UNIVERSIDADE DA CORUÑA

MÁSTER UNIVERSITARIO EN INVESTIGACIÓN QUÍMICA E QUÍMICA INDUSTRIAL

PABLO CORTÓN DEBÉN

Peptide-viologen conjugates as supramolecular tools for nanomaterials design

Directors: Elena Pazos Chantrero

Marcos Daniel García Romero

Centro de Investigacións Científicas Avanzadas (CICA)

Course 2018/2019

Evaluation period: July 2019

Report of the Master's thesis
presented by Pablo Cortón Debén
for the obtention of the Master's Degree in
Chemical Research and Industrial Chemistry
at the UDC in 2019.

Acknowledgements

To the directors of this work, Elena Pazos Chantrero and Marcos Daniel García Romero, for their amazing support, care, interest and guidance. Thank you for giving me the chance to participate in this new exciting project and for the opportunity to keep working on these topics during my future thesis.

To all my classmates and my doctoral fellows from the CICA, for their help, for all the good moments we shared together and for their valuable friendship.

To Paula Novo Valencia, for her unconditional friendship and her continuous interest and trust. Thank you for being an amazing teammate and friend.

Thank you all.

Index

Abbreviatures

Alloc	allyloxycarbonyl
Boc	*tert*-butoxycarbonyl
Bzl	benzyl
CB[*n*]	cucurbit[*n*]uril
CD	Circular Dichroism
DEDTC	diethyldithiocarbamate
DIEA	*N*,*N'*-diisopropylethylamine
DLS	Dynamic Light Scattering
Fmoc	9-fluorenylmethoxycarbonyl
HBTU	*N*,*N*,*N'*,*N'*-Tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
MS	Mass Spectrometry
Mtt	4-methyltrityl
NMM	*N*-methylmorpholine
NMR	Nuclear Magnetic Resonance
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
rt	room temperature
SPPS	Solid Phase Peptide Synthesis
*t*Bu	*tert*-butyl
TEM	Transmission Electron Microscopy
TFA	trifluoroacetic acid
TIS	triisopropylsilane
TLC	Thin Layer Chromatography
TNBS	2,4,6-trinitrobenzenesulfonic acid
t_R	retention time
Trt	triphenylmethyl

Abstract

One of the main challenges in nanotechnology is the development of complex and smart materials, whose properties can be modulated by application of external stimuli such as changes on pH or red/ox potential. Among all biomolecules, peptides are versatile and biocompatible platforms that constitute ideal starting points for the development of materials with biomedical applications. In this context, the aim of this project is the design and development of coiled coil peptide conjugates functionalized with pseudoviologen units. These conjugates are expected to form supramolecular nanostructures upon the addition of cucurbit[8]uril, which can be controlled by modifying the pH of the medium.

For this purpose, an activated pseudoviologen and two peptides, which are known to form a parallel heterodimeric coiled coil structure, were prepared. Both peptides were coupled with the pseudoviologen moiety following a solid phase Zincke reaction protocol. NMR and HPLC-MS were used to follow the reactions and characterize the products. The final conjugates were purified by reversed-phase semipreparative HPLC.

The formation of coiled coil heterodimers was confirmed by CD, and the self-assembly of the coiled coil oligomers upon addition of CB[8] was studied by UV spectroscopy, TEM and DLS. These techniques show good preliminary results that indicate the formation of nanometric fibrils.

Keywords: coiled coils, peptides, viologens, CB[8], supramolecular complexes, nanostructures.

Resumo

Un dos principais retos en nanotecnoloxía é o desenvolvemento de materiais complexos e intelixentes cuxas propiedades poidan ser modificadas mediante a aplicación de estímulos externos como cambios de pH ou de potencial red/ox. Entre todas as biomoléculas, os péptidos son plataformas versátiles e biocompatibles que constitúen puntos de partida ideais para o desenvolvemento de materiais con aplicacións biomédicas. Neste contexto, o obxectivo deste proxecto é o deseño e desenvolvemento de conxugados peptídicos con estrutura "coiled coil" funcionalizados con unidades pseudoviolóxeno. Espérase que estes conxugados formen nanoestruturas supramoleculares tras a adición de cucurbit[8]urilo, as cales poden ser controladas modificando o pH do medio.

Para este propósito, prepararonse un pseudoviolóxeno activado e dous péptidos coñecidos por formar unha estrutura de "coiled coil" paralela e heterodimérica. Ambos péptidos acopláronse coa fracción pseudoviolóxeno seguindo un novo protocolo de reacción de Zincke en fase sólida. Empregáronse técnicas de RMN e HPLC-MS para seguir as reaccións e caracterizar os produtos. Os conxugados finais foron purificados mediante HPLC semipreparativo en fase reversa.

A formación dos heterodímeros "coiled coil" confirmouse por DC. Finalmente, estudiouse a autoensamblaxe dos oligómeros "coiled coil" tras a adición de CB[8] mediante espectroscopía UV, TEM e DLS. Estas técnicas mostran bos resultados preliminares que indican a formación de fibras nanométricas.

Palabras chave: "coiled coils", péptidos, violóxenos, CB[8], complexos supramoleculares, nanoestruturas.

Resumen

Uno de los principales retos en nanotecnología es el desarrollo de materiales complejos e inteligentes cuyas propiedades se puedan modificar mediante la aplicación de estímulos externos como cambios de pH o de potencial red/ox. Entre todas las biomoléculas, los péptidos son plataformas versátiles y biocompatibles que constituyen puntos de partida ideales para el desarrollo de materiales con aplicaciones biomédicas. En este contexto, el objetivo de este proyecto es el diseño y desarrollo de conjugados peptídicos "coiled coil" funcionalizados con unidades pseudoviológeno. Se espera que estos conjugados formen nanoestructuras supramoleculares tras la adición de cucurbit[8]urilo, las cuales pueden ser modificadas modificando el pH del medio.

Para este propósito, se prepararon un pseudoviológeno activado y dos péptidos conocidos por formar estructuras "coiled coil" paralelas y heterodiméricas. Ambos péptidos se acoplaron con la fracción pseudoviológeno siguiendo un mecanismo de reacción de Zincke en fase sólida. Se emplearon técnicas de RMN y HPLC-MS para seguir las reacciones y caracterizar los productos. Los conjugados finales fueron purificados por HPLC semipreparativo en fase reversa.

La formación de heterodímeros "coiled coil" se confirmó por DC. Finalmente, se estudió el ensamblaje de los oligómeros "coiled coil" tras la adición de CB[8] mediante espectroscopía UV, TEM y DLS. Estas técnicas muestran buenos resultados preliminares que indican la formación de fibrillas nanométricas.

Palabras clave: "coiled coils", péptidos, viológenos, CB[8], complejos supramoleculares, nanoestructuras.

1. Introduction

Nanotechnology, as one of the most promising and challenging fields of innovation and research, has a wide range of potential applications in many areas as new materials, industry and medicine. Particularly, nanotechnology has the potential to be applied to the field of nanomedicine, whose research topics range from diagnostic and therapeutic tools to regenerative medicine, ultimately improving people's quality of life.

Biocompatible and bioactive molecules, capable of self-assembling into nanostructures that degrade over time *in vivo* after fulfilling their function, constitute ideal building blocks for developing systems for biomedical applications. Among biomolecules, peptides offer the greatest functional and structural versatility, combined with synthetic simplicity, intrinsic biocompatibility and high biodegradability. Most commonly, self-assembled peptide nanostructures arise from stackable motifs, typically β-sheet-like hydrogen bonding units, that form filamentous assemblies which are able to induce complex cellular processes.[1]

However, despite these advances on the development of peptide-based nanomaterials, one of the key challenges of nanotechnology is the development of smart materials whose properties may be modulated upon the application of external stimuli such as light, pH or red/ox changes. In this context cucurbit[*n*]uril-based host:guest chemistry offers great opportunities for the development of stimuli responsive assemblies based on organic molecular switches like viologens.[2]

In this work, we propose the development of responsive high aspect ratio nanostructures whose constitution can be controlled on demand, from a bottom-up approach using simple components. To this end, we intend to use coiled coil peptides (a well-known supersecondary structure found in proteins that shows a remarkable richness both in structure and function) in combination with viologen-cucurbituril host:guest complexes.

1.1. Peptides and proteins: structure and bonding

Peptides and proteins are natural biopolymers with a wide variety of functions and structures, made of monomeric units called amino acids. There are 20 standard amino acids with a common structure, formed by an α-carbon bonded to an amine group, a carboxylic group, a hydrogen atom and a side chain R, which is specific to each amino acid (**Figure 1**). Due to this disposition of the amine and the carboxylic groups, standard amino acids are also called α-amino acids. Moreover, they all share the same stereochemistry and belong to the L series.

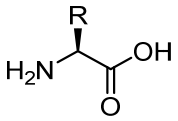


Figure 1. *Standard amino acid structure.*

All amino acids are named depending on their side chain and can be also identified by a three- or one-letter code. They are classified based on the nature of the functional groups at their side chains, being non-polar (G, A, V, L, I, M, F, P, W), polar (S, T, N, Q, Y, C), acid (D, E) or basic (K, R, H) standard amino acids.

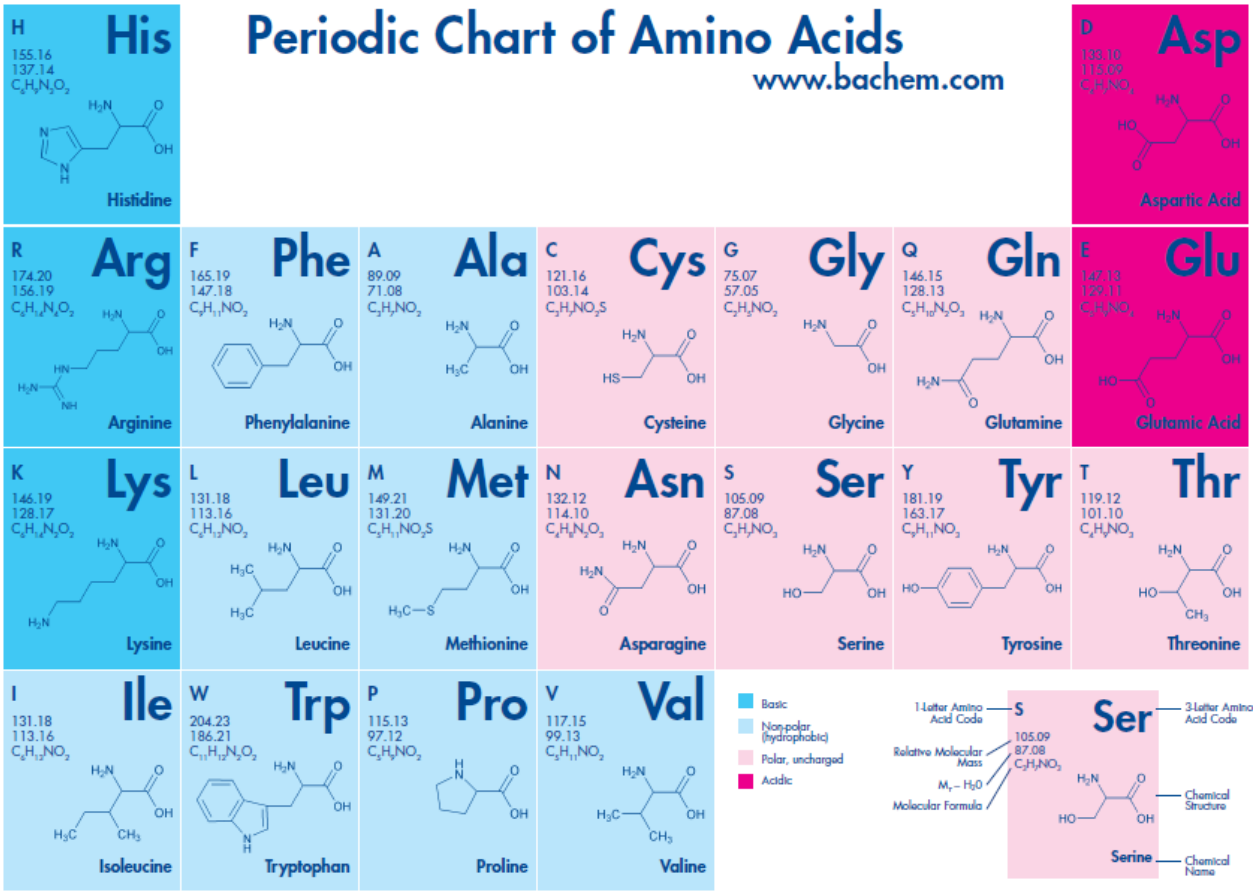


Figure 2. *Structure and classification of amino acids.*[3]

The amine and the carboxylic groups of amino acids imply that all of them are defined by at least two pK_as, whereas some of them present a third pK_a corresponding to a functional group of the side chain that may be ionized. The pH value where a certain amino acid presents net charge equal to zero is described as the isoelectric point (*pI*).

Name	pK			pI
	pK α-CO2H	pK NH3	pK R-group	at 25°C
Alanine	2.35	9.87		6.11
Arginine	2.18	9.09	13.2	10.76
Asparagine	2.18	9.09	13.2	10.76
Aspartic Acid	1.88	9.60	3.65	2.98
Cysteine	1.71	10.78	8.33	5.02
Glutamic Acid	2.19	9.67	4.25	3.08
Glutamine	2.17	9.13		5.65
Glycine	2.34	9.60		6.06
Histidine	1.78	8.97	5.97	7.64
Isoleucine	2.32	9.76		6.04
Leucine	2.36	9.60		6.04
Lysine	2.20	8.90	10.28	9.47
Methionine	2.28	9.21		5.74
Phenylalanine	2.58	9.24		5.91
Proline	1.99	10.60		6.30
Serine	2.21	9.15		5.68
Threonine	2.15	9.12		5.60
Tryptophan	2.38	9.39		5.88
Tyrosine	2.20	9.11	10.07	5.63
Valine	2.29	9.74		6.02

Figure 3. *pK_a and pI chart of standard amino acids.*[4]

Amino acids form the primary structure of peptides and proteins by successively bonding to each other through amide bonds, also called peptide bonds. Each amide bond is planar and presents restricted rotation, due to a certain double bond character at the $C_{carbonyl}$-N link. However, C_α-N and C_α-$C_{carbonyl}$ bonds rotate freely. Two angles are defined by these rotations, ψ and ϕ, which take values between -180º and +180º.

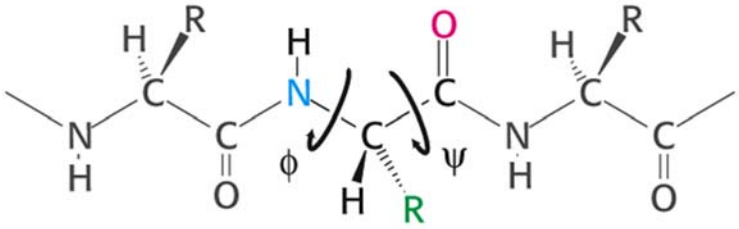


Figure 4. *Peptide bond rotation.*[5]

The folding of the primary polypeptide chains by angles ψ and ϕ constitutes the secondary structure of peptides and proteins. Hydrogen bonds and other intermolecular interactions allow different secondary structures, as α-helixes, β-sheets, twists or random coils. The first two were predicted by Linus Pauling and Robert Corey based on hydrogen bonding (HB) and cooperativity criteria.[6,7]

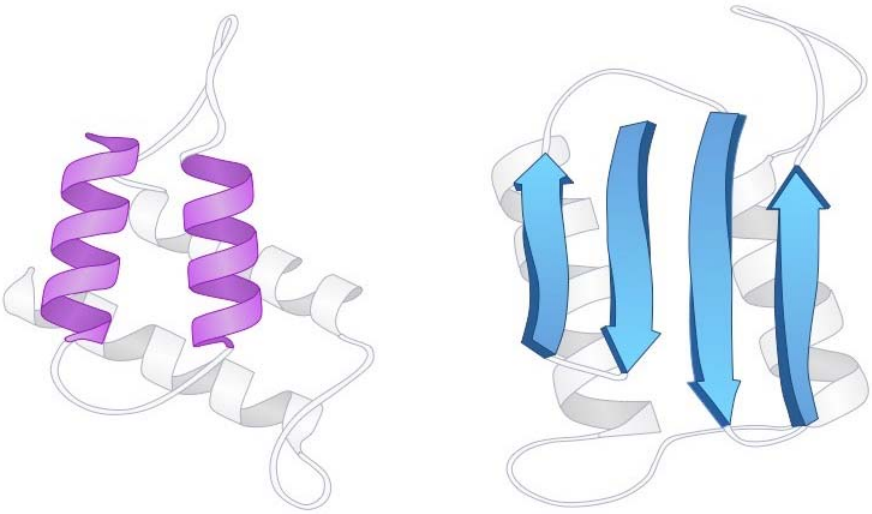


Figure 5. *α-helix (left) and β-sheet (right) secondary structures schematic representation.*[8]

α-helixes present a right-handed helicoidal shape with 3.6 residues per turn. These structures are stabilized by HBs between NH and C=O groups of consecutive turns and by Van der Waals interactions. HBs are parallel to the main axis of the helix, while side chains of all amino acids are disposed perpendicularly and facing outwards. β-sheets, on the other hand, are known for presenting HBs between adjacent chains and forming slightly wrinkled plates. Those bonds are in this case perpendicular to the axis of the sheets. Both chains can be oriented in a parallel or an antiparallel way, and may be joined and belong to the same sequence or not.

Proteins also present a tertiary structure, which consists on the spatial and geometrical distribution of the secondary structure. This may form new HBs, electrostatic, hydrophobic or Van der Waals interactions, establishing stable tridimensional conformations called protein domains. Protein domains are often enough to perform functions by themselves. However, some proteins are formed by more than one domain, which attach each other via non-covalent interactions and constitute the quaternary structure of proteins.

1.2. Coiled coils

As a combination of adjacent secondary elements, a very common supersecondary structure of peptides and proteins is the coiled coil structure, which is present in many natural proteins as transcription factors[9,10] or filaments[11]. These structures show great variety in function and properties, depending on their sequence, and are formed by from two to seven α-helixes oligomerized together, building a left-handed superhelix.

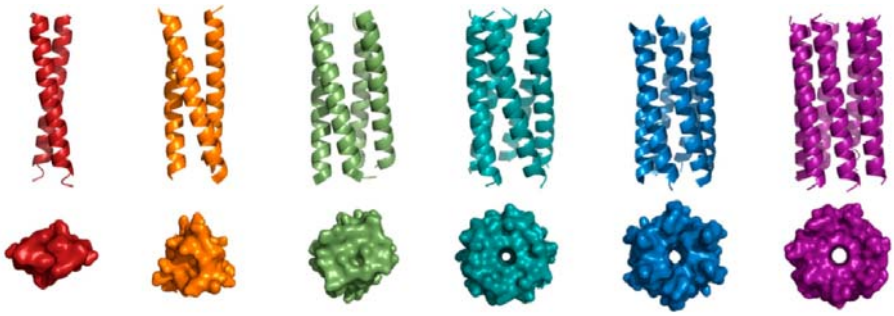


Figure 6. *X-ray crystal structures of coiled coils, from a dimer to a heptamer.*[12]

The general sequence of coiled coils is constituted by a periodic repetition of seven amino acid, also called heptad (-*a*-*b*-*c*-*d*-*e*-*f*-*g*-). The oligomerization is mediated by hydrophobic interactions between residues at the first (*a*) and fourth (*d*) positions of the heptad, which are typically isoleucine (Ile) and leucine (Leu) residues. Those side chains pack in the interhelical space in a knobs-into-holes manner. On the other hand, residues at *e* and *g* positions usually present charged side chains, as they face each other in opposite helixes and may stabilize the oligomerization via electrostatic interactions. Glutamic acid (Glu) and aspartic acid (Asp) are the typical negatively charged residues, whereas the usual positively charged amino acids are arginine (Arg) or lysine (Lys). Finally, the resting positions of the heptad (*b*, *c* and *f*) are facing outwards the superhelix. Therefore, residues at those positions play an important role in solubility and can have recognition functions.

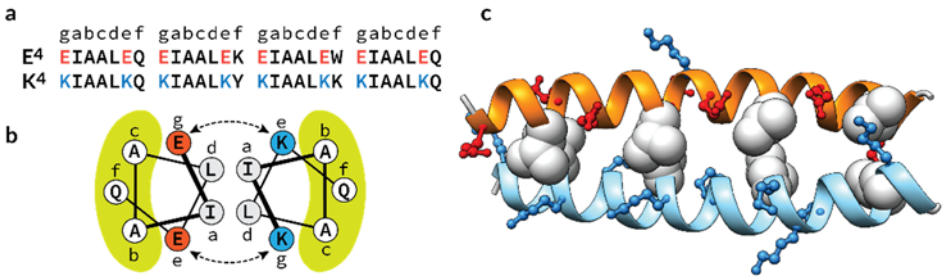


Figure 7*. Sequences of a coiled coil heterodimer (a), schematic diagram showing the disposition of the amino acids of the heptads (b) and model structure of the coiled coil (c).*

The most common coiled coil structures, which are dimers, vary from parallel to antiparallel, and from homo- to heterodimers. The coiled coil formation and the disposition of an artificial peptide can be programmed by following the above-mentioned criteria for the sequence design. In order to achieve a stable oligomerization, the sequence must be formed by three to five heptads.[13,14]

The programmability, stability and specificity of these coiled coil units make them a very powerful tool for the design of well-defined systems with tailored properties. For example, several systems have been developed in the recent years using coiled coils, as polyhedral

cages,[15] metal-modified oligomers[16], "sticky-ended" dimers that form fibers or nanotubes,[12,17,18] hexamers with mutable channels[19] or tridimensional crystals with incorporated guests.[20] However, despite these developments, the functionalization of these coiled coil based structures to produce stimuli-responsive materials has not yet been explored.

1.3. Solid Phase Peptide Synthesis (SPPS)

First developed by Merrifield in 1951,[21,22] solid phase peptide synthesis is the most widely used method for the preparation of peptides. SPPS consists in the addition of successive amino acids, which have been adequately modified, to a polymeric support. Residues are added following the order of the desired sequence from the *C*-terminus to the *N*-terminus, as the resin is bonded to the *C*-terminal amino acid, using a linker.

There are different polymeric supports available, which play a role on the efficiency of the coupling. A chosen resin must present the ability to swell and solvate appropriately with the coupling solvent, as most of the reactions occur inside the pellets of the resin and all reagents should have easy access. Besides, the linker must remain unbroken until the very end of the peptide synthesis, and it defines which functional group (carboxylic acid, amide, etc.) will remain at the *C*-terminus, once the peptide is cleaved from the resin.

To assure that they attach to the previously added residue and to avoid unwanted reactions, all added amino acids must have their amine group protected. Those protective groups are removed afterwards in order to perform the next amino acid coupling.[23] Functional groups of the side chains must also be protected. However, those protective groups must remain untouched until the final cleavage of the peptide synthesis, resisting both the coupling and the deprotection conditions for the other temporary protective groups.

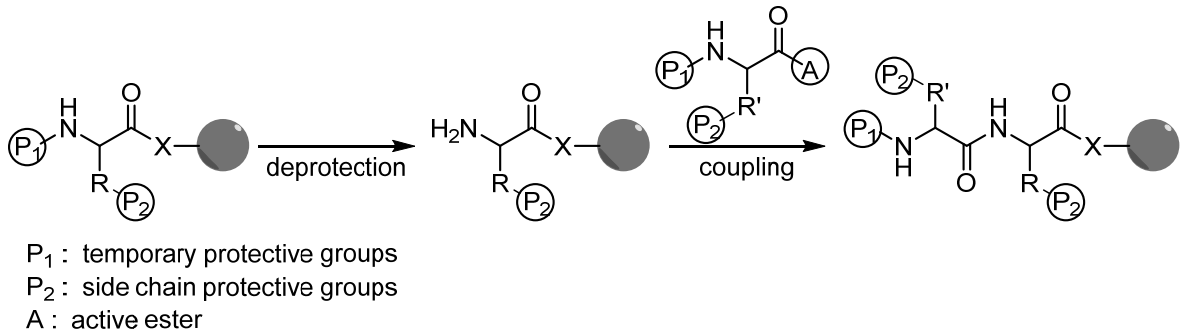


Figure 8. *General amino acid coupling scheme for SPPS.*

Keeping this in mind, there are two basic strategies for SPPS, using two different sets of orthogonal protective groups: Boc/Bzl and Fmoc/*t*-Bu.[24] The first strategy utilizes Boc protective groups for the α-amino groups, which can be deprotected by mild acidic conditions

(TFA). Bzl groups are reserved to the side chain functional groups, and can be removed at the final cleavage step under strong acidic conditions (HF). This method may be not very convenient, as the HF is very hazardous and the continuous use of TFA may cause trouble with some sensitive side chains.[25]

On the other hand, the second, more convenient, strategy protects the α-amino groups with Fmoc, which can be easily deprotected with basic media (piperidine). The functional groups of the side chains are protected with *t*-Bu or other similar functional groups like Trt, that can be removed by acidic media (TFA), along with the cleavage of the peptide from the resin.

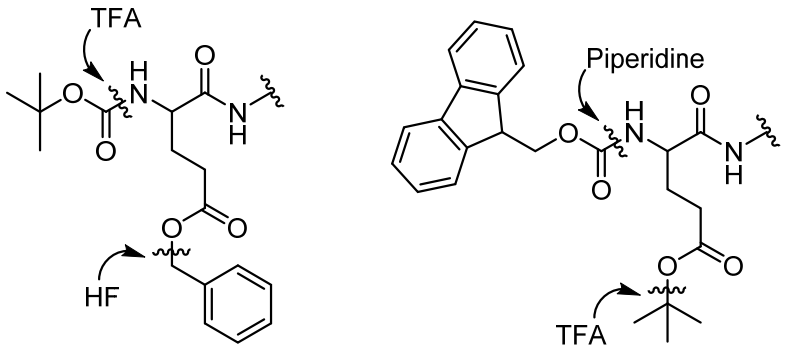


Figure 9. *Boc/Bzl (left) and Fmoc/t-Bu (right) strategies for amino acid protection.*

Additionally, carboxylic groups of the added residues must be activated so that the coupling occurs. There are four main ways of activation: carbodiimides, acid anhydrides, activated esters and activated esters generated *in situ*.

Although carbodiimides are widely used, they may dehydrate Asn and Gln residues. This problem can be avoided by the addition of HOBt to the reaction mixture. Acid anhydrides are a less efficient method, as they are usually generated *in situ* using two equivalents of amino acids. Activated esters are also very used and they are usually generated *in situ*, as it is a simpler method. Their biggest drawback is that they decompose when dissolved with DIEA, which is the most commonly used base for SPPS. Therefore, the activation is performed just before the amino acid coupling.[26]

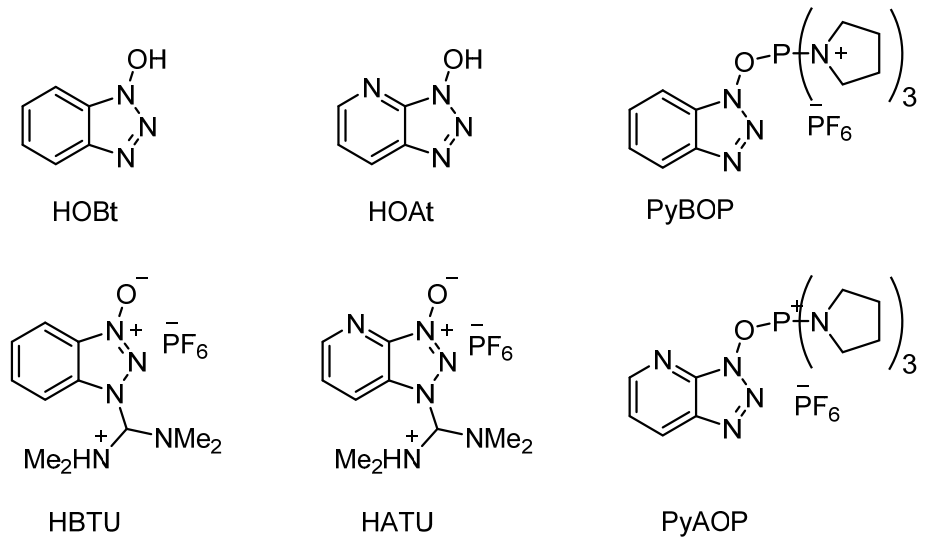


Figure 10. *Most commonly used coupling agents for the generation of activated esters in situ.*

1.4. Supramolecular chemistry and viologens

Viologens are *N*,*N*'-disubstituted organic salts derived from 4,4'-bypyridine.[27] Monosubstituted derivatives are sometimes called pseudoviologens. These compounds can be prepared by nucleophilic substitution or by the Zincke reaction with primary amines.[28]

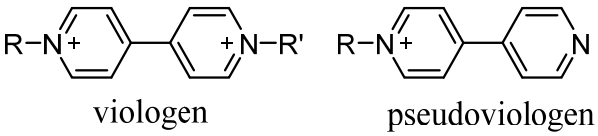


Figure 11. *Structure of viologens and pseudoviologens.*

Viologens and pseudoviologens have been widely used on the fields of materials and supramolecular chemistry,[29–31] as they show interesting properties like a rich red/ox activity and a π-deficient electronic behavior. In particular, these molecules can be encapsulated as guests inside cucurbituril units *via* supramolecular interactions, thanks to their hydrophobic inner part and their positive charges at the extremes.

Cucurbit[*n*]urils (CB[*n*]) are pumpkin-shaped cyclic oligomers generated from the reaction of n glycoluril units with formaldehyde.[32] These molecules interact with a variety of inorganic and organic molecules, and can act as hosts in inclusion complexes.

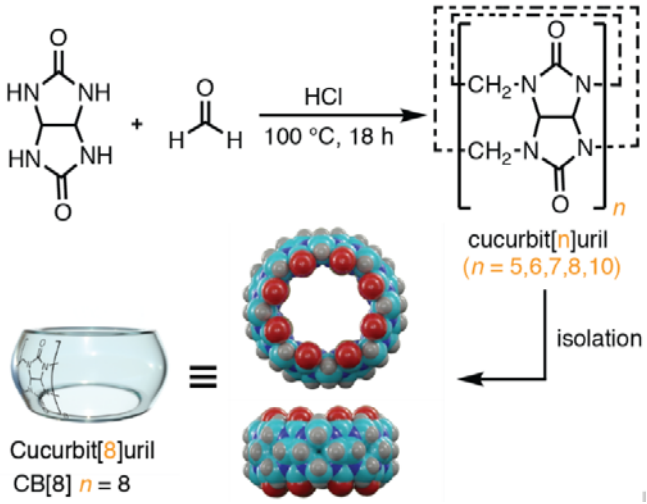


Figure 12. *Preparation and schematic representations of cucurbit[n]urils.*[32]

CB[*n*]s present so called portal areas of great electronic density, the carbonylated rims, that may bind *via* ion dipole interactions to positively charged molecules like viologens themselves. On the other hand, the hydrophobic part of the guest would remain inside the CB[*n*] cavity, displacing the water molecules that were encapsulated inside the host and enhancing the aggregation *via* hydrophobic effect.

In particular, CB[8] is a very interesting host, as it is the smallest member of the cucurbit[*n*]uril family that is capable of accepting two appropriate guests. In that way, it can predictably form binary 1:1 and homoternary 2:1 complexes with molecules like monocationic pseudoviologens.

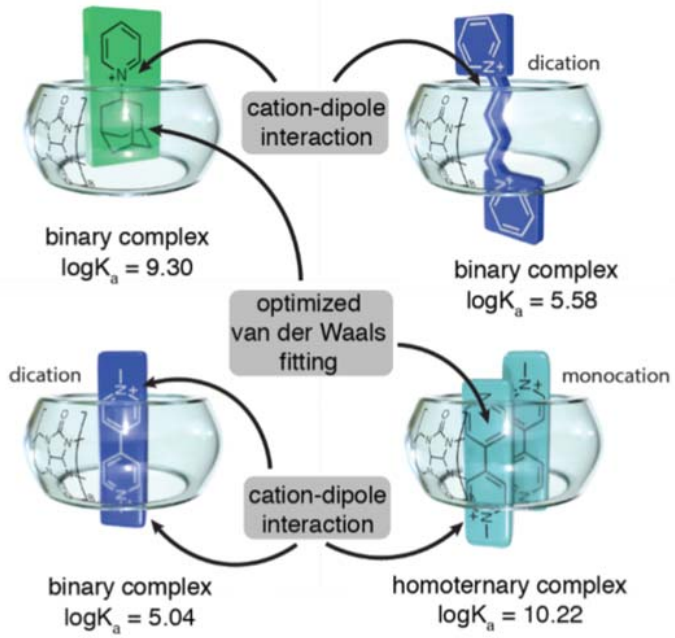


Figure 13. *Schematic representations of binary and homoternary complexes between CB[8] and amphiphilic guests.*

Those host:guest complexes may act as supramolecular switches, as they can respond to external stimuli. For example, the homoternary complex between CB[8] and two monocationic pseudoviologen units can be broken and reversibly turned into a binary 1:1 complex by changing the pH. Acidic conditions protonate the pseudoviologens converting them into dications, which cannot remain inside the host together, due to the repulsion between the positive charges. For the same reason, two viologen units do not fit inside one CB[8] molecule. However, the reversible reduction of these compounds turns them into radical cations, which assemble into homoternary 2:1 complexes.[33]

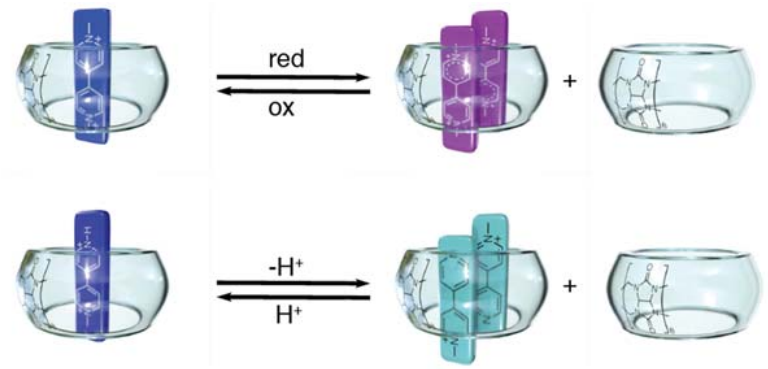


Figure 14. *Schematic representation of stimuli responstive supramolecular switches based on CB[8]:(pseudo)viologen host:guest complexes.*

Additionally, thanks to its previously mentioned characteristics, the hydrophobic yet polar cavity of CB[8] is capable to stabilize charge-transfer interactions. Therefore, heteroternary 1:1:1 can be formed by the inclusion of an electron-acceptor compound such as viologens and another electron-donor species that could not be encapsulated otherwise.[34] The charge-transfer interaction can be optimized by modulating the character of the donor/acceptor pair.

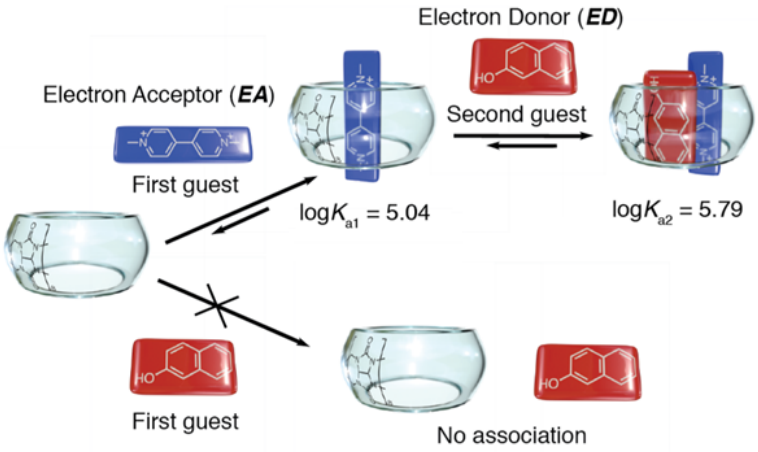


Figure 15. *Schematic representation of charge-transfer stabilization via heteroternary complex formation.*[35]

1.5. Peptide conjugates and other cucurbit[8]uril supramolecular systems

The synthesis of viologen-peptide conjugates was previously described by Reczek *et al.*[34,36] Their strategy consisted in the preparation of an aminoalkyl viologen and a Glu-containing peptide with those residues orthogonally protected. After the acetylation of the *N*-terminus and the selective deprotection of the Glu side chains, the carboxylic groups would be activated and the viologen units would be coupled in solid phase, forming amide bonds.

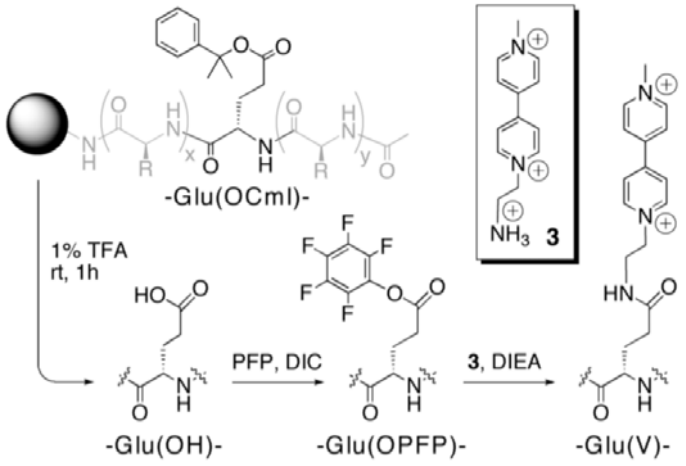


Figure 16. *Solid phase viologen-peptide conjugate synthesis via amide bond formation.*[36]

This method was used for the preparation of peptide scaffolds with viologen units at certain positions. This building block can be reversibly attached to another peptide scaffold containing Trp residues, *via* the formation of heterotrimeric 1:1:1 complexes with CB[8] where the Trp acts as the electron-donor.[34]

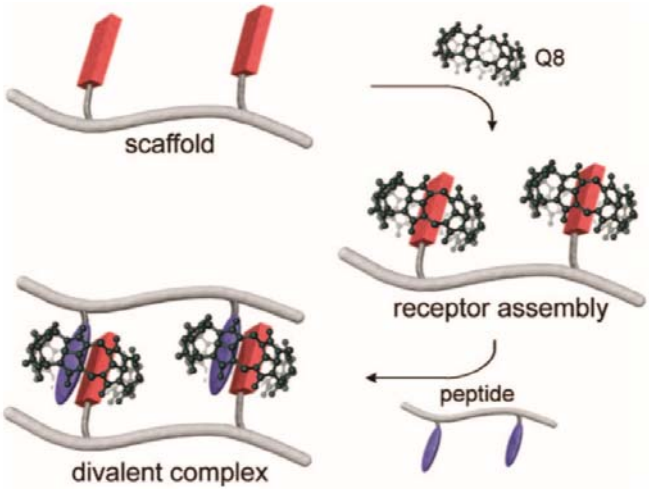


Figure 17. *Self-assembly of peptide scaffolds via inclusion heterocomplexes with CB[8] (Q8).*[34]

Other cucurbit[*n*]uril-peptide systems have been described, in which CB[8] recognizes a peptide with a *N*-terminal aromatic amino acid or a *N*-terminal Met, forming a complex with those residues and the immediate neighbors.[37] The inclusion of two side chains of two contiguous peptides, instead of one single chain, can yield to the formation of reversible dimeric systems, as described by Wu *et al*.[38] Additionally, Clarke *et al*. described the formation of conductive nanofibers from viologen-peptide conjugates *via β*-sheet self-assembly.[39]

Finally, some supramolecular polymers and frameworks which present viologen-cucurbit[8]uril complexes as binding units have been described. These systems may be generated *via* charge-transfer complexation[40] or by the homoternary complex formation with pseudoviologen rod-like monomers.[41]

2. Objectives

As part of a larger research project, which aims to build up smart modulable peptide-based materials whose properties may vary upon the application of external stimuli, this work is focused on the development of two viologen-peptide conjugates that will form coiled coil heterodimers and would reversibly assemble into high aspect ratio nanostructures after the complexation with CB[8].

In this context, the main goals of the present project are:

1. Synthesis of two peptides that are known to form parallel heterodimeric coiled coil structures using solid phase peptide synthesis protocols. Those peptides will present an orthogonally protected Lys residue at the *N*- and *C*-terminus, respectively, that will be used for pseudoviologen conjugation.

2. Synthesis of pseudoviologen-peptide conjugates from the previously synthesized peptides and an appropriately activated pseudoviologen salt. The coupling will be performed by following a solid phase Zincke reaction mechanism.

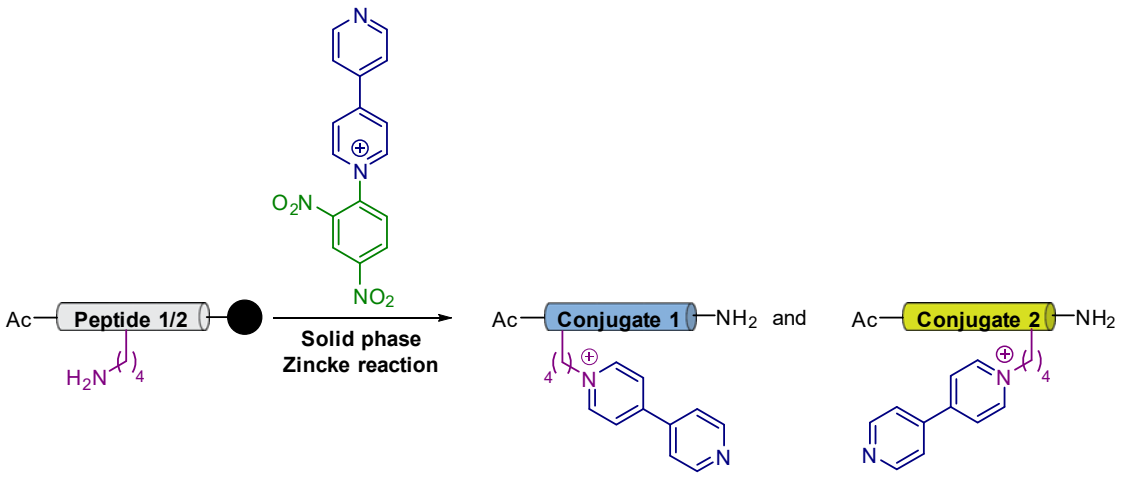


Figure 18. *General scheme of the pseudoviologen-peptide couplings through solid phase Zincke reaction.*

3. Study of coiled coil heterodimer formation and evaluation of their complexation and assembly upon the addition of CB[8].

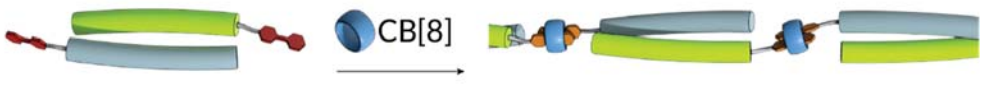


Figure 19. *Representation of the coiled coil assembly into high aspect ratio nanostructures.*

3. Results and discussion

3.1. Synthesis of the *N*-activated 4,4'-bipyridine derivative as precursor for the Zincke reaction

The conjugation of the pseudoviologen to the peptides was planned to be performed through a Zincke reaction adapted to a SPPS procedure. In order to achieve that, the first step of the synthesis was the activation of 4,4'-bipyridine as an *N*-arylated derivative. This salt would act as precursor for the Zincke reaction, which follows an ANROC (Addition of the Nucleophile, Ring Opening and Ring Closure) mechanism:[42]

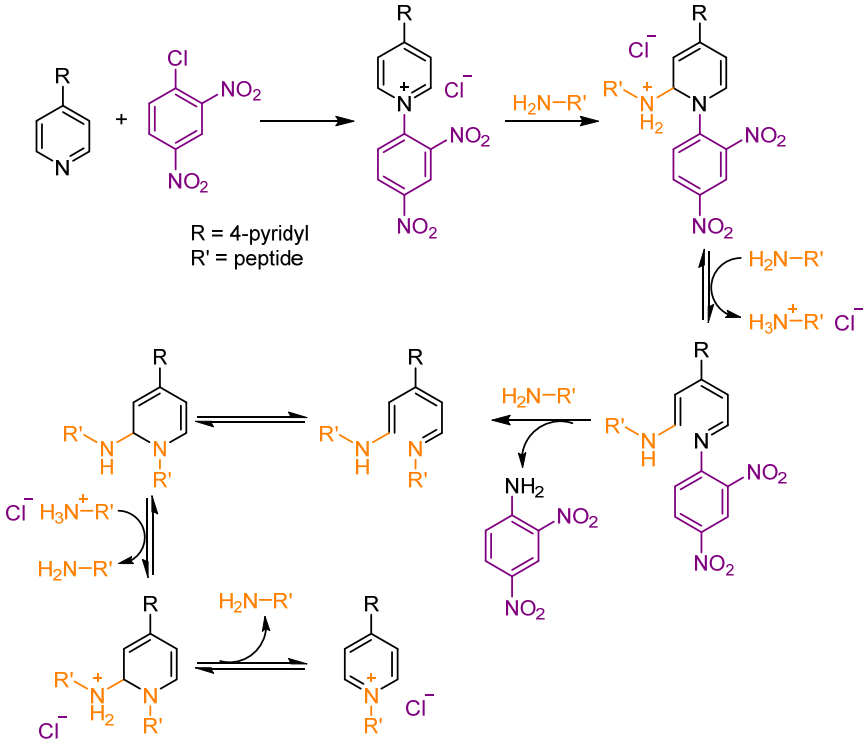


Figure 20. *ANROC mechanism for the Zincke reaction.*[42]

In this regard, compound **1** was appropriately synthesized by nucleophilic aromatic substitution of 1-chloro-2,4-dinitrobenzene with 4,4'-bipyridine in EtOH at reflux. After 24 h, the product was precipitated from the reaction mixture by the addition of Et_2O and washed with AcOEt. A brown crystalline solid was obtained with a yield of 83%. The product was considered virtually pure after its characterization by ^{1}H-NMR spectroscopy.

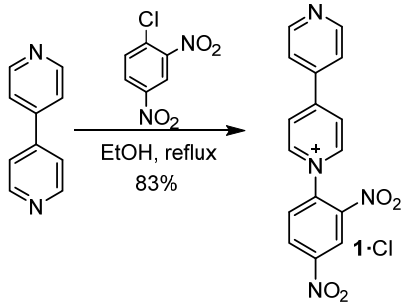


Figure 21. *Synthesis of compound* ***1****.*

3.2. Peptide design and synthesis

As previously commented, this work aims to develop self-assembled materials based on modified coiled coil units. Therefore, dimeric coiled coil peptide sequences must be designed. Since the pseudoviologen units would be placed in opposite extremes of the oligomers, there are two potential options for the coiled coil design. On one hand, the dimers can be formed by a single sequence (homodimers), with both peptides arranged in an antiparallel manner. On the other hand, dimers can be constituted by two different sequences (heterodimers), where both peptides can be disposed in a parallel or antiparallel fashion. Due to a lack of references dealing with antiparallel homodimeric coiled coils, we designed the conjugates starting with peptide sequences known to form stable heterodimers.[14]

Consequently, the two sequences were slightly modified by adding one Lys residue at their *N*- and *C*-terminus, respectively. The side chains of those residues would act as appropriate amine groups for the subsequent coupling with the pseudoviologen units. The final designed sequences are the following:

Fmoc-KGKIAALKQKIAALKYKNAALKKKIAALKQG-NH_2

Fmoc-GEIAALEQEIAALEKENAALEWEIAALEQGK-NH_2

Figure 22*. Peptide sequences*[14] *modified with a Lys residue (K, labelled in red) at the N-terminus (up,* ***P1****) and C-terminus (down,* ***P2****).*

The peptides were planned to be synthesized using the Fmoc/*t*-Bu strategy. Therefore, α-amino groups of all amino acids were temporarily protected with Fmoc groups, whilst the functional groups at the side chains were protected with TFA-labile moieties: Glu (E) and Tyr (Y) residues with *t*-Bu, Gln (Q) and Asn (N) with Trt, and Lys (K) and Trp (W) with Boc groups.

In addition, in order to perform the pseudoviologen coupling selectively at the terminal Lys residue side chains, those residues must be orthogonally protected. The Alloc group was chosen for this purpose, which can be selectively deprotected with Pd catalyst.

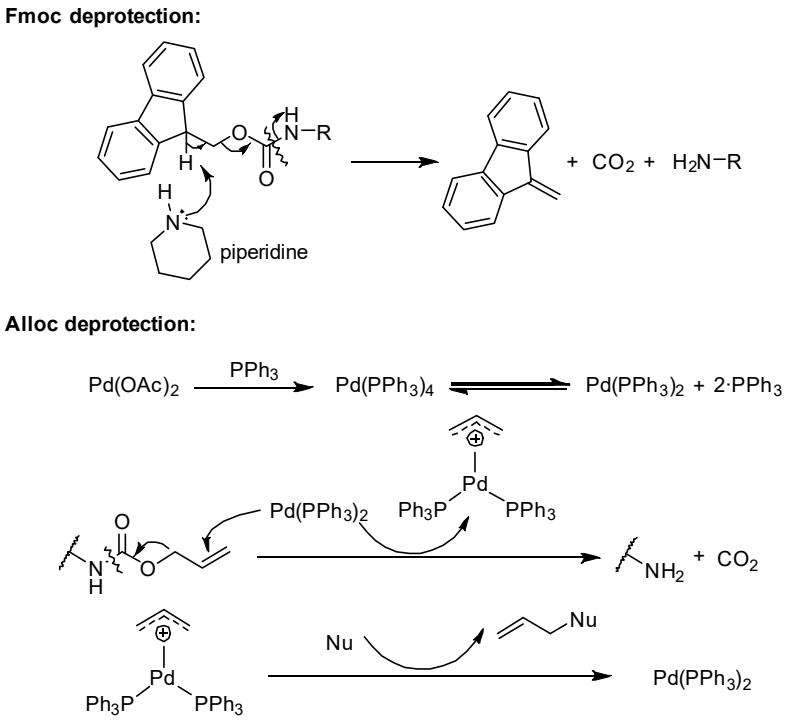


Figure 23. *Deprotection mechanisms of Fmoc (up) and Alloc (down) protecting groups.*

Moreover, one Lys residue of each chain was protected with Mtt, which can be selectively deprotected with diluted TFA. Those amino acids would occupy the *f* position of each coiled coil heptad, the most external one, and would serve as functionalization positions in future works.

The desired peptide sequences were successfully synthesized by following the standard procedure for solid phase synthesis, which will be further discussed at the experimental section. Both peptides were prepared at a 0.2 mmol scale and, after completing the full sequence, they were characterized by HPLC-MS, observing one main peak for each reaction crude that corresponds to the desired products **P1 (**t_R = 14.2 min) and **P2** (t_R = 18.0 min) (**Figure 24**).

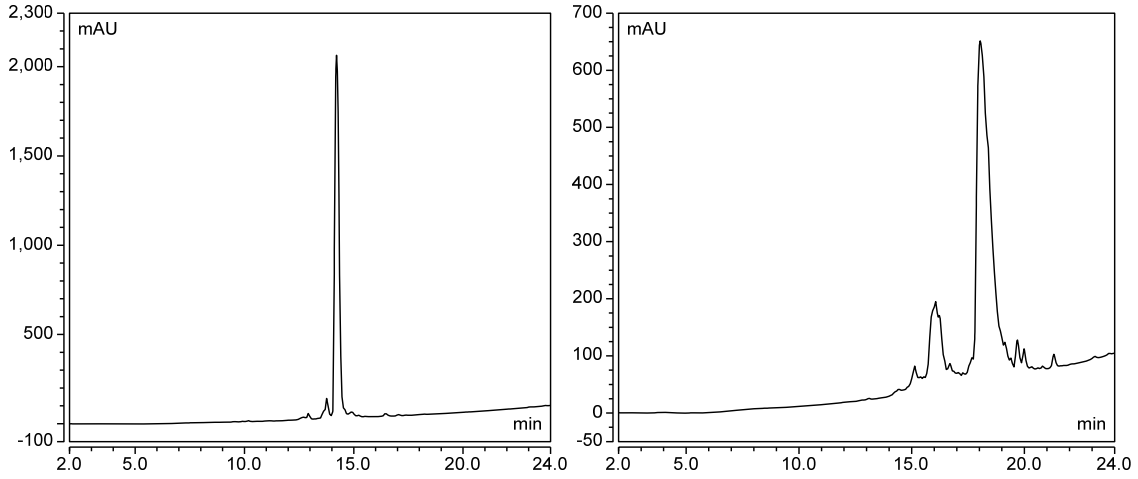


Figure 24. *HPLC chromatograms for peptides **P1** (left) and **P2** (right).*

3.3. Pseudoviologen-peptide conjugation

In order to avoid potential side reactions, the *N*-terminal Fmoc groups must be substituted, as they can be deprotected due to the basic character of the bipyridine derivatives. Therefore, the Fmoc groups were removed with an excess of 20% 4-methylpiperidine in DMF and both peptides **P1** and **P2** were acetylated with Ac_2O and DIEA in DMF.

Afterwards, Alloc groups were removed by reaction with Pd catalyst overnight., giving peptides **P5** and **P6**. Finally, the conjugation was performed by adding three equivalents of compound **1** and one equivalent of DIEA to the resin, and stirring the mixture for 90 min.

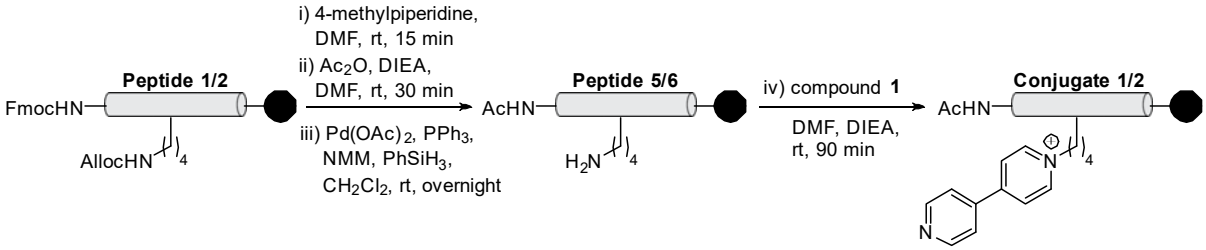


Figure 25. *Synthetic scheme for the acetylation and pseudoviologen-peptide conjugation.*

Both conjugates **C1** and **C2** were synthesized at a scale of 0.05 mmol. After the coupling step both resins were treated with the cleavage cocktail (2.5% TIS, 2.5% H_2O, 95% TFA), and the resulting reaction crudes were analyzed by HPLC-MS.

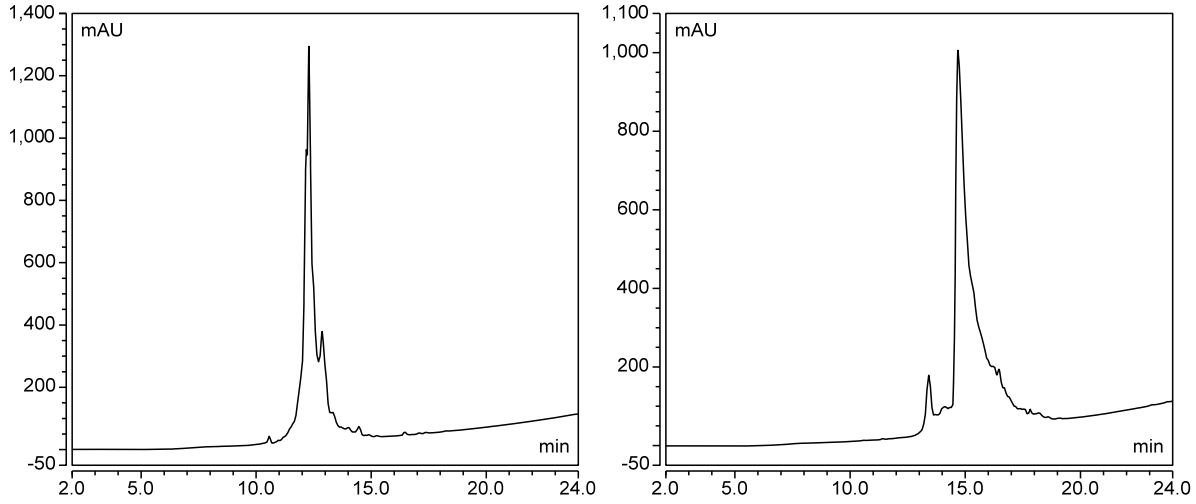


Figure 26. *HPLC chromatograms for the reaction crudes corresponding to conjugates* ***C1*** *(left) and* ***C2*** *(right).*

Both chromatograms (**Figure 26**) show major peaks that correspond to the desired products, at t_R = 12.3 min for **C1** and at t_R = 14.7 min for **C2**. The MS spectra (**Figure 27**) of both HPLC peaks confirm the identity of the conjugates:

C1: 1745.10 $[MH]^{2+}$, 1163.74 $[MH_2]^{3+}$, 873.05 $[MH_3]^{4+}$, 698.64 $[MH_4]^{5+}$.

C2: 1760.40 $[MH]^{2+}$, 1173.94 $[MH_2]^{3+}$.

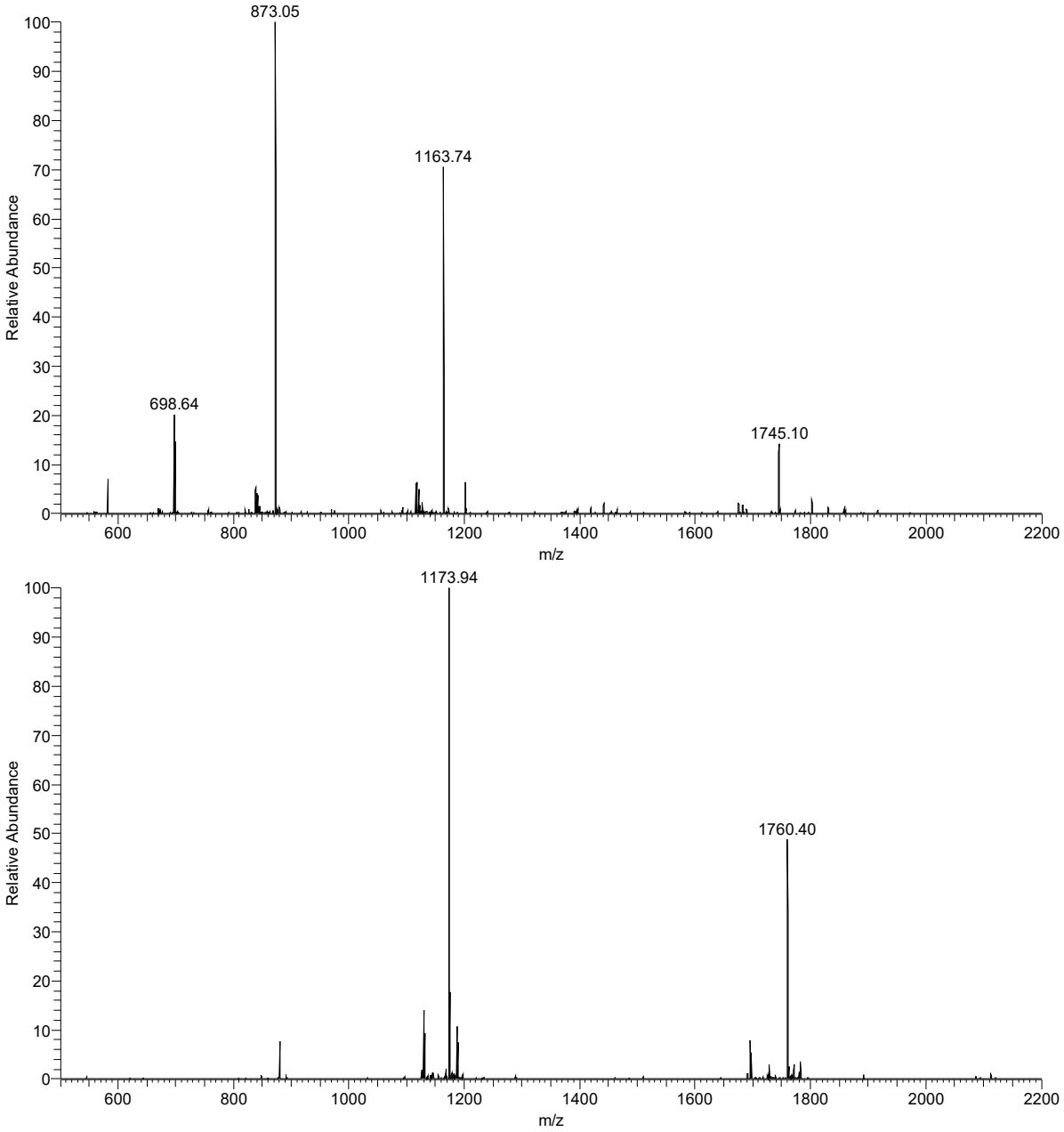


Figure 27. *MS spectra of the HPLC peaks at t_R = 12.3 min for **C1** (up) and t_R = 14.7 min for **C2** (down).*

Both peptide conjugates were purified by reverse-phase semipreparative HPLC and the collected fractions were lyophilized, obtaining fluffy white solids.

In order to calculate the concentration of conjugate **C1** and **C2** solutions, the molar extinction coefficient (ε) of the bipyridine moiety was calculated by UV spectroscopy. Therefore, the UV spectra of different solutions of methylpseudoviologen with known concentrations were recorded. After plotting the obtained absorbance values at 259 nm versus the concentration of

the solutions, and fitting the data using the Beer-Lambert law, the estimated ε was approximately 21243 $M^{-1}\cdot cm^{-1}$.

C1 and **C2** conjugates were dissolved in PBS, and their concentrations were calculated with the estimated ε, that takes into account the coefficients of the aromatic residues Tyr (ε = 21909 $M^{-1}\cdot cm^{-1}$ at 261 nm for **C1**) and Trp (ε = 26027 $M^{-1}\cdot cm^{-1}$ at 266 nm for **C2**).[43]

3.4. Assembly studies

Circular dichroism

First, in order to demonstrate that the pseudoviologen unit does not affect the formation of the coiled coil heterodimer, Circular Dichroism (CD) experiments were performed, since this technique allows the determination of the folding degree and secondary structure of peptides and proteins. From the intensity of the bands at 220 and 208 nm and their ratio, one can determine the helical content (100% if the molar ellipticity is below −32.000 $^{o}\cdot cm^{2}\cdot dmol^{-1}\cdot res^{-1}$), and if they are as isolated helixes or as coiled coils (a ratio > 1 indicates well-defined coiled coils while a ratio < 1 indicate a single helix).[44,45]

Using a 2 mm pathlength cuvette and solutions for each peptide with a concentration of 25 μM, the following spectra were obtained:

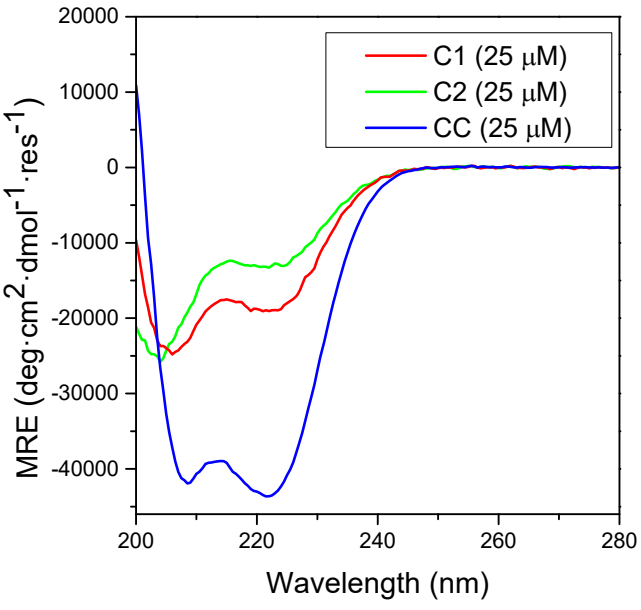


Figure 28. *CD spectra of a 25 μM solution of* ***C1*** *in PBS (10 mM. sodium phosphate, 137 mM NaCl, pH = 7.4), a 15 μM solution of* ***C2*** *in PBS (10 mM sodium phosphate, 137 mM NaCl, pH = 7.4), and for the equimolar mixture of both conjugates* ***C1*** *and* ***C2*** *(25 μM for each peptide) in PBS (10 mM sodium phosphate, 137 mM NaCl, pH = 7.4) labelled as* ***CC****.*

As it can be inferred from the negative bands at approximately λ = 208 and 220 nm, both conjugates **C1** and **C2** present certain α-helix contribution, but they appear as mainly disorder.

However, when both conjugates are together in solution, the α-helix contribution increases significantly, achieving an MRE value in the order of -40000 $^{o}\cdot cm^{2}\cdot dmol^{-1}\cdot res^{-1}$ at those wavelengths, with a ratio between both bands (220/208) > 1. Hence, it can be assumed that the coiled coil structures (**CC**) are fully formed in these conditions. The positive increment at 200 nm also indicates the presence of α-helixes, in contraposition with random coils, which would give in turn negative values around that wavelength value.

UV titration

In order to characterize the interaction of the coiled coils prepared with CB[8], a UV titration was performed. Absorbance measurements were registered upon the addition of small quantities of CB[8], while the concentration of coiled coil was kept constant at 15 μM. The absorbance data was taken at λ = 264 nm, the maximum of absorbance of the coiled coil units before the addition of CB[8].

As shown in **Figure 29**, the addition of CB[8] decreases the absorbance of the coiled coil heterodimer, as the inclusion of the bipyridyl units within the cucurbit[8]uril cavity substantially decreases the absorption. After the addition of a certain quantity of host, the absorbance stabilizes and does not decrease any further, meaning that full complexation of the pseudoviologen moieties has been achieved.

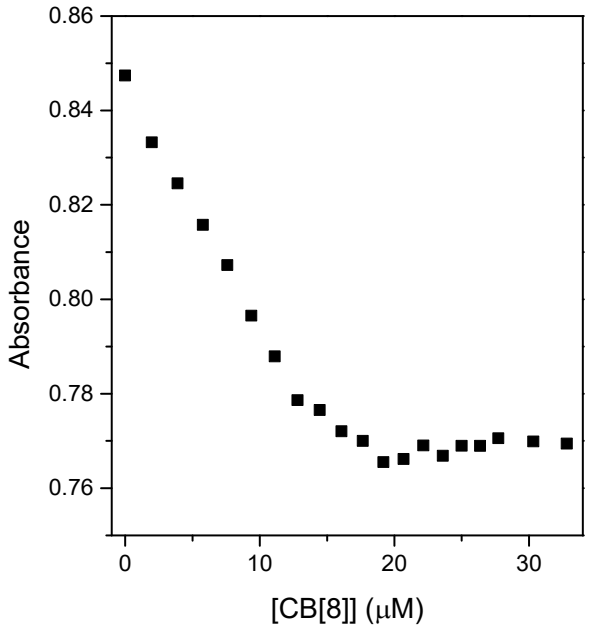


Figure 29. *Absorbance at 264 for a 15 μM solution of* ***CC*** *in PBS (10 mM sodium phosphate, 137 mM NaCl, pH = 7.4), with increasing concentrations of CB[8].*

In order to check whether CB[8] interferes with the formed coiled coil structure, circular dichroism experiments were repeated, this time comparing the coiled coil response in absence

and presence of CB[8]. As shown in **Figure 30**, there is no significant difference between both solutions, meaning that the host does not affect the coiled coil heterodimer.

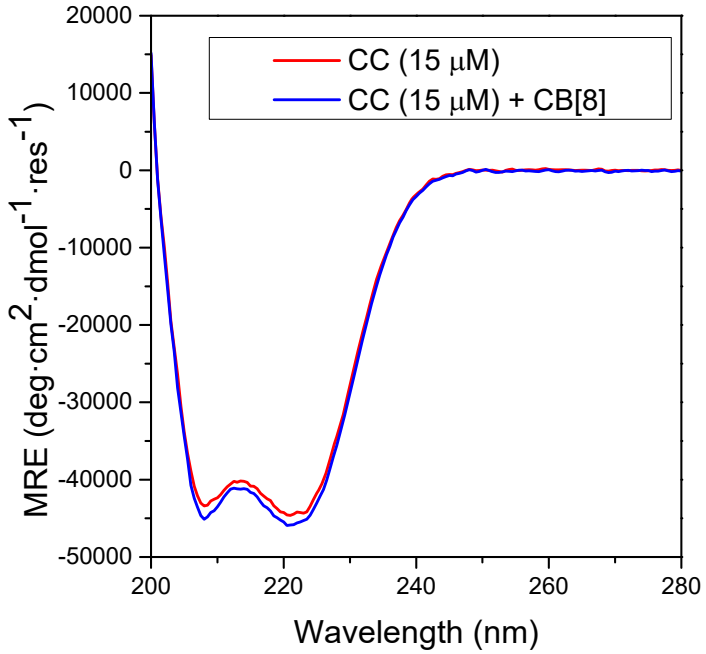


Figure 30. *CD spectra for the equimolar mixture of both conjugates **C1** and **C2** (15 μM for each peptide) in PBS (10 mM sodium phosphate, 137 mM NaCl, pH = 7.4) labelled as **CC** without and after the addition of a final CB[8] concentration of 68 μM.*

Microscopy

The resulting mixtures obtained after the UV titration (**CC** 15 μM, CB[8] 68 μM) were also characterized by TEM. The samples were deposited on Formvar coated Ni grids and stained with uranyl acetate. TEM images show the formation of small fibrils with a length between 40-70 nm and a diameter of 2-3 nm (**Figure 31**).

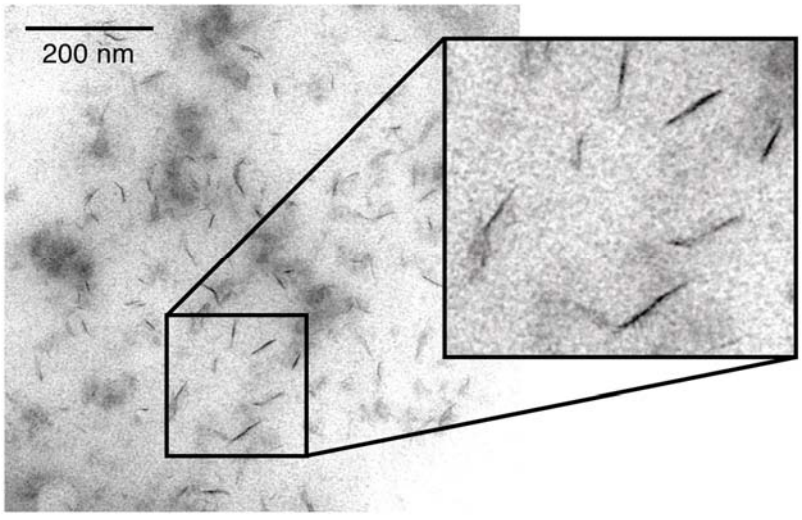


Figure 31. *TEM images of the nanofibrils formed by the assembly of **CC** with CB[8].*

DLS

Additionally, in order to corroborate an increase of the particle size due to aggregation, solutions of **CC** and **CC** plus CB[8] were analyzed by DLS experiments.

Results of the DLS analysis show a significant difference in size between **CC** with and without the addition of CB[8]. Hence, these experiments support the results obtained by UV titration and TEM.

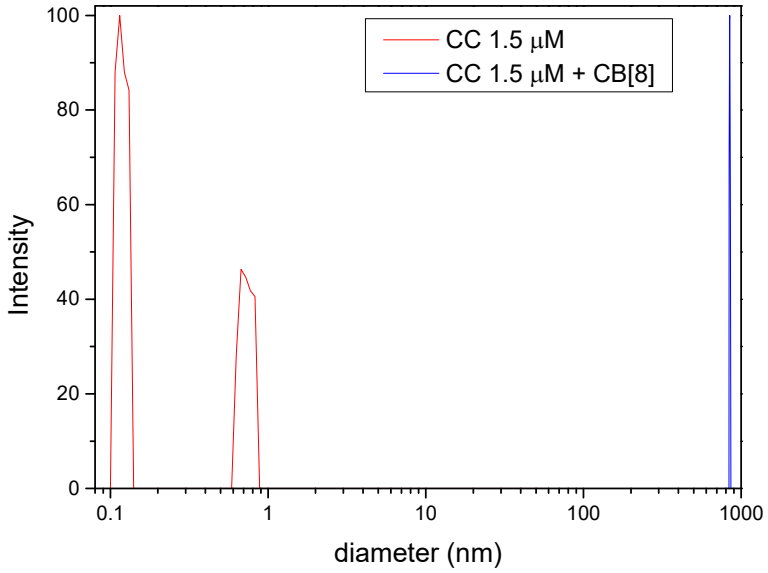


Figure 32. *DLS measurements for the equimolar mixture of both conjugates **C1** and **C2** (1.5 μM for each peptide) in PB (10 mM sodium phosphate, pH = 7)) labelled as **CC** without and after the addition of a final CB[8] concentration of 1.8 μM in PB (10 mM sodium phosphate, pH = 7).*

4. Experimental procedures

4.1. General information

All solvents used were synthesis grade, except for DMF, DIEA and TFA, which were peptide synthesis grade. Water was purified using a Milli-Q system (*Millipore*).

The reaction for the synthesis of the pseudoviologen (compound **1**) was followed by TLC. As the product is very polar and positively charged, the chosen eluent was a mixture of MeCN (600 mL), MeOH (150 mL), H_2O (150 mL) and NaCl (5 g). Compounds were identified with ultraviolet light at 254 nm and 360 nm.

Moreover, the identity and purity of compound **1** was checked by ^{1}H-NMR and ^{13}C-NMR spectroscopy, using a *Bruker Avance 300* (300 MHz) spectrometer. Samples were prepared with deuterated water (D_2O). Additionally, the signals were fully assigned by ^{1}H-NMR, ^{13}C-NMR, COSY, HSQC and HMBC using a *Bruker Avance III HD* (400 MHz) spectrometer.

Amino acid derivatives and coupling agents were purchased from *Iris Biotech GmbH*. Amino acids were purchased as protected Fmoc amino acids with the standard side chain protecting scheme.

Reversed-phase HPLC-MS was performed using a *Thermo Scientific UltiMate 3000* connected to a Photo-Diode Array (PDA) detector and a single quadrupole mass spectrometer *Thermo Scientific MSQ Plus*, or a UHPLC *Thermo Scientific Accela* connected to a PDA detector and a linear trap quadrupole mass spectrometer *Thermo Scientific Orbitrap Discovery*. An *Aeris* analytical column from *Phenomenex* (peptide XB-C18 stationary phase, 3.6 µm, 100 Å pore size, 150 × 2.1 mm) was used. HPLC-MS quality solvents were used to prepare the eluents, A: 0.04% TFA, H_2O and B: 0.04%TFA, MeCN. The standard method used for analytical HPLC was 5% B over 2 min followed by the gradient 5→95% B over 23 min.

The purification of the peptides was performed using an *Agilent Technologies 1200*, on an Aeris semipreparative column from Phenomenex (peptide XB-C18 stationary phase, 5 µm, 100 Å pore size, 250 × 10 mm). Peptide **P1** was purified with the same eluents as in the analytical method, whereas peptide **P2** was purified using eluents A: 20 mM NH_4Ac, pH = 7.8, H_2O and B: 1% buffer A, MeCN.

UV/Vis spectra were recorded on a *Jasco V-650* spectrometer, using a *Hellma* 114-10-40 QS semi-micro cuvette.

CD spectra were recorded on a CD-ORD *Jasco J-185* spectropolarimeter, coupled to a thermostatic water bath, using a *Hellma* 110-2-40 QS macro cuvette (2 mm pathlength)

TEM images were obtained on a *JEOL JEM 1010* 100 kV transmission microscope. Samples were prepared by depositing the solutions on Formvar coated Ni grids and stained with uranyl acetate.

DLS experiments were performed in particle size analyzer *Brookhaven NanoBrook Plus Zeta*.

4.2. Synthesis

N-(2,4-dinitrobenzil)bipyridinium chloride (1)

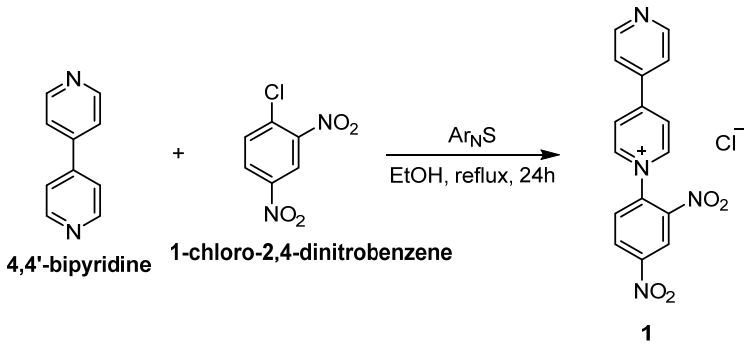


Figure 33. *Synthetic scheme for compound* ***1****.*

4,4-bipyridine (2 g, 12.8 mmol) and 1-chloro-2,4-dinitrobenzene (2.6 g, 12.8 mmol) were dissolved in EtOH (25 mL) and the mixture was refluxed and stirred for 24 h. The reaction was followed by TLC and quenched by cooling. After addition of Et_2O (185 mL) to the reaction crude, a yellowish precipitate was obtained. This solid was washed twice with AcOEt, dissolved in MeOH, and concentrated to dryness to give a brown solid (3.79 g, 83%).

^{1}H-NMR (D_2O, 400 MHz) δ = 9.41 (d, 1H, *J* = 2.5 Hz, CH-c), 9.26 (d, 2H, *J* = 6.9 Hz, CH-d), 8.95 (dd, 1H, *J1* = 2.5 Hz, *J2* = 8.7 Hz, CH-b), 8.86 (d, 2H, *J* = 6.3 Hz, CH-g), 8.70 (d, 2H, *J* = 6.9 Hz, CH-e), 8.28 (d, 1H, *J* = 8.6 Hz, CH-a), 8.05 (d, 2H, *J* = 6.3 Hz, CH-f).

^{13}C-NMR (D_2O, 100 MHz): δ = 157.1 (C-i), 150.0 (C-l), 149.6 (C-a), 145.8 (C-g), 142.9 (C-e), 142.3 (C-j), 138.4 (C-f), 131.1 (C-c), 130.6 (C-b), 126.2 (C-h), 122.8 (C-k), 122.7 (C-d).

MS (ESI): *m*/*z* = 323.08 $[M]^+$.

HRMS (ESI): *m*/*z* $[M]^+$ calcd for $C_{16}H_{11}N_4O_4$: 323.0775; found: 323.0792.

Peptide synthesis

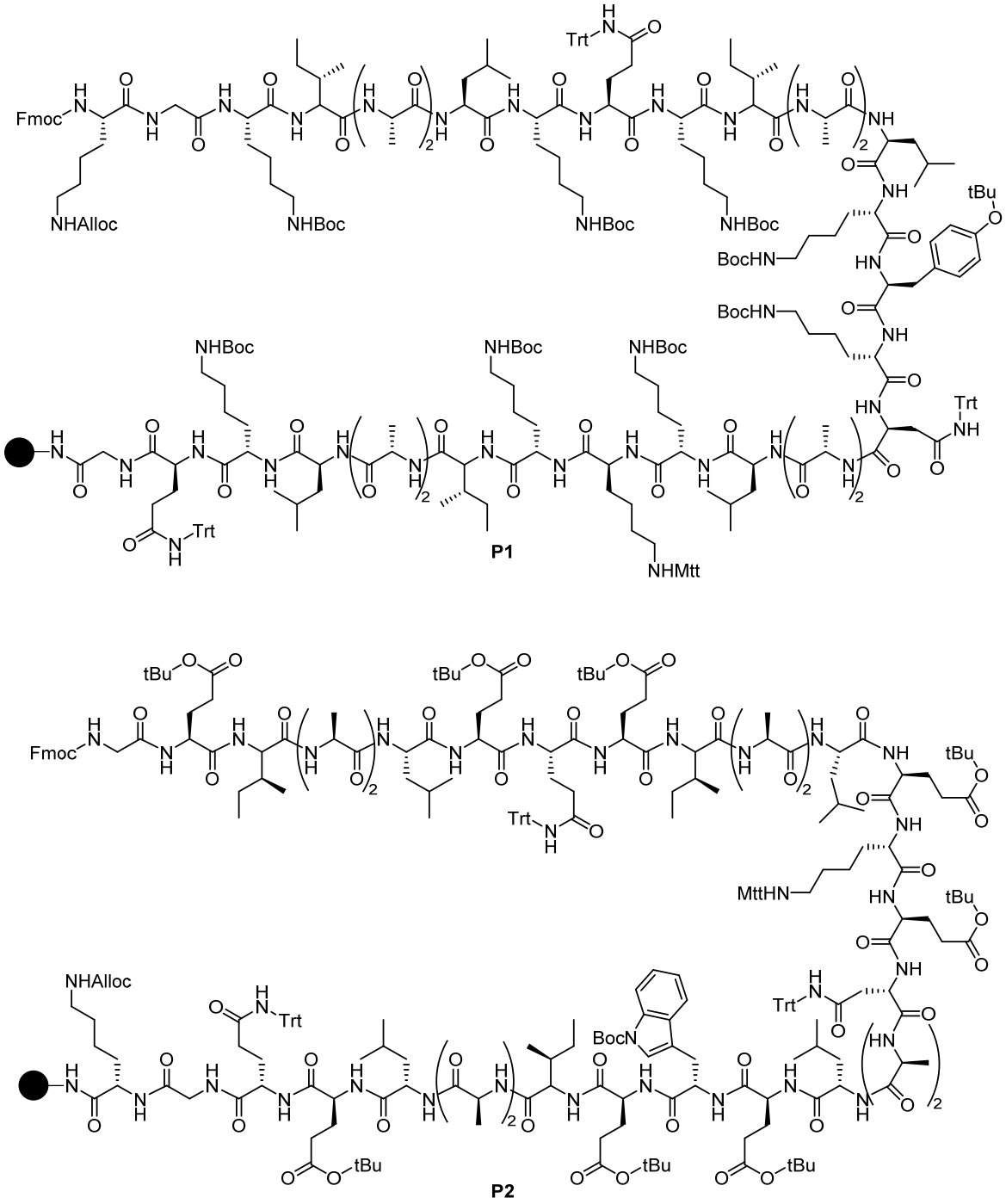


Figure 34. *Chemical structures of peptides* ***P1*** *and* ***P2****.*

- Resin: H-Rink amide ChemMatrix® resin (0.47 mmol/g)
- Coupling agent: HBTU/HOBt 0.2 M
- Base: DIEA 0.195 M
- TNBS test for checking the coupling/deprotection of the amino acids: 1 % TNBS/DMF + 10% DIEA/DMF

- Fmoc deprotecting agent: 20% 4-methylpiperidine in DMF
- Sequences:

  P1: Fmoc-KGKIAALKQKIAALKYKNAALKKKIAALKQG-NH_2

  P2: Fmoc-GEIAALEQEIAALEKENAALEWEIAALEQGK-NH_2

For the synthesis of **P1**, the resin (425.5 mg, 0.2 mmol) was placed in a plastic column and washed 3 times with DMF under N_2 bubbling (**Figure 34A**). The amine group was checked to be deprotected with the TNBS test (positive result). The protected amino acid Fmoc-Gly-OH (in excess, 4 eq) was dissolved in the coupling agent solution (4 eq, 4 mL) and then the DIEA solution (6 eq, 6 mL) was added. After 2 min of activation, the resulting solution was added to the resin and mixed under N_2 bubbling for 30 min. After solvent removal by filtration (**Figure 34B**), the resin was washed with DMF (2 × 10 mL × 2 min), and the amino acid coupling was corroborated by doing a TNBS test (no free amine, negative result). Then, the Fmoc protecting group was removed by treatment with an excess of 20% 4-methylpiperidine in DMF (10 mL) during 15 min. The resin was washed with DMF (3 × 10 mL × 2 min), and then a TNBS test was carried out to show a positive result. This procedure was repeated for each amino acid of the peptide sequence (from the *C*- to the *N*-terminus). The same method was followed for the synthesis of **P2**.

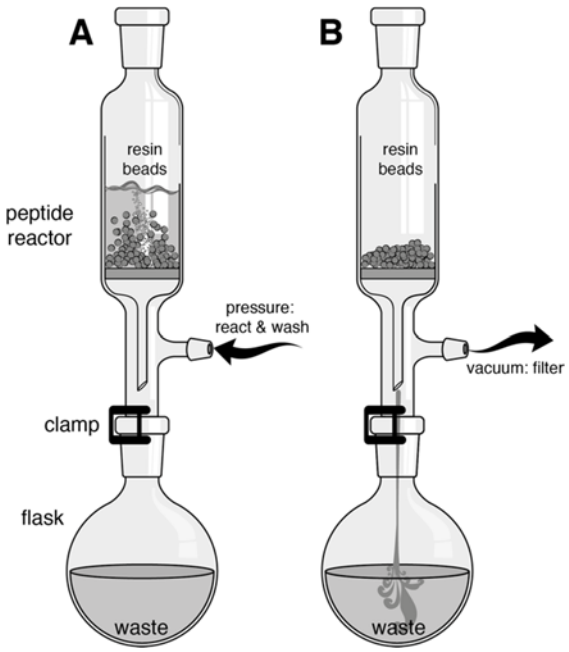


Figure 35. *General assembly for SPPS.*

Acetylation of the *N*-terminal amine

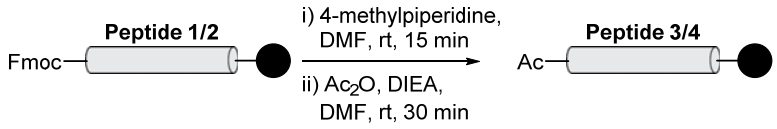


Figure 36. *Synthetic scheme for the acetylation of peptides* ***P1*** *and* ***P2****.*

After the final Fmoc deprotection step using standard conditions (20% 4-methylpiperidine/DMF), portions of each peptide (0.05 mmol) were acetylated by treatment with a mixture of Ac_2O (48 µL, 0.51 mmol) and DIEA/DMF (0.195 M, 3 mL, 0.59 mmol) for 30 min. After filtration, the resin was washed with DMF (3 × 5 mL × 3 min). A TNBS test was performed, giving a negative result.

Deprotection of the Alloc group

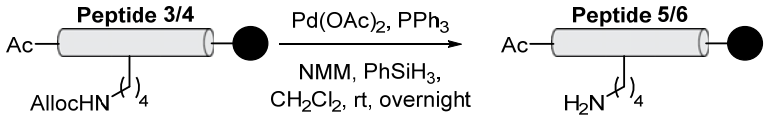


Figure 37. *Synthetic scheme for the deprotection of the Alloc group.*

Both resins **P3** and **P4** (0.05 mmol) were treated with a mixture of $Pd(OAc)_2$ (3.9 mg, 0.017 mmol), PPh_3 (20.8 mg, 0.079 mmol), $PhSiH_3$ (62 µL, 0.5 mmol) and NMM (55 µL, 0.5 mmol) in CH_2Cl_2 (2.5 mL) and the resulting mixture was stirred mechanically until the following day. The resin was then filtered and washed with: CH_2Cl_2 (2 × 2.5 mL × 5 min), DMF (2 × 2.5 mL × 5 min), sodium diethyldithiocarbamate (DEDTC, 25 mg/5 mL DMF × 15 min), DMF (2 × 2.5 mL × 5 min), and CH_2Cl_2 (2 × 2.5 mL × 5 min).

3-4 mg of the resin were treated with 150 µL of the cleaving cocktail (2.5% TIS (triisopropylsilane), 2.5% H_2O and 95% TFA) for 90 min. The resin was then filtered, the TFA filtrate was added to ice-cold ether (1.2 mL) and the precipitate centrifugated for 10 min. The solid residue was dissolved in 400 µL of H_2O and analyzed by reversed-phase HPLC-MS.

Coupling with compound 1

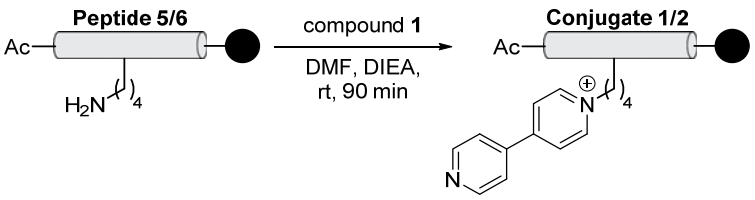


Figure 38. *Synthetic scheme for the conjugate formation.*

The resins **P5** and **P6** (0.05 mmol) were resuspended in DMF (2.5 mL) and compound **1** (55.4 mg, 0.15 mmol) was added to each mixture. The resin instantly turned brown-black and the resin suspensions were shaken for 90 min. After filtration, the resin was washed DMF (2 × 2.5 mL × 5 min) and CH_2Cl_2 (2 × 2.5 mL × 5 min).

Half of both resulting resins (0.025 mmol) were treated with 7 mL of the cleaving cocktail (2.5% TIS, 2.5% H_2O and 95% TFA) for 3.5 h. The resins were then filtered, the TFA filtrates were added to ice-cold ether (70 mL) and the precipitates centrifugated at 4 ºC for 15 min. The solid residues were dissolved in approximately 2 mL of MeCN:H_2O (1:1) each, filtered and purified by semipreparative reversed-phase HPLC. The resulting fractions were lyophilized, giving white fluffy solids (4% for **C1** and 3% for **C2**).

Conjugates **C1** and **C2** were dissolved in 1 mL and 1.3 mL of PBS (8.2 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, 137 mM NaCl, pH = 7.4), respectively. For the obtention of their concentrations, small portions of the solutions were successively added to a UV cuvette with 750 mL of PBS and the absorbance at the maximum wavelength (261 nm for **C1** and 266 nm for **C2**) was measured. The calculation of the concentration was performed using the molar extinction coefficient values of methylpseudoviologen (**Annex**) and the ones described for the residues Tyr and Trp[43] (ε = 21909 $M^{-1}cm^{-1}$ for **C1** and ε = 26027 $M^{-1}cm^{-1}$ for **C2**). Obtained values for the concentrations are 1.91 mM for **C1** and 1.17 mM for **C2**.

4.3. Self-assembly studies

Circular dichroism

CD spectra were recorded with a 2.00 mm pathlength cuvette, at 20 ºC and with 4 accumulations for each measurement. First, a PBS blank was recorded. Then, two solutions of **C1** and **C2** 25 μM were prepared, and an additional mixture solution of heterodimer at 25 μM (total peptide concentration = 50 μM) was prepared. All three solutions were measured, and the spectrum of the mixture solution was recorded again after waiting 5 min, finding no difference.

For the second CD experiment, a 15 μM solution of heterodimer (**CC**) was prepared and measured, along with another 15 μM solution of **CC** that had been previously titrated with CB[8] ([CB[8]] = 68 μM).

UV titration

UV spectra were recorded with a 1 cm pathlength cuvette and at room temperature. First, a PBS blank was recorded. Then, a 15 μM **CC** solution was prepared and used as first point. The titration was continued by adding small volumes of a 15 μM **CC** and 150 μM CB[8] solution in PB (8.2 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, pH = 7). In this way, the concentration of **CC** was kept constant during the whole titration. The absorbance was registered at λ = 264 nm, which is the maximum absorbance wavelength for the **CC**.

DLS measurements

A 15 μM **CC** stock solution in PB was prepared, and diluted 10-fold three times into 1.5 μM, 150 nM and 15 nM **CC** solutions, respectively. The four solutions were analyzed by DLS. This procedure was repeated for a 15 μM **CC** and 18 μM CB[8] solution.

Conclusions

Regarding the work done in this project, the following conclusions can be drawn:

- A pseudoviologen derivative, *N*-(2,4-dinitrobenzyl)bipyridinium chloride (compound **1**), was successfully synthesized as a precursor for the later Zincke reaction.

- Two peptides **P1** and **P2**, which are known to form parallel heterodimeric coiled coil structures, were synthesized following standard Fmoc solid phase peptide synthesis protocols. Both peptides presented orthogonally protected Lys residues at the *N*- and *C*-terminus respectively, as chosen amine moieties for the Zincke couplings.

- Both peptides were efficiently coupled to the pseudoviologen compound *via* solid phase Zincke reactions. Conjugated products **C1** and **C2** were purified by reversed-phase semipreparative HPLC.

- The formation of coiled coil heterodimers from the synthesized conjugates **C1** and **C2** was confirmed by CD.

- UV spectroscopy, TEM and DLS preliminary results indicate the self-assembly of the coiled coil oligomers into nanometric fibrils in presence of CB[8].

Conclusións

A partir do traballo realizado neste proxecto pódense extraer as seguintes conclusións:

- Preparouse con éxito un derivado pseudoviolóxeno, cloruro de *N*-(2,4-dinitrobenzil)bipiridinio (composto **1**), como precursor para a conseguinte reacción de Zincke.

- Sintetizáronse dous péptidos **P1** e **P2** coñecidos por formar estruturas de "coiled coil" paralelas e heterodiméricas mediante protocolos estándar de síntese de péptidos en fase sólida e seguindo a estratexia Fmoc. Ambos péptidos presentaban residuos de Lys protexidos ortogonalmente no *N*- e no *C*-terminal respectivamente, que constitúen os grupos amina escollidos para os acoplamentos de Zincke.

- Ambos péptidos foron acoplados eficientemente ao composto pseudoviolóxeno a través de reaccións de Zincke en fase sólida. Os produtos conxugados **C1** e **C2** purificáronse por HPLC semipreparativo en fase reversa.

- Confirmouse a formación de heterodímeros "coiled coil" a partir dos conxugados sintetizados **C1** e **C2** mediante DC.

- Os resultados preliminares de espectroscopía UV, TEM e DLS indican a ensamblaxe dos oligómeros "coiled coil" en presenza de CB[8] para formar fibras nanométricas.

Conclusiones

A partir del trabajo realizado en este proyecto se pueden extraer las siguientes conclusiones:

- Se preparó con éxito un derivado pseudoviológeno, cloruro de *N*-(2,4-dinitrobenzil)bipiridinio (compuesto **1**), como precursor para la consiguiente reacción de Zincke.

- Se sintetizaron dos péptidos **P1** y **P2** conocidos por formar estructuras "coiled coil" paralelas y heterodiméricas mediante protocolos estándar de síntesis de péptidos en fase sólida y siguiendo la estrategia Fmoc. Ambos péptidos presentaban residuos de Lys protegidos ortogonalmente en el *N*- y *C*-terminal respectivamente, que constituyen los grupos amina escogidos para los acoplamientos de Zincke.

- Ambos péptidos fueron acoplados eficientemente al compuesto pseudoviológeno a través de reacciones de Zincke en fase sólida. Los productos conjugados **C1** y **C2** fueron purificados mediante HPLC semipreparativo en fase reversa.

- Se confirmó la formación de heterodímeros "coiled coil" a partir de los conjugados sintetizados **C1** y **C2** mediante DC.

- Los resultados preliminares de espectroscopía UV, TEM y DLS indican el ensamblaje de los oligómeros "coiled coil" en presencia de CB[8] para formar fibrillas nanométricas.

Bibliography

(1) Sato, K.; Hendricks, M. P.; Palmer, L. C.; Stupp, S. I. Peptide Supramolecular Materials for Therapeutics. *Chem. Soc. Rev.* **2018**, *47* (20), 7539–7551.

(2) Pazos, E.; Novo, P.; Peinador, C.; Kaifer, A. E.; García, M. D. Cucurbit[8]uril (CB[8])-Based Supramolecular Switches. *Angew. Chemie - Int. Ed.* **2019**, *58* (2), 403–416.

(3) Obtained from: https://www.bachem.com (accessed Jun 17, 2019).

(4) Obtained from: https://www.anaspec.com (accessed Jun 17, 2019).

(5) Obtained from: http://www.nslc.wustl.edu (accessed Jun 17, 2019).

(6) Pauling, L.; Corey, R. B. Configurations of Polypeptide Chains With Favored Orientations Around Single Bonds: Two New Pleated Sheets. *Proc. Natl. Acad. Sci. U. S. A.* **1951**, *37*, 729–740.

(7) Pauling, L.; Corey, R. B. Atomic Coordinates and Structure Factors for Two Helical Configurations of Polypeptide Chains. *Proc. Natl. Acad. Sci. U. S. A.* **1951**, *37*, 235–240.

(8) Obtained from: https://ib.bioninja.com (accessed Jun 17, 2019).

(9) O'Shea, E. K.; Klemm, J. D.; Kim, P. S.; Alber T. X-Ray Structure of the GCN4 Leucine Zipper, a Two-Stranded, Parallel Coiled Coil. *Science* **1991**, *254*, 539–544.

(10) Glover, J. N. M.; Harrison, S. C. Crystal Structure of the Heterodimeric bZIP Transcription Factor c-Fos-c-Jun Bound to DNA. *Nature* **1995**, *373*, 257–261.

(11) Herrmann, H.; Aebi, U. Intermediate Filament Assembly: Fibrillogenesis Is Driven by Decisive Dimer-Dimer Interactions. *Curr. Opin. Struct. Biol.* **1998**, *8*, 177–185.

(12) Burgess, N. C.; Sharp, T. H.; Thomas, F.; Wood, C. W.; Thomson, A. R.; Zaccai, N. R.; Brady, R. L.; Serpell, L. C.; Woolfson, D. N. Modular Design of Self-Assembling Peptide-Based Nanotubes. *J. Am. Chem. Soc.* **2015**, *137*, 10554–10562.

(13) Jeffrey, Y. S.; Hodges, R. S.; Kay, C. M. Effect of Chain Length on the Formation and Stability of Synthetic α-Helical Coiled Coils. *Biochemistry* **1994**, *33*, 15501–15510.

(14) Thomas, F.; Boyle, A. L.; Burton, A. J.; Woolfson, D. N. A Set of de Novo Designed Parallel Heterodimeric Coiled Coils with Quantified Dissociation Constants in the Micromolar to Sub - Nanomolar Regime. *J. Am. Chem. Soc.* **2013**, *135*, 5161–5166.

(15) Ljubetič, A.; Lapenta, F.; Gradišar, H.; Drobnak, I.; Aupič, J.; Strmšek, Ž.; Lainšček, D.; Hafner-Bratkovič, I.; Majerle, A.; Krivec, N.; et al. Design of Coiled-Coil Protein-Origami Cages That Self-Assemble in Vitro and in Vivo. *Nat. Biotechnol.* **2017**, *35*, 1094–1101.

(16) Berwick, M. R.; Lewis, D. J.; Jones, A. W.; Parslow, R. A.; Dafforn, T. R.; Cooper, H. J.; Wilkie, J.; Pikramenou, Z.; Britton, M. M.; Peacock, A. F. A. De Novo Design of Ln(III) Coiled Coils for Imaging Applications. *J. Am. Chem. Soc.* **2014**, *136*, 1166–1169.

(17) Papapostolou, D.; Smith, A. M.; Atkins, E. D. T.; Oliver, S. J.; Ryadnov, M. G.; Serpell, L. C.; Woolfson, D. N. Engineering Nanoscale Order into a Designed Protein Fiber. *Proc. Natl. Acad. Sci.* **2007**, *104*, 10853–10858.

(18) Thomas, F.; Burgess, N. C.; Thomson, A. R.; Woolfson, D. N. Controlling the Assembly of Coiled-Coil Peptide Nanotubes. *Angew. Chemie - Int. Ed.* **2016**, *55*, 987–991.

(19) Zaccai, N. R.; Chi, B.; Thomson, A. R.; Boyle, A. L.; Bartlett, G. J.; Bruning, M.; Linden, N.; Sessions, R. B.; Booth, P. J.; Brady, R. L.; et al. A de Novo Peptide Hexamer with a Mutable Channel. *Nat. Chem. Biol.* **2011**, *7*, 935–941.

(20) Nepal, M.; Sheedlo, M. J.; Das, C.; Chmielewski, J. Accessing Three-Dimensional Crystals with Incorporated Guests through Metal-Directed Coiled-Coil Peptide Assembly. *J. Am. Chem. Soc.* **2016**, *138*, 11051–11057.

(21) Merrifield, R. B. Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154.

(22) Merrifield, R. B. Automated Synthesis of Peptides. *Science* **1965**, *150*, 178–185.

(23) Bachem. Bachem Peptide Guide. *BioConnect, Hüissen, Niederlande* **2014**.

(24) Abdelmoty, F. Albericio, L.A. Carpino, B.M. Foxman, and S. A. K. *Lett. Pep. Sci* **1994**, *1*, 1–55.

(25) Fields, G. B.; Noble, R. L. Solid Phase Peptide Synthesis Utilizing 9-Fluorenylmethoxycarbonyl Amino Acids. *Int. J. Pept. Protein Res.* **2009**, *35*, 161–214.

(26) Albericio, F.; Bofill, J. M.; El-Faham, A.; Kates, S. A. Use of Onium Salt-Based Coupling Reagents in Peptide Synthesis. *J. Org. Chem.* **1998**, *63*, 9678–9683.

(27) Monk, P. M. S. *The Viologens, Physicochemical Properties, Synthesis and Applications of the Salts of 4, 4'-Bipyridine*; Wiley, 1998.

(28) Zincke, T.; Heuser, G.; Moller, W. Dinitrophenylpyridiniumchloride and Its Conversion Products. *Justus Liebigs Ann. Chem.* **1904**, *333*, 296–345.

(29) Striepe, L.; Baumgartner, T. Viologens and Their Application as Functional Materials. *Chem. - A Eur. J.* **2017**, *23*, 16924–16940.

(30) Dale, E. J.; Vermeulen, N. A.; Juríček, M.; Barnes, J. C.; Young, R. M.; Wasielewski, M. R.; Stoddart, J. F. Supramolecular Explorations: Exhibiting the Extent of Exended Cationic Cyclophanes. *Acc. Chem. Res.* **2016**, *49*, 262–273.

(31) Peinador, C.; Blanco, V.; García, M. D.; Quintela, J. M. PdII and PtII Metal-directed Self-assembly of Supramolecular Structures Based on N-monoalkyl-4,4′-bipyridinium Derivatives. In *Molecular self-assembly: advances and applications*; Li, A. D. Q., Ed.; Pan Stanford Publishing, 2012.

(32) Masson, E.; Ling, X.; Joseph, R.; Kyeremeh-Mensah, L.; Lu, X. Cucurbituril Chemistry: A Tale of Supramolecular Success. *RSC Adv.* **2012**, *2*, 1213–1247.

(33) Ahmed, S.; Singha, N.; Pramanik, B.; Mondal, J. H.; Das, D. Redox Controlled Reversible Transformation of a Supramolecular Alternating Copolymer to a Radical Cation Containing Homo-Polymer. *Polym. Chem.* **2016**, *7*, 4393–4401.

(34) Reczek, J. J.; Kennedy, A. A.; Halbert, B. T.; Urbach, A. R. Multivalent Recognition of Peptides by Modular Self-Assembled Receptors. *J. Am. Chem. Soc.* **2009**, *131*, 2408–2415.

(35) Biedermann, F.; Scherman, O. A. Cucurbit[8]uril Mediated Donor–Acceptor Ternary Complexes: A Model System for Studying Charge-Transfer Interactions. *J. Phys. Chem. B* **2012**, *116*, 2842–2849.

(36) Reczek, J. J.; Rebolini, E.; Urbach, A. R. Solid-Phase Synthesis of Peptide-Viologen Conjugates. *J. Org. Chem.* **2010**, *75*, 2111–2114.

(37) Hirani, Z.; Taylor, H. F.; Babcock, E. F.; Bockus, A. T.; Varnado, C. D.; Bielawski, C. W.; Urbach, A. R. Molecular Recognition of Methionine-Terminated Peptides by Cucurbit[8]uril. *J. Am. Chem. Soc.* **2018**, *140*, 12263–12269.

(38) Wu, G.; Olesińska, M.; Wu, Y.; Matak-Vinkovic, D.; Scherman, O. A. Mining 2:2 Complexes from 1:1 Stoichiometry: Formation of Cucurbit[8]uril-Diarylviologen Quaternary Complexes Favored by Electron-Donating Substituents. *J. Am. Chem. Soc.* **2017**, *139*, 3202-3208.

(39) Clarke, D. E.; Olesinska, M.; Mönch, T.; Schoenaers, B.; Stesmans, A.; Scherman, O. A. Aryl-Viologen Pentapeptide Self-Assembled Conductive Nanofibers. *Chem. Commun.* **2019**, *55*, 7354-7357.

(40) Madasamy, K.; Velayutham, D.; Kathiresan, M. Facile Construction of a Supramolecular Organic Framework Using Naphthyl Viologen Guests and CB[8] Host via Charge-Transfer Complexation. *ACS Omega* **2019**, *4*, 8528–8538.

(41) Lin, F.; Zhan, T. G.; Zhou, T. Y.; Zhang, K. Da; Li, G. Y.; Wu, J.; Zhao, X. The Construction of Rigid Supramolecular Polymers in Water through the Self-Assembly of Rod-like Monomers and cucurbit[8]uril. *Chem. Commun.* **2014**, *50*, 7982–7985.

(42) Cheng, W.-C.; Kurth, M. J. The Zincke Reaction. A Review. *Org. Prep. Proced. Int.* **2002**, *34*, 585–608.

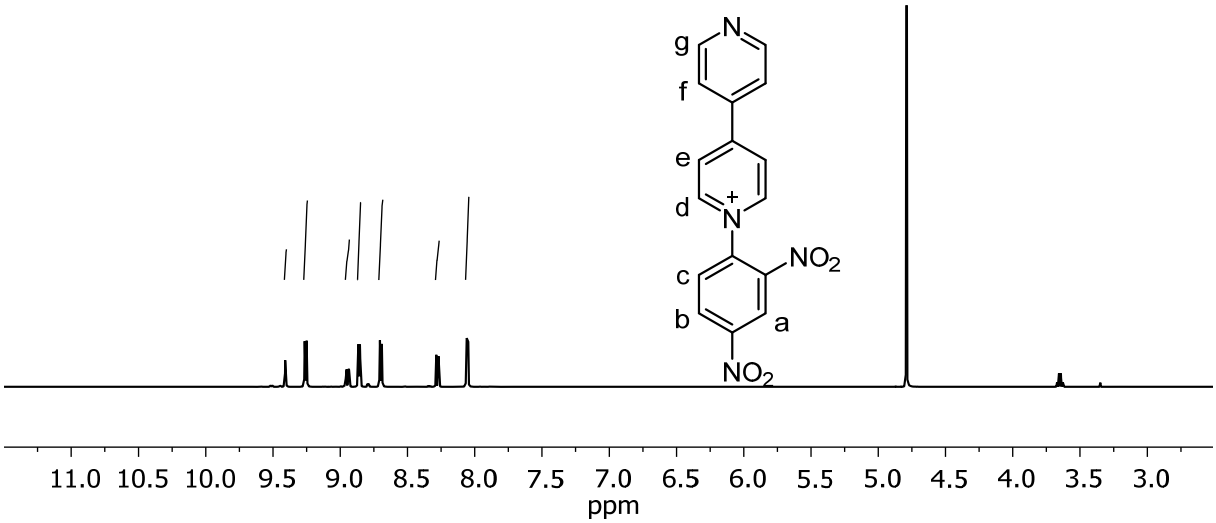
(43) Obtained from: https://omlc.org/spectra/PhotochemCAD/index.html (accessed Jul 2, 2019).

(44) Lau, S. Y. M.; Taneja, A. K.; Hodges, R. S. Synthesis of a Model Protein of Defined Secondary and Quaternary Structure Effect of Chain Length on the Stabilization and Formation of Two-stranded a-helical Coiled-Coils. *J. Biol. Chem.* **1984**, *259*, 13253–13261.

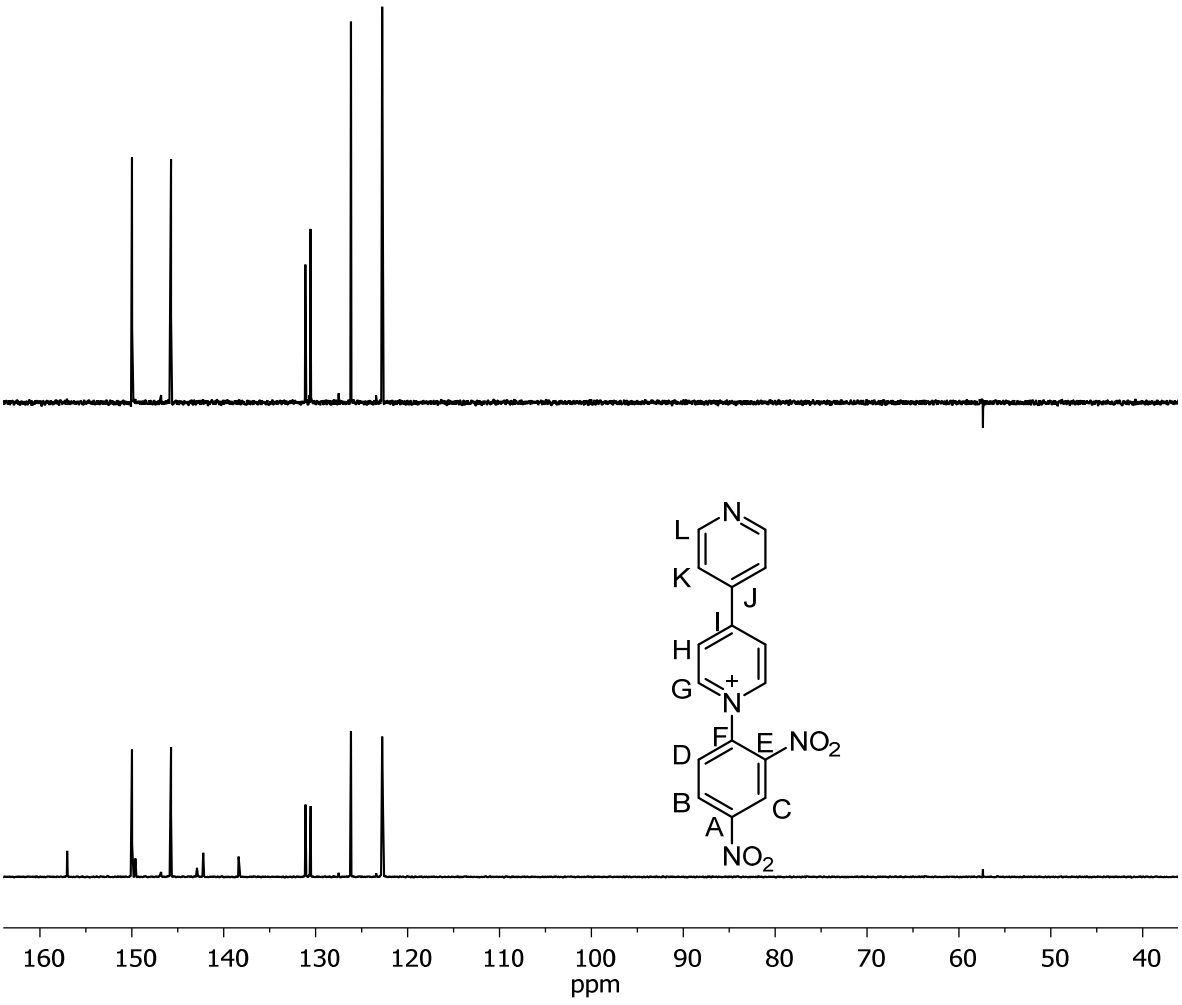
(45) Aronsson, C.; Dånmark, S.; Zhou, F.; Enander, K.; Su, H.; Aili, D. Self-Sorting Heterodimeric Coiled Coil Peptides with Defined and Tuneable Self-Assembly Properties. *Sci. Rep.* **2015**, *5*, 14063.

Annex

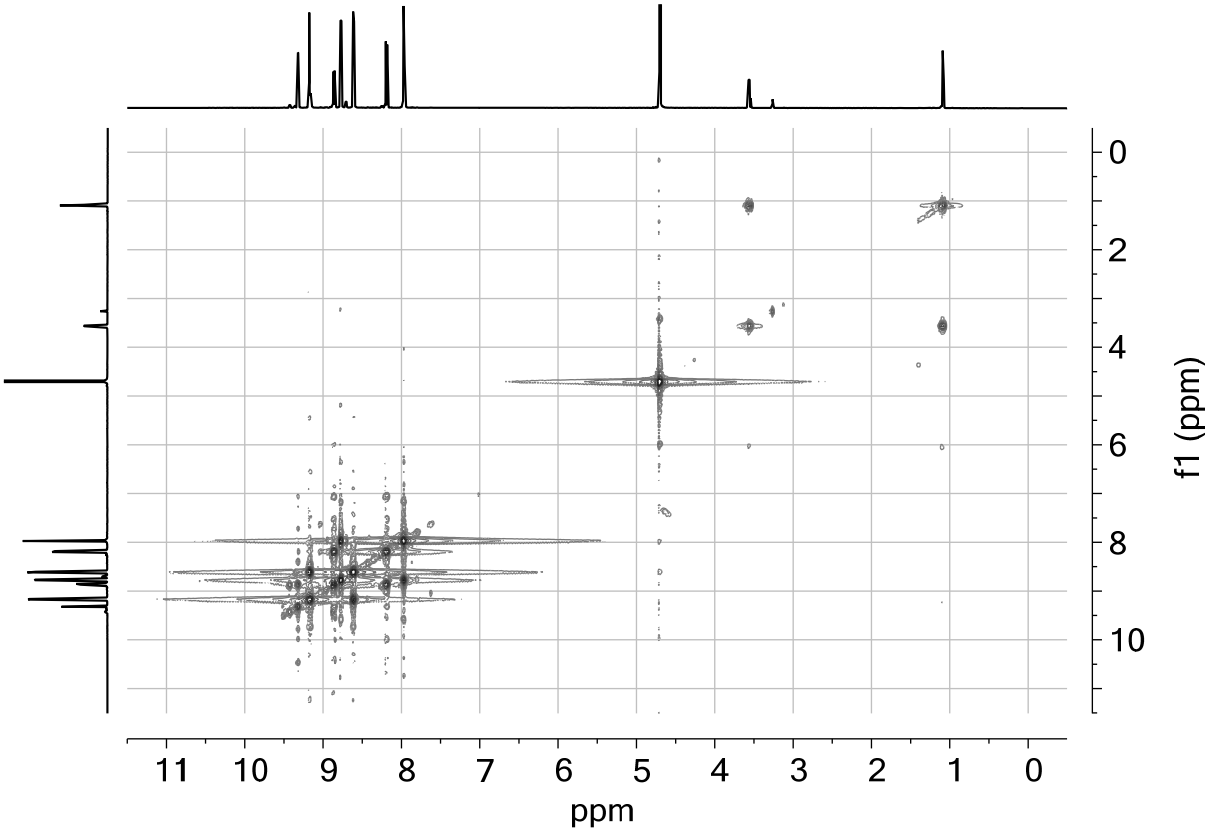
N-(2,4-dinitrobenzene)-bypiridinium chloride (1)



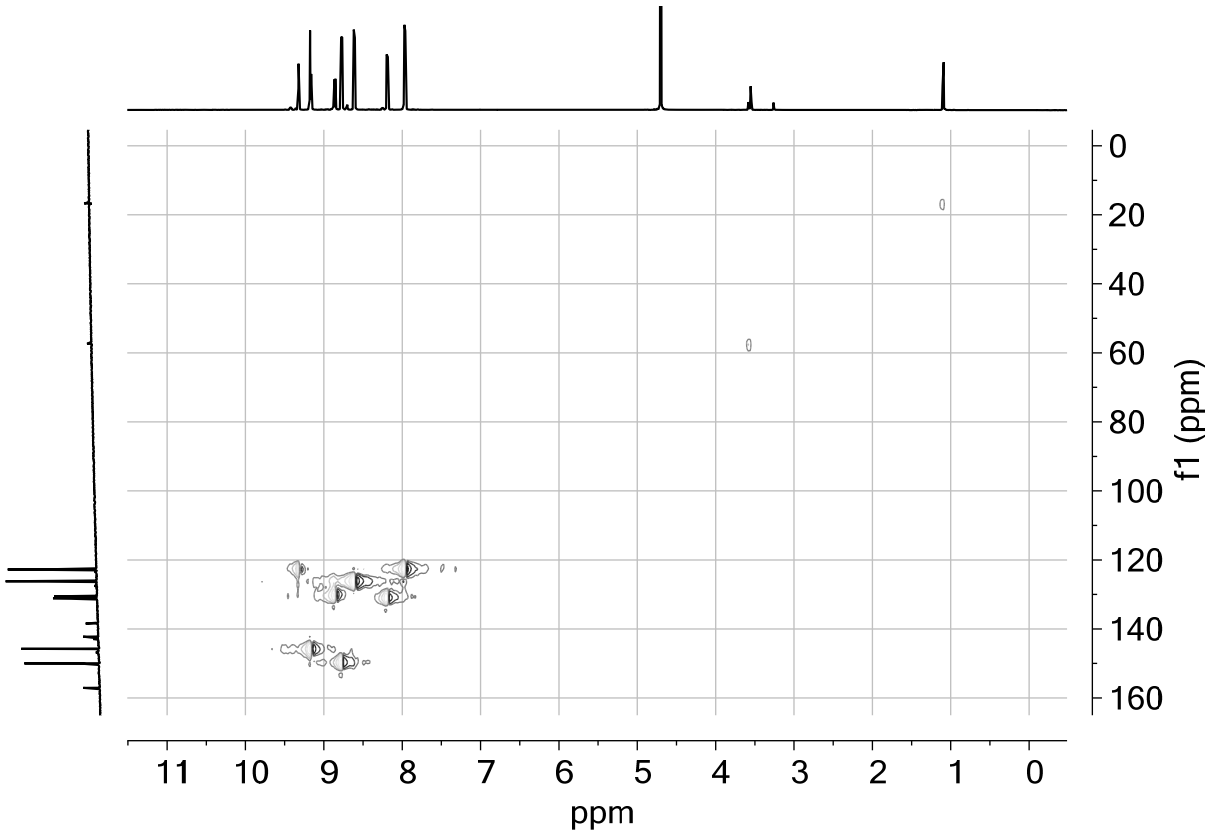
*^{1}H-NMR spectrum (D_2O, 400 MHz) for compound **1**.*



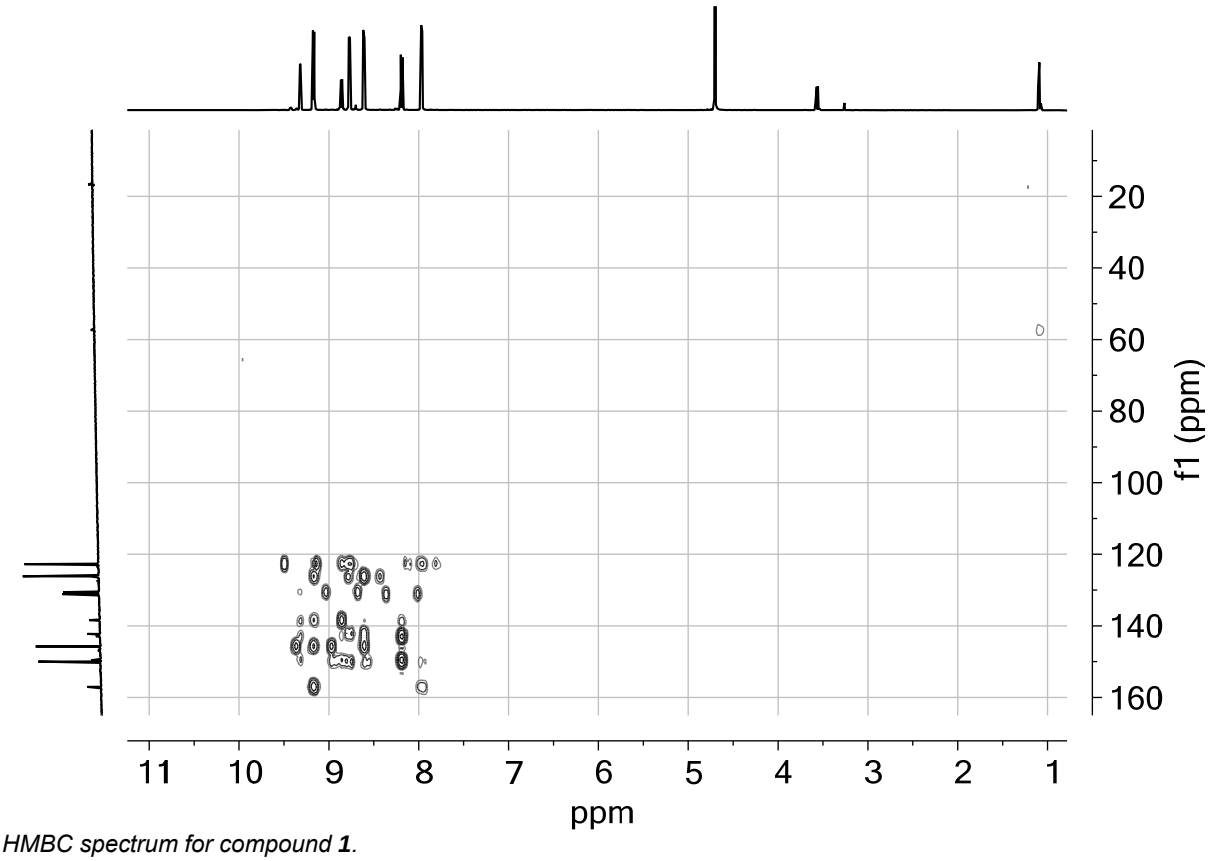
*DEPT and ^{13}C-NMR (D_2O, 100 MHz) for compound **1**.*



*COSY spectrum for compound **1**.*

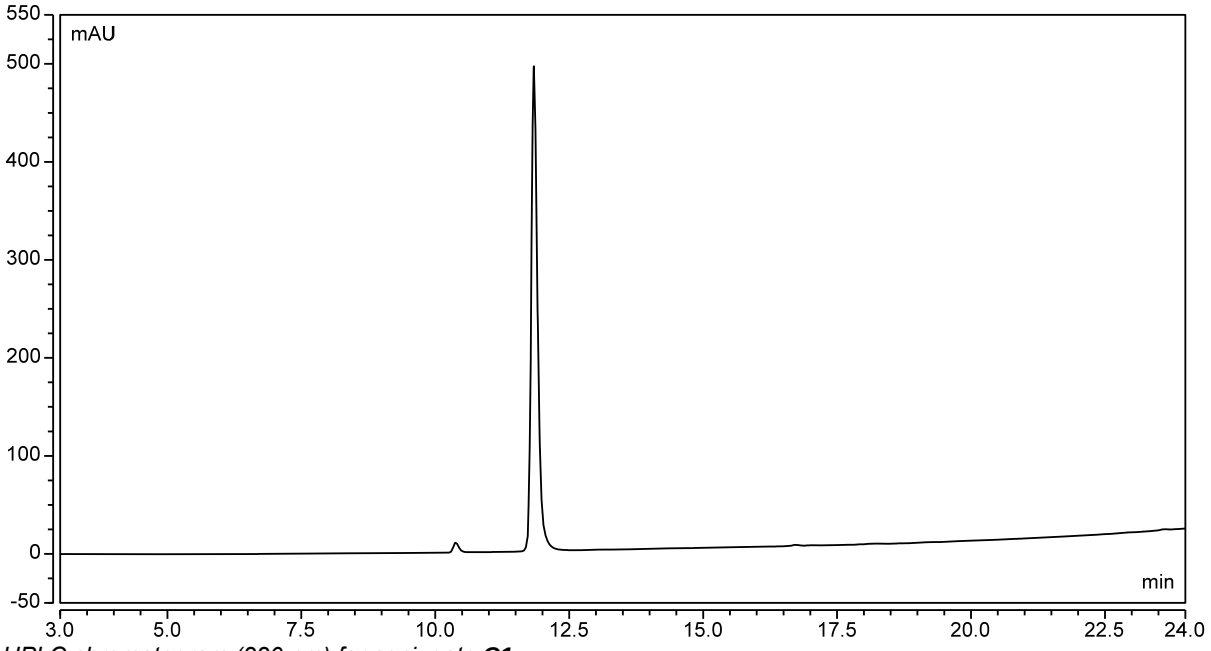


*HSQC spectrum for compound **1**.*

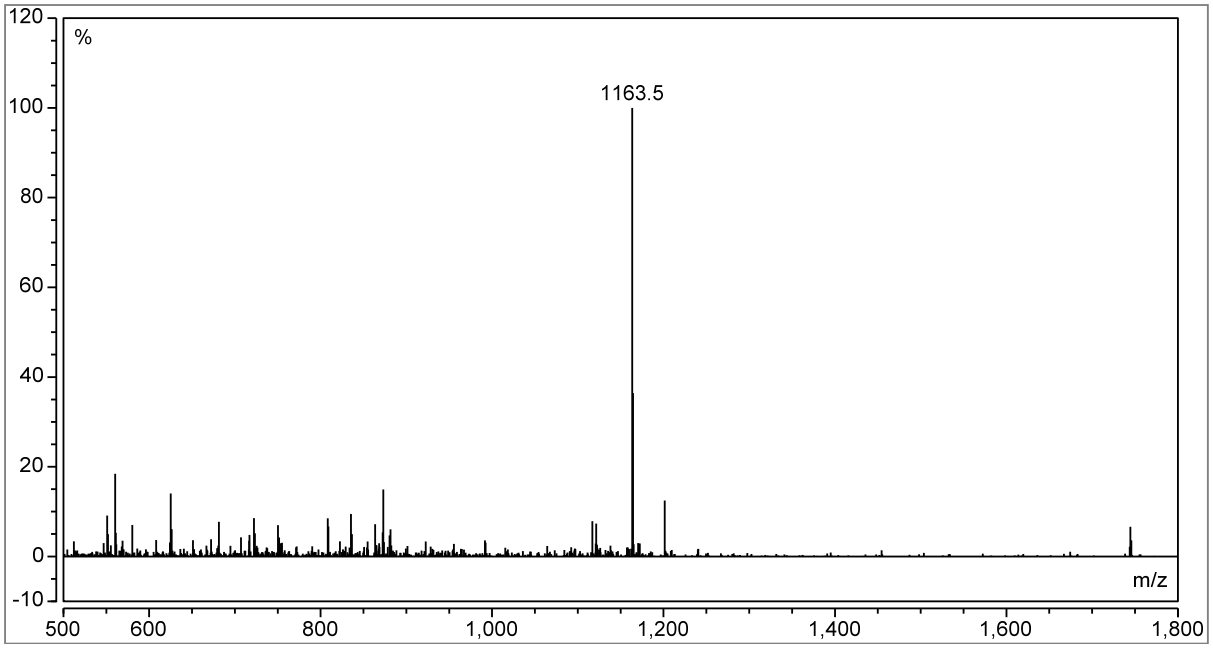


*HMBC spectrum for compound **1**.*

Conjugate C1

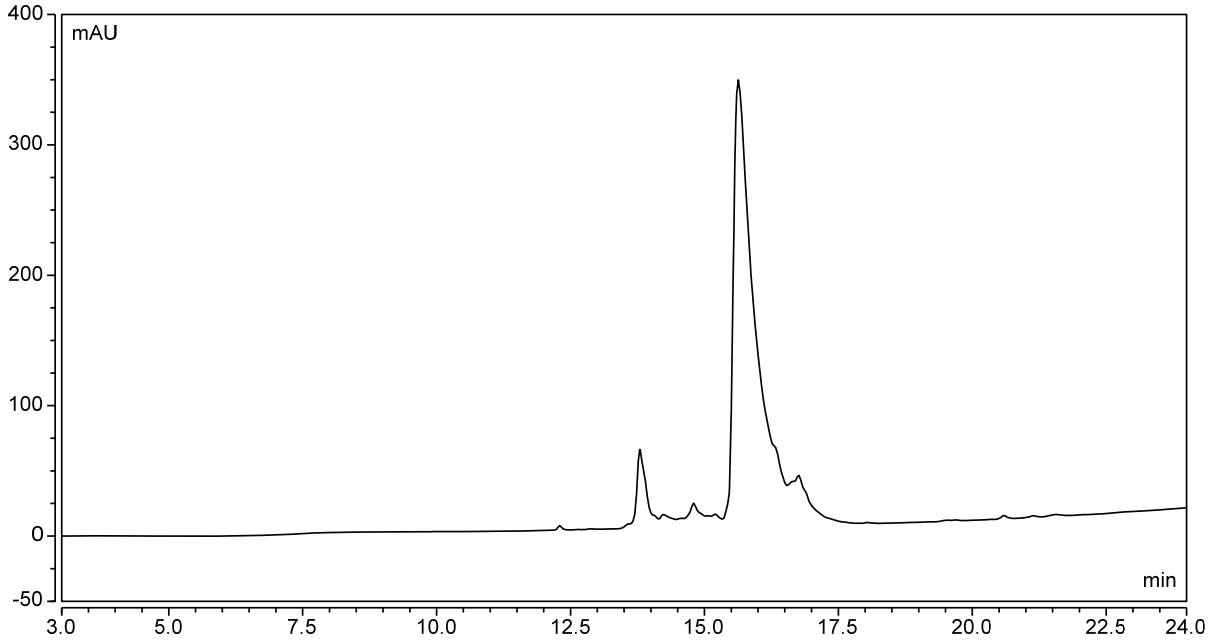


*HPLC chromatogram (220 nm) for conjugate **C1**.*

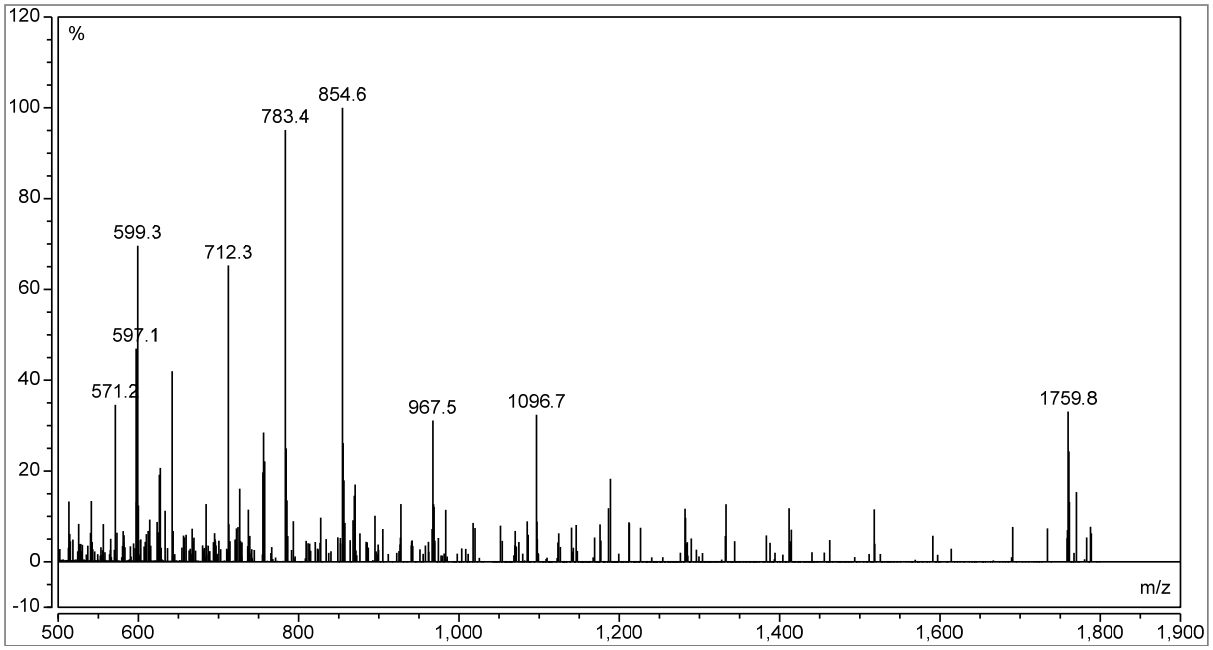


*ESI-MS of the HPLC chromatogram peak at t_R = 11.8 min, corresponding to conjugate **C1**.*

Conjugate C2

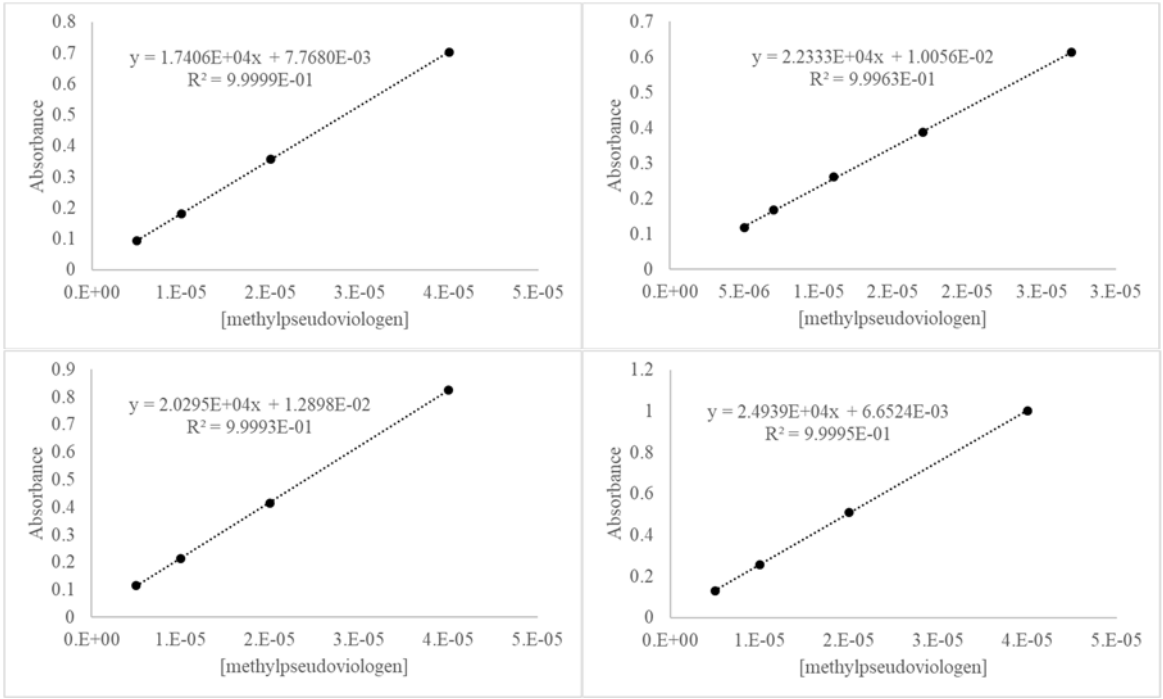


*HPLC chromatogram (220 nm) for conjugate **C2**.*



*ESI-MS of the HPLC chromatogram peak at t_R = 15.6 min, corresponding to conjugate **C2**.*

Calculation of the molar extinction coefficient (ε) of pseudoviologen moieties



Representation of the UV absorbance at 259 nm vs methylpseudoviologen concentration, for the calculation of the molar extinction coefficient (ε) of pseudoviologen moieties.