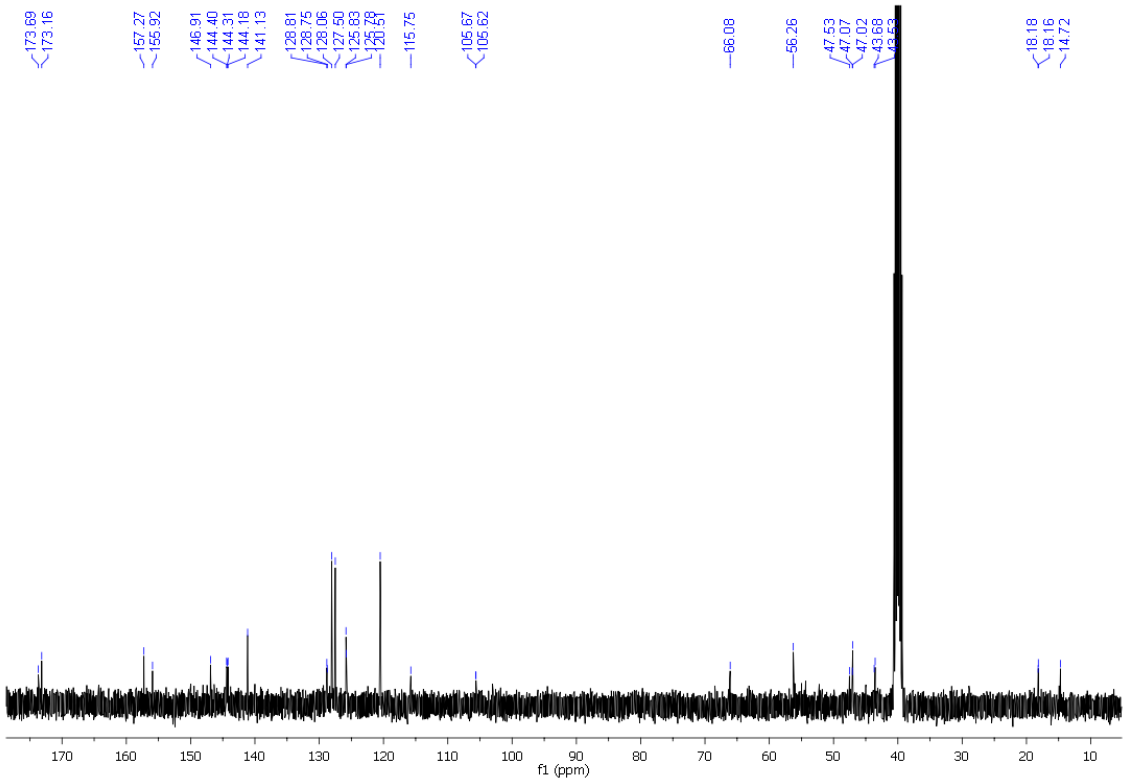
[Fmoc-Ala-*N*(Mmsb)-Ala-OH] (15) ^{1}H NMR (400 MHz, DMSO-d_6; Me_4Si)



^{13}C NMR (100 MHz, DMSO-d_6)



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CHAPTER 3.

Peptide-Based Solubilizing Tag Strategies

Introduction

1. Enhancement of Polarity by Peptide-Based Structures

Short peptide sequences may be used as fragment units to be attached to other molecules/peptides in order to induce physico-chemical changes. These peptides, which act as properties modulators, are named *tags* and the main purpose described regarding their function is enhancing the polarity or solubility of a certain molecule. Homooligo-peptides are the most studied short peptide tags, specifically those which comprise charged side-chains, such as the basic Lys/Arg and the acidic Glu/Asp.

The incorporation of **ionic short peptide-based solubilizing tags** into a non-polar peptide through the *C*-terminus has been reported for several insoluble sequences. As it was aforementioned in the general introduction section, when the tag and the non-polar molecule are conjugated permanently (for example through an amide bond), after the cleavage, the peptide-tag complex is isolated and used directly for the desired purpose. Examples of nanoparticles containing peptide-based solubilizing tags have been described as drug carriers or metal chelators, among others, because of their biocompatibility. On the other hand, when the tag is conjugated temporarily to the peptide through a bifunctional spacer (linker), after the detachment from the resin, the peptide-tag remains attached due to the stability of the spacer under the cleavage conditions. The isolated peptide-tag conjugate has its physico-chemical properties modified temporarily, facilitating not only its manipulation in solution in terms of chromatographic/spectrometric characterization, but also its purification. Finally, the short peptide tag is detached from the non-polar peptide by a single chemical treatment, rendering the native peptide sequence. When the tag, together with the linker, acts as a semipermanent group, it may be considered a ***C*-terminal protecting group**, which temporarily protects the carboxylic function and, in parallel, contributes to enhance the solubility.

1.1. Ionic Short Peptide-Based Solubilizing Tags

Principally, charged AAs (Glu, Asp, Lys, and Arg) are those selected to be conjugated and enhance the solubility of non-polar molecules. Most reported studies have focused on the synthesis of proteins, based on three principal applications: (i) drug delivery, (ii) structured material-based and (iii) as solubilizing tool. The biocompatibility of drug delivery systems is mandatory and conjugates that contain some of these charged AAs fit in this requirement. These anionic poly-AA comprising Glu (also named PGA) or Asp (also named PAA) have been designed to be used as drug carrier systems, principally, synthesized by polymerization with PEG[1,2] (Fig. 1a). The importance of their application

in medicine has become evident specially in those cases where an existent drug has demonstrated an increased efficiency against cancer when it is carried by a PEG-PolyAA complex.[3–5] These two drug delivery systems take advantage of its pH sensitivity associated to the poly-AA component, which allow the design of pH-dependent release systems.[6–8] Therefore, the drug is led through the biological system and specifically delivered to those tissues where the pH causes a chemical modification to the tag.

Regarding the second mentioned application, the micelles formation may be either composed by copolymers in conjugation with pegylated systems[6,9,10] (Fig. 1b), or in alternation with other AAs.[11,12] Moreover, other interesting materials composed by conjugating PEG, in this case to a poly-Arg, were studied as biodegradable hydrogels capable to encapsulate and release certain drugs.[13]

Finally, the subject of the present work, the connection of poly-AA to non-polar molecules to enhance its solubility in solution has been studied in different fields, such as the solubilization of: extremely non-polar organic compounds (graphene by poly-Lys);[14] or inorganic cations (calcium trough poly-Glu),[15] or even drugs with clear poor solubility in aqueous systems (by poly-Glu).[16] This last application would be directly associated to facilitating its characterization or purification, thereby large molecules, such as proteins conjugated permanent- or temporarily to these solubilizing poly-AA, become fruitfully manipulable. Different articles have reported interesting results about protein solubilization by poly-Asp,[17] poly-Glu,[18] poly-Arg,[19,20] or poly-Lys.[21]

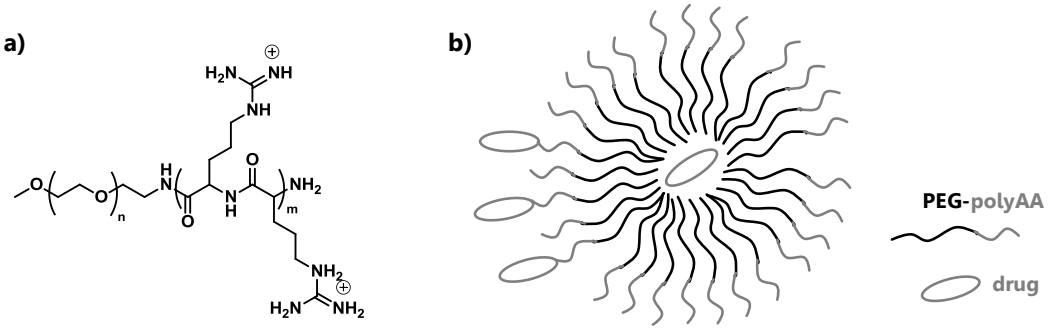

Figure 1. Two ionic peptide-based solubilizing structures formed by: (a) PEG-poly-Arg conjugate example; and (b) schematic representation of one micelle formed by the copolymer PEG and poly-AA with a conjugated drug on the surface and inside the core.

The spatial arrangement of AA side-chains into a branched morphology plays a significant role in the polarity, more than those that are exposed as linear primary structures. Short branched peptides, combined in some cases with PEG moieties, are easily synthesized and, depending on the charged AA selected as the branched

function, they are able to be used in biological systems, such as: cell internalization in case of the Arg-based tag;[22] drug carriers in case of the Glu-based tag[23] (Fig. 2a) or even bone regeneration in case of the Asp-based tag.[24] The charged lysine is one of the preferred AAs to form the core of branched structures because of the amino groups content, its ε-amino side-chain and in its α-amino, which allow the double amide bond formation per AA^2 (Fig. 2c). These highly branched structures are named in some cases dendrimers and, when peptide components are present in the structure, they are sub-classified as the peptide dendrimers family (Fig. 2b). Principal dendrimer characteristics rely on their globular structure, which allow them to functionalize the branches offering the maximum functional groups exposed in a minimum surface, feature that makes them suitable as drug carriers. Peptides integrated as dendrimer components confer to the system water-solubility, biodegradability and high branched versatility to form globular structures. Although the first, and most used dendrimer, is the known as PAMAM (formed by poly(amidoamine), it does not contain a peptide component, several publications reveal the successful improvements when peptide dendrimers are used as drug delivery systems.[25,26]

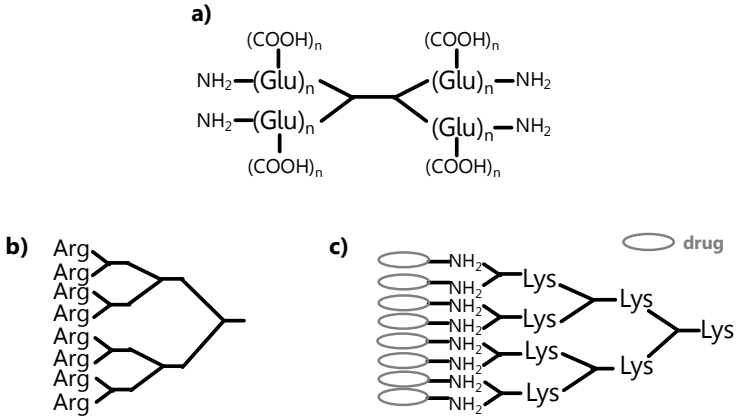

Figure 2. Three schematic representations of ionic peptide-based solubilizing structures composed of: (a) branched poly-Glu system; (b) dendrimer guanidinium functionalized, attributed to Arg; and (c) Lys-core branched structure conjugated with drug.

On the other hand, when linear or branched poly-peptides are designed to be temporarily conjugated to a non-soluble molecule, modulation of properties attributed to the connected tag is profitable basically to characterize or purify the target molecule. In this sense, although solubilizing proteins are well reported in the literature (previously mentioned in the general introduction section), only a few articles may be found regarding the solubility enhancement of non-polar peptides. In 1996, Englebretsen[27] and co-workers proposed, for the Boc/Bzl strategy, the SPPS of $(Gly\text{-}Arg)_4$ sequence as solubilizing tag connected to the hydrophobic dodecaalanine

peptide through the basic-labile hydroxyacetic acid linker. After cleaving the peptide from the resin by HF, the peptide remains connected to the sequence, being the purification a feasible step. Finally, a last treatment with triethylamine renders the dodecaalanine sequence. Later, in 1998, the same author proposed,[28] for the Fmoc/*t*Bu methodology, the basic-labile 4-hydroxymethylbenzoic acid (HMBA) linker for the identical purpose, this time with a series of Gly(Lys/Arg-Gly_{1-2})$_{3-4-6}$ tags. Again, after cleavage from the resin, the peptide remains connected to the solubilizing tag allowing its purification, and subsequently, under 0.1 M NaOH, the tag is hydrolyzed yielding the desired peptide. Later, in 2009, two parallel works developed by Wade[29] and Brimble,[30] by applying the strategy described in 1998, used the same HMBA linker to conjugate a non-soluble peptide to the solubilizing tags: $(Arg)_6$ or $(Lys)_5$, respectively. One of the limitations associated to these linkers is the fact that cleavages are performed under basic conditions, a media known because of favoring the aspartimide side-reaction. More recently, in 2014,[31] a $(Lys)_4$ tag connected to a non-polar peptide through the acid-labile 4-(hydroxymethyl)phenylacetic linker was described to be compatible only with the Fmoc/*t*Bu strategy, but with the advantage of being totally free of aspartimide formation.

2. *C*-Terminal Protecting Groups

When peptides are synthesized on solid-phase by standard sequential AA incorporations, carboxylic acid protecting groups are not required, since the α-carboxylic acid from the incoming AA is the only carboxylic moiety susceptible to react with the α-amino group from the peptidyl-resin. Moreover, when the AA is coupled to the resin, or to the peptidyl-resin, its carboxylic group becomes protected, thus the solid support acts itself as carboxylic acid protector. However, there are some specific cases where the use of a carboxylic acid protection is mandatory: (i) on-resin "head-to-tail" cyclizations (Fig. 3b), (ii) attachment of the AA to the resin mediated by its side-chain (Fig. 3a), and (iii) in the reverse *N*→*C* peptide synthesis (Fig. 3c).

On the other hand, when peptides are synthesized in solution, the amide bond formation entails the presence of carboxylic protectors, because the absence of resin promotes competition between the carboxylic acid from the incoming AA and those from the other fragment. Thereby, strategies that combine solid-phase peptide methods and solution approaches, such as enzymatic-catalyzed peptide synthesis[32] or peptide fragment condensation in solution, demand a wide range of carboxylic acid protecting groups. Specifically, those carboxylic acid protectors comprised in the *C*-terminus of a peptide fragment, are known as *C*-terminal protecting groups.

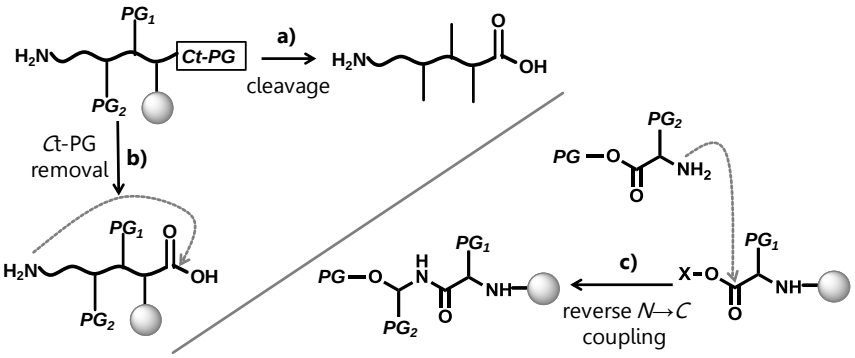

Figure 3. Solid-phase peptide synthesis situations when *C*-terminal protecting group is required. (a) Side-chain anchoring; (b) on solid-phase "head-to-tail" cyclization; and (c) reverse *N*→*C* peptide synthesis.

Some researchers have focused their attention on modifying the *C*-terminal function permanently[33–35] or temporarily.[36,37] Otherwise, several authors have centered their research on protecting the *C*-terminal of peptides when the resin does not act as a protector. Since peptide fragment condensation was initially described to reach large peptides, a broad range of *C*-terminal protectors emerged in the literature.[38,39] Obviously all described *C*-terminal protectors are suitable for carboxylic acid side-chains of AAs, such as Asp or Glu. These protectors, depending on the conditions which allow their removal, may be classified in *acid-labile*, *basic-labile* or *other lability conditions*. Based on these parameters, some of the most used carboxylic acid protecting groups are exposed below.

2.1. Removable Under Acidic Conditions

The *C*-terminal protecting groups removable under acidic conditions may be used in the Fmoc/*t*Bu SPPS strategy, since this carbamate is removed under basic conditions, and are also orthogonal to the Alloc moiety because of its lability to palladium conditions. The most used *C*-terminal protector belonging to the acid-labile group is the *tert*-butyl, ***t*Bu** (removed under 90% TFA in DCM, or 4 M HCl in dioxane) (Fig. 4).

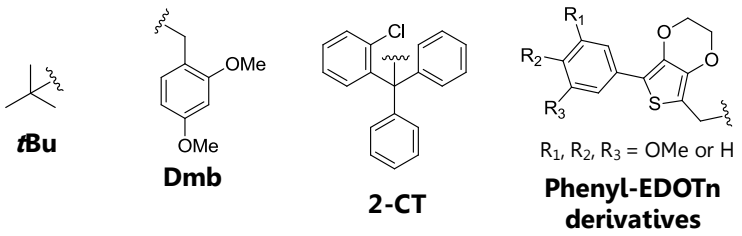

Figure 4. The most significant described *C*-terminal protecting groups removable under acidic conditions.

Although there are other acid-labile protectors removable under equivalent acidic proportion, in this section are highlighted those labile to low acidic conditions. These

groups permit the use of acid-resistant resins because, whereas the resin remains stable, these carboxylic protectors may be removed. An example of those is the 2,4-dimethoxybenzyl, **Dmb**[40] (removed under 1% TFA in DCM) (Fig. 4). Some reports described the preparation of this kind of groups, as well as the wide application of them in peptide syntheses, demonstrating to be suitable groups to assemble peptide fragments in solution.[32,33,41] Similarly, it is found the 2-chlorotrityl, **2-CT**[42,43] (removed under 1% TFA in DCM) (Fig. 4) and the 5-phenyl-3,4-ethylenedioxythenyl derivatives, **phenyl-EDOTn**[44] (removed under 0.01-0.5% TFA in DCM) (Fig. 4), described as a very acid-sensitive protecting group.

2.2. Removable Under Basic Conditions

The basic labile *C*-terminal protecting groups may be suitable for the Boc/Bzl SPPS strategy, since this carbamate is removed under acidic conditions, and these protectors are also orthogonal to the Alloc moiety. Principal basic-labile *C*-terminal protectors found in the literature are the methyl or ethyl, **Me**, **Et** (removed under NaOH, KOH, or LiOH) (Fig. 5) which are used on solid-phase, but also in solution to perform peptide fragment condensations.[32,45,46] The 9-fluorenylmethyl, **Fm**[47,48] (removed under 15% DIEA in DMF, or 20% piperidine in DMF or DCM) (Fig. 5) described also in those cases where the carboxylic moiety from AA side-chain is orthogonally protected by other groups,[49,50] or the carbamoylmethyl, **Cam**[51,52] (removed under 0.5 M NaOH, or Na_2CO_3 in H_2O/DMF) (Fig. 5). Specifically, Cam protector was exploited in enzymatic-catalyzed peptide synthesis,[53] and due to its biocompatibility with enzymes, together with its acyl-donating capacity, has become one of the preferred *C*-terminal protector for this kind of chemistry. Moreover, this protector has been used during the last decade contributing to the successful completion of large peptides.[54–56] Another significant basic-labile *C*-terminal protector is the 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyllamino}benzyl, **Dmab**[57] [removed under hydrazine in H_2O/DMF (2:98)] (Fig. 5) and, although some authors have reported side-reactions for the Dmab,[58] it has been widely used during last years'.[59–61]

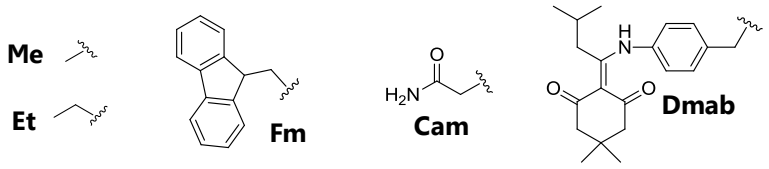

Figure 5. The most significant *C*-terminal protecting groups removable under basic conditions.

2.3. Removable Under Other Conditions

Other *C*-terminal protecting groups non-classified as acid- or basic-labile moieties have been employed because of their orthogonal feature which become them totally stable under mild acidic and basic conditions, being compatible with Fmoc/*t*Bu and Boc/Bzl strategies. One of the preferred protector belonging to these non-classified group is the allyl, **Al** [removed under $Pd(PPh_3)_4$ (0.1 equiv) and $PhSiH_3$ (10 equiv) as scavenger in DCM,[62] or $Pd(PPh_3)_4$ and morpholine as nucleophile in THF/DMSO/0.5 M HCl (2:2:1)[63]] (Fig. 6) used in SPPS[64,65] and in solution. During years this carboxylic protector has aided to elongate peptides and this is the best proof of its fruitfulness in terms of orthogonality, thereby Al has been described as a compatible protector in those peptides which contain saccharides[66,67] or lipids.[68,69]

Carboxylic acid protecting groups based on the benzyl function have introduced a broad range of versatility regarding the orthogonality. The most simple structure is the benzyl, **Bn**[70] (removed under cat. H_2; or HF; or aq. NaOH; or TFMSA) (Fig. 6), mainly used in solution-phase, specifically in dipeptide synthesis.[71–73] Nitrobenzyl derivatives were described as more stable towards acids as compared with the Bn moiety because of the deactivating effect of the nitro group. The most known principal protector based on nitrobenzyl moiety is the *para*-nitrobenzyl, ***p*-NB**[45,74] [removed under cat. H_2; or tetrabutylammonium fluoride (TBAF) in THF, DMF or in DMSO; or $SnCl_2$ in DMF; or $Na_2S{\cdot}H_2O$ in H_2O; or $Na_2S_2O_4$ in aq. Na_2CO_3] (Fig. 6), described to reach linear peptide sequences[75] and also to afford cyclic peptides.[76,77] On the other hand, the methoxy-containing nitrobenzyl analogue 4,5-dimethoxy-2-nitrobenzyl, **Dmnb**[78] (photolabile at 320 nm wavelength) (Fig. 6) which was designed as a totally orthogonal protector due to its already mentioned peculiar lability feature that becomes it stable under acidic conditions during SPPS.[79] Finally, the 2-phenyl-2-trimethylsilyl, **PTMSE**[80] (removed under $TBAF{\cdot}3H_2O$ in DCM) (Fig. 6) described as a linker[81,82] cleavable under almost neutral conditions by fluoride compounds, as well as the PTMSE has been reported as an effective carboxylic protecting group for certain peptide synthesis.[83]

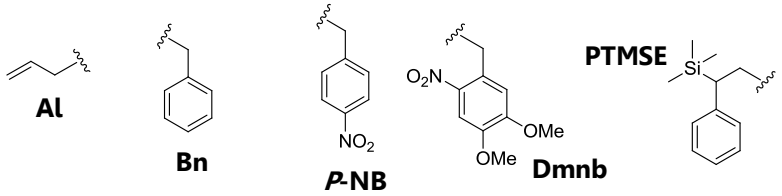

Figure 6. The most significant *C*-terminal protecting groups removable under other conditions.

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Linear *versus* branched poly-lysine/arginine as polarity enhancer tags†

Marta Paradís-Bas,[a,b] Maria Albert-Soriano,[a] Judit Tulla-Puche*[a,b] and Fernando Albericio*[a,b,c,d,e]

The design and synthesis of Lys- and Arg-containing peptides as solubilizing tags were studied to evaluate their influence on polarity. The relevance of spatial arrangement of polar groups, in α- or ε-amino positions, was confirmed by chromatographic analysis of a rational PolyLys-based synthesized structure. The most promising of the solubilizing tags here analyzed was conjugated to a commercial water-insoluble drug (indomethacin) as proof of concept.

The synthesis, purification, and manipulation of biological active molecules are often jeopardized by the poor polarity of the substrates, which is translated into low solubility for small molecules, peptides, and proteins alike. In the case of small molecules, a clear example is the preparation of antibody drug conjugates (ADCs). For the preparation of these molecules, the conjugation of the antibody and the drug in the final step must be performed in aqueous medium. With respect to peptides, the solubility of these molecules can be increased by introducing temporary chemical modifications such as *N*-(2-acetoxy-4-methoxy)benzyl (acyl-Hmb)[1,2] or *O*-acylisoacylpeptides;[3,4] however, these strategies are not straightforward. Another alternative is the introduction of solubilizing tags, mostly derived from oligoethyleneglycol or polycationic peptides. The former do not always render the desired solubility.[5] The groups of Aimoto,[6] Kent,[7] and Brimble[8] used $(Arg)_{5/6}$ as a part of the thioester moiety to increase the solubility of peptide fragments in a chemical ligation strategy. Furthermore, the groups of Englebretsen,[9] Wade,[10] and Brimble[11] attached $(Gly–Arg)_4$ and $(Lys)_5$ *via* a base linker to the C-terminus of the poorly soluble peptide. Although Kuroda[12] and coworkers reported that polyArg confers slightly more solubility than its Lys counterpart to the protein Bovine Pancreatic Trypsin Inhibitor-22 (BPTI-22), which contains 22 Ala residues, we were intrigued whether this was also valid for a peptide and, more importantly, by the contribution of the spatial arrangement disposition (linear *vs.* branched) to enhancing solubility.

To evaluate the polarity effect, several tags were assembled stepwise by the solid-phase technique on a di-naphthylalanine [H–$(Nal)_2$–NH_2, **1**] moiety, which was selected as a non-polar molecule. By means of an Fmoc/*t*Bu strategy, all the peptide sequences proposed (Fig. 1) were synthesized onto a Rink amide polystyrene resin using DIPCDI/OxymaPure® as coupling reagents.[13] All couplings were checked by the Kaiser test.[14] Peptides were analyzed by HPLC to determine the difference in polarity.

First, we performed a comparative study with the incorporation of four amino or four guanidinium groups, which were introduced into four peptides with distinct structures. The amino groups were introduced through Lys, resulting in compounds **2** (linear) and **8** (branched), while guanidinium groups were introduced through Arg, corresponding to peptides **3** and **4** (both linear). Fig. 2a shows the superposed HPLC chromatograms. As expected, all polycationic peptides conferred more

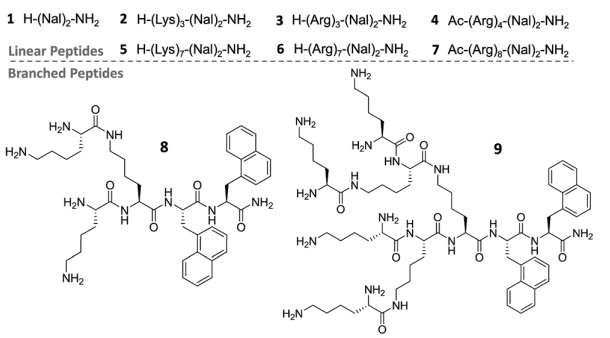

Fig. 1 Chemical structure of the peptides synthesized.

[a]*Institute for Research in Biomedicine Barcelona, Baldiri Reixac 10, 08028 Barcelona, Spain. E-mail: judit.tulla@irbbarcelona.org, albericio@irbbarcelona.org; Fax: +34 934037126; Tel: +34 934037088*
[b]*CIBER-BBN, Networking Centre for Address, Baldiri Reixac 10, 08028 Barcelona, Spain*
[c]*Department of Organic Chemistry, Martí i Franquès 1, 08028 Barcelona, Spain*
[d]*School of Chemistry & Physics, University of KwaZulu Natal, 4001 Durban, South Africa*
[e]*School of Chemistry, Yachay Tech, Yachay City of Knowledge, 100119 Urcuquí, Ecuador*

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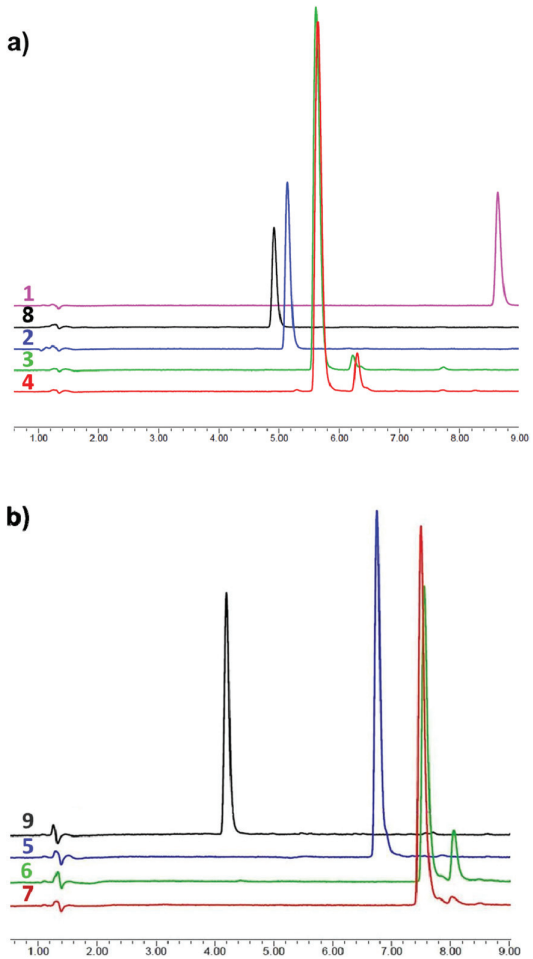

Fig. 2 Superposition of HPLC chromatographic profiles of: (a) first generation peptides (**1**, **2**, **3**, **4**, **8**); and (b) second generation peptides (**5**, **6**, **7**, **9**). Study of polarity influence of amino and guanidinium groups in linear (**2**, **3**, **4**, **5**, **6**, **7**) and branched (**8**, **9**) Poly-AA sequences.

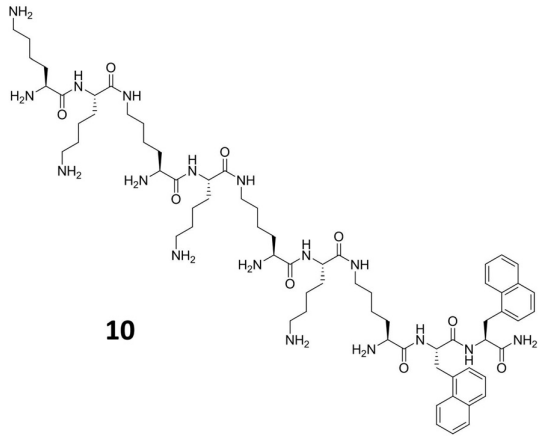

Fig. 3 Chemical structure of a synthesized linear PolyLys peptide.

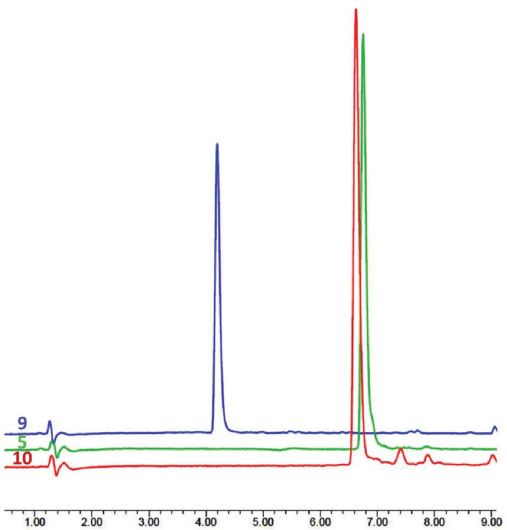

Fig. 4 Superposition of chromatogram analysis by HPLC of three lysine-based peptide sequences of linear (**5**)- and dendron (**9**, **10**)-based structures. Study of both α/ε-type amino groups influence and spatial arrangement disposition.

polarity to the –$(Nal)_2$ moiety. However, differences were noted depending on the nature of the tag. Thus, Lys-containing peptides induced greater enhancement of polarity than those holding Arg. In addition, branched peptide **8** (t_R = 4.9 min) showed a slightly greater polarity than the linear peptide **2** (t_R = 5.1 min).

In the second experiment, looking for a confirmation of the first results, we increased the number of cationic groups to **8**. Three linear sequences matching peptides **5** (Lys), **6** and **7** (Arg) and one branched peptide **9** (Lys) were synthesized. Analogous to the first set of peptides, PolyLys conferred higher polarity than PolyArg (Fig. 2b). Most importantly, the branched peptide (**9**, t_R = 4.2 min) gave much more polarity than its equivalent linear peptide (**5**, t_R = 6.8 min), thus confirming the trend observed with the first generation of peptides.

Encouraged and intrigued by the large differences in the behaviour of PolyLys compounds **5** and **9** and taking into account that peptide **5** contains 7ε- and 1α-amino groups and **9** bears 4ε- and 4α-, we synthesized the linear PolyLys **10** (Fig. 3), which contains 4ε- and 4α-amino groups similar to **9**.

The synthesis of **10**, which sandwiched α- and ε-amide bonds, was also performed using a Fmoc strategy by sequential addition of Fmoc-Lys(Boc)-OH and Boc-Lys(Fmoc)-OH. Comparative HPLC analysis of **5**, **9**, and **10** (Fig. 4) revealed significant polarity differences depending on the spatial arrangement disposition of the polar moieties. Thus, the two "linear" sequences showed very similar retention times (**5**, t_R = 6.8 min; **10**, t_R = 6.7 min). These times were higher than the most "spherical" structure (**9**, t_R = 4.2 min).

Once the chromatographic results confirmed that the "favorite" PolyLys structure (in terms of substantially increasing the polarity) was the branch shaped moiety (**9**), a commercial

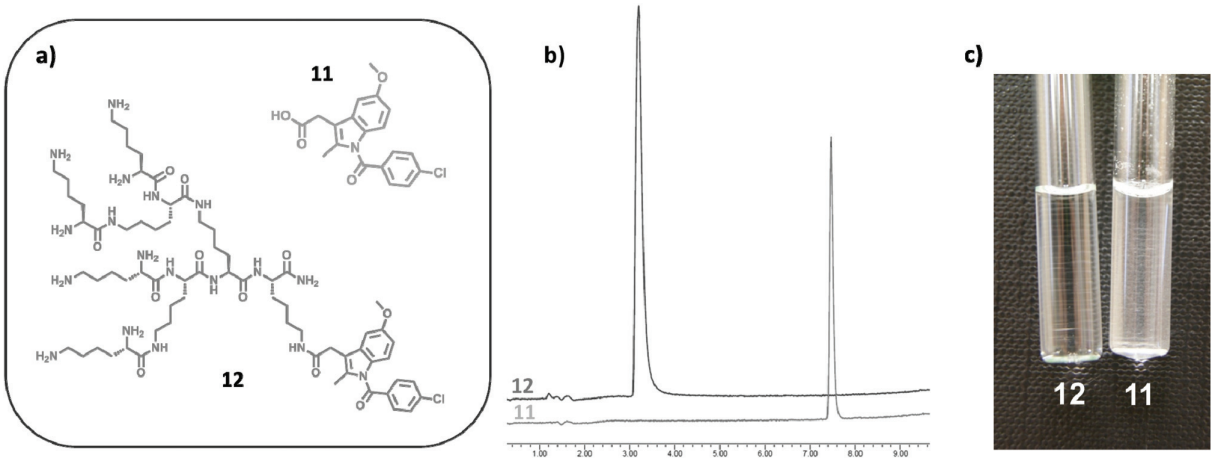

Fig. 5 Indomethacin (**11**) and branched PolyLys peptide conjugated to indomethacin (**12**) compared by: (a) HPLC chromatography, elution at a linear gradient of 20–90% MeCN containing 0.036% TFA in 0.045% aqueous TFA over 8 min; and (b) solubility in H_2O (0.72 mM) of indomethacin (dispersion) and conjugated indomethacin (soluble).

drug was selected to be conjugated with branched PolyLys. Indomethacin **11** (Fig. 5) is a non-polar drug which provided the water-insolubility feature suitable as a proof of concept to test the polarity/solubility modulation of the PolyLys conjugation. The ligation of both molecules was performed stepwise in the solid phase, by incorporating the drug on the side-chain of the C-terminal Lys. Once the conjugated PolyLys–indomethacin was built up on the resin and after its release, **12** (Fig. 5) was purified. HPLC analysis comparison between the standard indomethacin and the conjugated PolyLys–indomethacin (Fig. 5a) revealed that the polarity of indomethacin is impressively increased when it is combined with the branched PolyLys [retention time switches from (**11**) t_R = 7.5 min to (**12**), t_R = 3.2 min].

More importantly, the different solubility in water of the two molecules (Fig. 5b) gives the unequivocal evidence of the enhancing polarity power of the branched PolyLys when a non-polar moiety is conjugated (see ESI† for details).

In summary, linear PolyLys leads to a greater increase in polarity than its Arg counterpart. Moreover, this enhanced effect is much greater when the PolyLys adopts a branched arrangement. The conjugation of branched PolyLys with one commercial drug (indomethacin) with intrinsic characteristics of insolubility in water media is an example which confirms the capacity of branched PolyLys to increase the polarity and most importantly the water solubility of a non-polar molecule. We envisage that a branched PolyLys—which in dendrimer chemistry can be considered a dendron—will be crucial as a polarity enhancer tag to facilitate the purification of peptides and/or the manipulation of peptides and small molecules.

Acknowledgements

The work was partially supported by MINECO (MARINMAB, IPT-2012-0198-09000), CICYT (CTQ2012-30930), the *Generalitat de Catalunya* (2009SGR 1024), and the Institute for Research in Biomedicine (Spain). We thank Dr Carmen Cuevas [Pharma-Mar SA, Madrid (Spain)] for fruitful discussions.

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Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Electronic Supplementary Information

Linear *versus* Branched poly-Lysine/Arginine as Polarity Enhancer Tags

Marta Paradís-Bas,[a,b] Maria Albert-Soriano,[a,b] Judit Tulla-Puche,*[a,b] and

Fernando Albericio*[a,b,c,d,e]

[a] Institute for Research in Biomedicine Barcelona, Baldiri Reixac 10, 08028-Barcelona, Spain

[b] CIBER-BBN, Networking Centre for Address, Baldiri Reixac 10, 08028-Barcelona, Spain

[c] Department of Organic Chemistry, Martí i Franquès 1, 08028-Barcelona, Spain

[d] School of Chemistry & Physics, University of KwaZulu Natal, 4001-Durban, South Africa

[e] School of Chemistry, Yachay Tech, Yachay City of Knowledge, 100119.Urcuquí, Ecuador

MATERIALS AND METHODS

Fmoc-L-Nal(1)-OH was purchased from Neosystem (Strasbourg, France) and the rest of the Fmoc-L-AA-OH amino acids from Iris Biotech (Marktredwitz, Germany). Coupling reagents most commonly used: DIPCDI, OxymaPure®, DIEA, and formic acid were from Sigma-Aldrich (Schnelldorf, Germany), and other coupling reagents, such as PyBOP, HBTU and HOBt, for difficult amino acid incorporations were from Iris Biotech. Solvents used in SPPS were peptide synthesis-grade DMF and CH_2Cl_2 from Carlo Erba-SdS (Sabadell, Spain). For final acetylation steps and cleavage Ac_2O, TFA (HPLC-grade) and TIS were purchased from Sigma-Aldrich. The solid support for all peptide syntheses was Fmoc-Rink amide AM resin (Polystyrene, loading 0.69 mmol/g) from Iris Biotech. Indomethacin drug was purchased at Tokyo Chemical Industry Co. (TCI).

Analytical HPLC and HPLC/ESMS measurements used HPLC-grade MeCN purchased from Carlo Erba-SdS (Sabadell, Spain). The analytical HPLC were performed on a Waters instrument comprising a separation module (Waters 2695), an automatic injector (Waters 717 autosampler), a photodiode array detector (Waters 2998), and a software system controller (Empower). The reverse-phase C18 column (4.6 × 100 mm, 3.5 μm, XBridge BEH130 from Waters, Cerdanyola del Vallès, Spain) was used at flow rate of 1 mL/min and UV detection at 220 nm. Linear gradients (specified in every peptide synthesis) over 8 min of eluent B (MeCN + 0.036% TFA) into eluent A (H_2O + 0.045% TFA) were used. Analytical HPLC/ESMS were performed on a Waters Micromass ZQ spectrometer, comprising a separation module (Waters 2695), an automatic injector (Waters 717 autosampler), a photodiode array detector (Waters 2998), and a software system controller MassLynx v. 4.1). Linear gradients were performed over 8 min on a

reverse-phase C18 column (3.9 × 150 mm, 5 μm) with MeCN (+ 0.07% formic acid) into aqueous (+ 0.1% formic acid) at 0.3 mL/min, UV detection was at 220 nm and mass scans were acquired in positive ion mode.

Purification of compound **12** was performed in a semi-prep Waters equipment with 600 Delta system comprising a sample manager (Waters 2747) and a UV detector (Waters 2487) with a dual absorbance selected at 254 and 220 nm was used. The software used was a MassLynx version. A reverse-phase C18 column (XBridge™ Prep BEH130, Waters: 19 × 100 mm, 5μm) was used. Linear gradient of 15-90% MeCN containing 0.1% TFA into 0.1% TFA aqueous over 30 min. The tubes which contain the expected peptide were combined and lyophilized to afford the expected pure product.

EXPERIMENTAL PROCEDURES

General peptide synthesis procedure. Peptides were synthesized manually in polypropylene syringes fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. The standard SPPS 9-fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl (*t*Bu) strategy was followed using Fmoc-Rink amide AM resin (Polystyrene, loading 0.69 mmol/g) as a solid support. All peptides, except the branched ones (**8**, **9**, **10** and **12**), were synthesized using the following amino acids: Fmoc-L-Nal(1)-OH, Fmoc-L-Arg(Pbf)-OH, and Fmoc-L-Lys(Boc)-OH. In the case of the branched peptides **8** and **9**, Fmoc-L-Lys(Fmoc)-OH was used to build the sequences while the common Boc-L-Lys(Boc)-OH · DCHA was used for the last coupling. Compound **10** was achieved by means of an alternating coupling of Boc-L-Lys(Fmoc)-OH first and Fmoc-L-Lys(Boc)-OH second until a total of seven Lys couplings was reached. The branched Poly-Lys peptide conjugated to Indomethacin drug (**12**) was synthesized using the

appropriated Lys orthogonal protecting groups to allow attachment of the drug to the peptide chain. Compared with peptide **9**, an extra Lys (Fmoc-L-Lys(Mtt)-OH) was introduced on the resin as a first amino acid, where the drug would be attached through its side chain. Once this amino acid was coupled, the Mtt group was removed by treating the resin with TFA/triisopropilsilane (TIS)/CH_2Cl_2 (2:10:88) (2 × 5min, 1 × 20 min, 1 × 5 min). The Indomethacin (**11**), through its carboxylic acid, was coupled following the standard coupling system and the other Lys residues were coupled with the same protecting groups used to build compound **9**. The standard coupling system used in all peptide syntheses was Fmoc-AA-OH (3 equiv.) with *N,N*'-diisopropylcarbodiimide (DIPCDI)/ ethyl 2-cyano-2-(hydroxyimino)acetate (OxymaPure®)[13] (3 equiv., each) in DMF at room temperature for 1 h. The Kaiser[14] test was used to check the absence of free amino groups. When a positive test was obtained, the amino acid was re-coupled using first Fmoc-AA-OH (3 equiv.) with *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene] *N*-methylmethanaminium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) (3 equiv., each) and DIEA (6 equiv.) in DMF for 45 min; if the coupling was still not completed a last re-coupling was performed with Fmoc-AA-OH (3 equiv.), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (3 equiv.) and DIEA (6 equiv.) in DMF for 45 min. Resin washings after every coupling and after Fmoc removal were performed using DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min) and DMF (3 × 1 min). Fmoc removal after every coupling was done by piperidine/DMF (1:4) treatment (1 × 1 min, 2 × 5 min). When required (**4** and **7**), the final acetylation of compounds was carried out using Ac_2O/DIEA (10 equiv., each) in DMF for 30 min. After completion of peptide sequences, the resin was washed with DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min), and the peptide was cleaved from the resin by treatment with TFA/H_2O (95:5) for 1 h, conditions which removes also the

2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) and *tert*-butyloxycarbonyl (Boc) protecting groups. The cleavage time was increased to 4 h when the peptide contained Arg, because of the Pbf protecting group. The mixture was filtered in the same type of syringe used during peptide elongation, and the solvent was removed under N_2 (g). The peptide was precipitated with cold diethyl ether and centrifuged to remove the liquid phase. In the case of compound **12** the peptide crude was subjected to a purification step and the expected product was isolated with excellent purity (>99%). The crude peptides were dissolved in H_2O/MeCN (1:1), lyophilized, and characterized by HPLC and MS without further purification.

CHARACTERIZATION OF PEPTIDES

Peptide **1**– A white solid was obtained after lyophilization. HPLC characterization showed one main peak (t_R = 5.73 min, 99.9% purity, gradient (%B): 25-40% over 8 min). MS (ESI): *m/z* calcd. for $C_{26}H_{25}N_3O_2$, 411.19, found 412.17 $[M + H]^+$.

Peptide **2**– A white solid was obtained after lyophilization. HPLC characterization showed one main peak (t_R = 5.14 min, 98.4% purity, gradient (%B): 20-35% over 8 min). MS (ESI): *m/z* calcd. for $C_{44}H_{61}N_9O_5$, 795.48, found 796.54 $[M + H]^+$, 398.95 $[M + 2H]^+/2$.

Peptide **3**– A white solid was obtained after lyophilization. HPLC characterization showed one main peak (t_R = 5.61 min, 95.9% purity, gradient (%B): 20-35% over 8 min). MS (ESI): *m/z* calcd. for $C_{44}H_{61}N_{15}O_5$, 879.49, found 880.54 $[M + H]^+$.

Peptide **4**– A white solid was obtained after lyophilization. HPLC characterization showed one main peak (t_R = 5.64 min, 91.6% purity, gradient (%B): 20-35% over 8 min). MS (ESI): *m/z*

calcd. for $C_{53}H_{76}N_{18}O_7$, 1076.61, found 1078.71 $[M + H]^+$, 539.83 $[M + 2H]^+/2$, 360.29 $[M + 3H]^+/3$.

Peptide **5**– A white solid was obtained after lyophilization. HPLC characterization showed one main peak (t_R = 6.75 min, 98.2% purity, gradient (%B): 15-30% over 8 min). MS (ESI): *m/z* calcd. for $C_{68}H_{109}N_{17}O_9$, 1307.86, found 1309.00 $[M + H]^+$, 655.19 $[M + 2H]^+/2$.

Peptide **6**– A white solid was obtained after lyophilization. HPLC characterization showed one main peak (t_R = 7.55 min, 86% purity, gradient (%B): 15-30% over 8 min). MS (ESI): *m/z* calcd. for $C_{68}H_{109}N_{31}O_9$, 1503.90, found 753.42 $[M + 2H]^+/2$, 502.00 $[M + 3H]^+/3$.

Peptide **7**– A white solid was obtained after lyophilization. HPLC characterization showed one main peak (t_R = 7. 50 min, 96.6% purity, gradient (%B): 15-30% over 8 min). MS (ESI): *m/z* calcd. for $C_{76}H_{123}N_{35}O_{11}$, 1702.01, found 852.85 $[M + 2H]^+/2$, 568.67 $[M + 3H]^+/3$.

Peptide **8**– A white solid was obtained after lyophilization. HPLC characterization showed one main peak (t_R = 4.91 min, 97.7% purity, gradient (%B): 20-35% over 8 min). MS (ESI): *m/z* calcd. for $C_{44}H_{61}N_9O_5$, 795.48, found 796.54 $[M + H]^+$, 398.88 $[M + 2H]^+/2$.

Peptide **9**– A white solid was obtained after lyophilization. HPLC characterization showed one main peak (t_R = 4.20 min, 99.2% purity, gradient (%B): 15-30% over 8 min). MS (ESI): *m/z* calcd. for $C_{68}H_{109}N_{17}O_9$, 1307.79, found 1309.13 $[M + H]^+$, 655.26 $[M + 2H]^+/2$, 437.27 $[M + 3H]^+/3$.

Peptide **10**– A white solid was obtained after lyophilization. HPLC characterization showed one main peak (t_R = 6.66 min, 94.7% purity, gradient (%B): 15-30% over 8 min). MS (ESI): *m/z* calcd. for $C_{68}H_{109}N_{17}O_9$, 1307.86, found 655.32 $[M + 2H]^+/2$.

Peptide **12**– A white solid was obtained after purification. HPLC characterization showed one main peak (t_R = 3.19 min, 99.9% purity, gradient (%B): 20-90% over 8 min). MS (MALDI-TOF): *m/z* calcd. for $C_{67}H_{113}ClN_{18}O_{11}$, 1380.85, found 1381.96 $[M + H]^+$ and 1403.95 $[M + Na]^+$.

SOLUBILITY ANALYSIS

The two common solvents used to peptide analysis [H_2O and H_2O–MeCN (1:1)] were added, separately, to both, Indomethacin and the branched PolyLys peptide conjugated to Indomethacin. The different solubility of both samples in the mentioned solvents was observed (Fig. S1) and also checked by HPLC analysis (Fig. S2 and S3). Two different chromatographic analyses were performed: (a) addition of H_2O to a known quantity of sample until reaching a fixed concentration (0.72 mM), filtration of the solution (0.45 μm) and analysis by HPLC; (b) addition of H_2O–MeCN (1:1) to a known quantity of sample until reaching a fixed concentration (0.72 mM), filtration of the solution (0.45 μm) and analysis by HPLC. Measurements of the absorbance intensity obtained in HPLC in four cases (Fig. S2 and S3) allowed us to evaluate the different solubility behavior of two samples in the analyzed solvents. In the case of Indomethacin in H_2O, the absorbance in HPLC was 0.14 (Fig. S2a); however in a more non-polar media H_2O–MeCN (1:1), the solid was more soluble and, at the same concentration, showed a substantial increase of absorbance until 0.54 (Fig. S2b). This experiment confirms the poor solubility of Indomethacin in H_2O. In case of branched PolyLys peptide conjugated to Indomethacin, at the same concentration, the sample showed total solubility in H_2O, confirmed by the HPLC absorbance, which is in the same range in both solvents, in H_2O and in H_2O–MeCN (1:1) (Fig. S3).

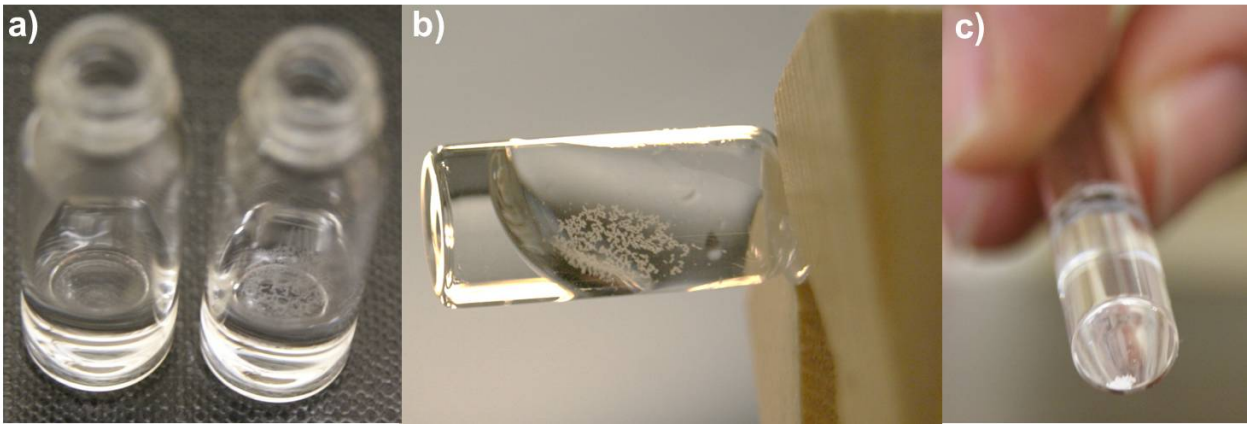

Fig. S1 Solubility trials in H_2O (0.72 mM) of: (a) Indomethacin (right) compared with branched PolyLys peptide conjugated to Indomethacin (left); (b) Indomethacin floating in aqueous media; (c) some Indomethacin solid precipitated at the bottom of the glass flask.

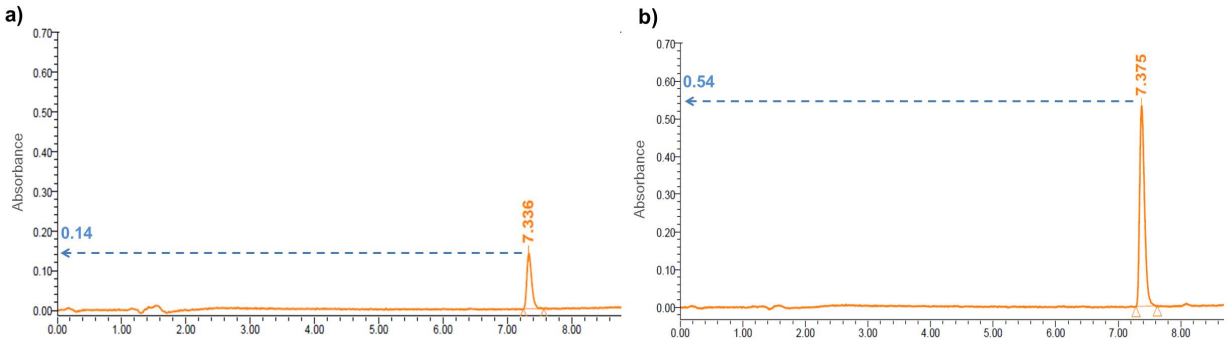

Fig. S2 HPLC chromatograms of Indomethacin at 0.72 mM in: (a) H_2O; (b) in H_2O–MeCN (1:1). Elution at a linear gradient of 20-90% MeCN containing 0.036% TFA into 0.045% aqueous TFA over 8 min.

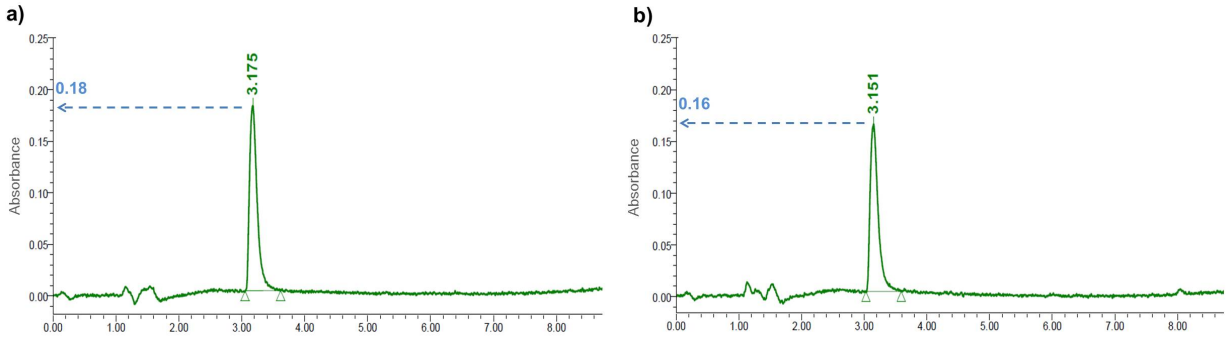

Fig. S3 HPLC chromatograms of branched PolyLys peptide conjugated to Indomethacin at 0.72 mM in: (a) H_2O; (b) in H_2O-MeCN (1:1). Elution at a linear gradient of 20-90% MeCN containing 0.036% TFA into 0.045% aqueous TFA over 8 min.

Publication IV

Organic LETTERS

Letter

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Semipermanent *C*-Terminal Carboxylic Acid Protecting Group: Application to Solubilizing Peptides and Fragment Condensation

Marta Paradís-Bas,[†,‡] Judit Tulla-Puche,*[,†,‡] and Fernando Albericio*[,†,‡,§,∥,⊥]

[†]Institute for Research in Biomedicine (IRB Barcelona) Barcelona, Baldiri Reixac 10, 08028 Barcelona, Spain

[‡]CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain

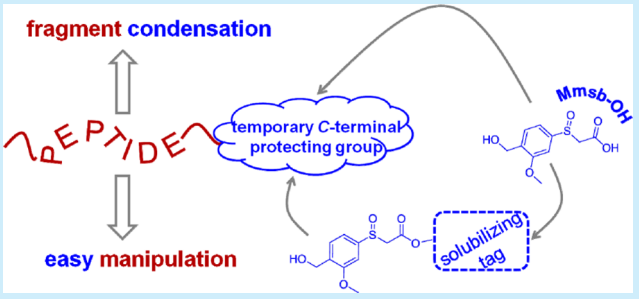
[§]Department of Organic Chemistry, University of Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain

[∥]School of Chemistry & Physics, University of Kwazulu-Natal, Durban 4001, South Africa

[⊥]School of Chemistry, Yachay Tech, Yachay City of Knowledge, Urcuquí 100119, Ecuador

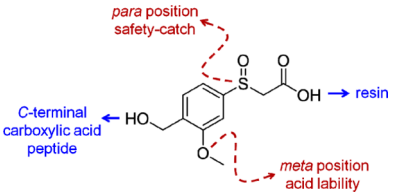
Supporting Information

ABSTRACT: The 2-methoxy-4-methylsulfinylbenzyl alcohol (Mmsb-OH) safety-catch linker has been described as a useful tool to overcome two obstacles in peptide synthesis: the solubility and fragment condensation of peptides. The incorporation of the linker into an insoluble peptide target, thereby allowing the conjugation of a poly-Lys as a "solubilizing tag", notably enhanced the solubility of the peptide. The selective conditions that remove that linker favored its incorporation as a semipermanent *C*-terminal protecting group, thereby allowing fragment condensation of peptides.



Peptides with a large number of nonpolar amino acids, self-assembling tendency, and highly structured or merely difficult sequences remain a challenge in terms of synthesis. One of the proposed strategies used to attain the synthesis of such molecules tackles the common limitation of insufficient solubility. In recent years, several synthetic modes aimed to enhance solubility have been described. Most are focused on the disruption of β-sheet interactions, which is achieved by using mainly backbone amide protecting groups, such as pseudoprolines[1] or others based on substituted benzyl group[2−4] or the depsipeptides grounded on the *O*−*N* intramolecular acyl migration strategies,[5] among others. Expensive presynthesized building blocks or the limitation of purification conditions associated with these strategies highlight the need to explore new tools to facilitate such syntheses. Although less studied, another peptide approach to increase solubility consists of the incorporation of a temporary *C*-terminal cationic peptide fragment attached to the native sequence by a cleavable linker, known as a "solubilizing tag". Although this kind of method has been used in the field of recombinant proteins for years,[6−8] only a few examples have been described for peptides.[9,10] There is currently no robust synthetic tool to address the increasing demand for peptide targets with inherent insolubility. In order to open the spectrum of applications in this area, and taking advantage of our previous experience in enhancing peptide solubility,[11] herein we sought to exploit the 2-methoxy-4-methylsulfinylbenzyl alcohol (Mmsb-OH) linker[12] (Scheme 1) as a *C*-terminal semipermanent protecting group.

Scheme 1. Mmsb-OH Linker Structure



Previous studies initiated by Englebretsen and Alewood[9] proposed the $(Gly\text{-}Arg)_4$ motif as a "solubilizing tag" linked to a sequence by the glycolic acid, and later Englebretsen[13] again, followed by Wade,[14] described a five Lys sequence to solubilize peptides linked through the base-labile 4-hydroxymethylbenzoic acid (HMBA) linker. Brimble[15] also used that last linker connected to a six Arg "solubilizing tag". However, the use of those linkers can lead to the formation of undesired aspartimide side products, which are favored by the basic cleavage conditions. To circumvent this issue and to increase the solubility of a given peptide, here we attached the sequence to a six Lys mer, as a "solubilizing tag", through the Mmsb-OH linker (Scheme 2). The peculiarity of this linker is that, in addition to being compatible with Boc/Bzl and Fmoc/*t*Bu solid phase peptide synthesis (SPPS) strategies, as a safety-catch[16,17] linker its cleavage is mediated after a selective chemical modification (in that case reductive acidolysis), thereby

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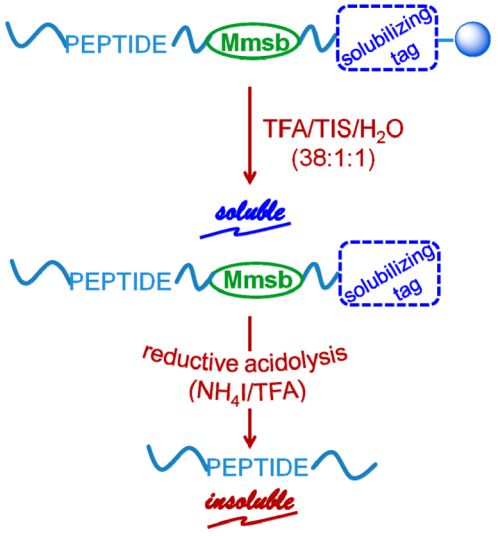
Organic Letters Letter

Scheme 2. Proposed Solubility Peptide Answer by Using a "Solubilizing Tag" Attached to the Mmsb-OH Linker



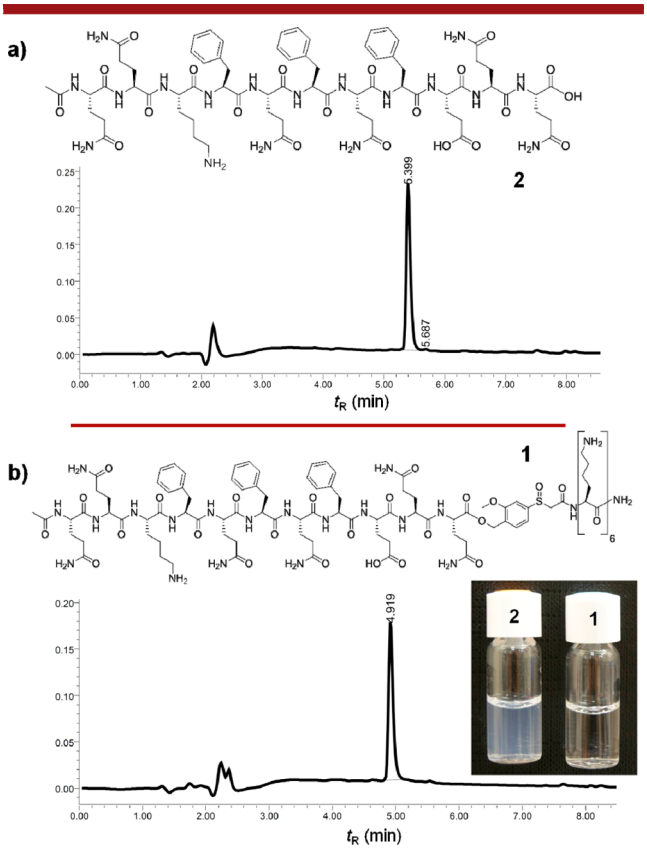
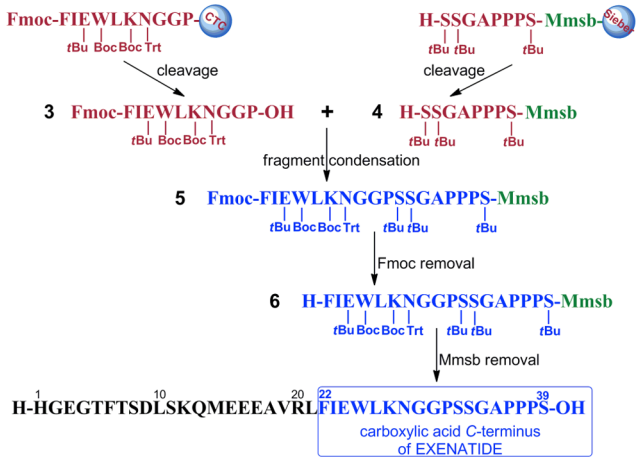


Figure 1. Structures and HPLC chromatograms, gradient from 10 to 60% B, of standard Q11 **2** (a) and solubilizing tag-linked Q11 peptide **1** (b). Solubility assays were done at 1 mg/mL in water.

avoiding basic conditions and consequently side reactions related to the same. Herein, we described for the first time its incorporation by the Fmoc/*t*Bu SPPS strategy. The insoluble target sequence Q11 (Ac-Gln-Gln-Lys-Phe-Gln-Phe-Gln-Phe-Glu-Gln-Gln-NH_2)[18] was selected to explore our proposal. This peptide is a self-assembling molecule with the capacity to form hydrogels. Extensive biomedical Q11 applications have been reported; therefore, synthetic methods to achieve the sequence are required.[19,20]

Scheme 3. Synthetic Design of Fragment Condensation to Obtain the Exenatide Fragment H-(^{22}Phe-^{39}Ser)-OH



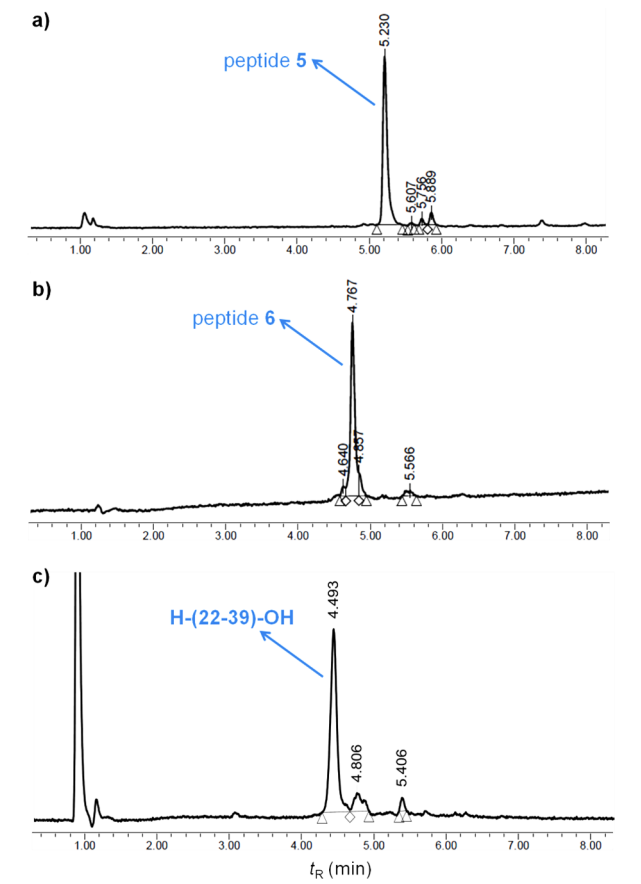


Figure 2. HPLC chromatograms of peptide crude products with unprotected side chains after fragment condensation, gradient from 30 to 80% B (a); Fmoc removal, gradient from 10 to 60% B (b); and Mmsb removal, gradient from 10 to 60% B (c).

The Mmsb-OH linker was synthesized by optimizing the strategy proposed by Thennarasu and collaborators[12] (see details in the Supporting Information). Only one purification step was required, and an overall yield of 28% was obtained.

Initially, the solubilizing tag-linked Q11 peptide (**1**) was synthesized by SPPS (see details in the Supporting Information) on a Rink-amide resin, after the coupling of six Fmoc-L-Lys(Boc)-OH amino acids using OxymaPure (3 equiv)

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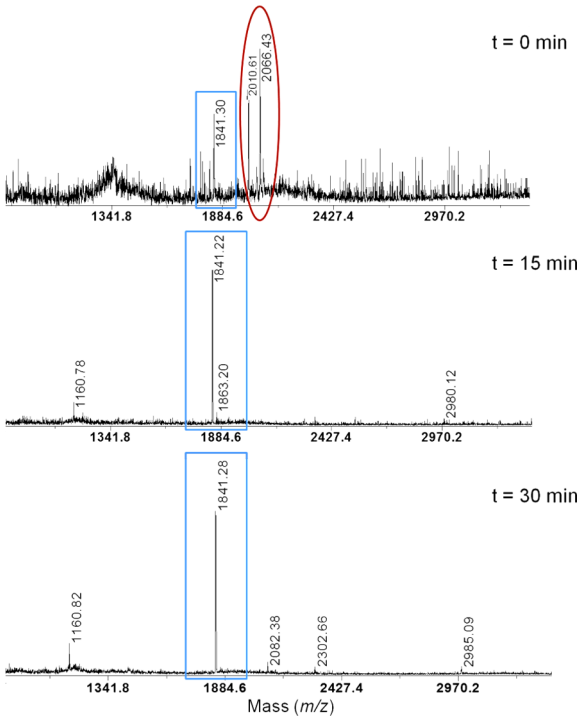

Figure 3. MS (MALDI-TOF) spectra of Mmsb removal monitored for peptide **6**. Starting material (**6**) (red) and final product H-(22−39)-OH (blue).

and DIPCDI (3 equiv) over 1 h. The Mmsb linker was introduced under mild conditions with HOSu (1.5 equiv) and DIPCDI (1.5 equiv) to avoid multiple incorporations. To complete the Q11 amino acid sequence, the same conditions used to couple the Lys were applied. After the final acetylation [by Ac_2O (5 equiv) and DIEA (5 equiv) over 30 min], the peptide was cleaved with TFA/TIS/H_2O (38:1:1) for 1 h, rendering the expected sequence with high purity (99.9%) (Figure 1b and Figure S2 in the Supporting Information), thereby confirming the stability of the Mmsb linker under the acidic cleavage conditions. Parallel to this synthesis, the carboxylic acid version of Q11, assigned as a standard (**2**), was synthesized on the same type of resin using the same coupling reagent conditions described before. After cleavage with TFA/TIS/H_2O (38:1:1) for 1 h, the peptide was precipitated with ether and lyophilized to afford the sequence, also with high purity (99.4%) (Figure 1a and Figure S1 in the Supporting Information). Although the HPLC chromatograms do not show big differences in polarity, the modified Q11 sequence (Figure 1b) showed enhanced solubility, which was attributed to the strategy used to attach the sequence to the Mmsb-$(Lys)_6$ "solubilizing tag".

In order to obtain the native Q11 from the solubilizing tag-linked Q11 peptide, the crude product was subjected to a reductive acidolysis treatment by NH_4I (30 equiv) in neat TFA (1 mg/mL) for 30 min. The ensuing MS analysis confirmed the total release of the Q11 sequence (Figure S8 in the Supporting Information) with high peptide purity (99.9%).

In addition to demonstrating the capacity of Mmsb to improve peptide properties, thereby facilitating its manipulation, and motivated by the possibility to have in our hands a potential *C*-terminal protecting group, we wanted to contribute with those already known carboxylic protecting groups for peptide synthesis.[21] Thereby, we addressed one of the SPPS's most important applications: fragment condensation, where the Mmsb would act as a *C*-terminal protecting group. Large peptide sequences sometimes require the use of sophisticated methods to assemble the peptide fragments in solution in order to reach the final target. One of the most common strategies for this purpose is to prepare peptide fragments for ensuing coupling in solution.[22] The need for an appropriate combination of protecting groups is crucial, and in this regard, the Mmsb-OH, as a carboxylic protecting group, is highly suitable for this strategy. We selected the well-known peptide Exenatide to test the conjugation. This compound is of relevance because of its use in diabetes therapy.[23] Specifically, the synthesized sequence was the acid carboxylic version of *C*-terminal Exenatide (NH_2-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-OH), which was assembled from two presynthesized peptide fragments (Scheme 3).

The separation of the fragments, in terms of retrosynthetic analysis, was chosen between ^{31}Pro and ^{32}Ser. First, Pro is a cyclic amino acid that does not allow racemization during fragment condensation; second, we took advantage of preceding literature regarding Exenatide synthesis.[24] Initially, peptide fragments Fmoc-(22−31)-OH (**3**) and H-(32−39)-Mmsb (**4**) were synthesized separately by the *t*Bu/Fmoc SPPS strategy on CTC[25] and Sieber[26] resin (see Supporting Information for details), respectively. Both resins allow synthesis of peptides with fully protected side chains. After cleavage with a reduced amount of TFA (1% in the case of CTC and 3% in the case of Sieber), the two peptides were lyophilized and analyzed by HPLC and MS, both techniques confirming the expected sequences were properly protected (see Figures S3 and S4 in the Supporting Information). Fragment condensation of the two peptides was performed using H-(32−39)-Mmsb (1.5 equiv) and Fmoc-(22−31)-OH (1 equiv) coupled to PyBOP (1 equiv) and HOBt (1 equiv) at pH 8−9 in DMF. The reaction was stopped at 7 h. After lyophilizing the crude product (**5**), the expected peptide was obtained with 92% HPLC purity (Figure 2a and Figure S5 in the Supporting Information).

Fmoc was removed with diethylamine to facilitate the analysis, rendering the expected protected sequence (**6**) with 91% HPLC purity (Figure 2b and Figure S6 in the Supporting Information). Finally, in a one-pot reaction by reductive acidolysis, the side chains and the Mmsb *C*-terminal protecting groups were removed (Figure 3) to afford the expected H-(22−39)-OH peptide fragment with 85% HPLC purity (Figure 2c and Figure S9 in the Supporting Information). In this case, because of the presence of Trp (an amino acid that is known to be susceptible to alkylation), diisopropyl sulfide was added as a scavenger.[27] Again, the total stability of Mmsb during fragment condensation and also its application in convergent strategies to attain large peptide sequences were demonstrated.

In summary, we synthesized the Mmsb-OH linker in a straightforward manner in five steps and only one purification step. The linker was later incorporated into a peptide sequence by SPPS, the first time applied in the Fmoc/*t*Bu strategy. Mmsb-OH has been exploited in two important aspects focused on the development of new tools for peptide synthesis. First, Mmsb-OH serves as a tool to enhance the solubility of peptides, such as Q11, through the incorporation of a "solubilizing tag". Second, based on the carboxylic acid protection, the linker has allowed the synthesis of a large fragment of Exenatide by fragment condensation. This

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achievement opens the possibility of using the Mmsb linker strategy to synthesize other peptides with difficult sequences.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, NMR characterization of Mmsb synthesis, HPLC and MS peptides characterization, and solubility data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: judit.tulla@irbbarcelona.org.
*E-mail: albericio@irbbarcelona.org.

Notes

The authors declare no competing financial interest.

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Supporting Information

Semipermanent *C*-Terminal Carboxylic Acid Protecting Group: Application to Solubilizing Peptides and Fragment Condensation

Marta Paradís-Bas,[†,‡] Judit Tulla-Puche,*[,†,‡] and Fernando Albericio*[,†,‡,§,∥,⊥]

[†]Institute for Research in Biomedicine (IRB Barcelona) Barcelona, Baldiri Reixac 10, 08028 Barcelona, Spain; [‡]CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain; [§]Department of Organic Chemistry, University of Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain; [∥]School of Chemistry & Physics, University of Kwazulu-Natal, Durban 4001, South Africa; [⊥]School of Chemistry, Yachay Tech, Yachay City of Knowledge, Urcuquí 100119, Ecuador

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Abbreviations

AA, amino acid; *t*Bu, *tert*-butyl; CTC, 2-chlorotrityl chloride; DIPCDI, *N,N'*-diisopropylcarbodiimide; DIEA, *N,N'*-diisopropylethylamine; DMF, *N,N'*-dimethylformamide; DMAP, *N,N'*-dimethylpyridin-4-amine; Fmoc, 9-fluorenylmethyl carbamate; HFIP (1,1,1,3,3,3-hexafluoro-2-propanol); HOBt, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; OxymaPure, ethyl 2-cyano-2-(hydroxyimino)acetate; PyBOP, benzotriazol-1-yloxytri(pyrrolidino)phosphonium hexafluorophosphate; TIS, triisopropylsilane ; TFA, trifluoroacetic acid.

Materials and General Methods

All Fmoc-L-AA-OH, HOBt and the CTC resin were purchased from Iris Biotech. Sieber resin and DMAP were purchased from Novabiochem. Coupling systems such as DIPCDI, OxymaPure, PyBOP, HOSu and DIEA were from Sigma-Aldrich. Solvents used in SPPS, CH_2Cl_2 and DMF were peptide synthesis-grade from Carlo Erba-SdS. Acetylation (acetic anhydride), cleavage reagents [TIS and TFA (HPLC-grade)] and also the solvent HFIP were purchased from Sigma-Aldrich.

Solid-Phase syntheses were performed manually in polypropylene syringes fitted with a polyethylene porous disc. Solvents and soluble reagents were removed by suction.

Peptide crudes were dissolved commonly in H_2O/MeCN (1:1) to be analyzed by HPLC, MALDI-TOF MS or HPLC-ESI MS. When peptides were not soluble in those conditions, the solvent used to dissolve them is specified in the peptide analysis section.

HPLC characterizations were carried out on Waters instrument comprising a separation module (Waters 2695) and a photodiode array detector (Waters 2998) with a software system controller Empower. The analytical column used to characterize the synthesized Mmsb-OH and the peptide crudes was a reverse-phase C18 column (XBridge^{TM} BEH130, 4.6 x 100 mm, 3.5 μm). Common column temperature analysis used was 25 ºC and when T = 60 ºC was required, it has been specified in the peptide analysis section. UV detection was measured at 220 nm for peptides and measured by a maximum of absorbance average in case of Mmsb-OH synthesis. Flow rate was at 1 mL/min. The eluent system used in all HPLC conditions was based in linear gradients of eluent B (MeCN + 0.036% TFA) into eluent A (H_2O + 0.045% TFA) over 8 min.

Mass analyses of peptides were performed by the matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) on an Applied Biosystems Voyager-DE RP instrument, using α-cyano-4-hydroxycinamic acid as a matrix dissolved in H_2O/MeCN (1:1) containing 0.1% of TFA. Mass analyses of synthesized Mmsb and its intermediates were carried out on a Waters instrument in an

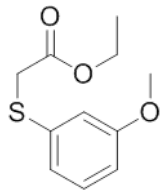
analytical RP-HPLC-ESMS with a Micromass ZQ spectrometer comprising a separation module (Waters 2695), an automatic injector (Waters 717 autosampler), a photodiode array detector (Waters 2998) and a software system controller MassLynx v. 4.1. The analytical column was a reverse-phase C18 column (SunFire^{TM}, 2.1 x 100 mm, 5 μm). UV detection was measured at 220 nm, mass scans were acquired in positive ion mode. Flow rate was at 0.3 mL/min. The eluent system used was based on linear gradients of eluent MeCN (+ 0.07% formic acid) into H_2O (+ 0.1% formic acid) over 8 min.

^{1}H and ^{13}C NMR spectra were recorded on a Varian MERCURY 400 (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR) spectrometer. Chemical shifts (δ) are expressed in parts per million downfield from tetramethylsilyl chloride. Coupling constants are expressed in Hertz.

Experimental Section

Synthesis of Mmsb-OH Linker

Ethyl 2-[(3-methoxyphenyl)thio]acetate



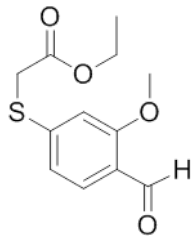
3-methoxythiophenol (1.77 mL, 14.3 mmol) was dissolved in CH_2Cl_2 (7 mL) and after cooling the solution in an ice-water bath, ethyl 2-bromoacetate (1.77 mL, 15.7 mmol) was added. Then, triethylamine (2.19 mL, 14.3 mmol) was added dropwise (to avoid dimerization of starting material) and the mixture was warmed to room temperature and stirred for 30 min (monitored by HPLC). After completion of the reaction, the mixture was washed with H_2O (3 × 10 mL) and brine (3 × 10 mL). The organic layer was dried ($MgSO_4$), filtered and then, the solvent was removed under reduced pressure to afford the expected compound as a yellow oil (3.07 g, 95%), pure enough (99%) to be used in the following reaction without further purification.

HPLC analysis (t_R = 3.61 min; gradient from 50% to 100% B over 8 min).

^{1}H NMR (400 MHz, $CDCl_3$; Me_4Si): δ 7.21 (t, J = 7.9 Hz, 1H), 6.99 – 6.94 (m, 2H), 6.76 (ddd, J = 8.3, 2.4, 1.0 Hz, 1H), 4.18 (q, J = 7.2 Hz, 2H, CH_2), 3.79 (s, 3H, OCH_3), 3.64 (s, 2H, SCH_2), 1.24 (t, J = 7.1 Hz, 3H, CH_3).

^{13}C NMR (100 MHz, CDCl3): δ 169.78 (C), 160.00 (C), 136.50 (C), 129.96 (CH), 121.86 (CH), 115.03 (CH), 112.87 (CH), 61.72 (CH_2), 55.42 (OCH_3), 36.65 (CH_2), 14.23 (CH_3).

Ethyl 2-[(4-formyl-3-methoxyphenyl)thio]acetate



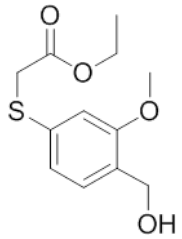
Freshly Vilsmeier reagent (6 equiv) was prepared as follows: in a 100 mL round-bottomed flask, phosphorous oxychloride (8 mL, 89.7 mmol) was added followed by dry CH_2Cl_2 (19 mL) under an argon atmosphere. The solution was cooled to 10 ºC with an ice-water bath and a solution of dry DMF (5.11 mL, 65.8 mmol) in dry CH_2Cl_2 (19 mL) was added dropwise. After the Vilsmeier reagent was prepared, a solution of ethyl 2-[(3-methoxyphenyl)thio]acetate (3.38 g, 14.9 mmol) in dry CH_2Cl_2 (4.7 mL) was added slowly. The ice bath was removed and the solution was heated under reflux to 60 ºC and stirred for 24 h. After the reaction mixture was cooled to room temperature, the mixture was poured carefully into crushed ice and the product was extracted with CH_2Cl_2 (3 × 30 mL). The organic layers were combined, dried ($MgSO_4$), filtered and then, the solvent was evaporated under reduced pressure. Residual DMF was removed by co-evaporation with toluene, CH_2Cl_2 and diethyl ether consecutively. The resulting pale brown solid contains as a major isomer the expected product in 66.7% purity. A portion of this crude (2.5 g) was subjected to purification by CombiFlash Rf 200 system (reverse phase C18 silica, linear gradient from (B/A) 5:95 to 45:55 over 45 min, then isocratic 45:55 over 15 min) to yield the expected compound, after lyophilization, as a white solid (974 mg, non optimized 39% yield).

HPLC analysis (t_R = 2.89 min; gradient from 50% to 100% B over 8 min). The expected product shows an HPLC-MS (ESI): *m/z* calcd for $C_{12}H_{14}O_4S$: 254.06, found: 183.21 $[M + H]^+$. The main impurity detected (HPLC t_R = 2.71 min; gradient from 50% to 100% MeCN over 8 min) corresponds to the other isomer [ethyl 2-[(2-formyl-5-methoxyphenyl)thio]acetate], HPLC-MS (ESI): *m/z* calcd for $C_{12}H_{14}O_4S$: 254.06, found: 254.85 $[M + H]^+$.

^{1}H NMR (400 MHz $CDCl_3$; Me_4Si): δ 10.34 (s, 1H, CHO), 7.73 (d, J = 8.2 Hz, 1H), 6.95 (d, J = 1.6 Hz, 1H), 6.92 (dd, J = 8.2, 1.6 Hz, 1H), 4.21 (q, J = 7.1 Hz, 2H, CH_2), 3.92 (s, 3H, OCH_3), 3.74 (s, 2H, SCH_2), 1.26 (t, J = 7.1 Hz, 3H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$): δ 188.86 (CHO), 169.16 (C), 161.87 (C), 146.15 (C), 129.08 (CH), 122.70 (C), 118.76 (CH), 109.85 (CH), 62.13 (CH_2), 55.88 (OCH_3), 34.84 (CH_2), 14.24 (CH_3).

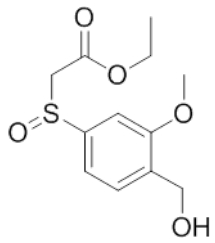
Ethyl 2-[(4-(hydroxymethyl)-3-methoxyphenyl)thio]acetate



Fresh sodium triacetoxyborohydride reducing agent (3 equiv) was prepared as follows: $NaBH_4$ (223 mg, 5.9 mmol) was suspended in ethyl acetate (45 mL), stirred and cooled with an ice-water bath. Then, glacial acetic acid (1 mL, 6 mmol) was added slowly and stirred for 10 min. After the reducing agent was prepared, the previously prepared aldehyde (500 mg, 1.9 mmol) was dissolved in the minimum quantity of ethyl acetate and the solution was added dropwise (over 20 min). The mixture was warmed to room temperature and stirred for 2 h (monitored by HPLC). After completion of the reaction, the mixture was washed with H_2O (3 × 50 mL) and brine (3 × 50 mL). The organic layer was dried ($MgSO_4$), filtered and then, the solvent was removed under reduced pressure to afford the expected compound as a white solid (475.7 mg, 95%), pure enough (98%) to be used in the following reaction without further purification.

HPLC analysis (t_R = 4.07 min; gradient from 30% to 80% B over 8 min).
^{1}H NMR (400 MHz, $CDCl_3$; Me_4Si): δ 7.21 (d, J = 8.0 Hz, 1H), 6.99 – 6.95 (m, 2H), 4.63 (s, 2H, CH_2OH), 4.17 (q, J = 7.1 Hz, 2H, CH_2), 3.85 (s, 3H, OCH_3), 3.63 (s, 2H, SCH_2), 1.24 (t, J = 7.1 Hz, 3H, CH_3).
^{13}C NMR (100 MHz, $CDCl_3$): δ 169.84 (CO), 157.65 (C), 135.81 (C), 129.23 (CH), 128.21 (C), 121.95 (CH), 112.06 (CH), 61.76 (CH_2), 61.67 (CH_2), 55.56 (OCH_3), 36.91 (SCH_2), 14.25 (CH_3).

Ethyl 2-[(4-(hydroxymethyl)-3-methoxyphenyl)sulfinyl]acetate



Acetic acid (27 mL) was added to dissolve the previously prepared alcohol (464.5 mg, 1.8 mmol). A solution of aqueous hydrogen peroxide (30%) (5.89 mL, 196.5 mmol) was slowly added to the reaction mixture while stirring until total conversion[1] (confirmed by HPLC). The mixture was poured onto crushed ice and the product was extracted with CH_2Cl_2 (3 × 40 mL). The organic layers were combined and dried ($MgSO_4$). The organic solvent was removed under reduced pressure. Acetic acid was removed by co-evaporation with toluene, dichloromethane and diethyl ether to afford the expected compound as a white solid (490 mg, 99.3%). This product was pure enough (99%) to be used in the following reaction without further purification.

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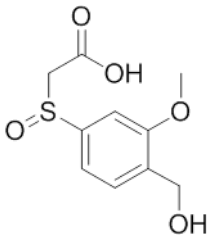
HPLC analysis (t_R = 2.98 min; gradient from 20% to 50% B for 8 min).
^{1}H NMR (400 MHz, $CDCl_3$; Me_4Si): δ 7.46 (d, J = 7.7 Hz, 1H), 7.28 (d, J = 1.5 Hz, 1H), 7.14 (dd, J = 7.7, 1.5 Hz, 1H), 4.71 (s, 2H, CH_2OH), 4.17 (q, J = 7.1 Hz, 2H, CH_2), 3.92 (s, 3H, OCH_3), 3.73 (dd, J = 76.4, 13.7 Hz, 2H, $SOCH_2$), 1.24 (t, J = 7.1 Hz, 3H, CH_3).
^{13}C NMR (100 MHz, $CDCl_3$): δ 164.87 (CO), 158.22 (C), 143.53 (C), 133.47 (C), 128.82 (CH), 116.59 (CH), 105.22 (CH), 62.25 (CH_2), 62.11 (CH_2), 61.16 (CH_2), 55.92 (OCH_3), 14.18 (CH_3).

2-[(4-(hydroxymethyl)-3-methoxyphenyl)sulfinyl]acetic acid [Mmsb-OH]



The compound previously prepared (464.8 mg, 1.7 mmol) was dissolved in THF/H_2O (2:1) (20 mL) and LiOH (215 mg, 5.1 mmol) was slowly added, and stirred for 30 min (monitored by HPLC). The mixture was acidified with the aid of HCl (0.2 M) to pH 1 and the product was extracted with CH_2Cl_2 (3 × 25 mL). Organic layers were combined, dried ($MgSO_4$), filtered, and the solvent was removed under reduced pressure affording the expected compound as a white solid (79.4%). This product was pure enough (99%) to be used on solid-phase peptide synthesis without further purification. The overall yield to convert **3-methoxythiophenol** to **Mmsb-OH** was 28%.

HPLC analysis (t_R = 3.90 min; gradient from 5% to 40% B for 8 min). The main product corresponds to the expected final Mmsb-OH, HPLC-MS (ESI): *m/z* calcd for $C_{10}H_{12}O_5S$: 244.04, found: 244.96 $[M + H]^+$.
^{1}H NMR (400 MHz, CD_3OD; Me_4Si): δ 7.60 (d, J = 7.8 Hz, 1H), 7.37 – 7.27 (m, 2H), 4.89 (s, 2H, CH_2OH), 4.67 (s, 2H, SCH_2), 3.92 (s, 3H, OCH_3).
^{13}C NMR (101 MHz, CD_3OD): δ 168.05 (COOH), 158.91 (C), 143.38 (C), 135.48 (C), 129.18 (CH), 117.52 (CH), 106.47 (CH), 62.33 (CH_2), 59.87 (CH_2), 56.22 (OCH_3).

Solid-Phase Peptide Synthesis

A) Synthesis of Q11 and the "solubilizing tag" Mmsb-analogue

The two peptides: the standard Q11 (**2**) and the solubilizing tag-linked Q11 (**1**) were synthesized by using standard Fmoc/*t*Bu strategy on aminomethyl-ChemMatrix resin (50 mg, 0.66 mmol/g). The resin was conditioned by washings with TFA/CH_2Cl_2 (1:99) (5 x 0.5 min), CH_2Cl_2 (5 x 0.5 min), DIEA/CH_2Cl_2 (1:19) (5 x 0.5 min) and CH_2Cl_2 (5 x 0.5 min). Previously to amino acids incorporation, the resin was functionalized by coupling the commercial [3-(4-hydroxymethylphenoxy)-propionic acid linker, in case of standard Q11 (**2**); and Fmoc-Rink amide linker, in case of solubilizing tag-linked Q11

(**1**)]. Both the commercial linker and the common amino acids (Fmoc-L-AA-OH) were incorporated using 3 equiv, DIPCDI (3 equiv) and OxymaPure (3 equiv) in DMF, with a 5-min preactivation and a total coupling time of 1 h. After every incorporation the resin was washed with DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min). Then, a Kaiser test was carried out to check which amino acid was not completely coupled. In the case of incomplete couplings, the same conditions were applied to repeat the incorporation. Fmoc removal was performed with piperidine/DMF (1:4) (25 mL/g resin, 1 × 1 min, 2 × 5 min), followed by resin washings with DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min) and DMF (3 × 1 min). The cycle of AA coupling/Fmoc removal was performed until complete elongation of peptides.

Incorporation of Mmsb-OH linker

The incorporation of the synthesized Mmsb-OH linker for the solubilizing tag-linked Q11 was carried out under mild conditions: Mmsb (1.5 equiv), *N*-hydroxysuccinimide (HOSu) (1.5 equiv) and DIPCDI (1.5 equiv) in DMF for 30 min. Kaiser test was carried out to check its complete incorporation.

Ester bond formation on previously incorporated Mmsb-OH linker

Ester bond formation between the amino acid and the alcohol functionalized linkers [commercial 3-(4-hydroxymethylphenoxy)-propionic acid or synthesized Mmsb-OH] was carried out using the symmetrical anhydride conditions: Fmoc-AA-OH (4 equiv), DIPCDI (2 equiv) catalyzed by DMAP (0.2 equiv) in CH_2Cl_2. After 3 h of reaction, a second incorporation using fresh reagents, was performed for 15 h.

Final acetylation and cleavage/global deprotection of peptides

After Fmoc removal of the last amino acid, acetylation of *N*-terminal amino acid was carried out with acetic anhydride (10 equiv) and DIEA (10 equiv) for 30 min, followed by resin washings with DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min). Kaiser test confirmed the completed reaction. Then, the resin was subjected to global deprotection/cleavage by treatment with TFA/TIS/H_2O (38:1:1) for 1 h. The mixture was partially evaporated under reduced pressure and the peptides were precipitated with cold diethyl ether. The liquid layer was removed by centrifugation and the solids were washed with cold diethyl ether to give white solids that were dissolved in H_2O/MeCN (1:1) and lyophilized. The two peptide crudes were analyzed by HPLC and MS (MALDI-TOF) (see Figure S1 and S2).

Removal of Mmsb moiety

The Mmsb moiety of solubilizing tag-linked Q11 (**1**) was removed under the following conditions: 1 mg of peptide crude **1** was dissolved in neat TFA (1 mL/mg) and NH_4I (1.7 mg, 11.9 μmol) was added and stirred at room temperature. The reaction was monitored by MALDI-TOF MS (see Figure S7) and, after 60 min, the reaction was stopped, although by MALDI-TOF no starting material was observed after 30 min. The work-up was accomplished by filtering the solution to remove iodine impurities, evaporating the solvent under reduced pressure, further precipitation and washings of the peptide with cold diethyl ether. Characterization of the final unprotected peptide was performed by HPLC and MS (MALDI-TOF) (see Figure S8).

B) Synthesis of Exenatide fragments Fmoc-(22-31)-OH and H-(32-39)-Mmsb

1) Synthesis of Fmoc-(22-31)-OH (3)

N-terminal fragment Fmoc-(22-31)-OH (**3**) was synthesized by using standard Fmoc/*t*Bu strategy on 2-chlorotrityl chloride resin (CTC) (100 mg, 1.55 mmol/g). The resin was conditioned by washings with DMF (3 × 2 min) and CH_2Cl_2 (3 × 1 min). The first Fmoc-Pro-OH (1 mmol/g resin) was incorporated in presence of DIEA (10 equiv), which was added in two portions: first 1/3 of the volume was added and after 10 min, the remaining 2/3. The mixture was allowed to react for 45 min. Next, a capping step with MeOH (0.4 mL/g resin) was performed for 10 min. Fmoc removal was performed with piperidine/DMF (1:4) (25 mL/g resin, 1 × 1 min, 2 × 5 min), followed by resin washings with DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min) and DMF (3 × 1 min). Other Fmoc-L-AA-OH were coupled using 3 equiv, DIPCDI (3 equiv) and OxymaPure (3 equiv) in DMF, with a 5-min preactivation and a total coupling time of 1 h. After each incorporation, the resin was washed with DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min). Then, a Kaiser test was carried out to check which amino acid was not completely coupled. In the case of incomplete couplings, the same conditions were applied to repeat the incorporation. The cycle of AA coupling/Fmoc removal was performed until the elongation of peptide was complete. The last Fmoc group was maintained at the *N*-terminal position for fragment condensation step.

Cleavage of N-terminal peptide fragment

The peptide **3** was cleaved from the resin with TFA/CH_2Cl_2 (1:99) (8 × 0.5 min) and CH_2Cl_2 (3 × 1 min), and collected onto H_2O to avoid side-chain deprotection. The solvent was removed under reduced pressure and finally, the peptide was lyophilized,

affording the fully protected peptide **3** as a white solid, which was analyzed by HPLC and MS (MALDI-TOF) (see Figure S3).

2) Synthesis of H-(32-39)-Mmsb (4)

The *C*-terminal fragment H-(32-39)-Mmsb (**4**) was synthesized by using standard Fmoc/*t*Bu strategy on Fmoc-Sieber-amide aminomethyl polystyrene resin (100 mg, 0.69 mmol/g). The resin was conditioned by washings with DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min). Fmoc removal of Sieber linker was performed with piperidine/DMF (1:4) (25 mL/g resin, 1 × 1 min, 2 × 5 min), followed by resin washings with DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min) and DMF (3 × 1 min). Common amino acids (Fmoc-L-AA-OH) were incorporated using 3 equiv, DIPCDI (3 equiv) and OxymaPure (3 equiv) in DMF, with a 5-min preactivation and a total coupling time of 1 h. After every incorporation, the resin was washed with DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min). Then, a Kaiser test was carried out to check which amino acid was not completely coupled. In the case of incomplete couplings, the same conditions were applied to repeat the incorporation.

Incorporation of Mmsb-OH linker

After removing the Fmoc group of Sieber resin, the incorporation of synthesized Mmsb linker was performed with the same conditions described for the Q11 Mmsb-containing linker analogue: Mmsb-OH (1.5 equiv), *N*-hydroxysuccinimide (HOSu) (1.5 equiv) and DIPCDI (1.5 equiv) in DMF for 30 min. Kaiser test was carried out to check its complete incorporation.

Ester bond formation on previously incorporated Mmsb-OH linker

Ester bond formation between the amino acid and the alcohol group of Mmsb-OH linker was carried out using the same conditions described by Q11 Mmsb-analogue by using symmetrical anhydride conditions: Fmoc-AA-OH (4 equiv), DIPCDI (2 equiv), catalyzed by DMAP (0.2 equiv) in CH_2Cl_2. After 3 h of reaction, a second incorporation for 15 h was performed.

Cleavage of the C-terminal peptide fragment

The peptide was cleaved from the resin with TFA/CH_2Cl_2 (1:32) (8 × 1 min) and CH_2Cl_2 (3 × 1 min), and collected onto H_2O to avoid side-chain deprotection. The solvent was removed under reduced pressure and finally, the peptide was lyophilized affording the fully protected peptide **4** as a white solid, which was analyzed by HPLC and MS (MALDI-TOF) (see Figure S4).

Fragment Condensation to Afford Exenatide Fragment H-(22-39)-OH

Synthesis of Fmoc-(22-39)-Mmsb (5)

To a solution of the *N*-terminal fragment Fmoc-(22-31)-OH (**3**) (11.5 mg, 6.1 μmol) with HOBt (1 mg, 6.1 μmol) in DMF (150 μL), a solution of *C*-terminal fragment H-(32-39)-Mmsb (**4**) (10 mg, 9.2 μmol) in DMF (40 μL) was added. Then, the coupling reagent PyBOP (4.1 mg, 7.9 μmol) dissolved in DMF (40 μL) was added to the mixture and finally the pH was adjusted with DIEA to 8-9. The mixture was stirred at room temperature for 7 h (monitored by HPLC). After completion of the reaction, the mixture was directly lyophilized to afford the Exenatide fragment Fmoc-(22-39)-Mmsb (**5**) as a white solid, which was analyzed by HPLC and MS (MALDI-TOF) (see Figure S5).

Synthesis of H-(22-39)-Mmsb (6)

The Fmoc removal of Fmoc-(22-39)-Mmsb (**5**) (7 mg, 2.45 μmol) was performed in solution by using diethylamine (5 μL, 73.5 μmol) in CH_2Cl_2 (30 μL). The mixture was stirred at room temperature for 4 h. After the solvent removal, the peptide was washed with diethyl ether (3 x 1 mL) to dissolve and remove the dibenzofulvene adduct. H_2O was added to wash the solid (3 x 1 mL) and to allow the removal of the residual HOBt from the previous reaction. The white solid was lyophilized and analyzed by HPLC and MS (MALDI-TOF) (see Figure S6).

Removal of the Mmsb moiety: Synthesis of Exenatide Fragment H-(22-39)-OH

1 mg of peptide **6** was dissolved in neat TFA (1 mL/mg). In this case diisopropyl sulfide (10 μL, 1%, 68.8 μmol) was added because of the presence of tryptophan in the sequence (susceptible to suffer alkylation) and then, NH_4I (1.6 mg, 11.4 μmol) was added and stirred at room temperature. The reaction was monitored by MALDI-TOF MS and after 45 min the reaction was stopped, although no starting material was observed by MALDI-TOF after 15 min. The work-up was accomplished by filtering the solution to remove iodine impurities, evaporating the solvent under reduced pressure, further precipitation and washings of the peptide with cold diethyl ether. Characterization of the final unprotected peptide was performed by HPLC and MS (MALDI-TOF) (see Figure S9).

Peptide Analysis

Standard Q11 (2)

HPLC analysis of Standard Q11 crude **2** after global deprotection/cleavage from the resin, and further dissolution in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (t_R = 5.39 min; gradient from 10% to 60% B over 8 min) shows one main product (99.4%, HPLC purity) which corresponds to the expected Standard Q11 (**2**), MS (MALDI-TOF) (Figure S1): *m/z* calcd for $C_{70}H_{98}N_{18}O_{21}$: 1526.71, found: 1527.77 $[M + H]^+$, 1549.76 $[M + Na]^+$.

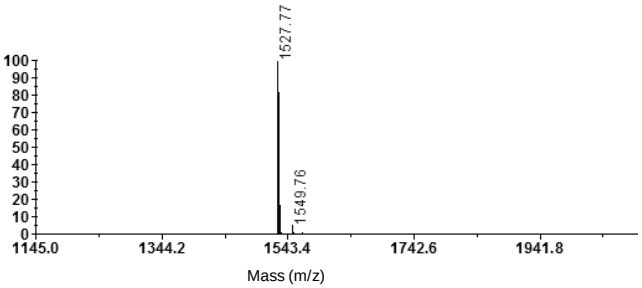


Figure S1. MALDI-TOF MS spectrum of Standard Q11.

Solubilizing tag-linked Q11 (1)

HPLC analysis of Solubilizing tag-linked Q11 crude **1** after global deprotection/cleavage from the resin, and further dissolution in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (t_R = 4.91 min; gradient from 10% to 60% B over 8 min) shows one main product (99.9%, HPLC purity) which corresponds to the expected Solubilizing tag-linked Q11 (**1**), MS (MALDI-TOF) (Figure S2): *m/z* calcd for $C_{116}H_{181}N_{31}O_{30}S$ 2520.33, found: 2521.60 $[M + H]^+$. Other *m/z* found: 828.68 $[M + H]^+$ is associated to a decomposition of Mmsb moiety linked to the peptide which releases the sulfonyl derivative and the Ac-$(Lys)_6$-NH_2 (calcd for $C_{38}H_{77}N_{13}O_7$ 827.61) caused by MALDI-TOF analysis (detected also in other Mmsb-analogues).

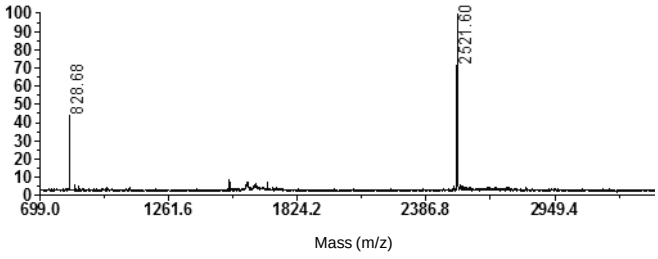


Figure S2. MALDI-TOF MS spectrum of Solubilizing tag-linked Q11.

N-terminal fragment Fmoc-(22-31)-OH (3)

HPLC analysis (Figure S3a) of fragment Fmoc-(22-31)-OH crude (**3**), with fully protected side-chains, dissolved in HFIP (t_R = 3.51 min; gradient from 90% to 100% B over 8 min) shows one main product (97%, HPLC purity). However, it was required to treat some portion with TFA/TIS/H_2O (38:1:1) for 1 h to remove the protecting groups and facilitate the detection by MS. HPLC analysis (Figure S3b) of fragment Fmoc-(22-31)-OH crude with unprotected side-chains (t_R = 5.91 min; column temperature at 60 ºC; gradient from 30% to 80% B over 8 min) shows one main product (99%, HPLC purity) which corresponds to the Fmoc-(22-31)-OH, MS (MALDI-TOF) (Figure S3c): *m/z* calcd for $C_{71}H_{91}N_{13}O_{16}$ 1381.67, found: 1382.81 $[M + H]^+$, 1404.82 $[M + Na]^+$. Some impurity, not observed in the HPLC, which corresponds to the peptide without the Fmoc protecting group is detected by MS (MALDI-TOF) (Figure S3c): *m/z* calcd for $C_{56}H_{81}N_{13}O_{14}$ 1159.60, found: 1160.7 $[M + H]^+$, 1182.71 $[M + Na]^+$.

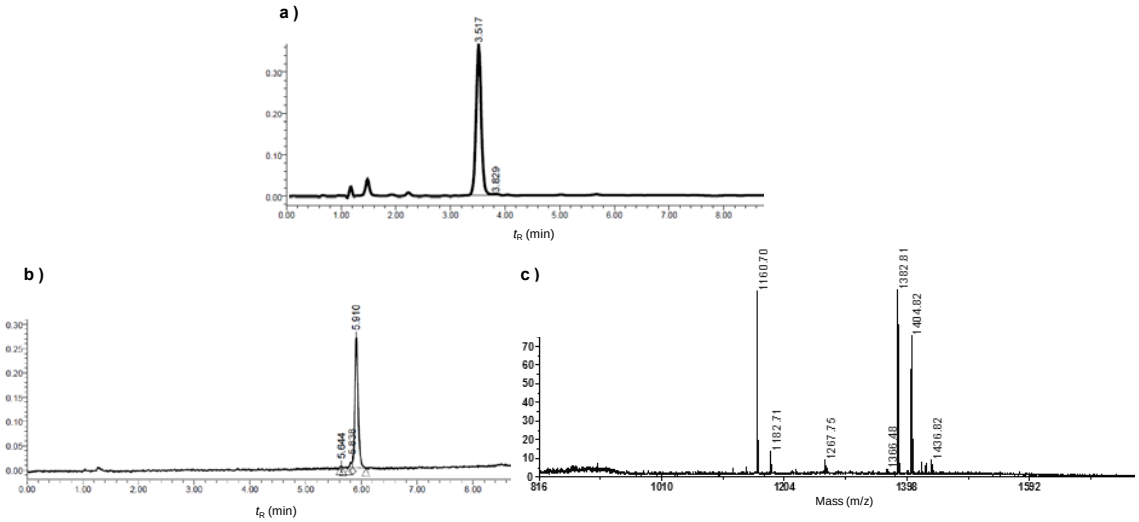


Figure S3. Analysis of *N*-terminal fragment Fmoc-(22-31)-OH: HPLC chromatogram with protected side-chains (a); HPLC chromatogram with unprotected side-chains (b); and MALDI-TOF MS spectrum of unprotected side-chains fragment (c).

C-terminal fragment H-(32-39)-Mmsb (4)

HPLC analysis (Figure S4a) of fragment H-(32-39)-Mmsb crude **4** with fully protected side-chains (t_R = 6.16 min; column temperature at 60 ºC; gradient from 10% to 60% B over 8 min) shows one main product (99%, HPLC purity) which corresponds to the expected *C*-terminal fragment H-(32-39)-Mmsb, MS (MALDI-TOF) (Figure S4b): *m/z* calcd for $C_{51}H_{81}N_9O_{15}S$ 1091.56, found: 1092.57 $[M + H]^+$, 1114.59 $[M + Na]^+$ and 1130.57 $[M + K]^+$. Other *m/z* found: 1036.51 is associated to a decomposition of Mmsb moiety linked to the peptide which releases the sulfonyl derivative (56 units less, calcd for $C_{49}H_{80}N_8O_{14}S$ 1036.55) caused by MALDI-TOF analysis (detected also in other Mmsb-analogues).

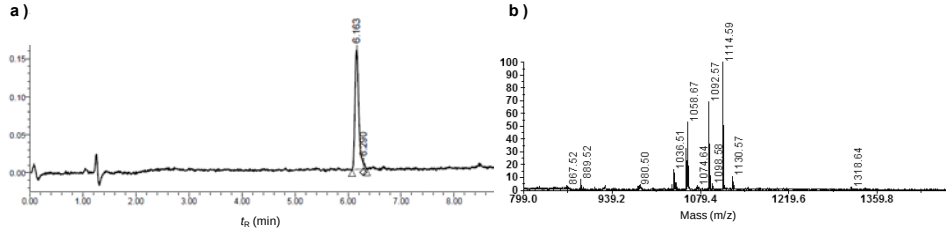


Figure S4. Analysis of *C*-terminal fragment H-(32-39)-Mmsb with fully protected side-chains: HPLC chromatogram (a); and MALDI-TOF MS spectrum (b).

Fragment Condensation: Fmoc-(22-39)-Mmsb (5)

HPLC analysis (Figure S5a) of fragment Fmoc-(22-39)-Mmsb crude **5**, with fully protected side-chains, dissolved in HFIP (t_R = 3.68 min; column temperature at 60 ºC; gradient from 80% to 100% B over 8 min) shows one main product (84%, HPLC purity). However, it was required to treat some portion with TFA/TIS/H_2O (38:1:1) for 1 h to remove the protecting groups and facilitate the detection by MS. HPLC analysis of fragment Fmoc-(22-39)-Mmsb crude with unprotected side-chains (t_R = 5.23 min; column temperature at 60 ºC; gradient from 30% to 80% B over 8 min) shows one main product (92%, HPLC purity) which corresponds to the Fmoc-(22-39)-Mmsb, MS (MALDI-TOF) (Figure S5b): *m/z* calcd for $C_{110}H_{146}N_{22}O_{30}S$ 2287.03, found: 2288.32 $[M + H]^+$. Other *m/z* found: 2232.54 is associated to a decomposition of the Mmsb moiety linked to the peptide which releases the sulfonyl derivative (56 units less, calcd for $C_{108}H_{145}N_{21}O_{29}S$ 2232.02) caused by MALDI-TOF analysis (detected in other Mmsb-analogues). The main impurity isolated at (5%, HPLC purity t_R = 5.89 min; column temperature at 60 ºC; gradient from 30% to 80% B over 8 min) corresponds to the *N*-terminal fragment [Fmoc-(22-31)-OH], MS (MALDI-TOF) (Figure S5c): *m/z* calcd for $C_{71}H_{91}N_{13}O_{16}$ 1381.67, found: 1382.83 $[M + H]^+$, 1404.85 $[M + Na]^+$ and 1420.83 $[M + K]^+$.

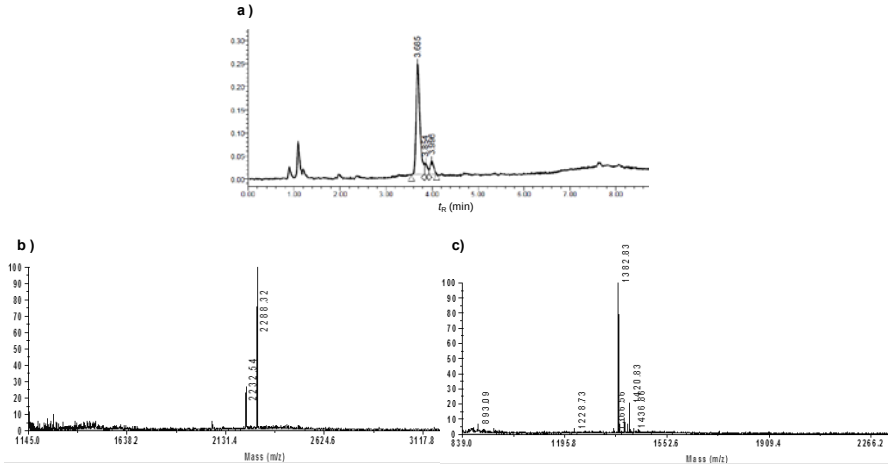


Figure S5. Analysis of Fmoc-(22-39)-Mmsb: HPLC chromatogram with protected side-chains (a); MALDI-TOF MS spectrum of collected t_R = 5.23 min product with unprotected side-chains (b); and MALDI-TOF MS spectrum of collected t_R = 5.89 min product with unprotected side-chains (c).

Fragment Condensation: H-(22-39)-Mmsb (6)

HPLC analysis (Figure S6a) of fragment H-(22-39)-Mmsb crude **6**, with fully protected side-chains, dissolved in HFIP (t_R = 5.22 min; column temperature at 60 ºC; gradient from 50% to 100% B over 8 min) shows one main product (91%, HPLC purity). However, it was required to treat some portion with TFA/TIS/H_2O (38:1:1) for 1 h to remove the protecting groups and facilitate the detection by MS. HPLC analysis of fragment H-(22-39)-Mmsb crude with unprotected side-chains (t_R = 4.77 min; column temperature at 60 ºC; gradient from 10% to 60% B over 8 min) shows one main product (85.5%, HPLC purity) which corresponds to the H-(22-39)-Mmsb, MS (MALDI-TOF) (Figure S6b): *m/z* calcd for $C_{95}H_{136}N_{22}O_{28}S$ 2064.96, found: 2066.22 $[M + H]^+$. Other *m/z* found: 2010.40, and its corresponding 2032.41 $[M + Na]^+$, are associated to a decomposition of the Mmsb moiety linked to the peptide which releases the sulfonyl derivative (minus 56 units, calcd for $C_{93}H_{135}N_{21}O_{27}S$ 2009.95) caused by MALDI-TOF analysis (detected in other Mmsb-analogues). The main impurity (3.7%, HPLC purity t_R = 5.56 min; column temperature at 60 ºC; gradient from 10% to 60% B over 8 min) corresponds to the *N*-terminal fragment [H-(22-31)-OH], MS (RP-HPLC-ESI): *m/z* calcd for $C_{56}H_{81}N_{13}O_{14}$ 1159.60, found: 1161.25 $[M + H]^+$.

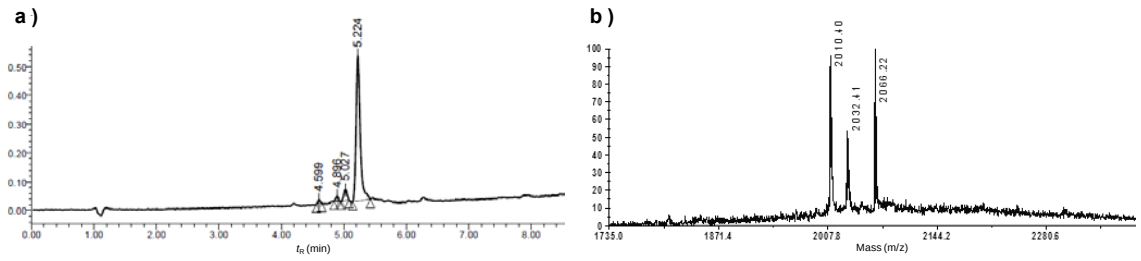


Figure S6. Analysis of H-(22-39)-Mmsb: HPLC chromatogram with protected side-chains (a); and MALDI-TOF MS spectrum of product with unprotected side-chains (b).

Analysis of Peptides after Mmsb Removal

Solubilizing tag-linked Q11

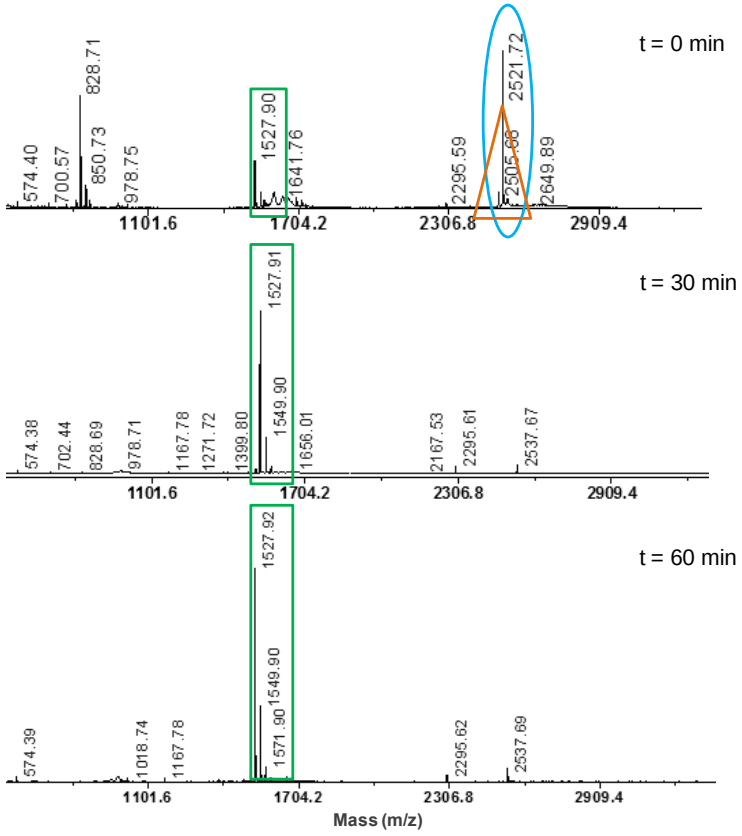


Figure S7. Monitoring the Mmsb removal of solubilizing tag-linked Q11 by MALDI-TOF MS. Starting material containing the Mmsb linker (blue circle); Intermediate of the reaction with the reduced sulfoxide from Mmsb (orange triangle); and Expected Q11 peptide without Mmsb (green rectangle).

HPLC analysis (Figure S8) of solubilizing tag-linked Q11, after Mmsb removal treatment, dissolved in HFIP (t_R = 4.37 min; gradient from 10% to 60% B over 8 min) shows one main product (99%, HPLC purity) which corresponds to the expected Q11, MS (MALDI-TOF) (Figure S8): *m/z* calcd for $C_{70}H_{98}N_{18}O_{21}$ 1526.71, found: 1527.91 $[M + H]^+$ and 1549.91 $[M + Na]^+$.

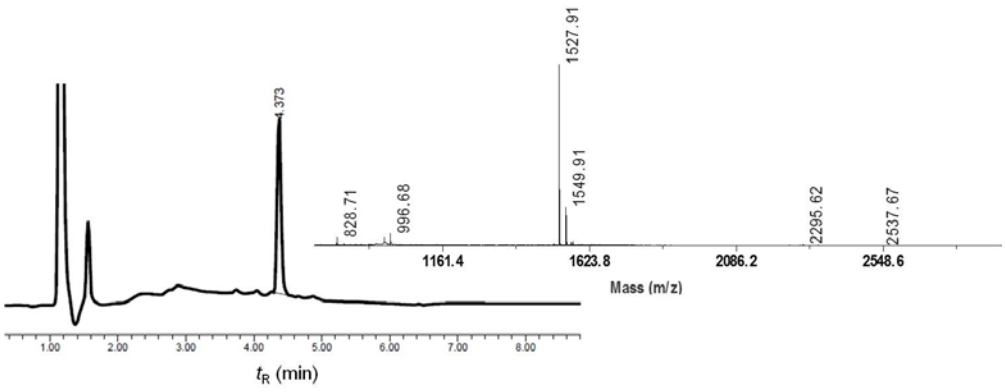


Figure S8. HPLC chromatogram and MALDI-TOF MS spectrum of Q11 released from solubilizing tag-linked Q11 after Mmsb removal.

Fragment Condensation: H-(22-39)-OH

HPLC analysis (Figure S9) of H-(22-39)-OH, after Mmsb removal treatment, dissolved in HFIP (t_R = 4.49 min; column temperature at 60 ºC; gradient from 10% to 60% B over 8 min) shows one main product (85%, HPLC purity) which corresponds to the expected H-(22-39)-OH, MS (MALDI-TOF) (Figure S9): *m/z* calcd for $C_{85}H_{125}N_{21}O_{25}$ 1839.91, found: 1841.11 $[M + H]^+$, 1863.09 $[M + Na]^+$. The impurity (3.4%, HPLC purity t_R = 5.40 min; column temperature at 60 ºC; gradient from 10% to 60% B over 8 min) corresponds to the *N*-terminal fragment [H-(22-31)-OH], MS (RP-HPLC-ESI):): *m/z* calcd for $C_{56}H_{81}N_{13}O_{14}$ 1159.60, found: 1160.68 $[M + H]^+$.

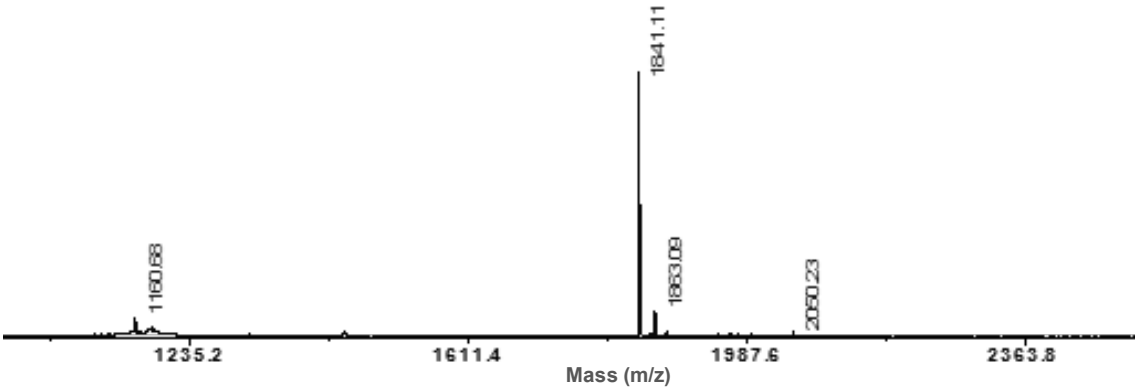


Figure S9. MALDI-TOF MS spectrum of Exenatide fragment H-(22-39)-OH after Mmsb removal.

Solubility Analysis of Q11 and its Mmsb-analogue

Standard Q11 and the Solubilizing tag-linked Q11

1 mg of Standard Q11 crude (**2**) and 1 mg of Solubilizing tag-linked Q11 crude (**1**) were dissolved separately in H_2O (1 mL) and compared. It was clearly observed that Standard Q11 is not soluble and, on the contrary, the Solubilizing tag-linked Q11 is completely soluble in water.

GENERAL CONCLUSIONS

"Difficult peptides", as well as those sequences that aggregate in solution, are widely present in a large range of areas such as biochemistry, nanomaterials or even medicine. The structural behavior is its most distinguished feature but unfortunately, is also the responsible to produce synthetic drawbacks. In response to the constantly increasing demand of finding straightforward methodologies to reach these peptides, we have contributed to overcome these difficulties. The strategies proposed and evaluated in the present thesis have expanded the scope of tools addressed to solubilize peptides not only during SPPS, but also after their elongations. The main conclusions, organized by chapters are the following:

Chapter 1. The RADA-16 self-assembling peptide, described as one of the most valuable sequences for its biomedicine applications, has been extensively studied in the present thesis. Three solid-phase peptide strategies were selected to address the RADA-16 synthesis. First, the most commonly used, the stepwise methodology; and later the two convergent approaches: the solid-phase and the fragment condensation in solution. The four times repeating Arg-Ala-Asp-Ala sequence comprised in this peptide was crucial for the retro-synthetic design of both convergent strategies. Fragment condensation in solution of two fully protected segments resulted in the most fruitful strategy to synthesize the RADA-16 peptide. Development of a new work-up protocol was essential to achieve the final peptide with a satisfactory purity. Several HPLC conditions of commercial RADA-16 samples were studied allowing to find the most significant parameters to achieve an accurate characterization, thus contributing to the current methods for the analysis of other similar sequences.

Chapter 2. The design of a new backbone amide protecting group, the Mmsb, was developed as a "safety-catch" protector in order to disrupt the β-sheet interactions present in "difficult peptides". The solid-phase incorporation of the Mmsb into a sequence was preceded by the synthesis of Mmsb α-amino protected alanine [Fmoc-*N*(Mmsb)-Ala-OH]. The safety-catch property of this sulfoxide derivative protecting group was confirmed by evaluating its resistance to acidic cleavage conditions, and its subsequent acid-labile conversion after reduction, to the thioether.
Initially, Decaalanine was the sequence of choice to verify the synthetic benefits attributed to the new backbone amide protection, and the structural modifications induced by Mmsb were confirmed by circular dichroism. The RADA-16 synthesis served as a second "difficult peptide" example to demonstrate, not only improvements on the synthesis, but also the enhancement of the solubility in solution which allowed its HPLC purification. Finally, the synthesis of the challenging self-assembling Aβ(1-42) peptide

evidenced that the Mmsb strategy permits to overcome synthetic and also purification troubles, even for large "difficult peptides".

Chapter 3. Several short cationic peptides based on Lys or Arg residues were synthesized and conjugated to a non-polar di-naphthylalanine moiety to evaluate their effect in terms of polarity. Linear and branched poly-Lys/Arg designed as solubilizing tags provided the following polarity features: (i) linear poly-Lys resulted more polar than its homologue poly-Arg; (ii) branched poly-Lys contributed to increase the polarity in a greater range than the linear form; (iii) amino-type (α or ε) exposed to the media influenced on a minor extent than the spatial distribution of those amino groups. The permanent ligation of a known insoluble drug to a branched poly-Lys solubilizing tag provided a proof of concept to demonstrate the tag contribution to enhance the solubility and polarity of the drug.

The Mmsb structure used in the second chapter was applied as a linker (Mmsb-OH). The first proposed application of this handle consisted of a temporary connection between the non-soluble Q11 sequence and a linear poly-Lys solubilizing tag. Enhancement of solubility, as well as the conjugated solubilizing tag resistance towards acidolytic cleavage, permitted the accurate peptide characterization. Reductive acidolysis released the peptide target in the similar manner than the Mmsb backbone amide protector.

The second application of Mmsb-OH led to the obtaining of a new *C*-terminal protecting group. Stability of Mmsb to cleavage conditions allowed reaching a fully protected peptide fragment, to be further conjugated in solution to another protected sequence. Fragment condensation of these sequences was carried out successfully and, the subsequent protecting groups removal rendered the expected peptide, validating the Mmsb-OH as a new *C*-terminal protector.

RESUM

Capítol 1: Avaluació Sintètica per a la Obtenció d'un "Pèptid Difícil"

La propietat d'autoensamblar que presenten certes molècules ha estat valorada en el camp de la medicina i la tecnologia per a la cerca de nous nano-materials.[1] La característica d'organització molecular que es dóna en aquests productes s'ha avaluat amb elevat interès, sent els més rellevants aquells destinats a la biomedicina.[2] Algunes de les aplicacions clíniques que s'han dut a terme amb aquests materials han estat per exemple en la distribució de fàrmacs dins l'organisme,[3] en la regeneració de teixits,[4] per a l'ajut de cristal·lització de proteïnes[5] o per facilitar la internalització en cèl·lules.[6] La medicina imposa a aquests productes a ser forçosament biocompatibles, així que la comunitat científica s'ha fixat en els pèptids com a clars candidats, en tant que compleixen aquest requisit. A la natura es troben exemples de pèptids que poden autoensamblar, i aquests han servit com a model per al disseny de noves seqüències amb la finalitat que presentin aquesta característica.[7] A part de ser biocompatibles, els pèptids permeten una senzilla combinació i modificació sintètica de la seqüència d'AAs (AAs) de manera que, *de novo*, es poden obtenir cadenes peptídiques que presentin autoensamblatge en solució.

A nivell molecular, s'ha pogut observar que un dels principals mecanismes a través del qual certs pèptids presenten la tendència a autoensamblar, es basa en la formació d'interaccions de tipus làmina-β. Aquestes associacions es donen quan el pèptid es troba totalment desprotegit i en solució, succeint tant a nivell intra- o com inter-molecular. De manera que, ja sigui entre diferents cadenes de pèptid, com entre una mateixa cadena, aquestes interaccions provoquen inicialment una associació en làmines-β, que en alguns casos es pot arribar a estendre en l'espai tridimensional donant supra-estructures com les fibres. La característica que presenten els pèptids que autoensamblen, considerada com un avantatge per crear nous materials, resulta ser un inconvenient pel què fa a la seva manipulació en solució.

Per altra banda, quan les forces d'unió làmina-β d'una seqüència es produeixen durant la síntesi del pèptid, ja sigui en fase sòlida o en solució, aquesta se l'anomena "pèptid difícil".[8,9] Aquestes seqüències concretament presenten enllaços de pont d'hidrogen entre l'esquelet de la cadena peptídica, específicament entre el protó de l'amida i l'oxigen del carbonil. Les dificultats durant el procés conegut de síntesi de pèptids en fase sòlida (SPPS) succeeixen perquè el suport polimèric sobre el qual creix la seqüència no evita la formació d'interaccions entre els enllaços amida. Per definició no tots els pèptids difícils d'obtenir són "pèptids difícils", però sempre es donarà aquesta relació a la inversa. Tot i que a la literatura s'han descrit estructures peptídiques que

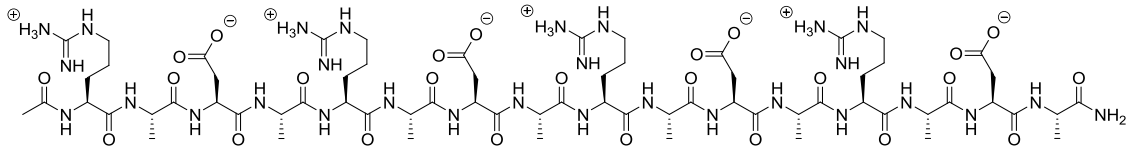
s'engloben dins d'aquest conjunt, no és fàcil *a priori* tenir la certesa que una determinada seqüència serà un pèptid d'aquest tipus. S'han identificat, en base a les dificultats sintètiques, una sèrie de conceptes comuns a tots els "pèptids difícils":[10,11] la constant impossibilitat d'incorporar certs AAs, malgrat l'augment del nombre d'intents; l'acusat problema sintètic quan la funcionalització de la resina és elevada; llargues o incomplertes desproteccions del grup amino abans de cada acoblament; i complicacions considerables quan hi ha AAs que contenen cadenes laterals voluminoses.

Entre les seqüències classificades a la literatura com a pèptids que autoensamblen en destaca un subgrup que s'anomenen pèptids amfifílics, ja que tenen una part de l'estructura que es comporta com a hidrofòbica i una altra part com a hidrofílica.[5,12] Alhora, els pèptids amfifílics se subdivideixen en un altre grup menor que es coneix com pèptids d'autoensamblatge iònics. La majoria de membres d'aquest tipus són seqüències no naturals sintetitzades *de novo*, de manera que es composen per AAs amb cadenes laterals carregades positiva i negativament en una mateixa seqüència. La complementarietat de càrregues afavoreix substancialment l'autoensemblatge, motiu pel qual aquests pèptids han estat extensament estudiats. Concretament, pertanyent en aquest subgrup es troba el pèptid conegut com RADA-16, dissenyat junt amb altres seqüències similars pel professor Zhang l'any 2000[13] i que ha estat subjecte d'estudi en aquest capítol. En concret, aquest pèptid també presenta interaccions de tipus làmina-β durant la seva síntesi, així que es pot dir que és una seqüència que s'agrupa dins dels coneguts com "pèptids difícils".

El RADA-16 [Ac-$(RADA)_4$-NH_2] (Fig. 1), com el seu nom abreujat indica, és una seqüència de 16 AAs amb una repetició de quatre vegades el tetrapèptid Arg-Ala-Asp-Ala. Precisament, aquesta continua alternança de càrregues (positiva atribuïda a l'Arg i negativa atribuïda a l'Asp) és el què confereix al pèptid la seva elevada tendència a formar l'autoensamblatge. Recents estudis detallats sobre la seva estructura terciària o quaternària han ajudat a entendre que les cadenes del RADA-16 es disposen en forma de làmina-β paral·leles que s'ensamblen exposant dues cares definides. Aquestes dues parts defineixen dos plans, en tant que pèptid amfifílic, un és l'hidrofòbic (format per les Ala) i l'altre l'hidrofílic (format per l'alternança dels dos AAs carregats, l'Arg i l'Asp). Quan diferents cadenes del RADA exposen aquests plans en un mateix medi, inevitablement s'orienten en l'espai tridimensional de manera que els dos plans hidrofòbics s'encaren entre sí i els plans carregats se situen contraposant les càrregues positives amb les negatives. Degut a la organització d'aquest pèptid en solució, s'ha descrit la seva formulació com a hidrogel, concretament en un 99.5% de contingut

d'aigua i l'addició d'una solució salina que afavoreix el seu autoensamblatge. Aquesta composició es comercialitza des del 2005,[14] inicialment per l'empresa BD Bioscience que l'ha anomenat PuraMatrix,® i posteriorment per altres empreses que la ofereixen amb diferents graus de puresa i amb altres noms comercials. Aquest compost, tot i no ser directament un fàrmac, s'ha utilitzat en estudis mèdics per tal de facilitar l'alliberament de fàrmacs en cèl·lules, com a regeneració de nombrosos teixits, o fins i tot en tractaments combinats pel càncer en cèl·lules i en assajos *in vivo*.[15]

Fruit de totes les aplicacions, que es troben en augment dins la biomedicina associades al RADA-16, així com la seva consideració com a bon exemple de "pèptid difícil", en aquest capítol s'ha abordat la seva síntesi en fase sòlida, la seva purificació i caracterització. Com a resposta a la manca en la literatura d'un mètode sintètic eficient amb una detallada caracterització per al RADA-16, s'ha fet el següent estudi, també amb l'objectiu d'aportar noves eines sintètiques extensibles a altres "pèptids difícils" de característiques semblants.



Ac-Arg^{1}-Ala^{2}-Asp^{3}-Ala^{4}-Arg^{5}-Ala^{6}-Asp^{7}-Ala^{8}-Arg^{9}-Ala^{10}-Asp^{11}-Ala^{12}-Arg^{13}-Ala^{14}-Asp^{15}-Ala^{16}-NH$_2$

Figura 1. Estructura del RADA-16.

Tot i que la SPPS de manera seqüencial és la primera estratègia utilitzada per a la obtenció de cadenes peptídiques llargues, s'han anat desenvolupat variants d'aquest mètode on es combina la SPPS i el tradicional mètode de síntesi en solució. Dins de l'objectiu principal d'obtenció del pèptid RADA-16 amb elevada puresa s'han establert les següents estratègies convergents: la *condensació de fragments en fase sòlida*[16,17] i la *condensació de fragments en solució*.[18] La primera es fonamenta en la obtenció del pèptid sencer en fase sòlida mitjançant la incorporació d'un segment peptídic d'aquest prèviament sintetitzat en fase sòlida. Per altra banda, la segona estratègia és portada a terme mitjançant la unió en solució de dos fragments peptídics sintetitzats prèviament de manera independent en fase sòlida.

Inicialment, mitjançant la **via seqüencial**, tant la manual com l'automàtica, es va obtenir la síntesi del RADA-16 amb un valor de puresa que s'utilitzarà com a referència per avaluar l'eficiència les altres estratègies proposades. Tot i la necessitat d'incorporar més d'una vegada certs AAs, el pèptid RADA-16 es va obtenir amb una puresa per

HPLC del 65-75%, amb la massa esperada identificada per espectrometria de masses MALDI-TOF. L'anàlisi de les impureses detectades per HPLC del cru peptídic va mostrar que aquestes tenien unes característiques de polaritat molt semblants al pèptid objectiu, trobant-se molt properes al producte desitjat pel què fa a temps d'elució en el cromatograma, fet que suposa una gran barrera a l'hora de purificar.

A través de la via de **condensació de fragments en fase sòlida**, aprofitant la naturalesa de la seqüència del RADA-16, format de 4 fragments peptídics exactament iguals units consecutivament fins a 4 vegades, es va escollir el fragment peptídic següent: Fmoc-Arg(Pbf)-Ala-Asp(*t*Bu)-Ala-OH (F1, Fig. 2). Aquest pèptid es va sintetitzar en fase sòlida i un cop escindit de la resina mantenia els seus grups protectors a les cadenes laterals d'aquells AAs funcionalitzats; la seva puresa va ser del 99% per HPLC. Tenint en compte que F1 presenta cert impediment estèric es va decidir evitar la seva incorporació directa a la resina realitzant l'acoblament del primer tetrapèptid seqüencialment, fent que el segon tetrapèptid (F1) es pogués incorporar sense problemes. Les dificultats d'introducció d'aquest es van veure incrementades a mesura que anava creixent el pèptid, de manera que el RADA-16 només es va obtenir amb un 46% de puresa.

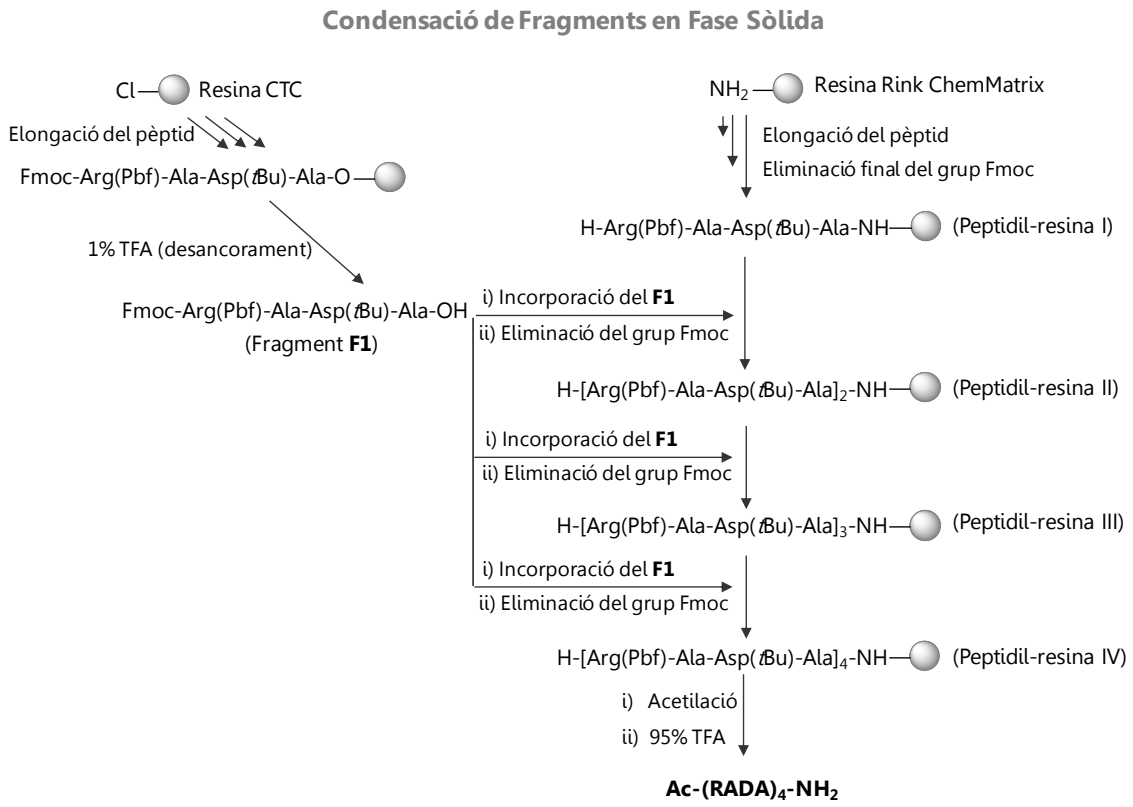


Figura 2. Esquema sintètic de l'estratègia convergent de condensació de fragments en fase sòlida.

Per últim, la via de **condensació de fragments en solució** va requerir d'una manera raonable i acurada una divisió d'aquest pèptid en els punts més òptims, que serien la unió de dos únics fragments de 8 AAs cadascun (Fig. 3). Els dos fragments peptídics, Ac-[Arg(Pbf)-Ala-Asp(*t*Bu)-Ala]$_2$-OH (F2, Fig. 3) i H-[Arg(Pbf)-Ala-Asp(*t*Bu)-Ala]$_2$-NH$_2$ (F3, Fig. 3), es van sintetitzar en gran escala (0.8 mmol) en fase sòlida. Les condicions per a la condensació en solució van ser equimolars i amb el sistema d'agents d'acoblament hexafluorofosfat de benzotriazol-1-il-*N*-oxi-tris(pirrolidino)fosfoni (PyBOP)/ *N*,*N*'-diisopropiletilamina (DIEA) amb l'additiu 1-hidroxi-7-azabenzotriazol (HOAt). Aquesta estratègia, junt amb les condicions descrites, va ser la via més òptima per a obtenir el pèptid desitjat amb una puresa considerablement superior a l'obtinguda amb les altres estratègies. Per altra banda, les impureses presents al cromatograma d'HPLC no es trobaven properes en temps al pèptid RADA-16, fet significant a l'hora de purificar un pèptid amb tendència a l'auto-ensamblatge. En aquest cas, la dificultat es va trobar en l'eliminació d'impureses, com els agents d'acoblament, sobretot l'additiu HOAt, que es va solucionar realitzant rentats del cru peptídic amb *tert*-butilmetiléter, obtenint-ne una puresa final del RADA-16 per HPLC del 85%.

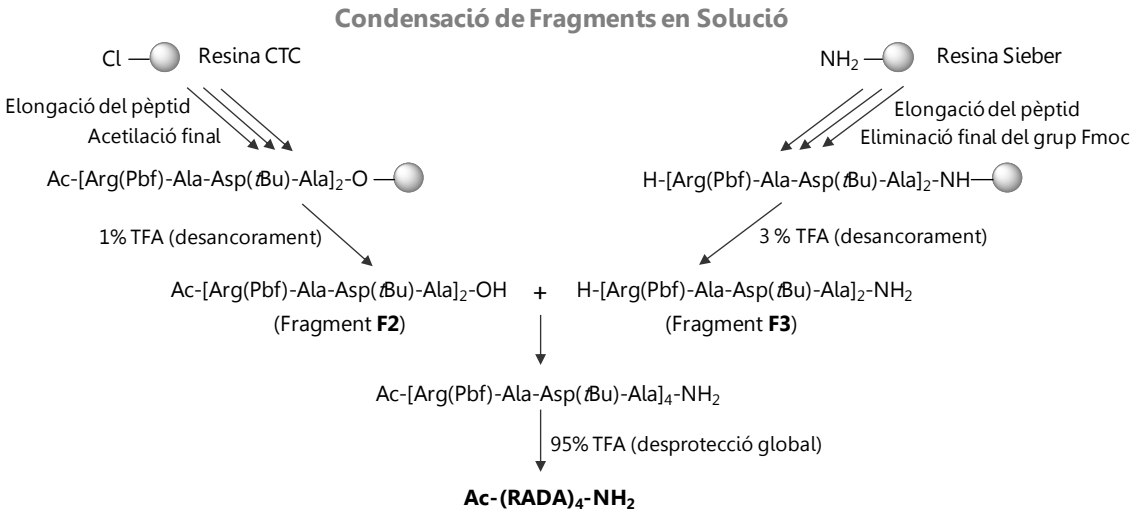


Figura 3. Esquema sintètic de l'estratègia convergent de condensació de fragments en fase sòlida.

Per tal de purificar el RADA-16, es va pensar en un disseny acurat basat en extraccions que pogués aprofitar la diferència de solubilitat dels diferents productes segons la polaritat del dissolvent. Comparat amb el mètode estàndard utilitzat per a la purificació de pèptids per HPLC semi-preparatiu, aquest nou procediment proposat va permetre obtenir el RADA-16 amb una puresa del 90%, notablement millorada respecte la inicial.

En paral·lel a l'avaluació d'estratègies sintètiques per obtenir el RADA-16 es va realitzar un treball d'optimització de paràmetres per caracteritzar aquest pèptid per HPLC, ja que el seu autoensamblatge en dificulta el tractament en solució. Sis mostres d'aquest pèptid proporcionades per cases comercials diferents van ser utilitzades per a dur a terme el següent estudi. Els factors avaluats van ser: la dissolució de la mostra, la concentració injectada a l'equip, el gradient cromatogràfic, la temperatura de la columna, així com la fase mòbil. Aquest estudi va demostrar la importància d'aquests paràmetres quan es vol caracteritzar un pèptid que presenta autoensamblatge, de manera que es va obrir el ventall de factors a tenir en compte per analitzar pèptids amb aquesta característica.

En aquest capítol es conclou que s'han avaluat diferents estratègies sintètiques en fase sòlida per a la obtenció del pèptid RADA-16, combinant tant les més comunament utilitzades com altres que combinen la condensació de fragments en fase sòlida o en solució. S'ha establert que la manera d'aconseguir aquest "pèptid difícil" amb la màxima puresa és mitjançant la condensació de dos fragments del RADA-16 en solució. L'eliminació de certes impureses del cru peptídic s'ha pogut realitzar amb un posterior protocol d'extraccions i rentats en diferents dissolvents que han permès purificar el pèptid sense la necessitat d'utilitzar l'HPLC semi-preparatiu. Estudis de cromatografia líquida amb mostres comercials del RADA-16 han permès identificar aquells paràmetres més crucials a tenir en compte quan s'han d'analitzar pèptids que autoensamblen en solució.

Capítol 2: Un nou Grup Protector per a Amides de Cadena Peptídica

Els anteriorment descrits "pèptids difícils" suposen un repte sintètic perquè la seva tendència a formar estructures tipus làmina-β és una barrera que impedeix que els grups funcionals que han de formar l'enllaç amida ho puguin fer de manera assequible. Aquest inconvenient es dóna tant en la síntesi de pèptids en solució com en la SPPS, ja que, com s'ha esmentat, aquestes interaccions organitzades es donen entre els grups amida de l'esquelet peptídic, presents en les dues estratègies. En els dos tipus de processos aquesta problemàtica es tradueix directament en un fenomen d'insolubilitat del pèptid durant la síntesi. La necessitat de solubilitzar seqüències quan aquestes es troben protegides, ha requerit el desenvolupament de noves tàctiques per solubilitzar-les i poder aconseguir la seva síntesi, especialment quan mètodes convencionals no serveixen. Donat aquest factor, en els darrers anys s'han proposat diverses estratègies

dirigides a disminuir aquesta associació de ponts d'hidrogen inter- o intramoleculars que afecta la solubilitat dels "pèptids difícils".[19–21]

Els factors que participen en solucionar la insolubilitat d'aquestes seqüències estan dirigits a solubilitzar pèptids protegits durant la seva síntesi o a solubilitzar-los desprotegits quan es troben en solució. Els dos tipus d'insolubilitats comparteixen el mateix grau d'importància, ja que el primer provoca la formació d'impureses i el segon impossibilita l'anàlisi i purificació. Degut a l'estudi realitzat en aquest capítol, ens centrarem en les estratègies dirigides a **solubilitzar seqüències durant la seva síntesi** per tal d'obtenir "pèptids difícils". Així doncs, els paràmetres que participen en la solució d'aquest inconvenient poden ser *externs* si s'afegeixen canvis del dissolvent, salts caotròpiques o detergents;[22] o poden referir-se a *modificacions químiques* quan es manipula internament l'estructura del pèptid. Pel què fa a les alteracions efectuades a la cadena peptídica, les més conegudes es classifiquen en: (i) síntesi d'un anàleg temporal de la seqüència que inclou un enllaç éster que posteriorment es transforma en amida;[23] i (ii) la incorporació d'un protector temporal per a l'enllaç amida de la cadena peptídica.[24,25] En aquest capítol ens hem centrat en la darrera estratègia per a solubilitzar "pèptids difícils", la protecció d'amida de cadena peptídica.

L'efecte d'incorporació d'un grup en el nitrogen amida evita la presència del protó amida i provoca a la seqüència una interrupció dels continuats ponts d'hidrogen, fent desestabilitzar l'estructura de làmina-β. Quan la molècula s'uneix a l'amida de manera temporal es parla de grup protector, així es pot dir que es protegeix un grup funcional que normalment es troba lliure en la síntesi de pèptids. Cal tenir en compte que aquests protectors, que generalment estan dissenyats per ser eliminats durant la desprotecció global del pèptid, només aporten solubilitat durant la síntesi, ja que quan la seqüència es troba desprotegida ells ja no hi són presents.

Des del 1992 que es van dissenyar les pseudoprolines[26] com a grups protectors per a amida, a la literatura se n'han descrit altres, tots encaminats a facilitar la síntesi de "pèptids difícils".[27–29] Els protectors d'enllaç amida per a pèptids proposats fins al moment presenten certes limitacions associades a una baixa reactivitat d'incorporació d'aquests; o a un elevat cost econòmic associat a la seva preparació; o a la necessitat que certs AAs siguin presents a la seqüència per tal d'introduir aquest protector.[30]

Dins del marc de la recerca associada a aquests protectors, i amb la intenció de millorar les limitacions mencionades, s'ha proposat, en el següent capítol, el desenvolupament de la síntesi d'un nou grup per l'amida de cadena peptídica que permeti la síntesi de "pèptids difícils" mitjançant la millora en la seva solubilitat.

Per tal d'assolir aquest objectiu es va proposar la molècula 2-metoxi-4-metilsulfinilbenzil (Mmsb) com a nou grup protector de l'esquelet peptídic. Aquesta molècula havia estat prèviament utilitzada, però en forma de carbamat (Mmsz)[31] per a la protecció d'amines i com a espaiador bifuncional. La peculiaritat de la molècula proposada és que es classifica dins del concepte de "safety-catch", que va ser definit el 1971 en base a aquells enllaços que després d'una modificació química, en un moment determinat, poden convertir-se en làbils a certes condicions.[32] Aquest comportament va permetre pensar en el disseny d'un grup protector de tipus semi-permanent: estable al medi bàsic utilitzat durant la síntesi del pèptid per 9-fluorenilmetoxicarbonil (Fmoc)/*tert*-butil (*t*Bu) en SPPS, i alhora estable al medi àcid associat a l'escissió del pèptid, però que després d'un tractament químic el grup es tornaria làbil a àcid (Fig. 4).

En tant que grup protector d'amida de cadena peptídica, l'Mmsb pretén facilitar la solubilitat de la seqüència en fase sòlida (durant la síntesi), permetent una incorporació millorada dels AAs. Però alhora, aquest grup aporta una nova avantatge que no la presenten la gran majoria dels grups protectors d'amida: la possibilitat d'obtenir la seqüència semi-protegida després de fer l'escissió del pèptid de la resina, que fa millorar la seva solubilitat en solució. Aquesta última qualitat evita l'agregació del pèptid en solució, facilitant-ne la caracterització i la seva purificació, etapa que amb l'absència d'aquest grup no hauria estat possible. Posteriorment a la escissió del pèptid, mitjançant un tractament reductiu del sulfòxid per donar un tioéter, el grup perd el caràcter electro-atractor fent que en medi àcid es torni làbil i s'afavoreixi la seva eliminació, alliberant-se la seqüència desitjada.

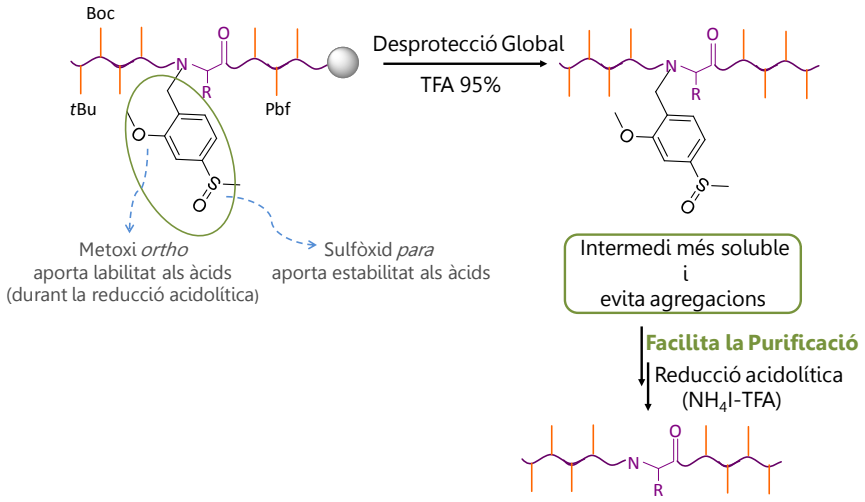


Figura 4. Esquema general de la metodologia d'utilització del grup protector d'esquelet peptídic Mmsb.

Per tal de dur a terme la incorporació de l'Mmsb a la seqüència peptídica es va decidir introduir-lo a un AA quiral representatiu, com va ser l'alanina (Fmoc-*N*(Mmsb)-L-Ala-

OH). La síntesi en solució d'aquest AA modificat es va basar en la preparació del Mmsz descrita per Thennarasu i col·laboradors,[31] amb certes modificacions per tal d'optimitzar-ne la puresa (Fig. 5). De manera que en cinc etapes i una única purificació (segona reacció per separar el compost 3 del seu regioisòmer formilat a la posició 5) es va obtenir el Fmoc-*N*(Mmsb)-L-Ala-OH amb una puresa del 98% i un rendiment, no optimitzat, del 19%. Sense cap més purificació aquest producte es va utilitzar directament en les estratègies de totes les síntesis de pèptids modificats amb l'Mmsb mitjançant fase sòlida Fmoc/*t*Bu.

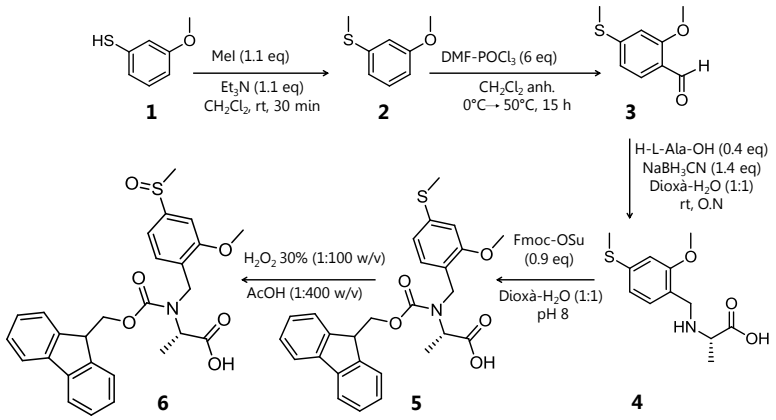


Figura 5. Procés sintètic per a la obtenció de Fmoc-*N*(Mmsb)-L-Ala-OH.

Per tal d'avaluar l'estratègia esmentada es va introduir l'Fmoc-*N*(Mmsb)-L-Ala-OH a tres models de pèptids coneguts com a "pèptids difícils": **H-(Ala)$_{10}$-NH$_2$** (decaalanina, utilitzat a la bibliografia com a model de seqüència problemàtica),[33,34] Ac-(Arg-Ala-Asp-Ala)$_4$-NH$_2$ (**RADA-16**),[13] i la **Aβ(1-42)** (H-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGL-MVGGVVIA-OH, pèptid de 42 AAs conegut per la seva implicació en la malaltia de l'alzheimer).[35] La síntesi d'aquests pèptids es va fer en fase sòlida i amb la incorporació de l'alanina protegida amb l'Mmsb en una única posició del pèptid: per a la decaalanina es van fer dos anàlegs amb introducció al tercer i cinquè AA, respectivament; per al RADA-16 en el cinquè AA; i finalment per la Aβ(1-42) en el vint-i-dosè AA. Tot i la confirmació de la completa incorporació dels AAs mitjançant el test qualitatiu Kaiser, les síntesis van presentar una dificultat comuna causada a l'impediment estèric del Mmsb, en el punt d'incorporació de l'AA consecutiu a l'Fmoc-*N*(Mmsb)-L-Ala-OH. Es va realitzar aquesta incorporació en diferents condicions i es van trobar que els millors resultats s'obtenien després de tres incorporacions consecutives de 2 h amb els agents d'acoblament *N*,*N*′-diisopropilcarbodiimida (DIPCDI) i 2-ciano-2-hidroxiiminoacetat d'etil (OxymaPure) en MeCN/DMF (3:1) a T= 45 ºC i augmentant els equivalents d'AA i agents d'acoblament.

Una alternativa proposada per tal de facilitar la incorporació de l'AA consecutiu a l'AA sintetitzat protegit amb l'Mmsb, va consistir en la síntesi en solució del dipèptid Fmoc-L-Ala-*N*(Mmsb)-L-Ala-OH per a la decaalanina. La formació de l'enllaç amida es va realitzar amb la prèvia activació de l'àcid carboxílic de l'alanina comercial com a fluorur d'àcid (Fmoc-L-Ala-F), per després fer-lo reaccionar amb el corresponent grup amino de l'*N*(Mmsb)-L-Ala-OH. L'acoblament del dipèptid per a fer la decaalanina en fase sòlida es va poder obtenir completament en condicions estàndards utilitzant PyBOP i DIEA. Després de l'escissió del pèptid de la resina es va poder confirmar la validesa del mètode sense incorporacions incompletes d'AA, amb la obtenció de la decaalanina amb una puresa molt elevada.

Un cop complerta l'elongació dels pèptids amb l'Mmsb, aquests es van escindir de la resina amb TFA 95% [combinat amb triisopropilsilà (TIS) 2.5%, i H_2O 2.5% quan els pèptids contenien cadenes laterals d'AA protegides], corroborant-ne per cromatografia i espectrometria de masses que tots ells contenien el grup Mmsb i la resta d'AAs desprotegits. Mitjançant anàlisis d'HPLC i espectrometria de masses es va confirmar que a nivell sintètic la incorporació de l'Mmsb a la seqüència produïa productes amb menys impureses que les síntesis que no tenien el grup protector, els quals majoritàriament contenien delecions de certs AAs. Per altra banda, l'anàlisi de solubilitat va permetre confirmar que els pèptids semi-protegits amb l'Mmsb eren notablement més solubles que els seus homòlegs totalment desprotegits.

Amb la decaalanina es van realitzar anàlisis amb dicroisme circular tant de la seqüència sense grup protector, com dels dos anàlegs protegits amb l'Mmsb, observant-se clarament que el pèptid desprotegit presentava una estructura secundària de làmina-β, mentre que els dos protegits mostraven una estructura desordenada "random coil". Aquesta diferència estructural pot entendre's com una demostració de l'efecte causat per la presència de l'Mmsb en el plegament del pèptid en solució, i per tant un motiu explicatiu de l'augment de solubilitat i la obstrucció en l'autoensamblatge.

Amb els altres dos pèptids modificats amb l'Mmsb, el RADA-16 i la Aβ(1-42), es van realitzar les seves purificacions per HPLC semi-preparatiu, i gracies a l'augment de la solubilitat associada al protector, els dos pèptids van poder ser aïllats amb una elevada puresa.

Finalment, l'etapa de desprotecció per a obtenir els pèptids diana va consistir en l'eliminació del grup Mmsb mitjançant la reducció acidolítica amb NH_4I en TFA, succeint dues reaccions alhora, la reducció i la desprotecció. Seguint la reacció per espectrometria de masses es va poder observar que en presència de NH_4I, el sulfòxid

de l'Mmsb es reduïa i es tornava làbil a àcid provocant la immediata alliberació del pèptid desprotegit.

En aquest capítol s'ha pogut concloure que la incorporació del nou grup protector per l'amida de l'esquelet peptídic, Mmsb, ha contribuït a facilitar la síntesi de "pèptids difícils" en fase sòlida, alhora que ha permès la seva manipulació en solució. La síntesi del grup protector s'ha realitzat mitjançant la seva inserció en el grup α-amino de l'Ala [Fmoc-*N*(Mmsb)-L-Ala-OH]. La seva incorporació s'ha dut a terme en condicions estàndards en fase sòlida en una posició de la seqüència racionalment seleccionada per als pèptids models: la decaalanina, el RADA-16 i la Aβ(1-42).

La peculiaritat de l'Mmsb, en tant que grup tipus "safety-catch", s'ha demostrat pel què fa a la seva estabilitat al medi bàsic durant la síntesi i al medi àcid durant l'escissió del pèptid de la resina. La millora en les síntesis en fase sòlida associades a la presència de l'Mmsb, comparat amb les que no el contenien, s'ha corroborat fent-se evident en tant que ha evitat la deleció d'AAs. La implicació de l'Mmsb en evitar associacions tipus làmina-β en solució s'ha demostrat per dicroisme circular per a la decaalanina. La solubilitat dels pèptids semi-protegits amb l'Mmsb ha estat notablement augmentada comparada amb els pèptids sense protegir, fet que ha permès la seva caracterització per HPLC, així com la purificació del RADA-16 i la Aβ(1-42). L'eliminació del grup protector "safety-catch" Mmsb s'ha dut a terme mitjançant una reducció acidolítica obtenint tots els exemples de "pèptids difícils" desprotegits mantenint-ne la puresa de síntesi.

Capítol 3: Estratègies de Conjugació amb Pèptids Solubilitzadors

En el marc de la investigació associada a la millora de la **solubilitat dels pèptids**, aquesta vegada s'ha dirigit cap a les seqüències obtingudes després de la desprotecció global, **en solució**. Donada la importància, comentada anteriorment, d'obtenir mostres solubles per a possibilitar la seva caracterització i purificació, s'han desenvolupat diferents eines sobre aquest camp. Centrant-nos en aquest sector tractat en aquest capítol trobem descrites estratègies basades en *factors externs* i altres en *modificacions químiques*. Pel què fa als primers trobem: la selecció de dissolvents, el pH, l'addició de sals caotròpiques, i l'addició de detergents.[38] En canvi pel què fa a les alteracions efectuades a la cadena peptídica, les més conegudes es classifiquen en: (i) incorporacions temporals o permanents de molècules polars com el polietilè glicol (PEG)[39] o els sucres;[40] (ii) conjugacions temporals o permanents de segments peptídics

curts i polars.[41] En aquest capítol concretament s'estudiarà aquesta última estratègia basada en la conjugació de pèptids de cadena curta amb capacitat solubilitzadora. Aquesta eina es pot aplicar tant a molècules orgàniques petites com a cadenes aminoacídiques.

Les seqüències curtes de pèptids utilitzades per a aquesta finalitat són homooligopèptids composats per AAs de cadena lateral carregada positiva- (Arg o Lys) o negativament (Asp o Glu).[36,37] De manera que la unió d'un d'aquests pèptids a una molècula/pèptid insoluble en aigua, aporta un component iònic que li fa augmentar la solubilitat i que pot estar connectat a la molècula d'estudi de manera temporal o permanent. Un dels avantatges per les quals aquesta estratègia suposa una alternativa a altres mètodes rau en la facilitat de conjugació sintètica, que s'aconsegueix mitjançant la síntesi en fase sòlida.

A la literatura es troba àmpliament estudiada aquesta estratègia per a proteïnes,[41] però menys aplicada als pèptids. S'ha descrit l'ús d'homooligopèptids que es connecten a proteïnes amb la finalitat d'aportar solubilitat, per facilitar sobretot la purificació d'aquestes.[42] Pel què fa als pèptids apolars amb **conjugació permanent** a aquestes seqüències curtes carregades, s'han descrit casos en combinació amb PEG[43] on s'aconsegueixen formació de micel·les o fins i tot dendrímers.[44] Aquestes estructures s'han utilitzat principalment com a sistemes d'alliberament de fàrmacs i han aprofitat l'avantatge d'AAs com la Lys que té un alt grau de ramificació.[45] La forma globular que presenten donen alhora un augment de punts d'enllaç per a les molècules apolars d'estudi, característica aprofitada en tractaments de transport de fàrmacs.

Per altra banda, quan el pèptid insoluble i el pèptid curt solubilitzador s'uneixen mitjançant una **conjugació temporal**, és necessari la incorporació d'un espaiador bifuncional entre la seqüència d'estudi i el pèptid solubilitzador. La síntesi d'aquest conjugats també es realitza íntegrament en fase sòlida, i un cop escindit el pèptid de la resina, la seqüència apolar s'obté connectada a través de l'espaiador bifuncional al pèptid solubilitzador. L'estabilitat del connector a les condicions d'escissió és imprescindible, permetent que la seqüència es pugui manipular en solució, ja que així n'haurà augmentat la seva solubilitat en aigua. Posteriorment, un tractament específic facilita la desconnexió entre pèptid apolar i l'espaiador junt amb el pèptid solubilitzador, alliberant-se així el pèptid desitjat.

A la literatura es troben pocs casos enfocats a solubilitzar pèptids amb l'estratègia de connexió temporal. Durant els darrers anys han destacat treballs on solubilitzadors del

tipus $(Gly\text{-}Arg)_4$,[46] $(Gly\text{-}Arg/Lys)_{2\text{-}6}$,[47] o homololigopèptids d'Arg[48] o de Lys[49,50] han estat connectats a través d'espaiadors làbils a àcid[50] o base.[46,48,49]

En el següent capítol, en base a l'estratègia de solubilitzar molècules/pèptids apolars amb la unió d'homooligopèptids s'han realitzat diferents estudis, (i) inicialment, comparar les diferències de polaritat aportades segons el tipus d'AA i la seva disposició a l'espai d'estructures peptídiques solubilitzadores. Per altra banda, (ii) avaluar l'eficàcia de l'estructura Mmsb-OH com a espaiador bifuncional per connectar un pèptid insoluble a una cadena curta solubilitzadora, de manera temporal. Com a darrer objectiu, de manera paral·lela, (iii) s'ha decidit ampliar l'aplicació de l'espaiador bifuncional Mmsb-OH com a grup protector *C*-terminal per a permetre la síntesi de pèptids mitjançant la condensació de fragments en solució.

El dipèptid di-naftilalanina [$H\text{-}(Nal)_2\text{-}NH_2$], va servir de model apolar per al disseny de **conjugació permanent** a un conjunt de cadenes curtes solubilitzadores que tinguessin 4 grups polars lliures (2, 3, 4 i 8, Fig. 6), o bé amino (aportats per les Lys) o bé guanidini (aportats per les Arg), combinant pèptids lineals o ramificats. La síntesi d'aquests pèptids units al model apolar es va dur a terme totalment en fase sòlida seguint l'estratègia Fmoc/*t*Bu. L'escissió dels pèptids de la resina es va fer amb TFA 95% per tal d'eliminar els grups protectors tant de les Lys com de les Arg. El posterior anàlisi cromatogràfic per HPLC va permetre avaluar la polaritat de cadascun d'ells.

1 $H\text{-}(Nal)_2\text{-}NH_2$ **2** $H\text{-}(Lys)_3\text{-}(Nal)_2\text{-}NH_2$ **3** $H\text{-}(Arg)_3\text{-}(Nal)_2\text{-}NH_2$ **4** $Ac\text{-}(Arg)_4\text{-}(Nal)_2\text{-}NH_2$

5 $H\text{-}(Lys)_7\text{-}(Nal)_2\text{-}NH_2$ **6** $H\text{-}(Arg)_7\text{-}(Nal)_2\text{-}NH_2$ **7** $Ac\text{-}(Arg)_8\text{-}(Nal)_2\text{-}NH_2$

Pèptids Lineals

Pèptids Ramificats

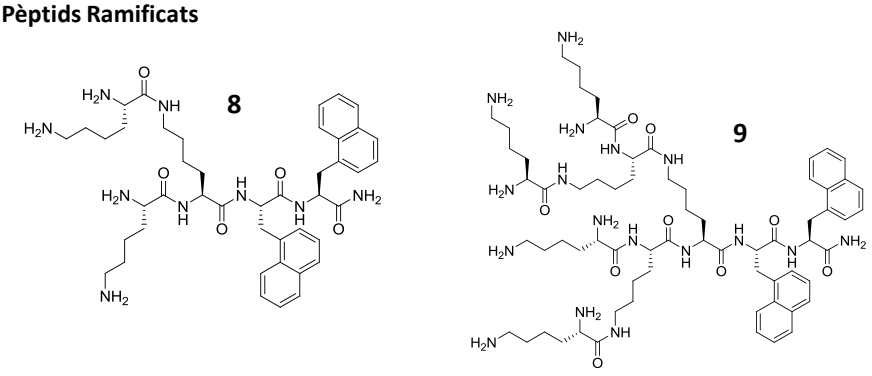


Figura 6. Estructura química dels pèptids sintetitzats.

La primera conclusió que es va extreure d'aquest experiment va ser que els grups amino aportaven una polaritat superior als grups guanidini; i per altra banda s'intuïa que el pèptid ramificat encara donava més polaritat que el seu homòleg lineal. Per tal de confirmar la tendència intuïda amb els primers pèptids, es va voler fer un pas més

ampliant el nombre de grups polars fins a 8, de manera que es va preparar una segona generació de pèptids (5, 6, 7 i 9, Fig. 6).

Un cop es van escindir els pèptids de la resina es van analitzar per cromatografia i es va observar que el poder per incrementar la polaritat de la molècula model s'accentuava més com més grups polars hi havia. De manera que la segona generació va permetre confirmar el què ja s'apuntava a la primera generació de pèptids, on el pèptid ramificat 9 era, amb diferència, el que més polaritat donava.

Un últim disseny peptídic (10, Fig. 7) va permetre comprovar la importància en termes de polaritat de la disposició espaial dels grups polars. Així que es va realitzar la síntesi d'un nou pèptid de 8 lisines disposades de manera que exposessin 4 grups amino de tipus α i 4 de tipus ε (comparable amb el 5 que en té 1 α i 7 ε i amb 9 que en té 4 α i 4 ε). De manera que el 10 compartia semblances amb el 9 pel què fa a la naturalesa dels grups amino exposats al medi, i semblances amb el 5 pel què fa a la disposició espaial lineal. L'anàlisi per HPLC va resoldre el dubte portant a la conclusió que, entre els tres, el ramificat 9 seguia sent el que aportava més polaritat, i que el 10 tenia una polaritat semblant a 5. Es demostrava la importància de la disposició a l'espai dels grups polars, més que no pas la naturalesa d'aquests grups.

Per tal de tenir donar una aplicació utilitzant molècula coneguda es va decidir agafar el pèptid que millors resultats de polaritat havia donat (9) i unir-lo a un fàrmac comercial insoluble en aigua (Indometacina, 11, Fig. 7). La síntesi es va fer íntegrament en fase sòlida, unint el fàrmac a través de la cadena lateral de la primera lisina introduïda com Fmoc-Lys(Mtt)-OH, després de l'eliminació del 4-metiltritil (Mtt). L'escissió del pèptid conjugat al fàrmac de la resina i posterior purificació va permetre la comparativa cromatogràfica que demostrava el notable increment de la polaritat del fàrmac. De la mateixa manera, es va analitzar la solubilitat en aigua a la mateixa concentració del fàrmac sol i d'aquest, conjugat, i es va observar clarament la millora de solubilitat quan aquest estava conjugat a les lisines ramificades.

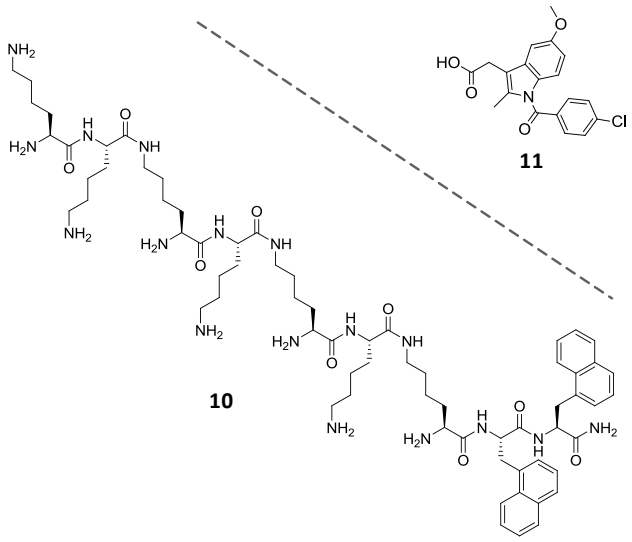


Figura 7. Estructura química de: (10) el pèptid amb 8 Lys i 4 grups amino tipus α i 4 tipus β; i (11) la Indometacina.

En relació al segon objectiu, aprofitant els avantatges obtinguts al capítol segon amb l'estructura Mmsb, en aquest capítol es van estudiar els beneficis d'utilitzar-lo com a espaiador bifuncional. La característica principal que l'Mmsb-OH comparteix amb el grup protector d'amida de cadena peptídica estudiat al capítol segon és relativa a la seva estructura, que el fa estable a condicions acídiques elevades, però làbil un cop reduït el sulfòxid. L'estudi realitzat amb aquesta molècula es va fer amb la química de pèptids en fase sòlida Fmoc/*t*Bu, aportant una primera novetat, ja que a la literatura es descriu l'ús de l'Mmsb-OH només en la química *tert*-butiloxicarbonil (Boc)/benzil (Bzl). La síntesi d'aquest espaiador bifuncional es va dur a terme seguint la mateixa ruta sintètica descrita per Thennarasu i col·laboradors, optimitzant-ne aquells passos que donaven rendiments baixos o que obligaven a fer una purificació.

Per tal de demostrar els avantatges d'introduir aquest espaiador com a grup protector semi-permanent es va escollir un pèptid conegut (Q11, Fig. 8) amb una seqüència amb característiques d'insolubilitat en medi aquós. Entre aquest espaiador bifuncional i la resina es va decidir introduir un conjunt de sis lisines com a pèptid solubilitzador, pretenent connectar un element de grups polars que aportés solubilitat (poly-Lys) a un pèptid clarament insoluble (Fig. 8). D'aquest mode, temporalment es té una seqüència soluble fàcilment tractable en solució, caracteritzable i purificable que permet, mitjançant un últim pas, separar aquesta unió i tenir el pèptid objectiu (Q11).

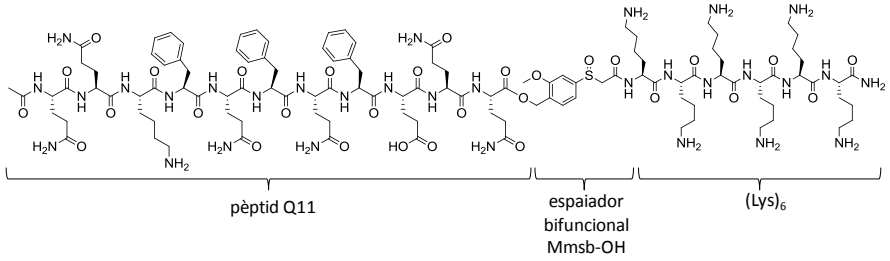


Figura 8. Seqüència del pèptid Q11 unit a través de l'espaiador bifuncional Mmsb a la poly-Lys.

La síntesi d'aquesta molècula es va fer totalment en fase sòlida i l'escissió d'aquesta de la resina es va dur a terme en condicions fortament àcides TFA/TIS/H_2O (95:2.5:2.5), de manera que es va obtenir l'estructura de la Figura 8. Posteriorment es va realitzar un seguiment amb HPLC i MALDI-TOF per tal de corroborar que, amb les mateixes condicions utilitzades per eliminar el grup Mmsb (NH_4I/TFA), es podia separar la seqüència Q11 del conjunt de lisines, quedant així el pèptid desitjat lliure.

Pel què fa al darrer objectiu, relatiu a una altra aplicació del mateix espaiador bifuncional, l'Mmsb-OH es va avaluar la possibilitat que formés part d'una seqüència com a grup protector *C*-terminal. Donada la necessitat de grups protectors d'àcid carboxílic en les estratègies de condensació de fragments en solució quan es volen obtenir pèptids àcid carboxílic en el seu extrem *C*-terminal, es va voler contribuir en aquest camp.[51] L'avaluació com a tal es va fer amb la condensació de dos fragments per a obtenir un segment de la seqüència d'un pèptid conegut de propietats terapèutiques per a la diabetis, l'Exenatide (NH_2-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-OH). Aprofitant la prèvia síntesi de l'Mmsb-OH, i la seva posterior introducció en fase sòlida, es va preparar el fragment *C*-terminal sense complicacions i amb una puresa elevada. Paral·lelament, també en fase sòlida es va sintetitzar el segon fragment, l'*N*-terminal, que contenia el grup Fmoc en el seu extrem amino de la cadena. Els dos pèptids van ser escindits de la resina independentment en condicions de TFA 1% que permetien la obtenció d'aquests amb les cadenes laterals protegides, així com la presència de l'Mmsb al fragment *C*-terminal.

La condensació de fragments en solució es va dur a terme amb l'agent d'acoblament PyBOP en presència de base, aconseguint el fragment desitjat amb una puresa acceptable del 92% per HPLC. La posterior eliminació del grup Fmoc en l'amino de l'*N*-terminal es va realitzar mantenint-se la puresa de l'etapa anterior. Finalment, l'eliminació del grup protector d'àcid carboxílic de l'extrem *C*-terminal mitjançant les condicions de reducció acidolítiques descrites pel grup protector d'amida Mmsb, van

permetre aïllar-ne el segment d'Exenatide desitjat sense haver augmentat el grau d'impureses.

En aquest capítol, en base als objectius plantejats amb les estructures poly-Lys i poly-Arg com a pèptids solubilitzadors units permanentment a un model de pèptid apolar, les polaritats obtingudes per HPLC van permetre deduir que: i) la seqüència poly-Lys lineal aporta més polaritat que la poly-Arg lineal; ii) l'estructura ramificada de poly-Lys contribueix en major grau a l'augment de la polaritat que la seva homòloga lineal; iii) el tipus de grup amino (α or ε) exposat al medi aquós influencia menys en la polaritat que no pas la distribució espaial d'aquest grups. Finalment, l'exemple de la seqüència solubilitzadora més polar de les proposades es va conjugar a un fàrmac apolar validant així l'eficàcia de l'augment de polaritat i solubilitat atribuït a aquesta estratègia.

Pel què fa a la mateixa estratègia de conjugació a una seqüència curta de pèptid solubilitzador es va dissenyar amb l'ús de l'espaiador bifuncional Mmsb-OH una unió temporal per a pèptids insolubles. L'estabilitat i labilitat posterior de l'espaiador bifuncional es va demostrar durant la síntesi, escissió del pèptid i trencament de l'enllaç d'unió amb la seqüència solubilitzadora. La cadena curta de 6 Lys va ser l'escollida per a facilitar l'anàlisi d'un pèptid insoluble en aigua, i el seu augment de solubilitat va corroborar l'objectiu assolit amb aquesta l'estratègia.

Com a darrer punt, pel mateix espaiador bifuncional Mmsb-OH s'ha ampliat el rang d'aplicacions, aquesta vegada emprat com a protector d'àcid carboxílic per al *C*-terminal. La condensació de dos fragments en solució d'un segment d'un pèptid conegut en va confirmar la seva validesa. L'escissió dels fragments mantenint les cadenes laterals dels AAs protegides i l'estabilitat també del protector Mmsb van permetre unir-los en solució amb una puresa adequada. Per finalment obtenir el segment desitjat es van eliminar alhora els protectors de les cadenes laterals d'AAs, així com el protector *C*-terminal, mitjançant una reducció acidolítica, fet que va validar la proposta d'aquest nou grup protector per a àcids carboxílics.

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