UNIVERSIDADE DA CORUÑA

FACULTADE DE CIENCIAS

Chemistry Degree

Final Project Report

Viologen-peptide conjugates in supramolecular chemistry

Conxugados violóxeno-péptido en química supramolecular

Conjugados viológeno-péptido en química supramolecular

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Abbreviatures

Å	armstrong
Ac	acetyl
Ac_2O	acetic anhydride
ACN	acetonitrile
AcOEt	ethyl acetate
Alloc	allyloxycarbonyl
Boc	tert-butoxycarbonyl
Bz	benzyl
CB[n]	cucurbit-[n]-uril
CH_2Cl_2	dichloromethane
DCC	*N*,*N'*-dicyclohexylcarbodiimide
DEDTC	diethyldithiocarbamate
DIEA	*N*,*N'*-diisopropylethylamine
DMF	dimethylformamide
Et_2O	diethyl ether
EtOH	ethanol
Fmoc	9-fluorenylmethoxycarbonyl
HBTU	N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate
HF	fluorhydric acid
HOBt	1-hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
KPF_6	potassium hexafluorophosphate
MeI	iodomethane
MeOH	methanol
MS	Mass Spectrometry
NMM	*N*-methylmorpholine
NMR	Nuclear Magnetic Resonance
$Pd(OAc)_2$	palladium acetate
$PhSiH_3$	phenylsilane
PPh_3	triphenylphosphine
rt	room temperature
SPPS	Solid Phase Peptide Synthesis
*t*Bu	tert-butyl
TFA	trifluoroacetic acid
TIS	triisopropylsilane
TLC	Thin Layer Chromatography
TNBS	2,4,6-trinitrobenzenesulfonic acid
t_R	retention time
Trt	triphenylmethyl
δ	chemical shift

Abstract

The aim of this project is to develop the methodology for the coupling of (pseudo)viologens to peptides using solid-phase protocols in order to study, in the future, the supramolecular aggregation of the products into controllable *β*-sheet architectures. It is expected that the formation and breaking of those secondary structures can be controlled by the addition of cucurbit[8]uril molecules and the variation of the redox and pH conditions.

For this purpose, in this work two activated (pseudo)viologen derivatives and a model peptide have been prepared. The bipyridinium derivatives were prepared following well established synthetic methodologies, whilst the β-sheet forming peptide has been generated via standard Fmoc solid-phase peptide synthesis protocols. Solid phase couplings between the peptide and both (pseudo)viologen derivatives were performed following a Zincke reaction between the activated salts and amine moieties placed in two different positions of the peptide chain: the *N*-terminus and the side chain of a Lys residue.

NMR and HPLC-MS were the techniques used to follow the reactions and to characterize the products. Semipreparative HPLC was used as purification method for some of the obtained peptide-(pseudo)viologen conjugates.

Keywords: supramolecular chemistry, solid phase peptide synthesis, viologens, Zincke reaction.

Resumo

O obxectivo deste proxecto é desenvolver a metodoloxía para o acoplamento de (pseudo)violóxenos a péptidos usando protocolos de fase sólida para estudar, nun futuro, a agregación supramolecular dos produtos en forma de estruturas de lámina-*β* controlables. Espérase que a formación e a disociación desas estruturas secundarias pódase controlar coa adición de moléculas de cucurbit-[8]-uril e coa variación das condiciones redox e de pH.

Para este propósito, neste traballo preparáronse dous derivados (pseudo)violóxeno activados e un péptido modelo. Os derivados de bipiridinio foron preparados seguido metodoloxías sintéticas establecidas, mentres que o péptido que forma de láminas-*β* foi xerado mediante protocolos estándar de síntese de péptidos en fase sólida empregando a estratexia Fmoc. Os acoplamentos en fase sólida entre o péptido e ambos derivados (pseudo)violóxeno leváronse a cabo seguindo unha reacción de Zincke entre os sales activados e as partes amina localizadas en dúas posicións diferentes da cadea peptídica: a *N*-terminal e a cadea lateral dun residuo de Lys.

As técnicas empregadas para o seguimento das reaccións e para caracterizar os produtos foron RMN e HPLC-MS. Empregouse HPLC semipreparativo como método de purificación para algúns dos conxugados péptido-(pseudo)violóxeno obtidos.

Palabras chave: química supramolecular, síntese de péptidos en fase sólida, violóxenos, reacción de Zincke.

Resumen

El objetivo de este proyecto es desarrollar la metodología para el acoplamiento de (pseudo)viológenos a péptidos usando protocolos de fase sólida para estudiar, en un futuro, la agregación supramolecular de los productos en forma de estructuras de lámina-*β* controlables. Se espera que la formación y disociación de estas estructuras secundarias se pueda controlar con la adición de moléculas de cucurbit-[8]-uril y con la variación de las condiciones redox y de pH.

Para este propósito, en este trabajo se prepararon dos derivados (pseudo)viológeno activados y un péptido modelo. Los derivados de bipiridinio fueron preparados siguiendo metodologías sintéticas establecidas, mientras que el péptido que forma de láminas-*β* fue generado mediante protocolos estándar de síntesis de péptidos en fase sólida utilizando la estrategia Fmoc. Los acoplamientos en fase sólida entre el péptido y ambos derivados (pseudo)viológeno se llevaron a cabo siguiendo una reacción de Zincke entre las sales activadas y las partes amina localizadas en dos posiciones diferentes de la cadena peptídica: la *N*-terminal y la cadena lateral de un residuo de Lys.

Las técnicas empleadas para el seguimiento de las reacciones y para caracterizar los productos fueron RMN y HPLC-MS. Se usó HPLC semipreparativo como método de purificación para algunos de los conjugados péptido-(pseudo)viológeno obtenidos.

Palabras clave: química supramolecular, síntesis de péptidos en fase sólida, viológenos, reacción de Zincke.

Introduction

Structure and bonding of proteins and peptides

Peptides and proteins are biopolymers with a wide variety of structures and functions. They are formed by monomeric subunits named amino acids. There are 20 standard amino acids, which share a common structure, formed by an *α*-carbon bonded to a hydrogen atom, an amine group, a carboxylic group and a R side chain, which distinguishes one amino acid from another (**Figure 1**). Besides, standard amino acids are also called *α*-amino acids, as the amino group is attached at the *α*-position respect to the carboxylic acid. They also belong to the L series, having all of them the same stereochemistry.

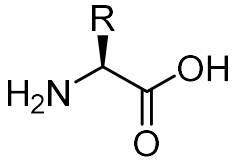


***Figure 1**. Standard amino acid structure.*

R side chains can be completely aliphatic or present different functional groups, and all the different amino acids can be named by a three- or one-letter code. Depending on the R side chains, the 20 standard natural amino acids can be classified as 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).

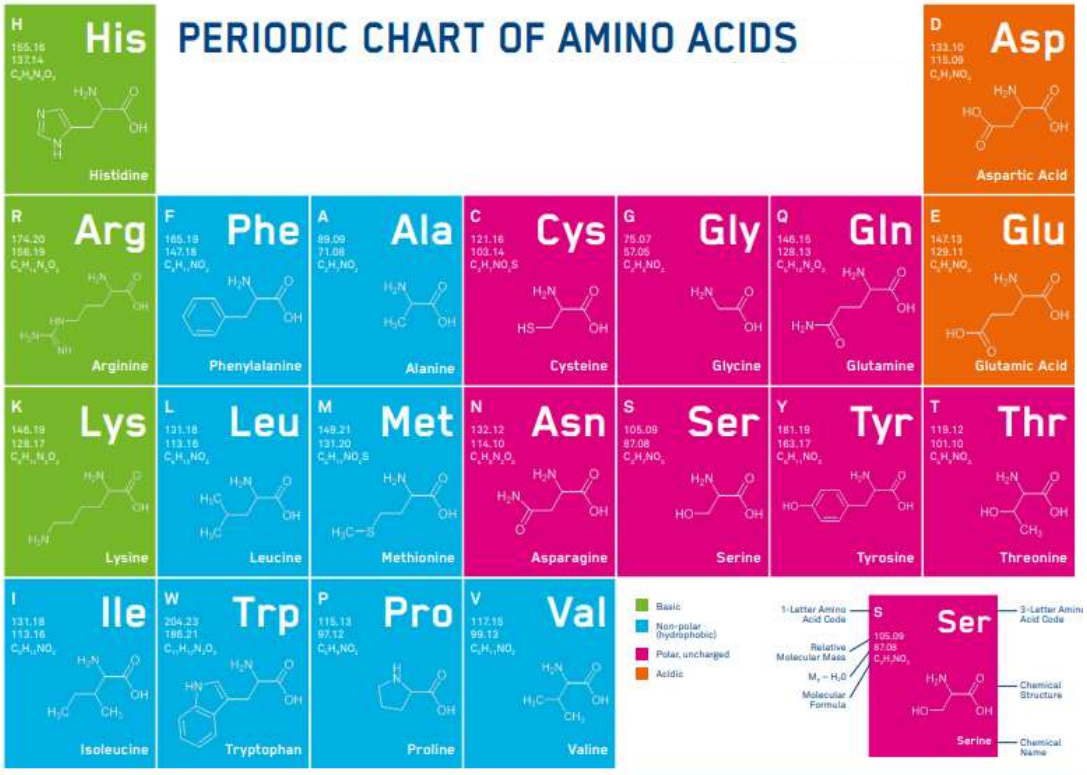


***Table 1**. Structure and classification of the 20 natural amino acids, including their one- and three-letter codings.*[1]

As all amino acids are formed by at least two ionizable groups (amine and carboxylic acid), they present two pK_as. Some amino acids are defined by an extra pK_a, corresponding to an

ionizable group present at the side chain. The isoelectric point (*pI*) is defined as the pH where a certain amino acid is found to have net charge equal to zero.

Amino acids in peptides and proteins are bound to each other through an amide bond, also called peptide bond. Successions of multiple amino acids through peptide bonds form the primary structure of peptides and proteins.

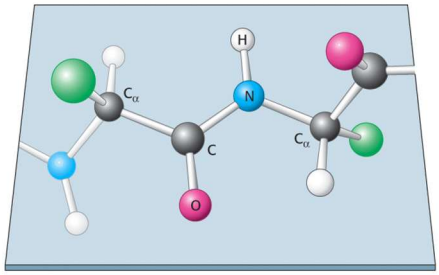


***Figure 2**. Planar structure of the peptide bond.*

The $C_{carbonyl}$-N link formed has certain double bond character, due to the resonance of the nitrogen with the C-O double bond. Therefore, there is not any free rotation inside the peptide bond. On the contrary, both C_α-N and C_α-$C_{carbonyl}$ σ bonds present free rotation respect to the contiguous bond. These two rotations define two angles, ψ and ϕ, which take values between -180º and +180º.

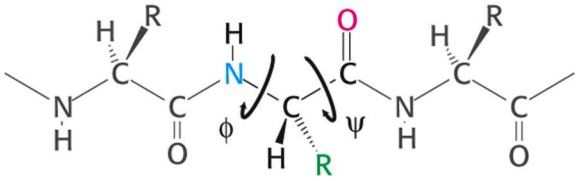


***Figure 3**. Peptide bond rotation.*[2]

The polypeptidic chains form the primary structure of peptides and proteins, with these chains folding to establish the secondary structures defined by angles ψ and ϕ. Secondary structures can be classified on the basis of different patterns of hydrogen bonds and other intramolecular interactions, including α-helixes, β-sheets, random coils, twists, etc. α-helixes and β-sheets were predicted by Linus Pauling and Robert Corey based on H-bonding and cooperativity criteria.[3,4]

α-helixes present an helicoidal shape (**Figure 4**), where each turn has 3.6 residues. The structure is bound together by H bonds between C=O and NH groups of consecutive turns and by Van der Waals interactions. Those H bonds are parallel to the main axis of the helix, while the side chains are oriented outwards and perpendicular to the axis. On the other hand, β-sheets are defined for having H bonds between different chains (**Figure 4**), forming plates that are not plain, but slightly wrinkled. In the case of β-sheets, H bonds are placed perpendicular to the axis of the macromolecule, with the chains being oriented either in a parallel or an antiparallel fashion.

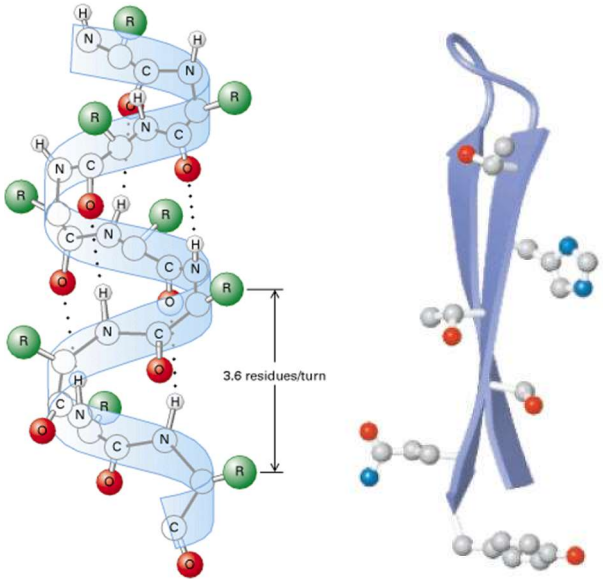
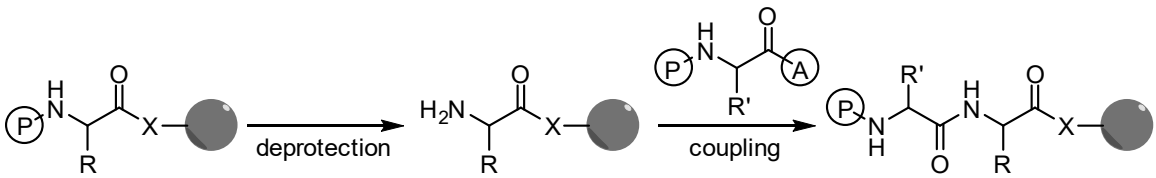


***Figure 4**. α-helix (left) and β-sheet (right) structures.*[2]

Proteins also present tertiary and quaternary structures. The tertiary structure is described by the spatial and geometrical distribution of the secondary structure. Due to this distribution, new electrostatic, hydrophobic or Van der Waals inter-chain interactions, or H bonds may be formed. Therefore, different stable tridimensional conformations are established, forming the so-called protein domains, which are usually enough to perform a certain activity by themselves. Some proteins are formed by more than one domain, which assemble via non-covalent interactions leading to the quaternary structure of proteins.

Solid Phase Peptide Synthesis (SPPS)

Solid phase peptide synthesis, first developed by Merrifield in 1951,[5,6] is the most widely method used for the preparation of peptides. SPPS consists in the consecutive addition of appropriately modified amino acids to a support polymeric resin. The resin is bound through a linker to the so-called *C*-terminal amino acid, thus the synthetic order of amino acids is from the *C*-terminus to the *N*-terminus. All amino acids must have their amine groups protected when added, so that they attach by forming an amide bond to the last amino acid of the peptide chain. Those protective groups are cleaved afterwards, in order to perform the next amino acid addition.[7]

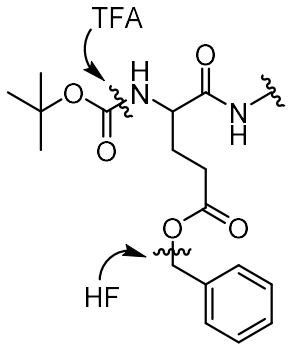


P : temporary protective groups
A : active ester

***Scheme 1**. General scheme of amino acid coupling in SPPS.*

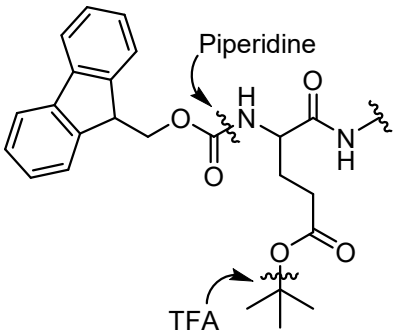
Functional groups at the side chains must be also protected. In this case, those protective groups should not be cleaved until the very end of the peptide synthesis. Therefore, they should be resistant to the coupling conditions, but labile to the conditions used for the final cleavage of the macromolecule from the resin.

Having this in mind, there are two basic SPPS strategies based on orthogonal protective groups: Boc/Bz and Fmoc/*t*Bu.[8] In the first strategy (**Scheme 2**), the Boc protective group would be used for the α-amino groups, while the Bz group protects the side chain groups. Boc can be deprotected by mild acidic conditions (TFA), whilst Bz requires more harsh conditions, like HF.



***Scheme 2**. Boc/Bz strategy scheme.*

In the second strategy (**Scheme 3**), *α*-amino groups are protected by Fmoc groups, whereas the side chain groups would be protected by the TFA-labile groups such as *t*Bu or Trt. Fmoc is easily deprotected with basic media (piperidine). On the other hand, other functional groups, as well as the cleavage of the peptide from the resin, require the addition of TFA. This method is more convenient than the Boc-based approach, because it avoids the use of hazardous HF, and the continuous addition of TFA used in the Boc/Bz strategy can also cause problems with some sensitive side chains.[9]



***Scheme 3**. Fmoc/tBu strategy scheme.*

The chosen polymeric support plays also a significant role on the SPPS, as the efficiency of the couplings depends on the ability of the resin to solvate and swell in an appropriate solvent. Most of the coupling reactions occur inside the resin pellets, where all the reagents used should have easy access. The linker that joins the resin with the C-terminal amino acid is also important. The bond formed must be breakable under the chosen final deprotection conditions. The linker also defines the functional group that is eventually obtained at the *C*-terminus.

In order to perform a coupling and form a peptide bond, it is not enough to add the corresponding amino acid to the resin. It is mandatory to activate the carboxylic group of the added amino acid using a coupling agent in basic media. There are four main ways of performing this activation: carbodiimides, acid anhydrides, activated esters and activated esters generated *in situ*.

Carbodiimides like DCC are widely used (**Figure 5**), with its main drawback being the potential dehydration of Asn and Gln residues. However, this issue can be avoided by adding HOBt to the reaction mixture. Conversely, anhydrides are usually generated *in situ* using two equivalents of the amino acid, which makes it a less efficient method.

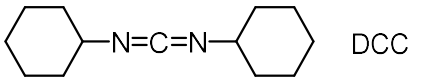


***Figure 5**. N,N'-Dicyclohexylcarbodiimide (DCC).*

Amino acid derivatives with highly reactive esters are sometimes used. However, it is much more common to generate those esters *in situ*, as it is a simpler method. Activated esters suffer degradation when dissolved in the presence of DIEA (the most used base in SPPS). Therefore, activation of the amino acids must be performed just before the coupling.[10]

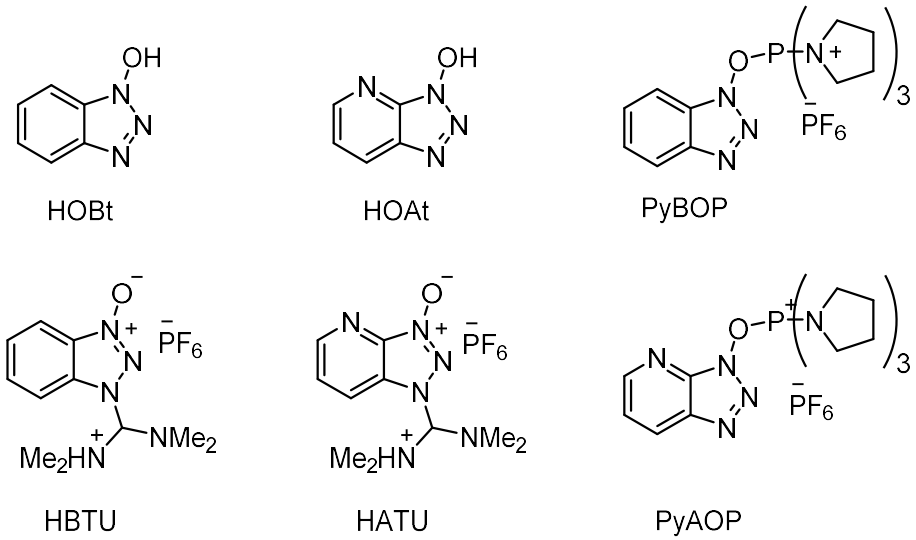


***Figure 6**. Common coupling agents used to produce activated esters in situ.*

Viologens in supramolecular chemistry

Viologens are *N*,*N*'-disubstituted derivatives of 4,4'-bipyridine (**Figure 7**).[11] There are several methods for the preparation of these organic salts, as the nucleophilic substitution of alkyl halides or the Zincke reaction with primary amines.

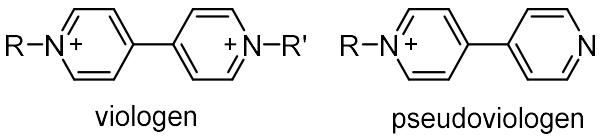
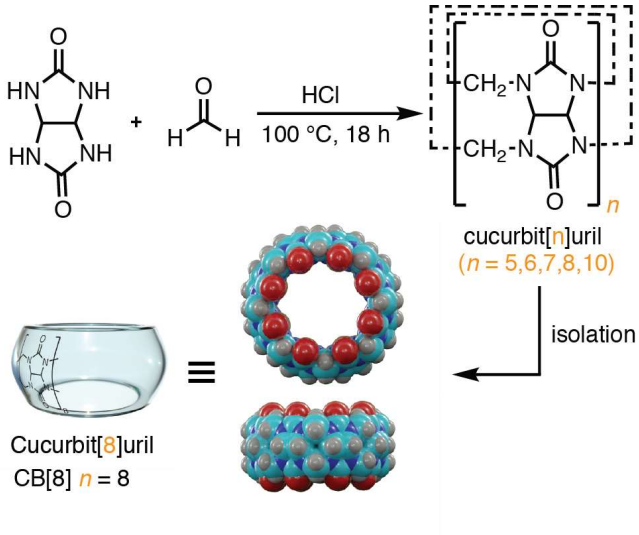


***Figure 7.** General structure of viologens and pseudoviologens.*

Due to their rich red/ox chemistry and electronic properties as π-deficient aromatic molecules, viologens and pseudoviologens (*N*-monosubstituted 4,4'-bipyridine derivatives) have been extensively used on the fields of materials and supramolecular chemistry.[12–14] In particular, in the context of the work presented herein, it should be considered that, thanks to the positive charges placed at both extremes of the (pseudo)viologens units, those can be encapsulated as guests *via* supramolecular interactions inside cucurbituril molecules.

Cucurbit[*n*]urils (CB[n]) are pumpkin-shaped macromolecules formed by the cyclic oligomerization of n glycoluril units with formaldehyde (**Scheme 4**).[15] CB[n]s interact with a wide variety of inorganic and organic species. In the particular case of organic molecules, CB[n]s are capable of acting as hosts and encapsulate them *via* extremely strong non-covalent interactions, forming 1:1 binary complexes.



***Scheme 4**. Preparation and schematic depictions of cucurbit[n]urils.*[15]

Positively charged amphiphilic compounds, like viologens, are ideal guests for CB[n]s. Positive charges bind through ion-dipole interactions to the carbonylated rims (portal areas) of the cucurbituril molecules, whilst the hydrophobic part is placed inside the host's cavity. Therefore, cation-dipole interactions are key interactions for the self-assembly of host and guest. Moreover, the hydrophobic effect is also extremely important on those host:guest aggregates, as the release of high energy water molecules that are encapsulated inside the cucurbituril's cavity in aqueous solution immensely enhance the binding.

CB[8], having a cavity of 493 Å^3, is the first member of the cucurbituril family capable of accepting two appropriate guests (e.g. like monocationic pseudo-viologens), forming in a predictable fashion binary 1:1, and homoternary 2:1 complexes based on hydrophobic and cation-dipole interactions with appropriate guests (**Figure 8**).

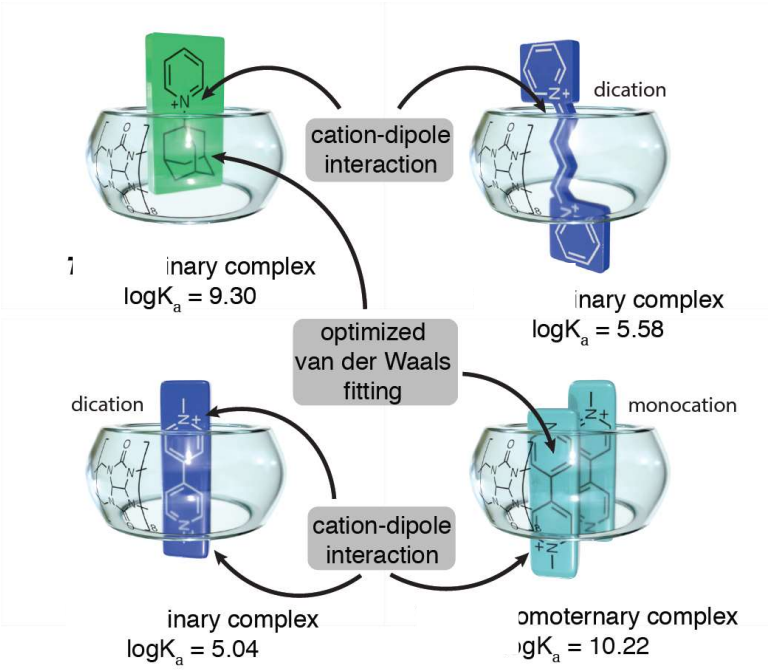


***Figure 8**. Schematic depictions of binary and homoternary host:guest complexes between amphiphilic guests and cucurbit[8]uril.*

(Pseudo)viologen:CB[8] aggregates are good examples of stimuli-responsive complexes (**Figure 9**). In the case of viologens, the reversible reduction of this type of molecule to a radical cation allows the reversible formation of a 2:1 ternary homocomplex. Similarly, the CB[8] ternary homocomplex with monocationic pseudoviologens can be reversibly broken by diminishing the pH. The acidic conditions protonate pseudoviologens, transforming them into dications, which assemble with the CB[8] in the form of 1:1 binary complexes.

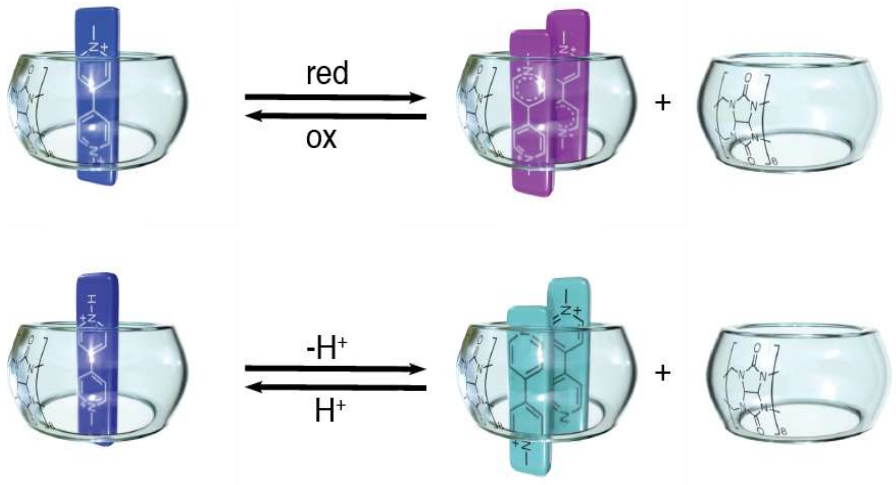


***Figure 9**. Redox and pH-controlled CB[8]:(pseudo)viologen-based supramolecular switches.*

Finally, it should be noted that CB[8] shows a largely negative electrostatic potential inside its cavity, with more electron density around the portal areas than smaller cucurbiturils. Due to this fact, the hydrophobic and yet polar cavity of the CB[8] is capable of stabilizing charge-transfer interactions. Consequently, the inclusion of an electron-donor compound (which could not act as guest otherwise) is facilitated, forming a 2:1 heterocomplex with the

corresponding electron-acceptor species (**Figure 10**). The electron-donor/acceptor character can be easily modulated for the guests in order to optimize this charge-transfer interaction.

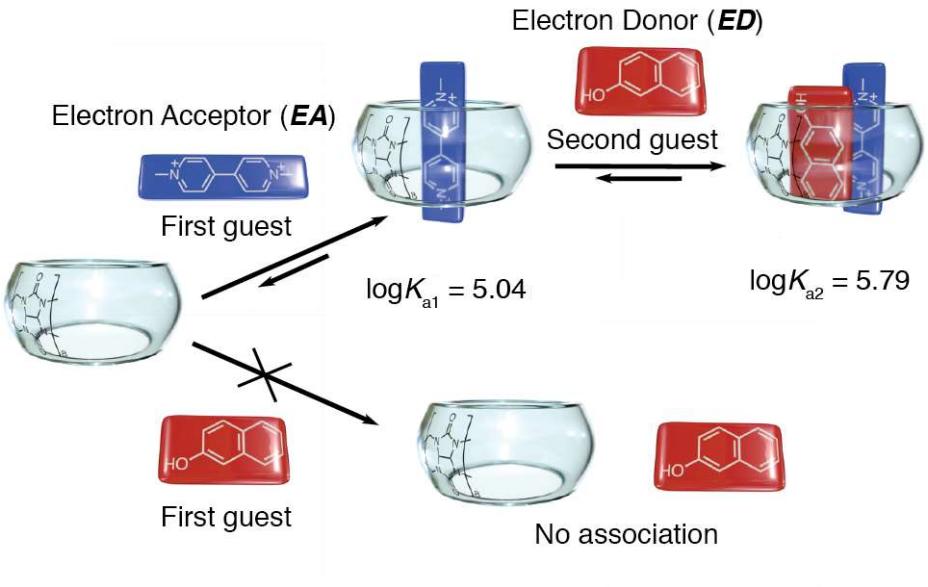
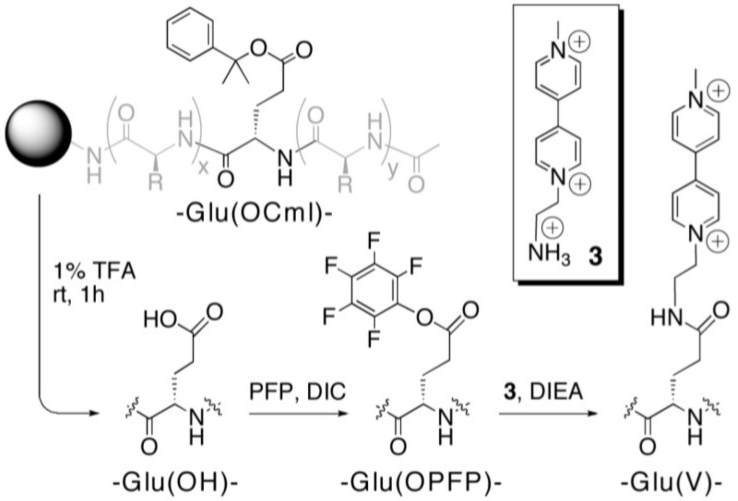


***Figure 10**. 2:1 heterocomplexes formed by charge transfer interactions within CB[8].*[16]

Background

The synthesis of viologen-peptide conjugates by SPPS has been previously described.[17,18] In that case, the group of Urbach prepared an aminoalkyl viologen and a peptide containing Glu residues with orthogonal protecting groups, in which the viologen unit was attached. After the acetylation of the *N*-terminal amine, Glu side chains were selectively deprotected. Subsequently, corresponding carboxylic groups were activated and amide bonds were formed between those groups and the amine groups from the aminoalkyl viologens (**Scheme 5**).



***Scheme 5**. Viologen-peptide conjugate synthesis using Glu side chains.*[17]

As previously discussed, viologens have shown the capability of forming 2:1 inclusion heterocomplexes within CB[8] with electron-donors.[18] Viologens would act as electron acceptors, while other molecules such as tryptophan side chains in peptides would act as electron donors.

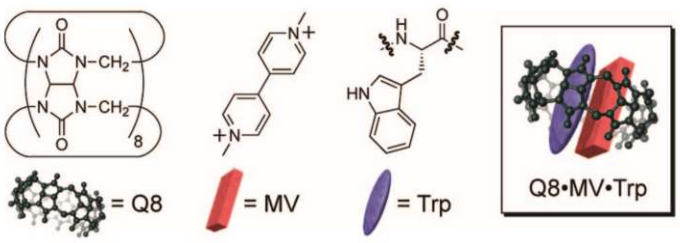


***Figure 11.** Charge transfer Inclusion heterocomplex between methylviologen and tryptophan.*[18]

These complexes can be also assembled with both guests, the viologen and the Trp residue of a peptide, bound to different scaffolds, allowing the temporary attachment of macromolecular building blocks on the basis of host:guest chemistry (**Figure 12**).

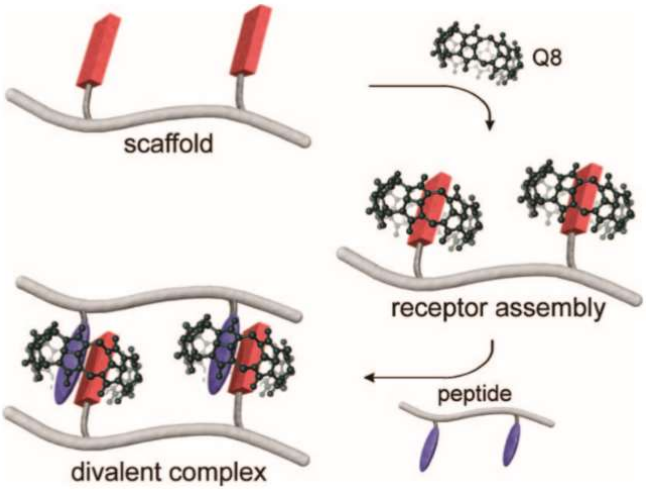


***Figure 12**. Self-assembly of inclusion heterocomplexes attached to scaffolds.*[18]

The formation of 2:1 homocomplexes containing two viologen units attached to peptides has also been reported.[19] This complexation occurs under reductive conditions, as the viologen molecules must be reduced to radical cations in order to form the 2:1 complex, in which the guests place themselves in an antiparallel position.

Objective

In the context of a larger research project, focused on the reversible control of peptide aggregation by CB[8]:(pseudo)viologen host:guest chemistry, the main aim of this project is the development of viologen-peptide conjugates that would be studied in the future as models of β-sheet reversible aggregation.

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

1. Synthesis of appropriately-functionalized *N*-activated (pseudo)viologens **1** and **2**, as adequate precursors for the synthesis of the peptide-(pseudo)viologen conjugates by implementation of the Zincke reaction on the SPPS methodology.

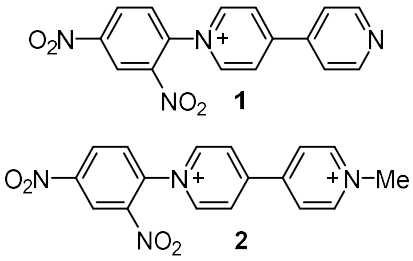


***Figure 13**. N-activated (pseudo)viologens **1** and **2**.*

2. SPPS of the model peptide Fmoc-VQIVYK-NH_2[20], which contains two amino groups, one at the Lys (K) side chain and the other at the *N*-terminus, that can be used for the (pseudo)viologen conjugation through solid-phase Zincke reactions.

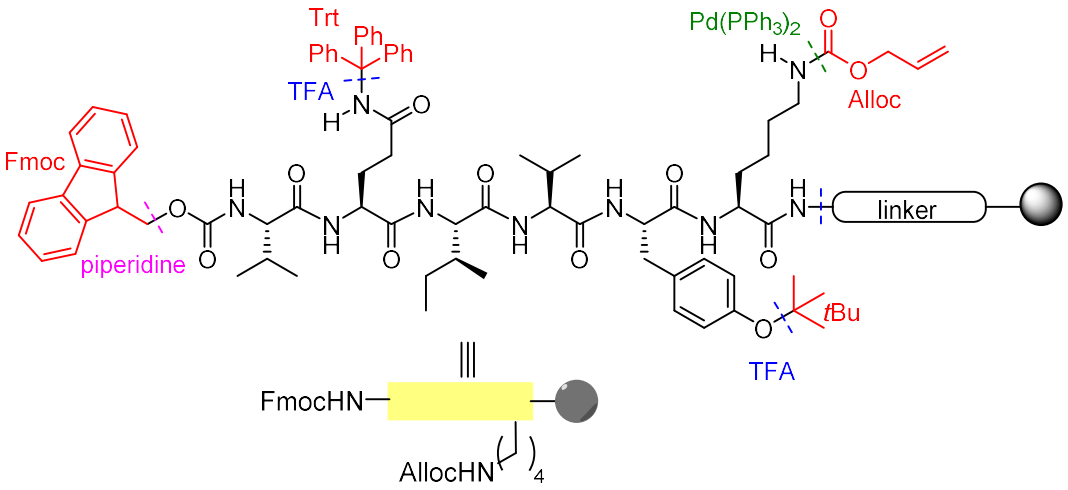
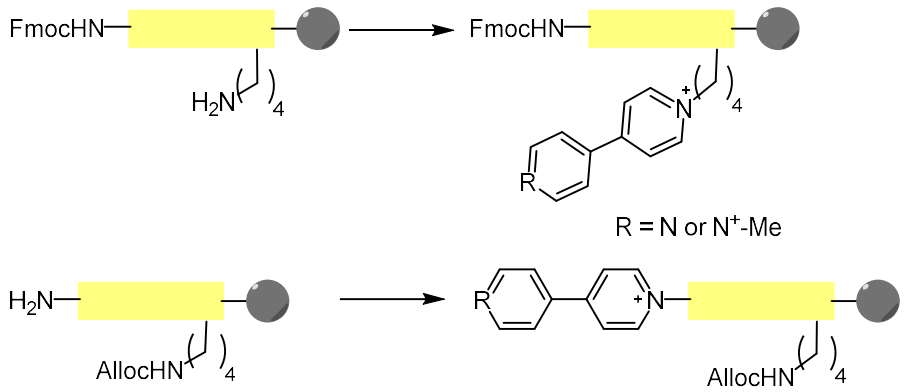


***Figure 14**. Model peptide Fmoc-VQIVYK-NH_2.*

3. Coupling of the (pseudo)viologen moieties to the peptides through solid-phase Zincke reactions.

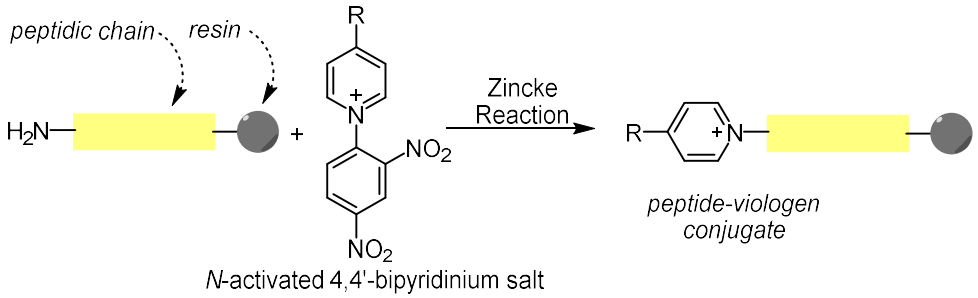


***Scheme 6**. (pseudo)viologen-peptide couplings.*

Results and discussion

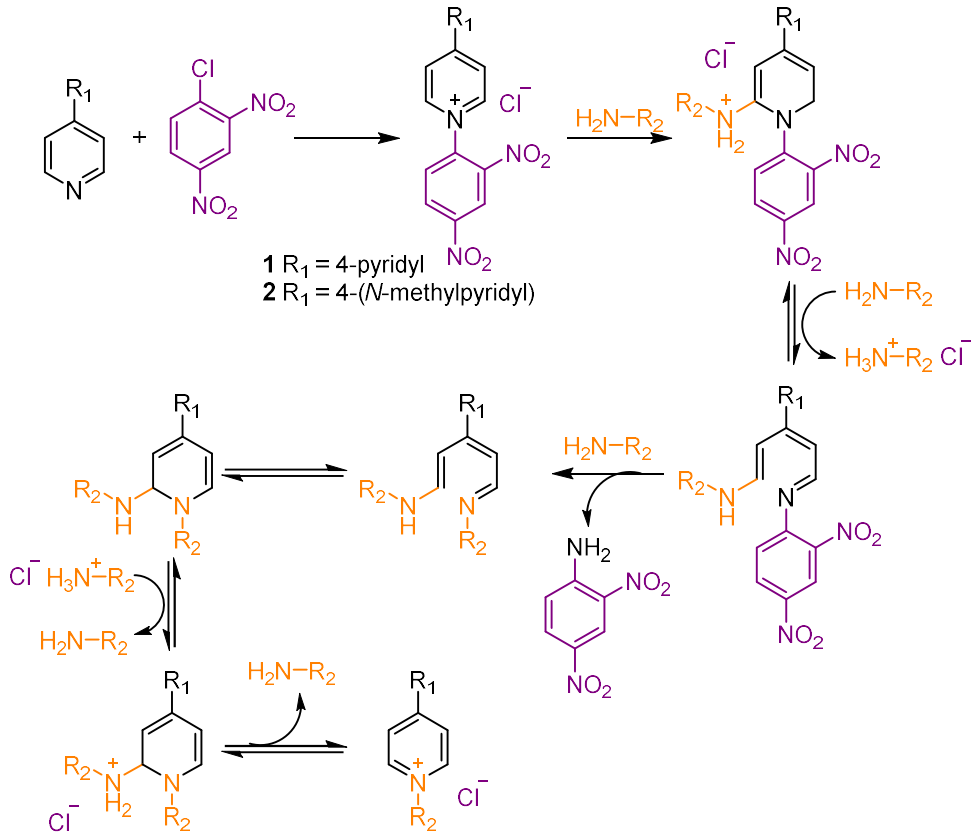
Synthesis of *N*-activated 4,4'-bipyridinium salts as precursors for the Zincke reaction

As shown on **Scheme 7**, the designed (pseudo)viologen-peptide conjugates were initially planned to be synthesized through Zincke reactions as the key step;[21] implemented on the last step of standard SPPS protocols.



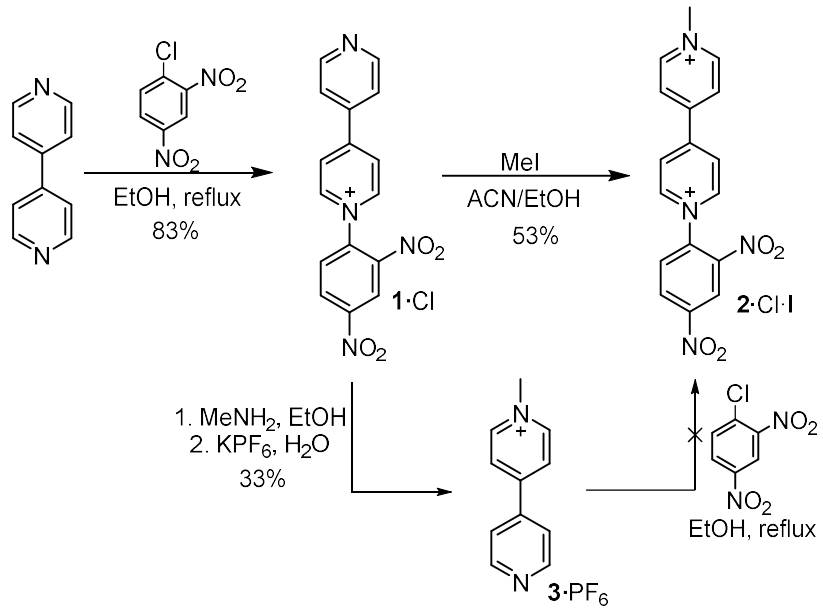
***Scheme 7**. Depiction of the solid phase synthesis of the designed peptide-viologen conjugates.*

For this purpose, the first step of the intended work was the synthesis of the *N*-activated 4,4'-bipydinium derivatives **1** and **2** (**Scheme 8**), compounds that would be able to produce the desired conjugates through Zincke reactions following an ANROC (Addition of the Nucleophile, Ring Opening and Ring Closure) mechanism:[22]



***Scheme 8**. ANROC mechanism for the Zincke reaction.[22]*

In this context, compound **1** was successfully synthesized in a 83% yield by nucleophilic aromatic substitution of 1-chloro-2,4-dinitrobenzene with 4,4'-bipyridine in refluxing EtOH (**Scheme 9**). Following the reported methodology,[11,23] after 24 hours, the product was precipitated from the reaction mixture with Et_2O and washed with AcOEt, rendering the desired compound virtually pure, as confirmed by ^{1}H-NMR spectroscopy.



***Scheme 9**. Synthesis of N-activated pyridinium salts **1** and **2**.*

For the synthesis of compound **2**, two options were considered. The first one involved the activation, by nucleophilic aromatic substitution in refluxing ACN of the already methylated derivative **3**, which unfortunately did not proceed. Following the second approach, consisting in the methylation of compound **1** with excess of MeI in ACN/EtOH at room temperature, **2** was successfully obtained in a satisfactory yield.

Peptide design and synthesis

As discussed before, the main goal of this work is to achieve the synthesis of a (pseudo)viologen-modified peptide, which would be used in the future to control *β*-sheet aggregation by means of CB[8]:(pseudo)viologen host:guest chemistry.

In this context, the peptide Fmoc-VQIVYK-NH_2 was chosen both as a well-known *β*-sheet forming peptide sequence,[20] and as an appropriate peptide model for the Zincke reactions. Those reactions with the (pseudo)viologen precursors **1** and **2** were intended to be performed in two amino groups placed at different positions of the peptide chain: the *N*-terminus and the Lys residue side chain. Therefore, to appropriately proceed with the synthesis of the peptide, and in order to achieve selectivity for the subsequent Zincke reactions planned to be carried out in the final steps of the processes, orthogonal protecting groups must be considered and introduced on the structure of the targeted peptide **P1**/compound **4** (**Figure 15**).

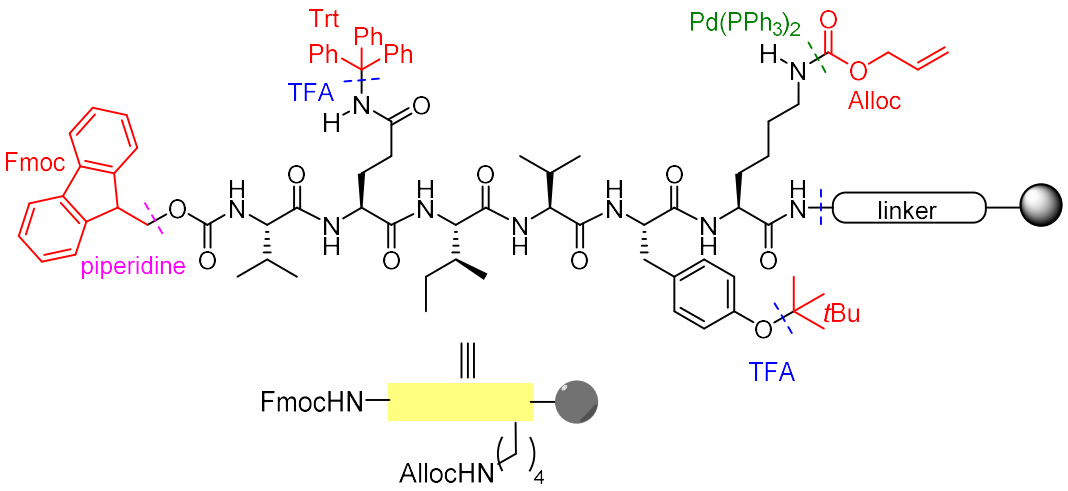
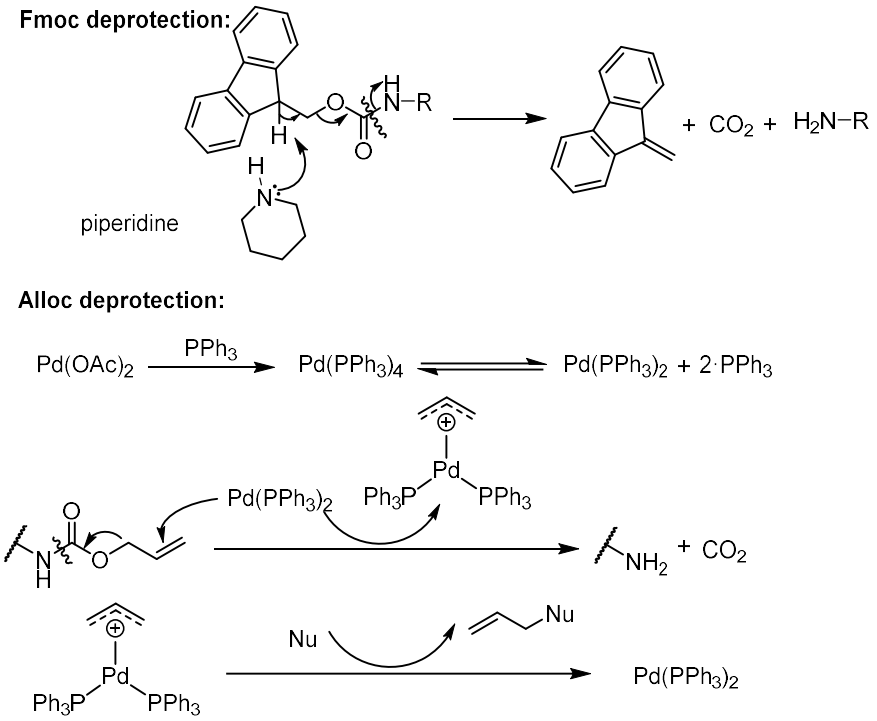


Figure 15. Designed model peptide for its conjugation with (pseudo)viologens.

Hence, from the chosen sequence (Fmoc-VQIVYK-NH_2), Gln (Q), Tyr (Y) and Lys (K) residues needed protective groups at their side chains. Gln was protected with Trt and Tyr with *t*Bu, both being TFA labile moieties for the final deprotection step. Conversely, with the goal of achieving the conjugation of the (pseudo)viologens on the Lys residue side chain, this residue was protected with an Alloc group for its subsequent orthogonal deprotection regarding to the Fmoc-protected *N*-terminus (**Scheme 10**).



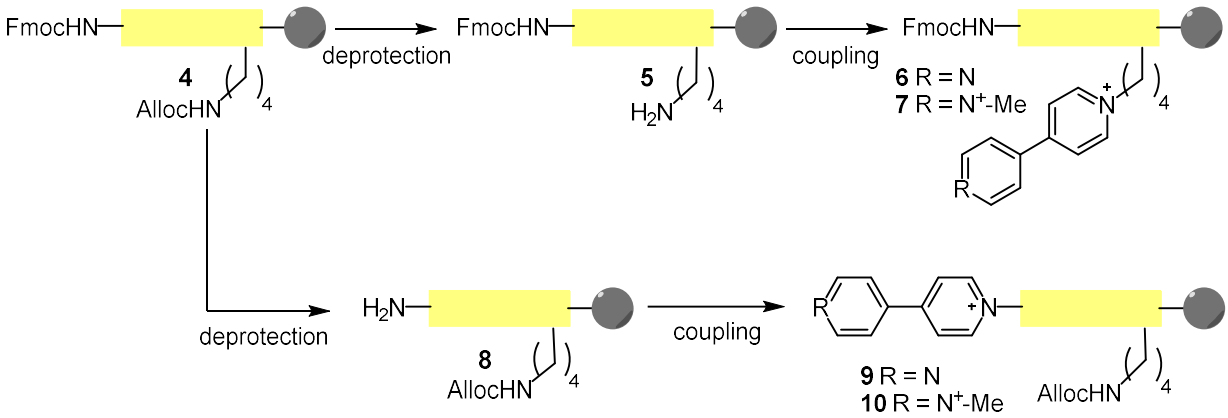
Scheme 10. Deprotection mechanism of Fmoc/Alloc containing peptides.

The designed peptide **P1** was successfully synthesized using standard Fmoc solid-phase peptide synthesis protocols, consisting on the addition of the corresponding activated Fmoc-protected amino acids, removal of the Fmoc protecting group, and repetition of the process. Couplings were performed in DMF, which is an appropriate solvent for the swelling of the polymeric resin, taking each coupling 30 min to be completed. The synthetic protocol is more extensively described at the experimental procedure section. It should be noted that peptide **P1**, when fully protected, had not any easily ionizable groups that allowed its

identification by mass spectrometry. Therefore, it was necessary to carry out the deprotection of at least one amine group for the identification of the peptide by mass spectrometry.

(Pseudo)viologen-peptide conjugation

In order to perform the solid phase Zincke couplings between the Val/Lys-deprotected analogues of **P1** and compounds **1** and **2**, a new SPPS-based methodology had to be developed. Therefore, when the coupling is performed at the *N*-terminus, the deprotection step is carried out with piperidine as base, following the SPPS standard protocol. However, different conditions must be used when the coupling is done at the Lys side chain, with the Alloc protecting group being selectively deprotected with palladium catalysis,[24,25] in the presence of other acid and base-sensitive protecting groups. The deprotection/coupling steps carried out are depicted in the **Scheme 11** below:

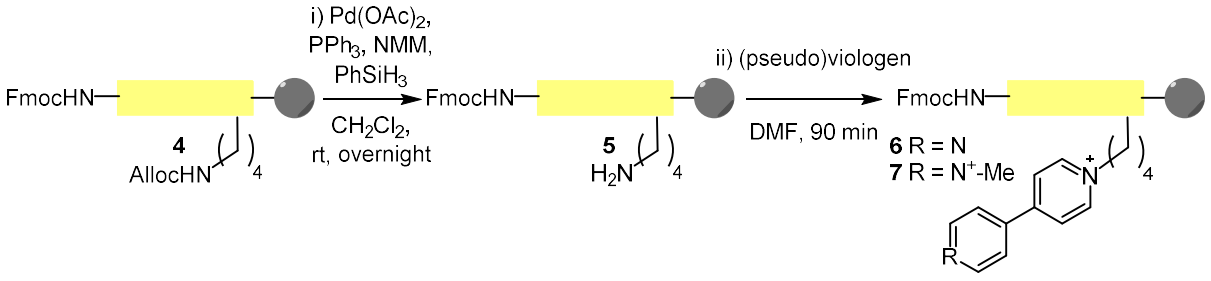


***Scheme 11**. General scheme of the (pseudo)viologen-peptide coupling steps.*

The (pseudo)viologen coupling steps were performed in DMF, with compounds **1** or **2** added as solids and dissolved with the aid of N_2 stirring. In contrast to the standard amino acid couplings, these Zincke reactions in solid phase take 90 min to be completed. More specifically:

A) Coupling at the Lys side chain.

The first conjugates were prepared by the coupling of the (pseudo)viologen at the Lys residue side chain (**Scheme 12**). Therefore, the first step was the selective removal of the Alloc group with Pd to obtain compound **5**. *N*-methylmorpholine and phenylsilane were the nucleophiles chosen for scavenging the allyl groups and regenerating the Pd catalyst.



***Scheme 12**. (pseudo)viologen-peptide coupling at the Lys side chain.*

Afterwards, two tests of the coupling of **5** with compound **1** were performed, with or without the addition of one equivalent of DIEA as base, in order to assure that the amine group of the

peptide was not protonated. Both tests gave the same result, as shown on the HPLC traces in **Figure 16**, so the rest of the couplings reported here were performed without base.

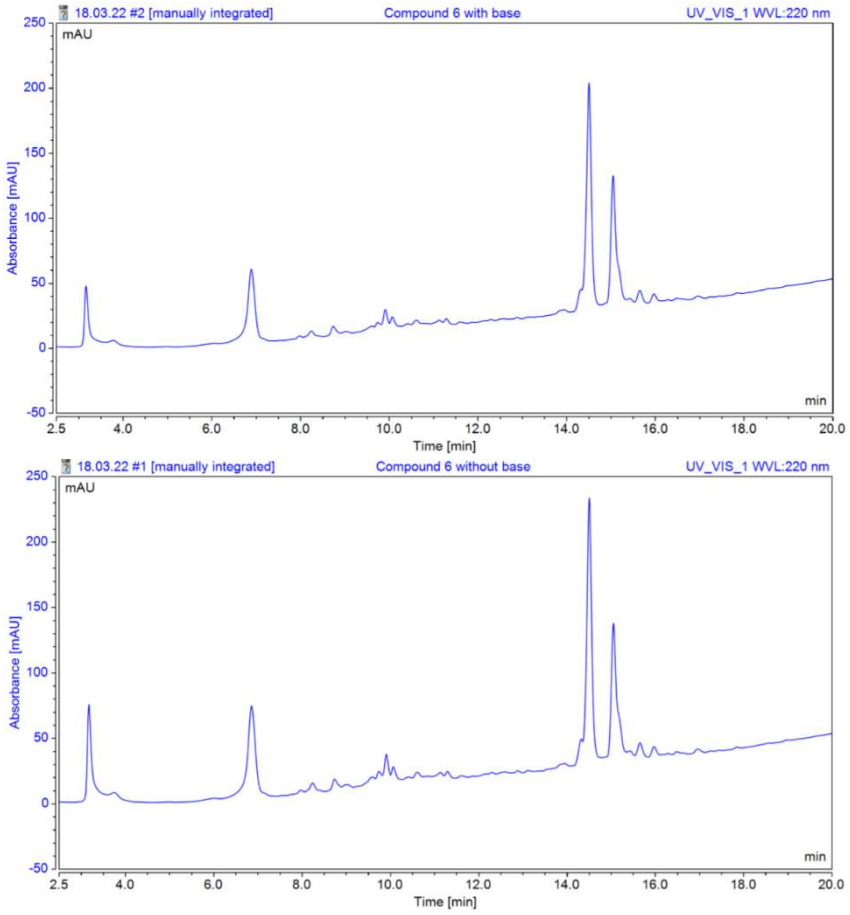


***Figure 16**. HPLC chromatograms of the reaction crude with 1 eq of base (up) and without base (down), in which the peak at t_R = 14.5 min corresponds to the desired product **6**.*

Although three equivalents of compound **1** were used, the reaction was not completed. Subsequent attempts with more equivalents of pseudoviologen **1** (6 eq and 10 eq) and re-couplings did not improve the result. HPLC analysis showed signals corresponding to the product (t_R = 14.5 min) and the starting material (t_R = 15.1 min), as well as other unidentified peaks. However, it was noted that those later signals increased respect to the others with the amount of compound **1** added.

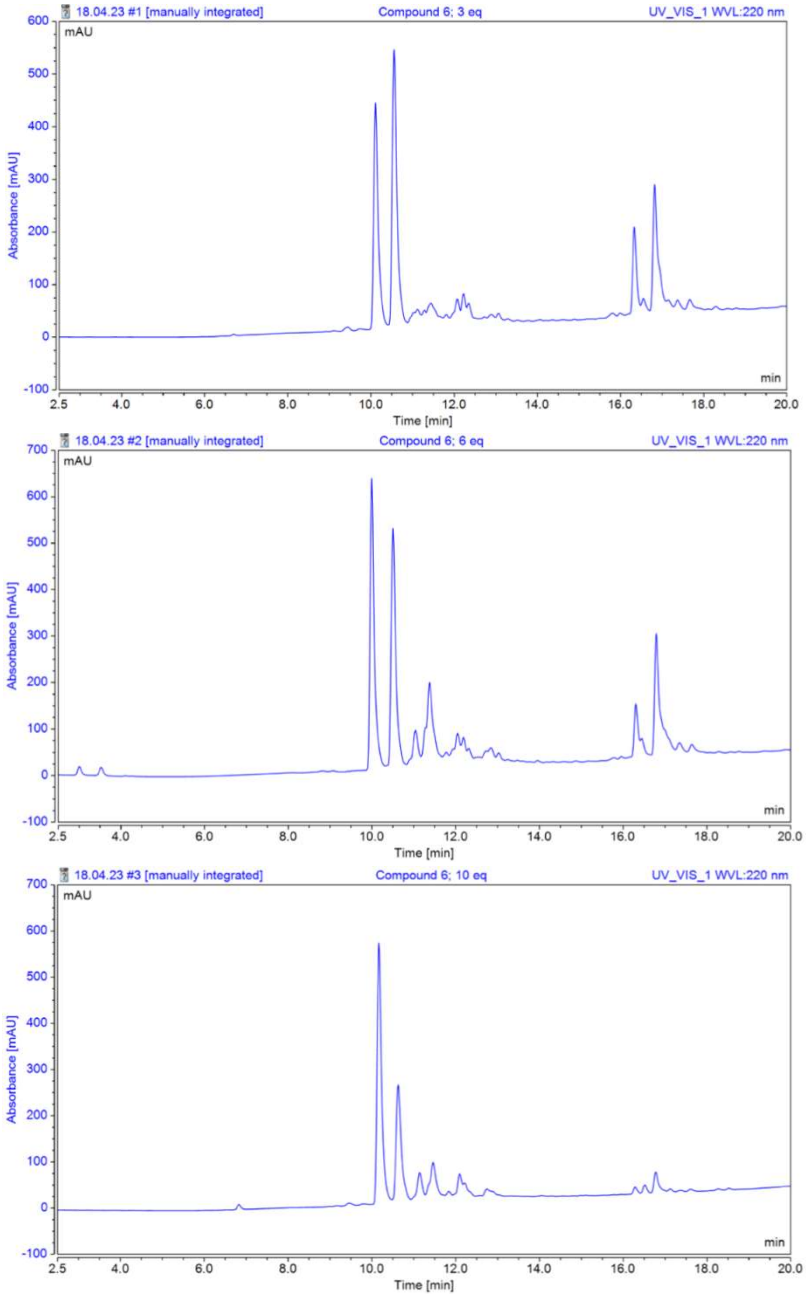
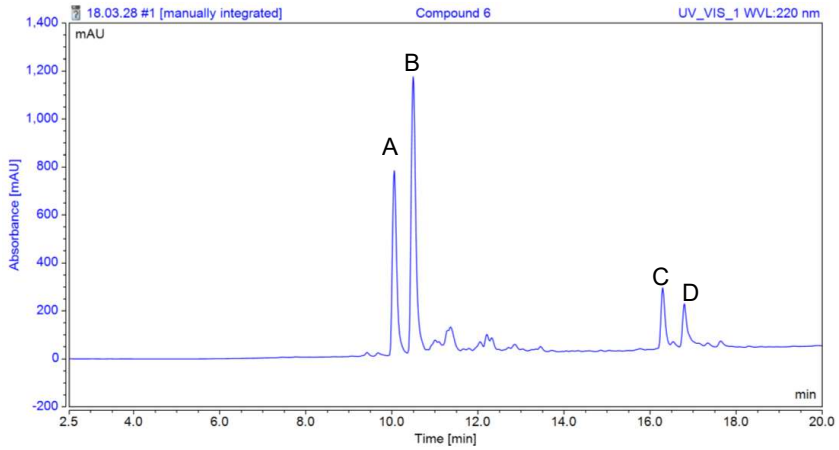


***Figure 17**. HPLC chromatograms of the reaction crude when adding 3 eq (up), 6 eq (middle) and 10 eq (down) of pseudo-viologen* **1**, in which the peak at t_R = 16.3 min corresponds to the desired product **6**.

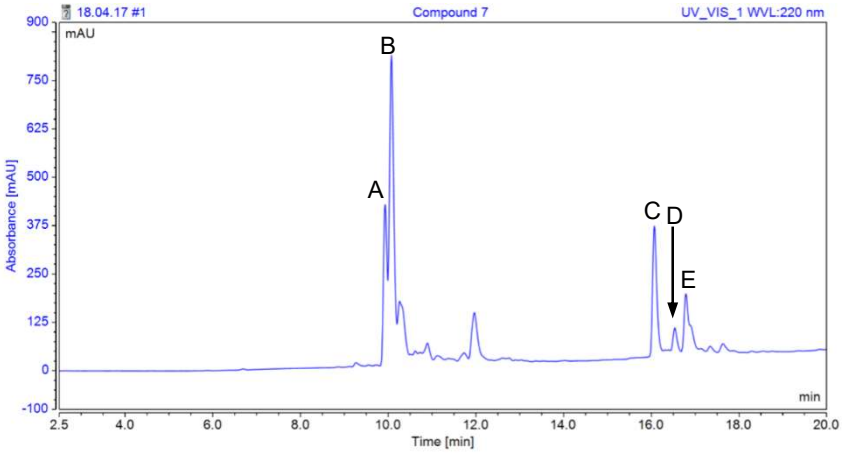
After MS analysis of the main peaks resolved on the HPLC chromatogram and a thorough analysis of the problem at hands, it was found out that the excess of compound **1** (having a pyridine moiety on its structure) acted as a base strong enough to remove the Fmoc group. HPLC chromatograms showed peaks identified as the desired product (t_R = 16.3 min), the unreacted peptide (t_R = 16.9 min), the product without Fmoc (t_R = 10.1 min), and the peptide with two pseudoviologens coupled (t_R = 10.6 min) (**Table 2**).



Signal	*m/z*	*Assignation*
A	444.2603 $[MH]^+$; 887.5147 $[M]^+$	Product without Fmoc
B	513.7815 $[M]^{2+}$; 1140.5511 $[M+TFA]^+$	Double-coupling product
C	555.2940 $[MH]^{2+}$; 1109.5841 $[M]^+$	Product
D	970.5413 $[MH]^+$	Starting material

***Table 2**. Peak analysis of the reaction crude chromatogram for the synthesis of compound **6**.*

Coupling between **2** and peptide **5** gave very similar results, with the HPLC chromatograms of both couplings being analogous to those obtained for the reactions of **1** and **5** (**Table 3**). This similarity on the chromatograms may be explained on the basis of a demethylation process of compound **2** under the coupling conditions used.



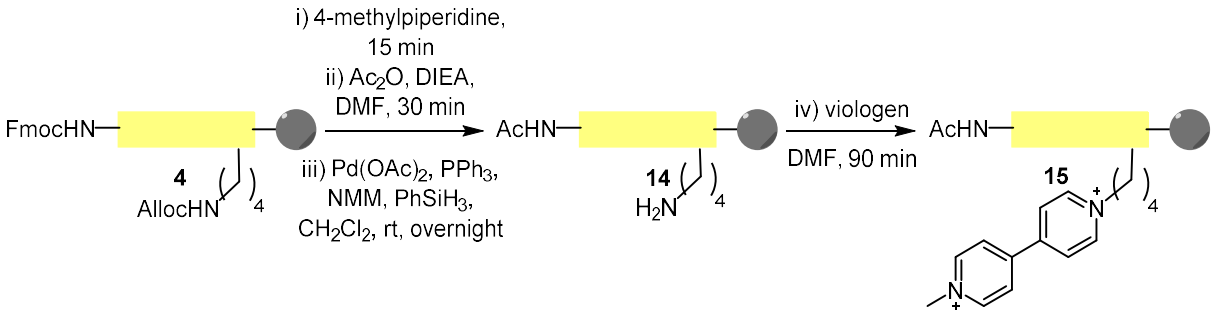
Signal	*m/z*	*Assignation*
A	451.2680 $[M]^{2+}$; 1015.5240 $[M+TFA]^+$	Product without Fmoc
B	641.7903 $[M+2TFA]^{2+}$; 1397.5712 $[M+3TFA]^+$	Double-coupling product
C	562.3021 $[M]^{2+}$; 1237.5917 $[M+TFA]^+$	Product
D	555.2940 $[MH_2]^{2+}$; 1109.5837 $[M]^+$	Demethylated product
E	970.5407 $[MH]^+$	Starting material

***Table 3**. Peak analysis of the reaction crude chromatogram for the synthesis of compound **7**.*

B) Coupling at the Lys side chain on a *N*-terminal-acetylated peptide

Because of the above-mentioned problems on the coupling reactions at the side chain of the Lys residue, due to the basic (pseudo)viologen reagents being able to remove the Fmoc protecting group from the peptide, it was decided to substitute this Fmoc group by an acetyl moiety at the *N*-terminus. In order to do so, the Fmoc group was removed with 4-methylpiperidine, and the peptide was acetylated with excess of Ac_2O. The reaction was

accomplished without any problem and the acetylated derivative **13** was generated (**Scheme 13**).



***Scheme 13**. (pseudo)Viologen-peptide coupling at the Lys side chain of the N-acetylated derivative* **13**.

Next, in order to proceed with the (pseudo)viologen coupling, the Alloc group at the Lys residue side chain was removed as previously described (compound **14**), and the coupling with 3 equivalents of compound **2** was performed. This time, only one major peak was detected on the HPLC chromatogram (t_R = 11.4 min) and identified as the desired product (compound **15**). Other smaller peaks, not absorbing at 280 nm, correspond to side products without the viologen chromophore (**Figure 18**).

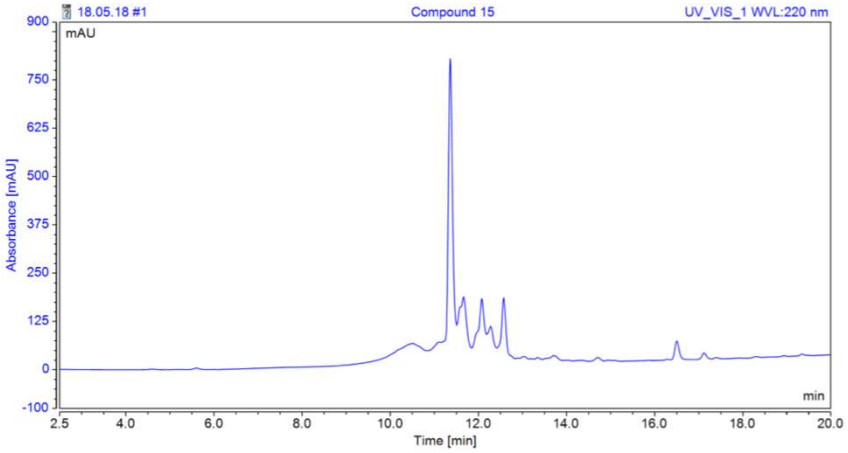
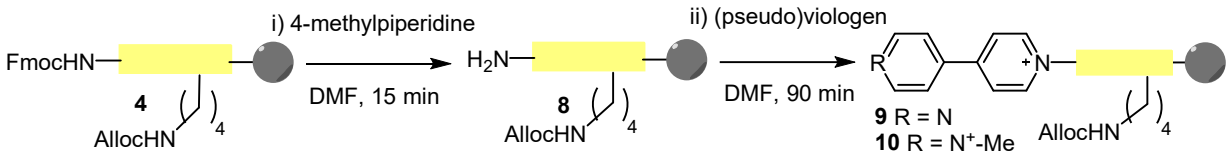


***Figure 18**. HPLC chromatogram of the reaction crude, in which the signal at t_R = 11.4 min corresponds to the desired product **15**.*

The acetylation strategy can be considered an appropriate and efficient way of solving the Fmoc-related problem and performing the Zincke coupling at the Lys residue side chain without any important side product.

C) Coupling at the N-terminus.

For the synthesis of the conjugates at the *N*-terminus, the Fmoc group had to be removed selectively in the presence of the Alloc group protecting the Lys side chain. This was achieved by using excess of 4-methylpiperidine, successfully generating compound **8** (**Scheme 14**).



***Scheme 14**. (pseudo)viologen-peptide coupling at the N-terminus* **8.**

For the reaction with compound **1**, three equivalents of the pseudoviologen were used, with the analysis of the product by HPLC giving just one big peak in the chromatogram (t_R = 12.7 min) corresponding with compound **9**, and a small peak corresponding to the starting material (t_R = 14.5 min) (**Figure 19**).

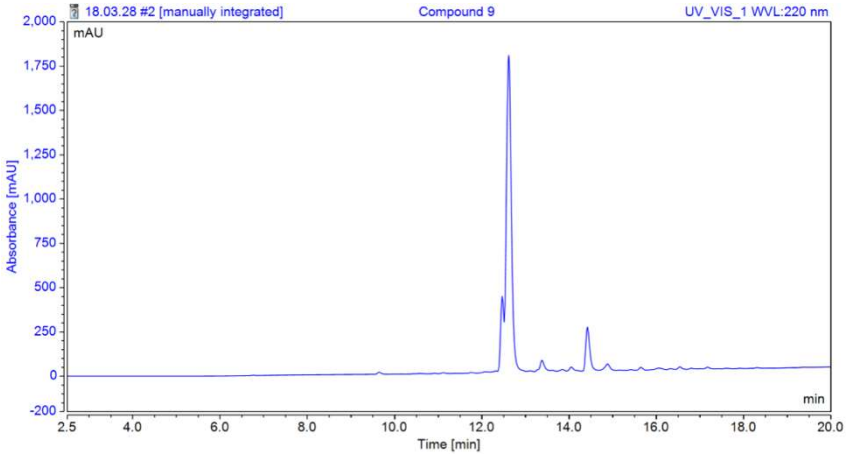


***Figure 19**. HPLC chromatogram of the reaction crude, in which the peak at t_R = 12.7 min corresponds to the desired product **9**.*

This reaction was scaled up from 4.4 µmol to 19.6 µmol, and the same result was obtained. For a greater similarity with the β-forming peptide described on the bibliography, the Alloc group was removed using Pd catalysis, yielding compound **11**. The crude was purified by reversed-phase semipreparative HPLC, and the collected fractions were lyophilized, obtaining a fluffy white solid.

Coupling of **8** with the activated salt **2** was carried out in the same way as with **1**, with three equivalents of viologen. Only one significant peak was detected on the HPLC chromatogram (t_R = 12.3 min), corresponding to the desired conjugate **10** (**Figure 20**).

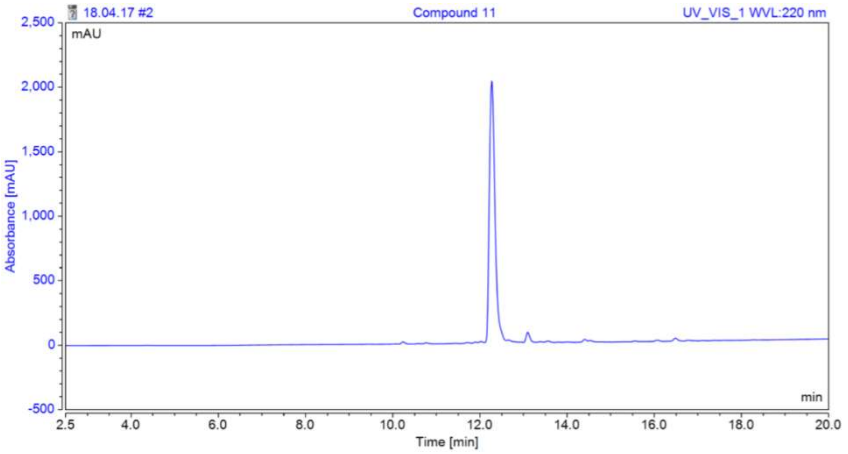


***Figure 20**. HPLC chromatogram of the reaction crude, in which the peak at t_R = 12.3 min corresponds to the desired product **10**.*

This reaction was scaled up as well, from 4.6 µmol to 12.5 µmol. The Alloc group was removed with Pd catalysis (giving compound **12**), and the crude was purified by reversed-phase semipreparative HPLC and the collected fractions were lyophilized, obtaining a fluffy white solid.

Future work

Several viologen-peptide conjugates have been successfully synthesized by implementation of the Zincke reaction in solid phase, and some of them have also been purified in an adequate scale. These compounds will be used in the future for controlling the β-sheet aggregation of the model peptide by reversible CB[8]:(pseudo)viologen host:guest chemistry.

Experimental procedure

General information

All solvents used were synthesis grade, unless specifically noted. Water was purified using a Milli-Q system (*Millipore*).

All reactions performed for the formation of viologens or pseudo-viologens were followed by TLC. Since these compounds are very polar and positively charged, the eluent chosen for the chromatography was the mixture formed by ACN (600 mL), MeOH (150 mL), H_2O (150 mL) and NaCl (5 g). The same eluent was used for the compound purification by flash chromatography when needed. Products were identified with ultraviolet light at 254 nm and 360 nm.

Moreover, all products identities and purities were checked by ^{1}H-NMR spectroscopy, using a *Bruker Avance 300* (300 MHz) spectrometer. Deuterated water (D_2O) and deuterated ACN (CD_3CN) were the solvents used for the preparation of the samples.

Reversed-phase HPLC-MS analyses were performed using a *Thermo Scientific UltiMate 3000* connected to a single quadrupole mass spectrometer *Thermo Scientific MSQ Plus,* and a PDA (Photo-Diode Array) detector. HPLC-MS quality solvents were used to prepare the eluents, A: 0.1% TFA, H_2O and B: 0.1%TFA, ACN. The column used for these analyses was *Phenomenex Aeris* 3.6 µm peptide XB-C18 100 Å; 150 × 2.1 mm.

The gradient used for reversed-phase HPLC-MS experiments was the following:

Time (min)	Flow (mL/min)	% B
0	0.300	5
2	0.300	5
25	0.300	95
26	0.300	100
32	0.300	100
33	0.300	5
40	0.300	5

***Table 4**. Gradient for HPLC and HPLC-MS analysis*

Two semipreparative reversed-phase HPLC purifications were also performed using the same instrument without the mass spectrometer. In those cases, the column used was: *Phenomenex Luna* 5 µm C18 100 Å; 250 × 10 mm.

The amount of solution in each injection was increasingly raised from 5 µL to 100 µL. The gradient was also changed, in order to make sure that the peaks were well resolved and that the elution time was not too high. The starting gradient was the same showed above, but with a flow of 2.500 mL/min. The final gradient (for 100 µL injections) was the following:

Time (min)	Flow (mL/min)	% B
0	2.500	10
2	2.500	10
35	2.500	50
42	2.500	100
43	2.500	10
50	2.500	10

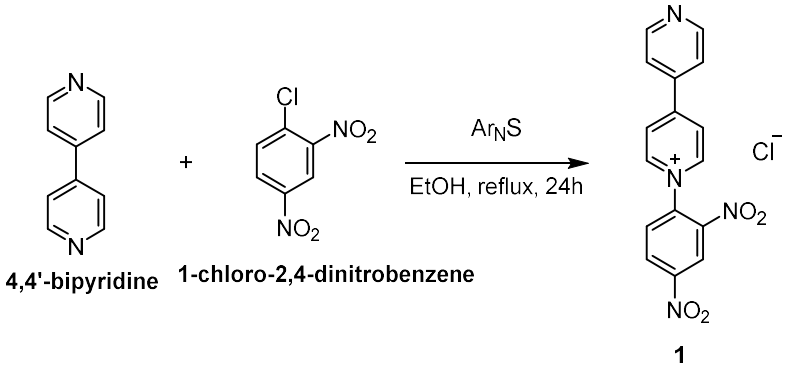
***Table 5**. Final gradient for semipreparative HPLC purification*

Purified compounds were lyophilized using a *Teslar Cryodos -80* device.

For a better characterization of the final conjugates MS and reversed-phase HPLC-MS analysis were performed with higher resolution instruments and by the staff of the SAI (Research Support Facilities) of the UDC. The instruments used were a *Bruker Avance 500* (500 MHz) spectrometer for NMR and a *Thermo Scientific Accela* connected to a mass spectrometer *Thermo Scientific LTQ-Orbitrap Discovery* for HPLC-MS.

Synthesis

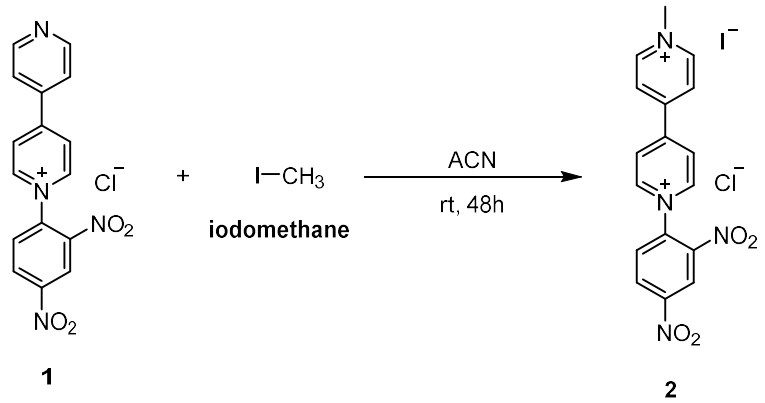
N-(2,4-dinitrobenzil)bipyridinium chloride (1)



4,4-bipyridine (12.8 mmol, 1.9973 g) and 1-chloro-2,4-dinitrobenzene (12.8 mmol, 2.6067 g) 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 air-cooling. The product was precipitated (yellowish) by adding Et_2O (185 mL aprox.). The precipitate was washed with AcOEt, dissolved in MeOH, and concentrated to dryness to give a brown solid (3.7932 g, 82.6 %).

^{1}H-NMR (300 MHz, D_2O) δ: 8.08 (d, 2H, J = 5.8 Hz), 8.30 (d, 1H, J = 8.7 Hz), 8.72 (d, 2H, J = 6.5 Hz), 8.88 (d, 2H, J = 5.4 Hz), 8.97 (dd, 1H, J_1 = 2.5 Hz, J_2 = 8.6 Hz), 9.28 (d, 2H, J = 6.4Hz), 9.43 (d, 1H, J = 2.5 Hz).

N-(2,4-dinitrobenzene)-*N*'-methylbipyridinium chloroiodide (2)

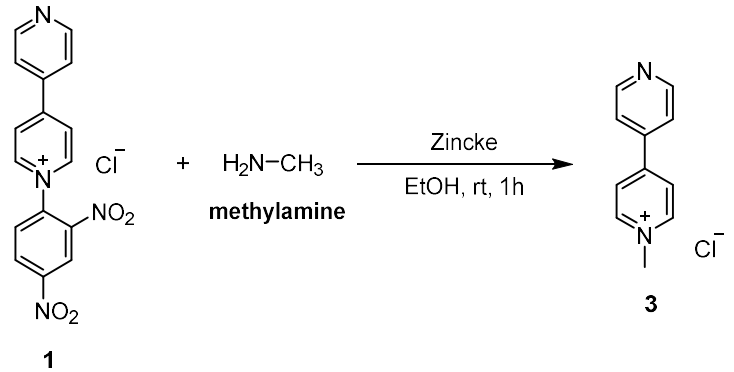


1 **2**

Approximately 3-4 mL of EtOH were added over a mixture of compound **1** (0.697 mmol, 250.9 mg) and ACN (15 mL), until the solid was completely dissolved. Then, MeI (13.94 mmol, 0.870 mL) was added, and the resulting solution was stirred at rt until the following day. The reaction was checked by TLC and it was found to be not completed. Therefore, another portion of MeI (13.94 mmol, 0.870 mL) was added and the solution was left again until the following day. A precipitate was formed and the mixture turned red. The mixture was vacuum filtered and the precipitate was washed with ether. 184.5 mg of a reddish-orange solid were obtained (184.5 mg, 52.9 %).

^{1}H-NMR (300 MHz, D_2O) δ: 4.58 (s, 3H), 8.34 (d, 1H, J = 8.7 Hz), 8.68 (d, 2H, J = 6.4 Hz), 8.87 (d, 2H, J = 6.7 Hz), 9.00 (dd, 1H, J_1 = 2.5 Hz, J_2 = 8.7 Hz), 9.17 (d, 2H, J = 6.5 Hz), 9.46 (m, 3H).

N-methylbipyridinium chloride (3)



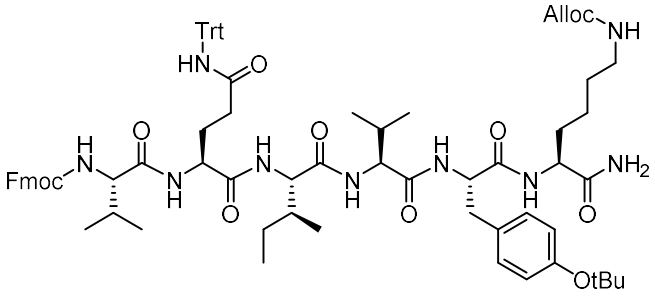
1 **3**

Compound **1** (1.4 mmol, 0.5039 g) was dissolved in EtOH (20 mL). Then, methylamine (3.5 mmol, 300 µL, 40 %) was added and the solution turned immediately black. The resulting mixture was stirred at rt for 1 h and followed by TLC. The solution was concentrated to dryness and the product was extracted with H_2O (40 mL) and AcOEt (40 mL). The aqueous phase was washed with AcOEt (100 mL) and concentrated to dryness.

Change of counterion: The product was dissolved in the minimum amount of H_2O and KPF_6 (3.4 mmol, 0.630 g) was added to the solution. The mixture was stirred, and the precipitated salt was filtered, washed with H_2O (25 mL), dissolved in ACN, and concentrated to dryness to give a pale brown solid (140 mg, 33 %).

^{1}H-NMR (300 MHz, D_2O) δ: 4.43 (s, 3H), 7.91 (d, 2H, *J* = 5.3 Hz), 8.38 (d, 2H, *J* = 6.2 Hz), 8.77 (d, 2H, *J* = 5.2 Hz), 8.89 (d, 2H, *J* = 6.5 Hz).

Peptide synthesis (4)



4/P1

- 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 the protection/deprotection of the amine group: 1 % TNBS/DMF + 10% DIEA/DMF
- Fmoc deprotecting agent: 4-methylpiperidine
- Sequence: Val-Gln-Ile-Val-Tyr-Lys (Fmoc-VQIVYK-NH_2)

The resin (0.1 mmol, 216.6 mg) was placed in a plastic column and washed 3 times with DMF with N_2 bubbling (**Figure 21**). The amine group was checked to be deprotected with the TNBS test (positive result). The protected amino acid Fmoc-Lys(Alloc)-OH (in excess, 4 eq) was dissolved in the coupling agent solution (4 eq, 2 mL) and then the DIEA solution (6 eq, 3 mL) was added. After 2 min of activation, the resulting solution was added to the resin and mixed by N_2 bubbling for 30 min. After solvent removal by filtration (figure XB), the resin was washed with DMF (2 × 5 mL), and then the Fmoc protecting group was removed by treatment with an excess of 4-methylpiperidine (5 mL) during 15 min. The resin was washed three times with DMF, and then a TNBS test showed a positive result. This procedure was repeated for each amino acid of the peptide sequence (from the *C*-terminus to the *N*-terminus).

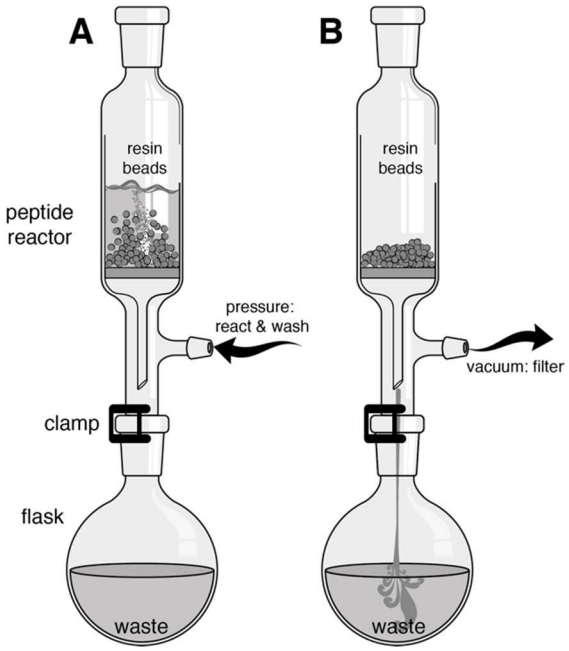
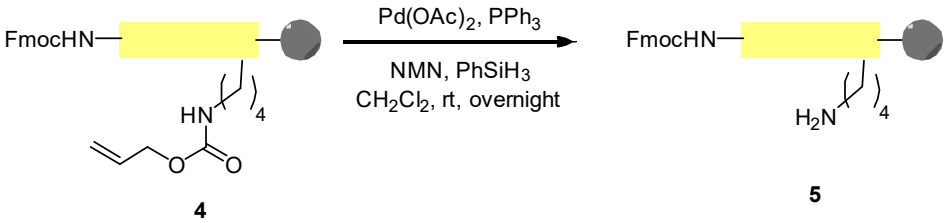


Figure 21. Assembly for SPPS.

Coupling at the Lys side chain

- Deprotection of the Alloc group:

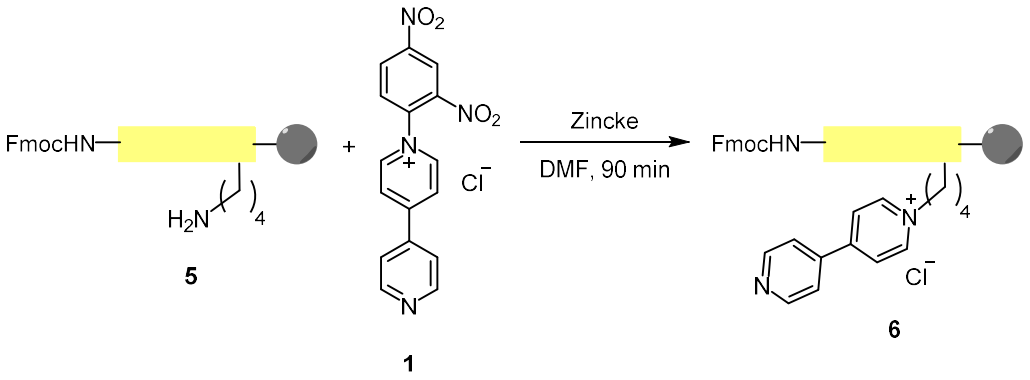


4 **5**

The resin (0.05 mmol) was treated with a mixture of $Pd(OAc)_2$ (0.015 mmol, 3.4 mg), PPh_3 (0.075 mmol, 19.7 mg), $PhSiH_3$ (0.5 mmol, 62 µL) and NMM (0.5 mmol, 55 µL) 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), 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).

Experimental procedure

- Coupling with compound **1**:



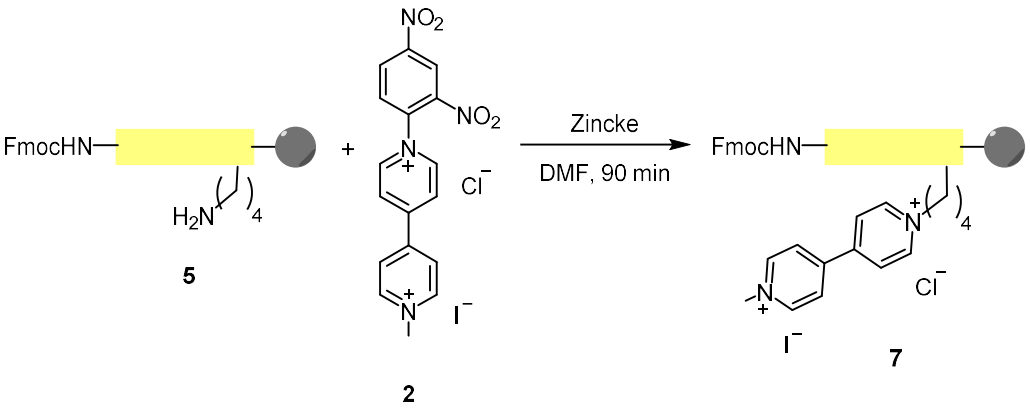
A small amount of the resin (4.4 µmol, 15.0 mg) was resuspended in DMF (320 µL) and compound **1** (13.2 µmol, 4.8 mg) was added to the mixture. The resin instantly turned black and the mixture was mechanically stirred for 90 min. After filtration, the resin was washed DMF (2 × 0.5 mL × 5 min) and with CH_2Cl_2 (2 × 0.5 mL × 5 min).

3-4 mg of the resin were treated with 150 µL of the cleaving cocktail (2.5% triisopropylsilane, TIS, 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.

t_R = 16.3 min (column *Phenomenex Aeris* peptide XB-C18, lineal gradient 5→95 % ACN, 0.1 % TFA / H_2O, 0.1 % TFA in 23 min).

ESI-MS (*m/z*): $[M]^+$ calc. for $C_{61}H_{77}N_{10}O_{10}^+$ = 1109.6, found = 1109.6 $[M]^+$; 555.3 $[MH]^{2+}$.

- Coupling with compound **2**:



A small amount of the resin (4.4 µmol, 15.0 mg) was resuspended in DMF (320 µL) and compound **2** (13.2 µmol, 6.6 mg) was added to the mixture. The resin instantly turned brown-black and the mixture was mechanically stirred for 90 min. After filtration, the resin was washed DMF (2 × 0.5 mL × 5 min) and with CH_2Cl_2 (2 × 0.5 mL × 5 min).

3-4 mg of the resin were treated with 150 µL of the cleaving cocktail (2.5% triisopropylsilane, TIS, 2.5% H_2O and 95% TFA) for 90 min. The resin was then filtered, the

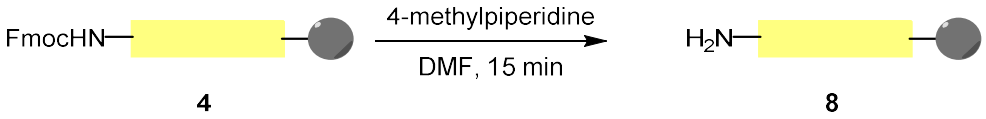
TFA filtrate was added to ice-cold ether (1.2 mL) and the precipitate centrifugated for 10 min. The solid residue was dissolved in 400 µL of H_2O and analyzed by reversed-phase HPLC-MS.

t_R = 16.1 min (column *Phenomenex Aeris* peptide XB-C18, lineal gradient 5→95 % ACN, 0.1 % TFA / H_2O, 0.1 % TFA in 23 min).

ESI-MS (*m/z*): $[M]^{2+}$ calc. for $C_{62}H_{80}N_{10}O_{10}^{2+}$ = 562.3, found = 562.3 $[M]^{2+}$; 1237.6 $[M+TFA]^+$.

Coupling at the *N*-terminus

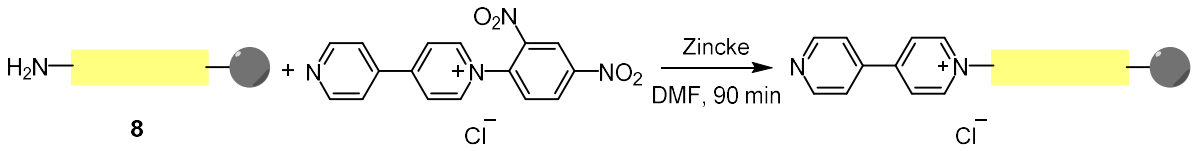
- Deprotection:



4 **8**

The resin (0.05 mmol) was treated with a solution of 20% 4-methylpiperidine in DMF (2.5 mL) with N_2 bubbling for 15 min, and then was filter and washed with DMF (3 × 2.5 mL × 5 min). A TNBS test was performed, giving a positive result. The resin was washed with CH_2Cl_2 (2 × 2.5 mL × 5 min) and dried under nitrogen.

- Coupling with compound **1**:



8 **1** **9**

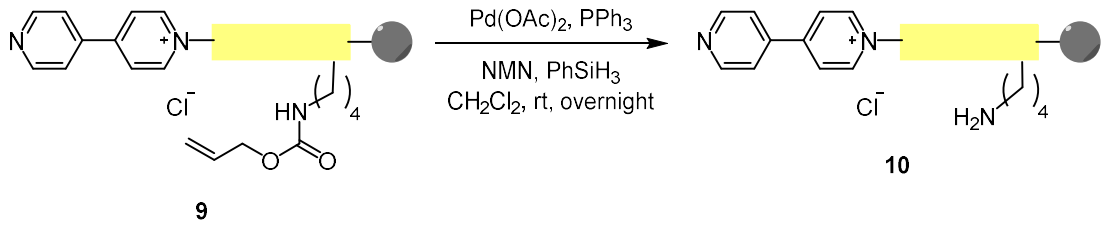
A small amount of the resin (4.6 µmol, 15.0 mg) was resuspended in DMF (320 µL) and compound **1** (13.2 µmol, 4.7 mg) was added to the mixture. The resin instantly turned black and the mixture was mechanically stirred for 90 min. After filtration, the resin was washed DMF (2 × 0.5 mL × 5 min) and with CH_2Cl_2 (2 × 0.5 mL × 5 min).

3-4 mg of the resin were treated with 150 µL of the cleaving cocktail (2.5% triisopropylsilane, TIS, 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.

t_R = 12.6 min (column *Phenomenex Aeris* peptide XB-C18, lineal gradient 5→95 % ACN, 0.1 % TFA / H_2O, 0.1 % TFA in 23 min).

ESI-MS (*m/z*): $[M]^+$ calc. for $C_{50}H_{71}N_{10}O_{10}^+$ = 971.5, found = 971.5 $[M]^+$; 486.3 $[MH]^{2+}$.

- Deprotection of the Alloc group:



9 10

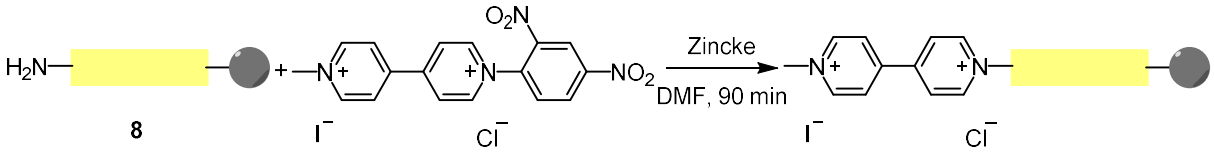
The resin (19.6 µmol) was treated with a mixture of $Pd(OAc)_2$ (5.64 µmol, 1.3 mg), PPh_3 (28.2 µmol, 7.4 mg), $PhSiH_3$ (0.188 mmol, 23 µL) and NMM (0.188 mmol, 21 µL) in CH_2Cl_2 (1.5 mL) and the resulting mixture was stirred mechanically until the following day. The resin was then filtered and washed with: CH_2Cl_2 (2 × 2.5 mL × 5 min), DMF (2 × 2.5 mL × 5 min), 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).

The resin was treated with 3 mL of the cleaving cocktail (2.5% triisopropylsilane, TIS, 2.5% H_2O and 95% TFA) for approximately 4 h. The resin was then filtered, the TFA filtrate was added to ice-cold ether (30 mL) and the precipitate centrifugated at 4 ºC for 15 min. The solid residue was dissolved in 1 mL of ACN:H_2O (1:1), filtered and purified by semipreparative reversed-phase HPLC.

t_R = 10.6 min (column *Phenomenex Aeris* peptide XB-C18, lineal gradient 5→95 % ACN, 0.1 % TFA / H_2O, 0.1 % TFA in 23 min).

ESI-MS (*m/z*): $[M]^+$ calc. for $C_{46}H_{67}N_{10}O_8^+$ = 887.5, found = 887.5 $[M]^+$; 444.3 $[MH]^{2+}$.

- Coupling with compound **2**:



8 2 11

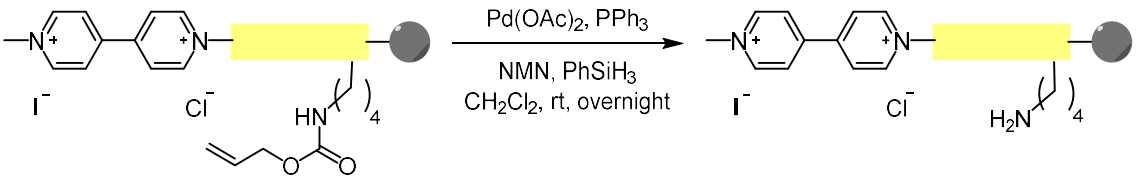
A small amount of the resin (4.6 µmol, 15.0 mg) was resuspended in DMF (320 µL) and compound **2** (13.8 µmol, 6.9 mg) was added to the mixture. The resin instantly turned brown-black and the mixture was mechanically stirred for 90 min. After filtration, the resin was washed DMF (2 × 0.5 mL × 5 min) and with CH_2Cl_2 (2 × 0.5 mL × 5 min).

3-4 mg of the resin were treated with 150 µL of the cleaving cocktail (2.5% triisopropylsilane, TIS, 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.

t_R = 12.3 min (column *Phenomenex Aeris* peptide XB-C18, lineal gradient 5→95 % ACN, 0.1 % TFA / H_2O, 0.1 % TFA in 23 min).

ESI-MS (*m/z*): $[M]^{2+}$ calc. for $C_{51}H_{74}N_{10}O_{10}^{2+}$ = 493.3, found = 493.3 $[M]^{2+}$; 1099.6 $[M+TFA]^+$.

- Deprotection of the Alloc group:



11 **12**

The resin (12.5 μmol) was treated with a mixture of $Pd(OAc)_2$ (3.75 μmol, 0.8 mg), PPh_3 (18.8 μmol, 4.9 mg), $PhSiH_3$ (0.125 mmol, 16 μL) and NMM (0.125 mmol, 14 μL) in CH_2Cl_2 (1.5 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), 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).

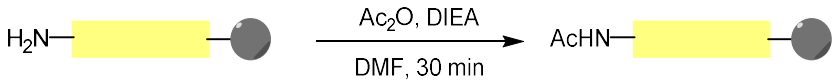
The resin was treated with 2 mL of the cleaving cocktail (2.5% triisopropylsilane, TIS, 2.5% H_2O and 95% TFA) for approximately 2.5 h. The resin was then filtered, the TFA filtrate was added to ice-cold ether (20 mL) and the precipitate centrifugated at 4 ºC for 15 min. The solid residue was dissolved in 1 mL of ACN:H_2O (1:1), filtered and purified by semipreparative reversed-phase HPLC.

t_R = 10.3 min (column *Phenomenex Aeris* peptide XB-C18, lineal gradient 5→95 % ACN, 0.1 % TFA / H_2O, 0.1 % TFA in 23 min).

ESI-MS (*m/z*): $[M]^{2+}$ calc. for $C_{47}H_{70}N_{10}O_8^{2+}$ = 451.3, found = 451.3 $[M]^{2+}$; 1015.5 $[M+TFA]^+$.

Coupling to the Lys side chain of the *N*-terminus acetylated peptide

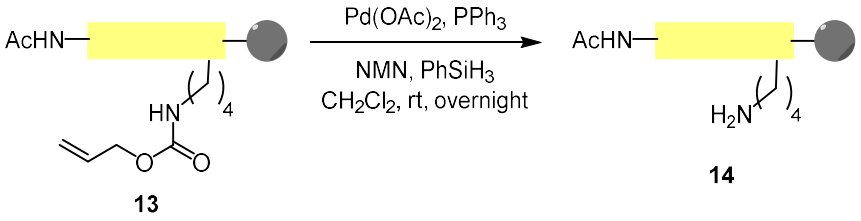
- Acetylation:



8 **13**

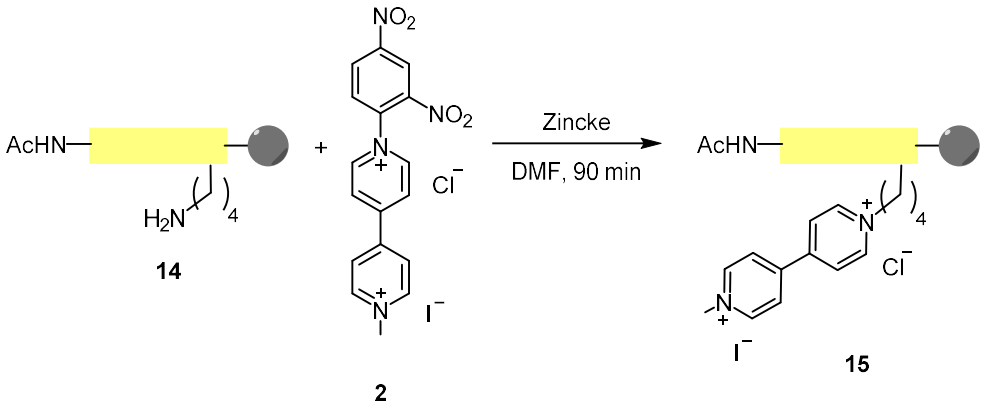
Ac_2O (0.146 mmol, 14 μL) and DIEA (0.175 mmol, 900 μL, 0.195 M in DMF) were mixed and added to the resin (14.6 μmol, 47.6 mg). The mixture was stirred by N_2 bubbling for 15 min, and then was filter and washed with DMF (3 × 2.5 mL × 5 min). A TNBS test was performed, giving a negative result.

- Deprotection of the Alloc group:



The resin (14.6 µmol) was treated with a mixture of $Pd(OAc)_2$ (4.38 µmol, 1.0 mg), PPh_3 (4.4 µmol, 5.7 mg), $PhSiH_3$ (0.146 mmol, 18 µL) and NMM (0.146 mmol, 16 µL) in CH_2Cl_2 (1.5 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), 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).

- Coupling with compound **2**:



The resin (14.6 µmol) was resuspended in DMF (2.5 mL) and compound **2** (43.8 µmol, 21.9 mg) was added to the mixture. The resin instantly turned dark maroon and the mixture was mechanically stirred for 90 min. After filtration, the resin was washed DMF (2 × 0.5 mL × 5 min) and with CH_2Cl_2 (2 × 0.5 mL × 5 min) and dried under nitrogen.

3-4 mg of the resin were treated with 150 µL of the cleaving cocktail (2.5% triisopropylsilane, TIS, 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.

t_R = 11.4 min (column *Phenomenex Aeris* peptide XB-C18, lineal gradient 5→95 % ACN, 0.1 % TFA / H_2O, 0.1 % TFA in 23 min).

ESI-MS (*m/z*): $[M]^{2+}$ calc. for $C_{49}H_{72}N_{10}O_9^{2+}$ = 472.3, found = 472.3 $[M]^{2+}$; 1057.6 $[M+TFA]^+$.

Conclusions

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

- Two (pseudo)viologens derivatives were successfully prepared: *N*-(2,4-dinitrobenzil)bipyridinium chloride (compound **1**) and *N*-(2,4-dinitrobenzil)-*N'*-methylbipyridinium chloroiodide (compound **2**) as appropriate precursors for Zincke reactions.

- A model *β*-sheet-forming peptide was synthesized via standard Fmoc solid-phase peptide synthesis protocols: Fmoc-VQIVYK-NH_2. According to the bibliography, this peptide forms β-sheet secondary structures in aqueous solutions.

- A new SPPS-based protocol has been developed for the coupling, *via* Zincke reactions, of (pseudo)viologen moieties to an amine group placed in two different positions of the model peptide: the *N*-terminus and the side chain of the Lys residue.

- Couplings at the *N*-terminus of the Val residue worked properly, whereas couplings at the Lys side chain were found more problematic, due to the deprotection of the Fmoc protective group under the mild basic conditions produced by the (pseudo)viologen reagents. Conversion of the Fmoc protecting group into a more base-resistant acetyl group was found a good strategy for the coupling of the (pseudo)viologen units at the Lys side chain, resulting in the desired conjugates without side products.

- The series of (pseudo)viologen-peptide conjugates prepared will be used in the future for the reversible host:guest-controlled β-sheet aggregation of the peptides.

Conclusións

Considerando o traballo realizado neste proxecto, pódense enunciar as seguintes conclusións:

- Preparáronse con éxito dous derivados (pseudo)violóxenos: cloruro de *N*-(2,4-dinitrobenzil)bipiridinio (composto **1**) e cloroioduro de *N*-(2,4-dinitrobenzil)-*N*'-metilbipiridinio (composto **2**) como precursores apropiados para reaccións de Zincke.

- Sintetizouse un péptido modelo que forma láminas-*β*: Fmoc-VQIVYK-NH_2, usando protocolos de síntese de péptidos en fase sólida e seguindo a estratexia Fmoc. De acordo coa bibliografía, dito péptido forma estruturas secundarias de lámina-*β* en disolucións acuosas.

- Desenvolveuse un novo protocolo baseado na SPPS para o acoplamento, mediante reaccións de Zincke, de fragmentos (pseudo)violóxeno cos grupos amina de dúas posicións diferentes do péptido modelo: a *N*-terminal e a cadea lateral do residuo de Lys.

- Os acoplamentos no *N*-terminal do residuo de Val funcionaron adecuadamente, mentres que os acoplamentos na cadea lateral da Lys atopáronse máis problemáticos, debido á desprotección do grupo protector Fmoc baixo as condicións lixeiramente básicas producidas polos reactivos (pseudo)violóxenos. A sustitución do grupo protector Fmoc por un grupo acetilo, que é máis resistente a medios básicos, resulta ser unha boa estratexia para o acoplamento de unidades de (pseudo)violóxeno á cadea lateral da Lys, dando como resultado os conxugados desexados sen produtos secundarios.

Os conxugados (pseudo)violóxeno-péptido preparados serán empregados nun futuro na agregación reversible de péptidos en láminas-*β* controlada por fenómenos receptor:hóspede.

Conclusiones

Considerando el trabajo realizado en este proyecto, se pueden enunciar las siguientes conclusiones:

- Se prepararon con éxito dos derivados (pseudo)viológenos: cloruro de N-(2,4-dinitrobenzil)bipiridinio (compuesto 1) y cloroyoduro de N-(2,4-dinitrobenzil)-N'-metilbipiridinio (compuesto 2) como precursores apropiados para reacciones de Zincke.

- Se sintetizó un péptido modelo que forma láminas-β: Fmoc-VQIVYK-NH2, usando protocolos de síntesis de péptidos en fase sólida y siguiendo la estrategia Fmoc. De acuerdo con la bibliografía, dicho péptido forma estructuras secundarias de lámina-β en disoluciones acuosas.

- Se desarrolló un nuevo protocolo basado en la SPPS para el acoplamiento, mediante reacciones de Zincke, de fragmentos (pseudo)viológeno con los grupos amina de diferentes posiciones del péptido modelo: la N-terminal y la cadena lateral del residuo de Lys.

- Los acoplamientos en el N-terminal del residuo de Val funcionaron adecuadamente, mientras que los acoplamientos en la cadena lateral de la Lys se encontraron más problemáticos, debido a la desprotección del grupo protector Fmoc bajo las condiciones ligeramente básicas producidas por los reactivos (pseudo)viológenos. La sustitución del grupo protector Fmoc por un grupo acetilo, que es más resistente a medios básicos, resulta ser una buena estrategia para el acoplamiento de unidades de (pseudo)viológeno a la cadena lateral de la Lys, dando como resultado los conjugados deseados sin productos secundarios.

- Los conjugados (pseudo)viológeno-péptido preparados serán empleados en un futuro en la agregación reversible de péptidos en láminas-β controlada por fenómenos receptor:huésped.

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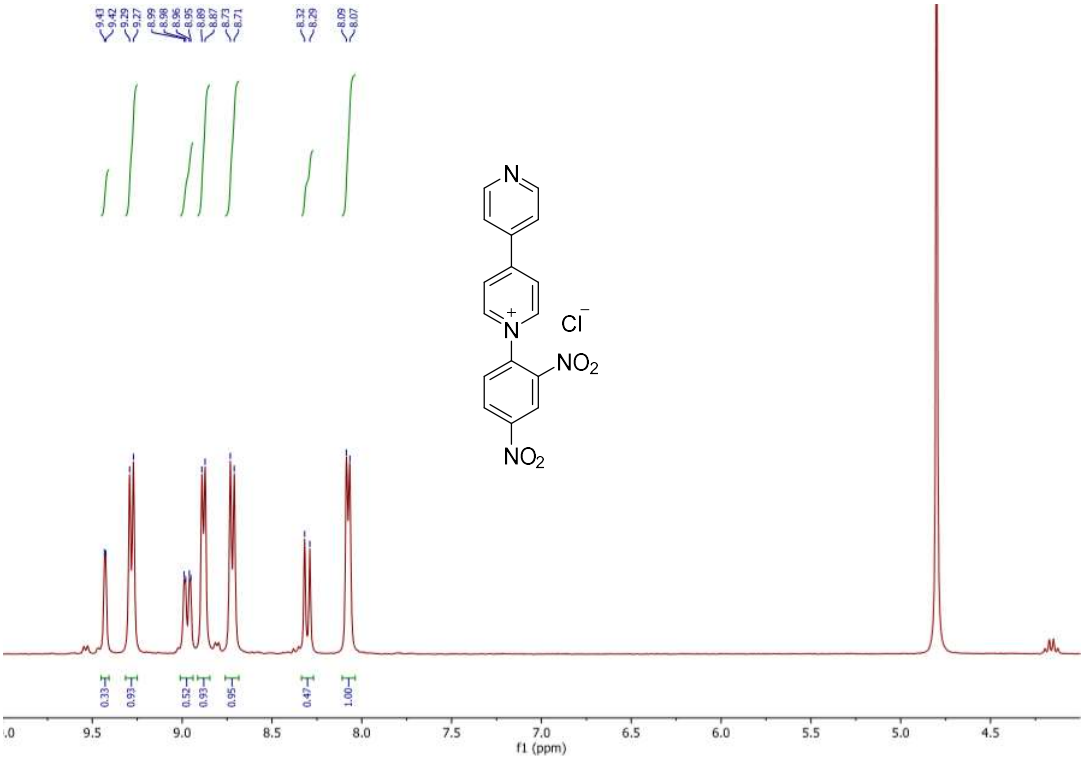
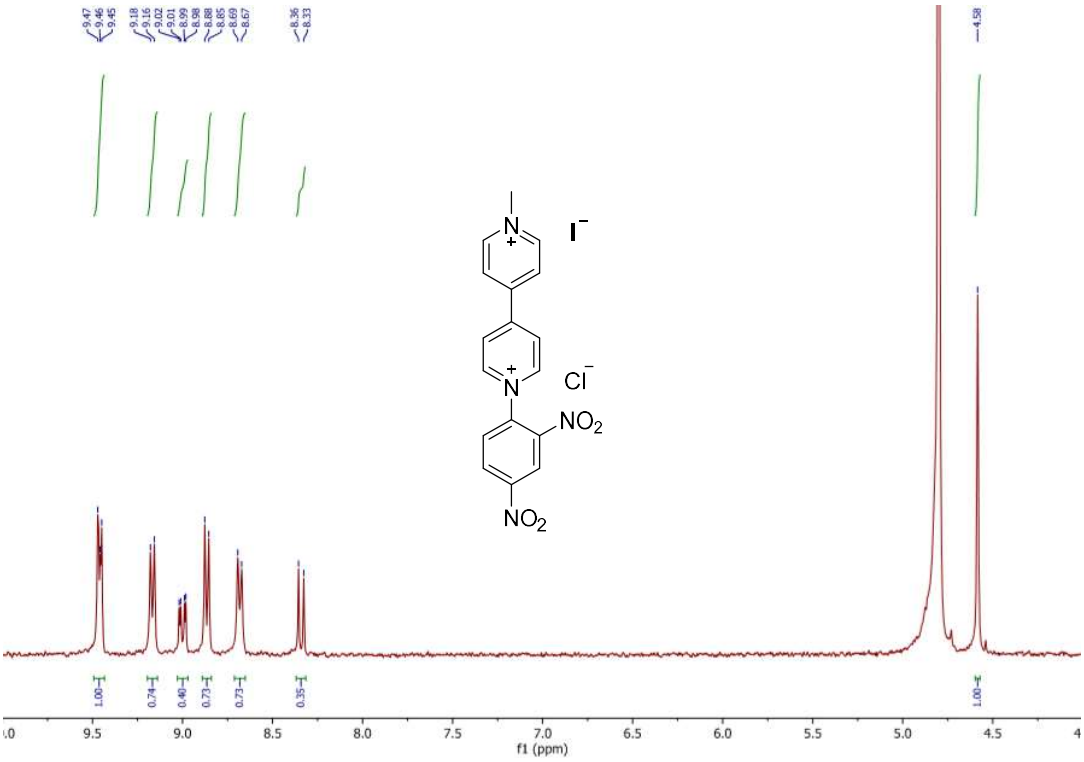
Annex

Compound 1



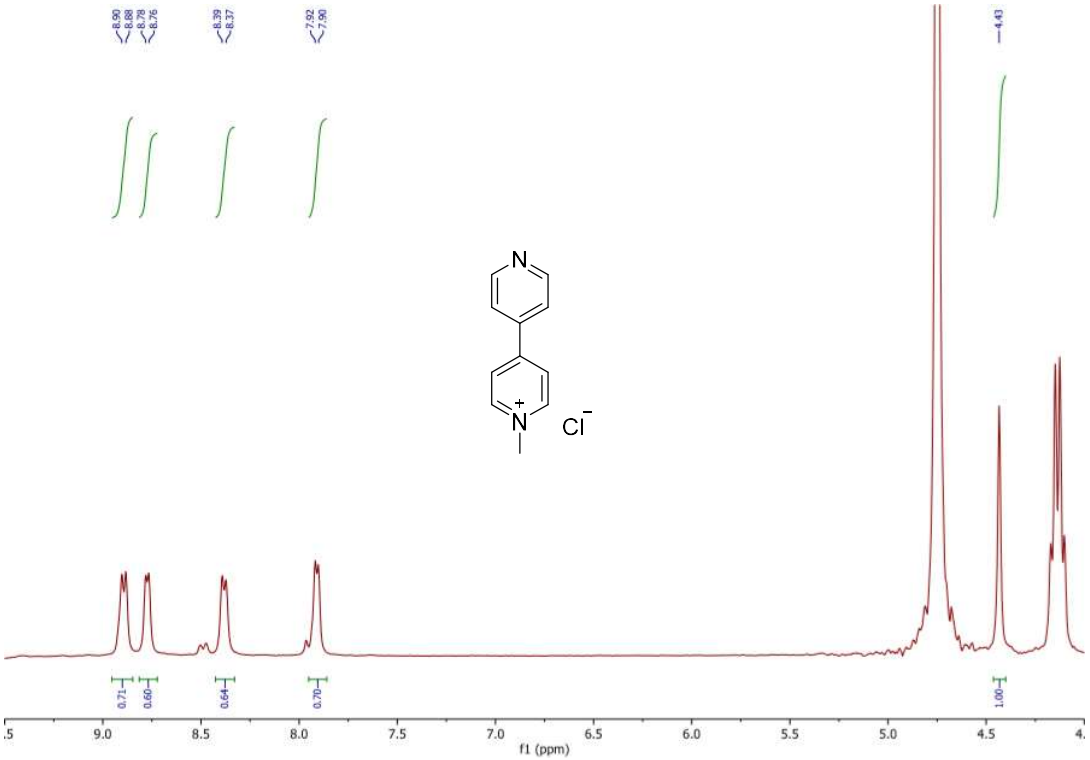
*^{1}H-NMR spectrum (300 MHz, D_2O) for compound **1**.*

Compound 2



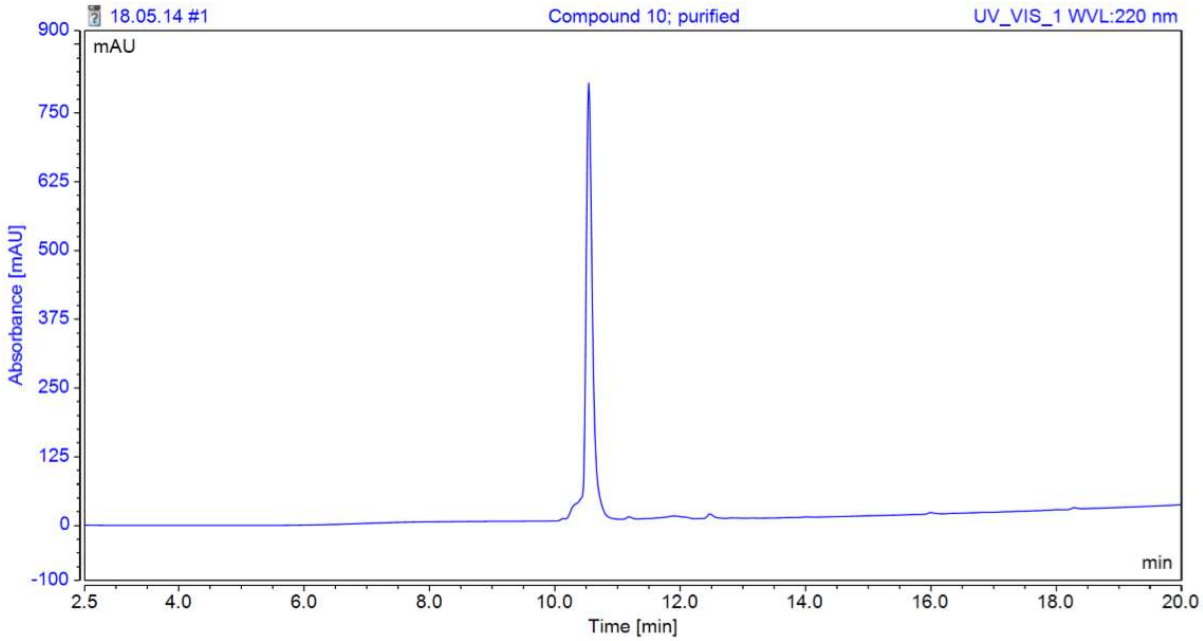
*^{1}H-NMR spectrum (300 MHz, D_2O) for compound **2**.*

Compound 3

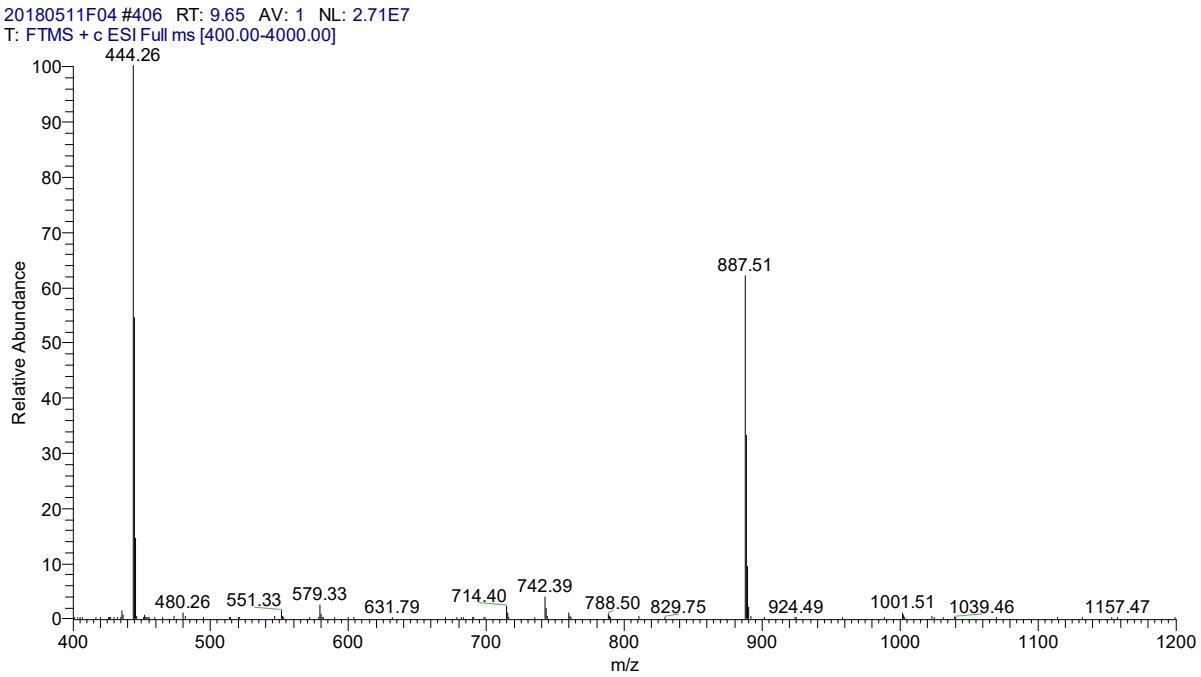


*[1]H-NMR spectrum (300 MHz, D_2O) for compound **3**.*

Compound 10

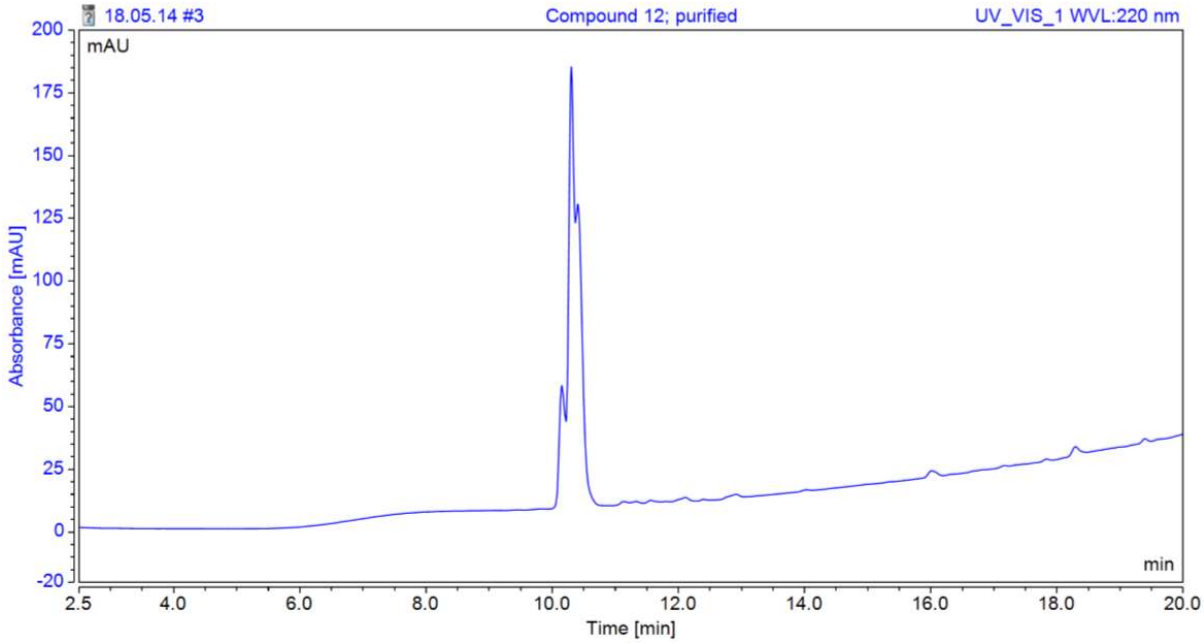


*HPLC chromatogram of compound **10**.*

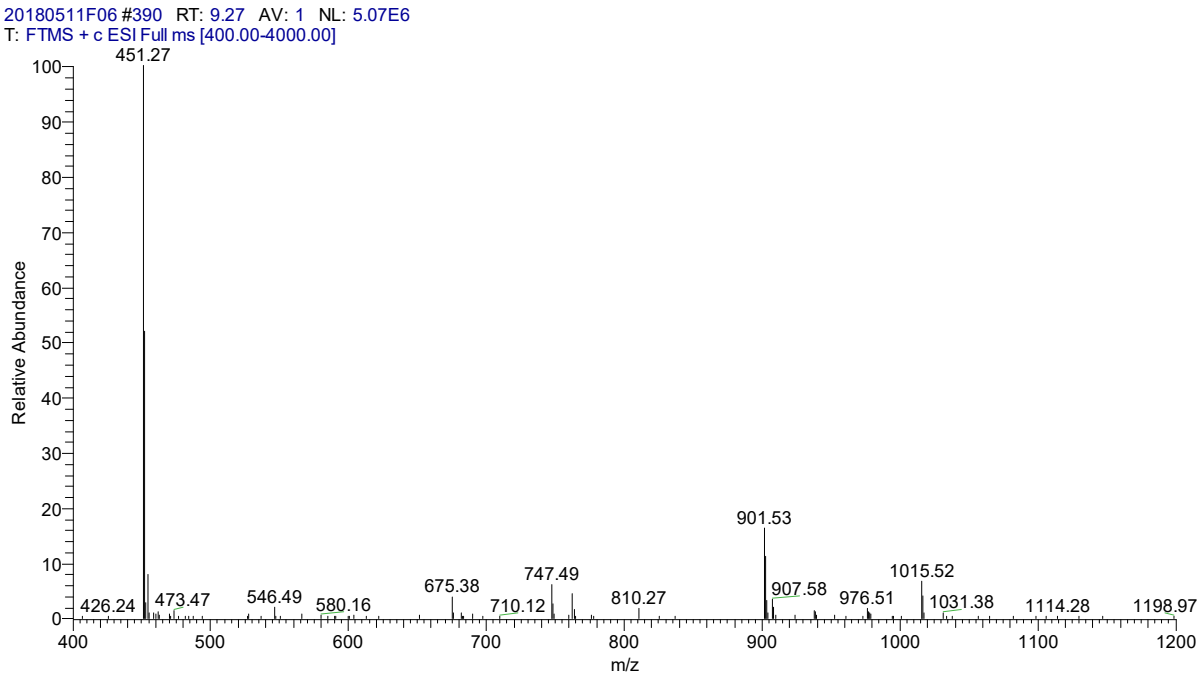


*ESI-MS spectrum of the HPLC chromatogram peak at 11.6 min corresponding to compound **10**.*

Compound 12



*HPLC chromatogram of compound **12**.*



*ESI-MS spectrum of the HPLC chromatogram peak at 10.3 min corresponding to compound **12**.*