HPLC analysis of the organometallic modification of synthetic peptides: An evaluation of the indole and iClick conjugations

Research Project

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To conclude, I would also like to thank all my relatives and friends that have been supportive but due to space reasons, not all of them could be named.

1. Introduction

1.1. Abbreviations

DMF: *N,N*-dimethylformamide
TFA: trifluoroacetic acid
TIS: triisoprophylsilane
Fmoc: 9-fluorenylmethoxycarbonyl
t-Bu: tert-butyl ether
HBTU: 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt: hydroxybenzotriazol
DIPEA: Diisopropylethylamine
PyBOP: benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
TOTU: O- [(Ethoxycarbonyl)cyanomethylenamino]-*N,N,N´,N´*-tetramethyluronium tetrafluoroborate
NMM: N-Methylmorpholine
COMU: (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate
EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
DMAP: 4-dimethylaminopyridine
DCM: dichloromethane
DIC: Diisopropylcarbodiimide
MeOH: methanol
DMSO: dimethyl sulfoxide
HPLC: high performance liquid chromatography

1.2. Peptides

Peptide research has experienced a high increase in the past decades due to its importance not only in biochemistry but also in other scientific fields like chemistry, biology, biotechnology, pharmacology, among others.

A peptide can be defined as an amino acid chain being linked through a covalent bond known as peptide bond or amide bond, mostly of naturally occurring amino acids (Figure 1). The formation of this bond would be from the carboxylic group of an amino acid to the amino group of the other amino acid, producing the release of one water molecule. Because of this release it would be a condensation reaction. For this reaction to take place an activation of both the amino and the carboxylic group would be needed, at the same time as a protection of the other amino and carboxylic groups which are not wanted to make a bond, leading to an unwanted peptide.

The chemical synthesis of peptides has changed many biological processes. They are now synthesized by a method called solid phase peptide synthesis (SPPS) which consists of repeated cycles of N-terminal protecting group removal on the last amino acid and a following coupling of the new amino acid which needs to have a previous activation to obtain a quick and smooth peptide coupling. All side chains need to be chemically protected to avoid side reactions and undesired products. [10]

The protecting group for both amino and carboxylic group should be chemically stable in the reaction´s conditions and have an easy removal at the end or intermediate stage of the reaction. Carboxybenzyl (Z), tert-butoxycarbonyl (Boc), Fluorenil-9-metoxycarbonyl (Fmoc) among more can be used as protecting groups for the amino group. The first one can de separated by a high acid concentration like HBr, the second one would be deprotected with TFA (trifluoroacetic acid) at room temperature and the last one with piperidine in DMF (*N,N*-dimethylformamide) at room temperature.

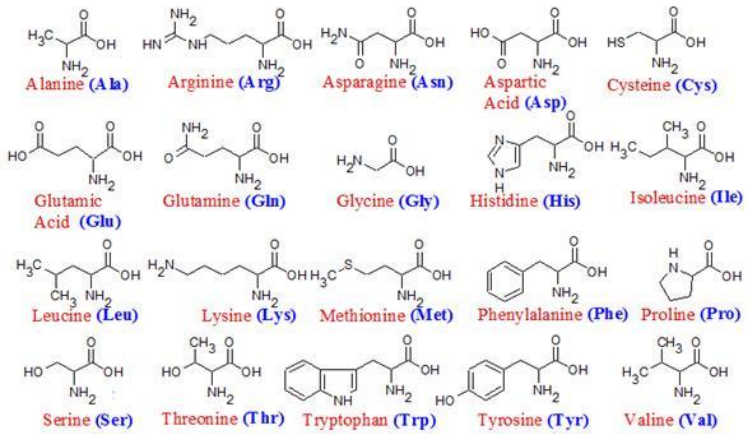


Figure 1: Naturally occurring amino acids

Being the carboxylic group less nucleophilic than amino group, the protection is less needed although it is recommended for the increase of the solubility of the peptide in organic solvents and to avoid side reactions. Carboxylic protecting groups can be: benzyl ester, tert-butyl ester (t-Bu) and phenyl acetate. Benzyl ester can de deprotected by HBr/AcOH or catalytic hydrogenenation but not with TFA (trifluoroacetic acid), therefore they would work well with deprotected amino groups in the same amino acid. Tert-butyl ester is more stable than the previous and can be deprotected by phosphoric acid or NaH in DMF (*N,N*-dimethylformamide). The latter protective group is stable in acid and catalytic hydrogenation, being deprotected with hydrogen peroxide in DMF.

Side chains can also be deprotected like for lysine whose amino group is more nucleophilic than the α-amino group of the principal chain thus the synthesis of the peptide could be produced through this amino acid leading to a ramified peptide. Some of the named above could be used for its protection like Carboxybenzyl and tert-butoxycarbonyl. Aspartic acid, Threonine and Serine should also be protected for the same reason as Lysine. They can be protected with benzyl or tert-butyl ester. Cysteine will be protected to prevent or in contrary to form sulfur bridges, with benzyl ester or tert-butyl ester.

SPPS is a sequential synthesis of linear peptides that is developed from amino acids with a solid support, a resin. It is normally an organic polymer with high mechanical stability which is needed as it is agitated during the synthesis. One common resin is Wang resin. It should be swollen in the solvent of choice at the beginning of the process to make sure that all reactive groups are exposed to the reagents which need to be able to diffuse freely inside the resin. Polar, non-protic solvents like DMF (*N,N*-dimethylformamide) in which all reagents should be soluble are normally used. At the end of the SPPS the peptide is cleaved from the resin through hard conditions (usually with the addition of TFA (trifluoroacetic acid), water and TIS (triisoprophylsilane)). Unless a protected peptide is required for later reactions, all protecting groups are also removed during the detachment of the resin. [10]

For a fast and complete peptide coupling the amino acids should be activated, before they are added to the resin, with a wide possibility of coupling reagents such as dicyclohexylcarboiimide (DCC) which is the most commonly used but has problems removing side products, therefore other reagents like phosphonium (BOP, pyBOP) or uronium salts (HBTU, TBTU, HATU). [10]

1.3. Function of peptides

Molecular chaperones are a type of polypeptide that binds in order to stabilize unfolded protein intermediates, preventing aggregation and enhancing the transport of newly formed proteins. They prevent the formation of abnormal protein structures by hindering nonspecific liaisons with inappropriate polypeptides throughout the binding of peptide sequences in partially unfolded proteins. Chaperones can also be used for cell repair by solubilizing aggregated or damaged proteins via a non-steric mechanism. [5]

Peptides have a big amount of different biological functions like signalling or acting as mediator molecules. [5] The presence of cell-wall material that involves the cells to separate them from the neighbour cell produces the appearance of mobile ligands to transport information through the cells, tissues, organs and with their environment. Some classical plant hormones like cytokinins, auxin and ethylene are used to regulate growth and developmental processes as extracellular signalling molecules, therefore peptides are well known as regulators of plant´s signalling. [13]

Peptides also act as mediator molecules, for example growth hormone-releasing hormone (GHRH), vasoactive intestinal peptide (VIP) and adrenocorticotrophic hormone (ACTH), among others. In some ways, neuropeptide-mediated transmission closely resembles transmission of non-peptide mediators, although the mechanism for peptide vesicular storage and calcium-activated release and the receptor mechanisms where these effects are shown, are essentially the same. The difference is that vesicles are full of peptide precursors in the cell whereas the active peptides are being formed within the vesicles as they are transported to the nerve terminals.

Many natural small peptides have their effects in small concentration, such as hormones in vertebrates like oxytocin which has an important role in reproduction, stimulating uterus contractions and the nipples for breastfeeding. Some poisons from very toxic mushrooms like α-amanitin which is a selective inhibitor of RNA polymerase II and III are also peptides. As well as some antibiotics which can be divided into two classes, non-ribosomally synthesized peptides, such as the gramicidins and glycopeptides, and ribosomally synthesized (natural) peptides. The first ones are produced by bacteria whereas the last one is produced by all species including bacteria as a high host defence molecule. Another example of non-ribosomally synthesized peptide is colimycin which is used for lung infections. Many lipopeptides are chemically modified to decrease toxicity. Lastly, an important example of ribosomally synthesized peptides are defensins that are found in mammals and in the fat body in insects and have acts as protection against bacteria, fungi and viruses. The pancreatic hormone is bigger than the peptides already named. Glucagon is a pancreatic hormone that inhibits the production of insulin and the corticotropic inhibits the adrenal glands. [3,12]

1.4. Proteins

The structure of proteins can be divided into four levels. The primary structure describes the sequence of amino acids connected together through peptide bonds. The secondary structure describes the possible three-dimensional stable arrangements of the amino acids, α-helix or β-pleated sheet. The α-helix refers to a compacted polypeptide into a twist around the longitudinal axe of the molecule whereas the β-pleated sheet describes the zig-zag conformation of the polypeptide chain, which could be antiparallel or parallel located; hydrogen bonds would be formed between the parallel or antiparallel chains. Helices and sheet structures are unidirectional; therefore, loops are required to reverse the direction of a polymeric chain. A polypeptide cannot fold into compacted globular structure without tight turns.

The tertiary structure refers to the folding and the intramolecular interactions of the three dimensional structure of polypeptide. Finally the quaternary structure describes the spatial arrangement of two or more polypeptides through noncovalent bonds, leading to oligomers and further to proteins, although some polypeptides are already a protein without the need of a quaternary structure like the lysozyme.

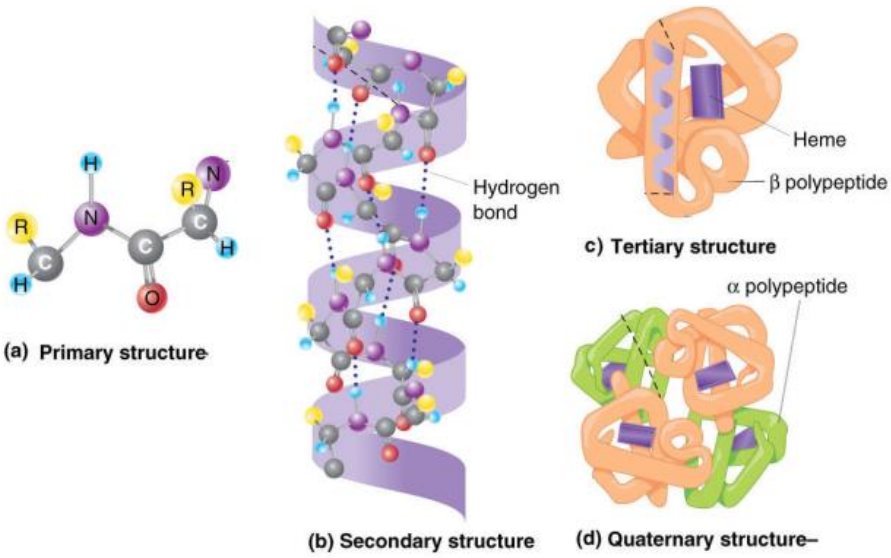


Figure 2: Levels of compaction of proteins

1.5. Functions of proteins

The functions of proteins can be summarised in structural and functional molecules like enzymes, signalling molecules, transport and storage proteins, and antibodies.

Proteins can be allosteric, meaning that they can reversible alternate between two stable conformations. [5] Some of these allosteric proteins are a part of enzymes containing active sites to react with substrates which attach to it. Many enzymes consist of a protein, which has a globular shape, and a non-protein (cofactor). Enzymes provide an alternative reaction pathway, speeding it but are not producing reactions which are not reacting without the catalysis. Enzymes are usually highly selective only catalysing specific reactions.

Proteins can undergo non-proteolytic alterations affecting their chemical reactivity and cellular location. These modifications known as post-translational modifications of amino acid side chains take place in the lumen of the endoplasmic reticulum (ER) or in the Golgi complex. The latter consists of fibrous coiled proteins and stacking proteins which create a structural scaffold around its functional membrane that contains enzymes. Some of the post-translational modifications are: phosphorylation, the addition of a phosphate group to hydroxylated amino acids, glycosylation, the addition of sugar to a protein sequence and ADP-ribosylation, the addition of ADP-ribosyl groups to a protein, among others. The most common modification is the first one, as the phosphate is an important molecule for the nucleic acid structure and for the cellular metabolism. Serine, threonine and tyrosine have free hydroxyl groups capable of accepting high-energy phosphates. Processes of repetitive phosphorylation and dephosphorylation are responsible for the transfer of a phosphate residue from ATP or GTP to the substrate of interest. [5]

Most post-translational modifications have a higher life than phosphorylation in the polypeptide´s life. These modifications might not always be important for molecular signalling, but for protein localization or stability. Proteins being modified by carbohydrates (glyco-) are glycoproteins, and this modification is known as glycosylation. This method applied to polymeric extracellular proteins, which are being modified by sugars, produces proteoglycans.

Oligosaccharides are attached to proteins through glycosyltransferases, which transfer the sugar group to nitrogen (amino) on the asparagine side-chains, producing an N-glycosylation, or to oxygen on serine or threonine, known as O-glycosylation.

Glycosylation is important for protein trafficking, cell adhesion and receptor-ligand interactions, and abnormalities might be related to the failure of cell surface glycoprotein expression because of the retention within the ER. Differentiation produced by agents like the retinoic acid may modify terminal glycosylation sequences by changing the expression of glycosyltransferase. Protein precursors could be activated by deglycosylation.

Translation is not the only important act of protein; also an efficient protein degradation system is needed to remove damaged or misfolded proteins from the cell, to enable the fast flux of intracellular signalling molecules and to recycle constituent amino acids for new protein synthesis.

1.6. Biological membranes

Membranes are protein-lipid sandwiches being a biological interface between cellular compartments. They are formed of lipid layers whose non-polar groups are facing the inside, away from the water and the polar group is in the outside part of the membrane. All organelles like the nucleus are surrounded by additional membranes. The plasma membrane which separates the cytoplasm from the extracellular space has an important role in cell membrane due to the transmembrane proteins that act as signalling conduits between the cell interior and the external word. Such transmembrane proteins have hydrophobic amino acid sequences which links biochemical waits, enabling them to fit into the membrane as if into a corset. [5]

Proteins of biological membranes can be extrinsic or intrinsic. The first one which are also known as peripheral proteins are loosely attached to one side of the membrane, whereas the second type of proteins are located inside the membrane and act as part of the transport system as transmembrane proteins. Cystic fibrosis (CF) is a human disorder produced by a modification of the transmembrane protein which affects the mostly the lungs but also the pancreas, liver, kidneys and intestines. It occurs due to a loss of the amino acid phenylalanine in a cystic fibrosis transmembrane protein (CFTR). This loss modifies the primary structure of a protein that normally helps the transport of salt and water through the cell (Figure 3).

The modification of the primary structure enables the protein from working properly, causing the body to produce unusually thick mucus that blocks the lungs and forms the accumulation of sticky mucus, which would obstruct the pancreas and stops natural enzymes from helping the body break down of food and absorption of vital nutrients. In the lungs of people with cystic fibrosis, this mucus produces an environment perfect for bacteria, whose colonization forms biofilms in the small lung´s airways. The most common pathogens that occurs the lungs of cystic fibrosis patients are *Pseudomonas aeruginosa* and *Burkholderia.*

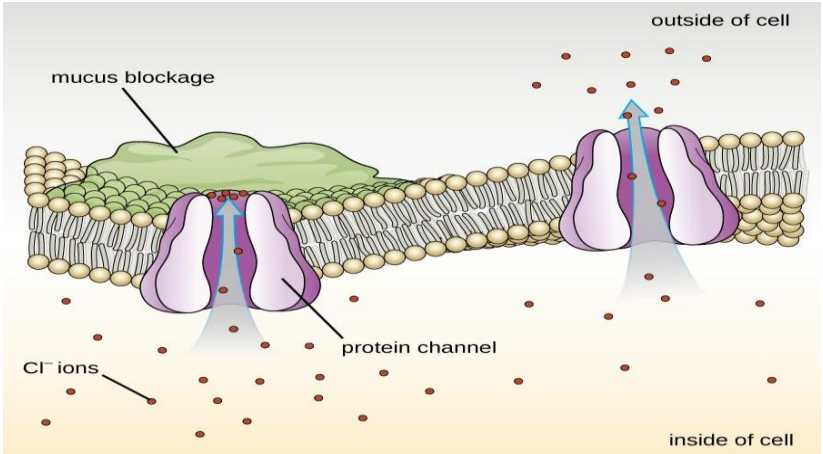


Figure 3: Transport of molecules through the membrane

The majority of ion channels are very selective about the ions that pass through; the ones that are conductive for potassium ions are non-conductive for sodium ions or vice versa, although some ion channels allow the transport of both ions. The differences between selective and non-selective ion channels are structural and dynamic. The concentration of potassium is higher inside the cell than in the extracellular fluid, whereas the concentration of sodium ions is the opposite, higher outside the cell than inside. This difference of concentration in both sides of the membrane produces a gradient of concentration, which is maintained by transporter proteins. The selectivity of Na^+ and K^+ channels with the concentration gradient produces a difference in voltage across the plasma membrane. [9]

Channels are assembled in supramolecular clusters where the protein´s functions are coupled to achieve an optimal response to a single stimulus. Cluster and coupled gating can be observed in a high number in ion channels like for sodium, potassium and calcium. The potassium channel is regulated by a cytoplasmic activation gate and an extracellular inactivation gate. The potassium channels form clusters in native-like lipid membranes, which provides structural details on the channel-channel interference. The open and closed gates of the potassium channels depend on the pH, in neutral pH the channels are closed forming nanoclusters, whereas with acid conditions the gate is opened. [7-8]

1.7. Antimicrobial peptides

The increase of antibiotic resistance has produced a high interest in antimicrobial peptides (AMPs) which target membranes by a disruption of the lipid bilayer leading to the growth inhibition and death of the bacteria. The most important thing about this mechanism is the structural properties to recognize, self-assemble and interact with a particular lipid membrane, which can be studied with solid-state NMR. AMPs are a type of host defence peptides only active in membrane environments. [15]

The *maculatin*, an AMP from the skin of Australian tree frogs that acts against *Gram-positive bacteria*, adopts a transbilayer orientation and an α-helix in model membranes (Figure 4) which have bigger interactions with anionic phospholipids. In lipid environments it forms an amphipathic α-helix. Although the structure, location and activity are related to the membrane´s composition, it is normally not very complex as in bacteria and eukaryotic cells. [15]

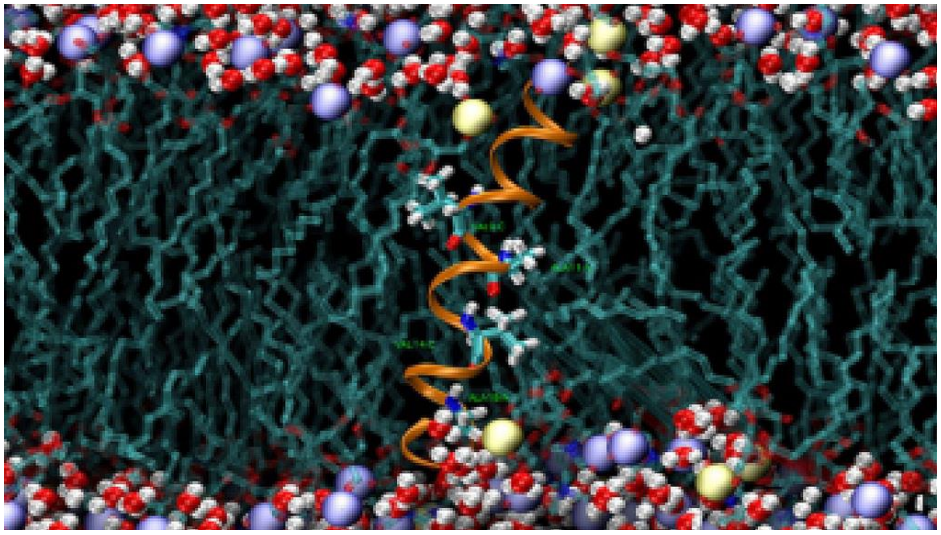


Figure 4: A single *maculatin*, more specific a maculatin 1.1, in a mixed phospholipid bilayer [15]

Another important AMP is *gramicidin Dubos* that was the first clinically tested antibiotic used for penicillin and acts as an ionophore transferring monovalent cations, being used as membrane ion channel. The peptide is composed of 15 residues with alternating sequence L- and D- amino acids. The ion channel is formed as two helical *gramicidin A* (gA) acting as a "pore" in a lipid bilayer membrane. Like the majority of AMP, the structure of gA in detergent micelles was analysed by NMR. [15]

The composition of cell membranes is much more complex than a phospholipid bilayer, which contains glycolipids, integral and peripheral membrane proteins, cholesterol, sphingomyelin and gangliosides. As it is known, NMR signals are related to the orientation of the chemical shielding tensor relative to the magnetic field; therefore, the angle of the bond of a specifically labelled atom can be defined to an oriented peptide. The channel form by the gA is head to head forming al helical dimer like in micelles. [15]

As said before, the resistance to antibiotics has produced the appearance of new AMPs like *maculatin* which shows more selectivity being more reactive against *Gram-positive bacteria*, resulting in a dye leakage from phospholipid vesicles. Different models for membrane lysis like carpet mechanism, barrel-stave and toroidal pore were studied. Mostly, longer peptides form transmembrane or toroidal pores whereas smaller peptides form a carpet or surface mechanism (Figure 5), depending on the type of lipid and on the peptide concentration.

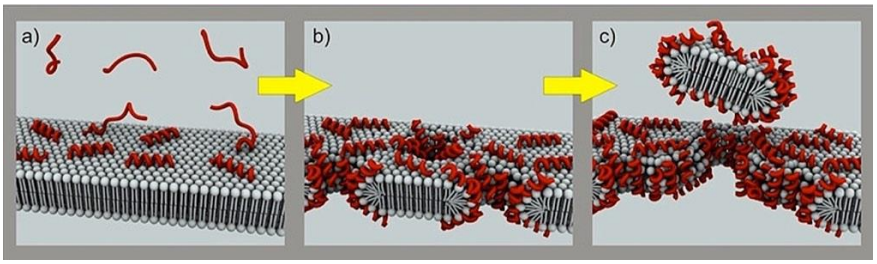


Figure 5: "Carpet" or surface mechanism [15]

In Figure 5 in the first picture (a) the peptides without a specific structure attach to the surface as α-helix. When the concentration is very high the peptides structure as toroidal pores (b) and finally the membrane disintegrates (c).

These last AMPs are introduced into bacterial membranes which are anionic phospholipid monolayers like α-helical peptides but being immiscible in zwitterionic monolayers. Normally, these AMPs are situated in aqueous phase of neutral membranes but they have higher interaction with negatively charged lipids. In fact, their activity is related to the lipid content with longer peptides forming pores whereas smaller peptides produce the disintegration of membranes. [15]

A very useful type of maculatin is maculatin 1.1 (Mac1) whose sequence has 21 amino acids. It produces more lysis against neutral in comparison to negatively charged phospholipid vesicles, although in a competitive lipid environment with both different charged vesicles, Mac1 does not interact strongly with neutral bilayers in the presence of the last one. In conclusion, even though Mac1 inserts into neutral bilayers, it has a higher affinity for anionic membranes which would result in a more targeted interaction with bacterial membranes. With NMR it can be seen that Mac1 is unstructured in buffer and α-helical in zwitterionic and anionic micelles and also bicelles.

1.8. Click chemistry

The first time a click reaction was studied was in 1961 by Dr. R. Huisgen when he performed a 1,3-dipolar addition, although he didn´t name the reactions in his investigation as click reactions. He studied the addition of phenyl azide to double bonds and its attack from the negative end of the dipole, the positive end or both charge centres at the same time. The azide is neither strong electrophilic nor strong nucleophilic. The first two mechanisms form zwitterions, for which polar solvents would be good, and the last one has a loss of dipolar character therefore its formation would be decreased with polar solvents. A 1,3-system is the union of a 1,3-dipole with a multiple bond system, called dipolarophile, and obtaining a five membered ring. One side of the dipole would be positive and the other side negative, whose loss would form the ring. When the positive centre of the dipole is an electron-deficient carbon, nitrogen or oxygen, it is not stable. Many different products could be obtained, for example, when using nitrogen anion as the carbanion nitrilimines were formed. Alkenes and alkynes can also act as acceptors. One type of these additions would be the Diels-Alder reaction. Other additions he researched would be the addition of nitrile oxides, benzonitrile and azomethine imine, among others. [19]

Two years later Dr. R. Huisgen gave importance to the stereoselectivity of cis-trans isomeric dipolarophiles, to the solvent, substitutients, the activation parameters and the orientation for the 1,3-cycloadditions. The solvent´s influence was studied with two cycloadditions of diphenyldiazomethane, one with cyclopentadiene-azodicarboxylic ester and the other with dimethyl fumarate. Different solvents like benzene, dioxane, acetone, etc were used, and it was seen that the polarity of the solvent didn´t influenced significally. Contrary to this, when the charge compensation of the 1,3-dipole is not as complete as in phenyl azide, like in azomethines imines and in nitrone, there is a solvent dependence where the higher the polarity the lower the product. After the addition, if the two new σ-bonds are closed the mechanism it would be cis-stereoselective, but if the process is done in two steps the intermediate would be a single bond that could rotate. Therefore if the initial dipolarophile is a cis-trans isomeric alkene, the mechanism would not be stereoselective, being obtained both diastereoisomers. A stereoselective mechanism could be the cycloaddition of benzonitrile-N-oxide to fumaric and maleic esters, producing diastereoisomeric isoxazolines. An example of this mechanism would be the stereoselective addition of diazomethane to dimethyl dimethylfumarate (trans-isomer) and dimethyl dimethylmaleate (cis-isomer) (Figure 6) [22]

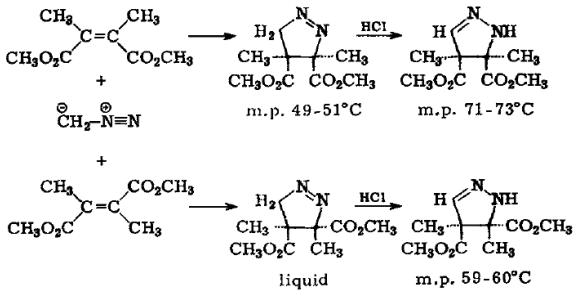


Figure 6: A cycloaddition reaction of diazomethane with dimethyl dimethylfumarate (bottom reaction) and dimethyl dimethylmaleate (top reaction) [22]

Another parameter to take into consideration would be the activation. At the peak of the activation barrier the transition state should have a high order degree and for that to happen the reactants must be aligned to each other to be able to overpass the barrier which is controlled by the entropy and enthalpy. With high negative activation entropy values the product is obtained in very small amounts. Some cycloadditions of diphenyldiazomethane to alkenes have high negative activation entropies. In relation to the electronic substituent effect, when the two bonds which should be form simultaneously but do not, because one is faster than the other, the centre of the dipolarophile is the carrier of a partial negative or positive charge, resulting on a reduction of the overall energy level (including the barrier) when neighbour substituents, that overtake the charge, are present. [22]

Not both cis- and trans- isomers are obtained equally, trans-isomers are highly formed. A comparison of fumarate and malate with different reactants sp^2 and sp^3 was done. Although the length from sp^2 to sp^3 increases, the van der Waals radius of the eclipsed cis-substituents is compressed. This produces an increase in the van der Waals repulsion, obtained during the activation process, which results on higher activation energy for the cycloaddition of the cis-isomer whereas it does not occur in the trans-isomer. [22]

The last parameter would be the orientation which would be influenced by the nature of the new σ-bond. In dipolarophiles with more than one bond including a heteroatom normally the addition is produced in one of the two possible directions. The formation of the structurally isomeric heterocycle would produce a gain of σ-bond energy, which would be smaller by 52 kcal. When the dipolarophiles are alkenes or alkynes both directions of the addition involve the same amount of σ-bond energy. In molecular orbitals, in nitrile ylides (Figure 7) in the right molecule (28), the accommodation of the ion electron pair at the “a” carbon in a sp^2 is favoured over the p orbital. This would produce the bending of the bond in the carbon “a”, in comparison to the linear sp-hybridized σ-system of the molecule on the left (27). [22]

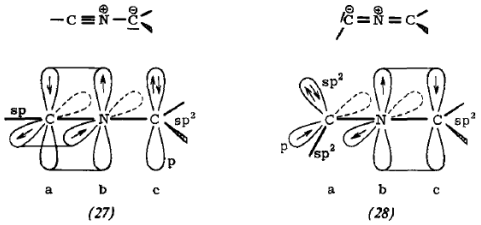


Figure 7: Molecular orbital of nitrile ylides [22]

Click reactions which do not need difficult separations or strong conditions, have the ability to form, modify and control the structure of materials of different sizes. These reactions are being used for polymer functionalisation, surface modifications, block copolymers and dendrimer synthesis, biomaterials fabrications and biofunctionalization.

These reactions, that involve heteroatoms linkage and thermodynamic driving forces, appeared firstly in reference to the creation of stereospecific carbon-carbon bonds, for which the protection of certain groups was needed, but also sequence reactions and various purifications techniques were performed. It is important that these reactions have high thermodynamic driving force, bigger than 20 kcal mol^{-1}. Natural products can be obtained using iclick reactions through simple and mild reaction conditions, for which the simple product´s synthesis has high yields with simple purifications. Through iclick reactions, synthesis and chemical modifications are more accessible and easier to perform. [18]

A type of click reactions would be inorganic click or iClick reactions which consist on the presence of metal ions in cycloaddition, in all organic alkyne/azide cycloaddition. The first iClick reaction was performed in 2011 between PPh_3AuN_3 (1) and $PPh_3AuC{\equiv}CPh$ (2) to obtain the product $[PPh_3Au]_2(1,5\text{-}\mu\text{-}N_3C_2Ph)$ (Figure 8) where the metal ion was linked to the triazolate bridge. Mostly iClick reactions were done with gold but later on it was also performed with other elements like Rh(I), Ir(I) and Pt(II). Crystals would be obtained from the product in a benzene solution, resulting of a single crystal X-ray diffraction experiment. The 1,5-digold substituent would be form instead of the 1,4-digold isomer. [17]

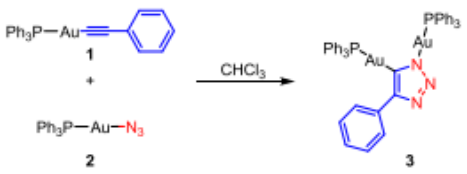


Figure 8: First iClick synthesis of the digold triazolate complex [17]

The synthesis of heterotrimetallics and tetranuclear gold complex were being done through an iClick reaction, a metal-azide-metal-acetylide cycloaddition like in Figure 8. Another iClick reaction would be between the PPh_3AuN_3 (5-Et) and PEt_3AuN_3 (1-Et) (Figure 9). There is a homo Au-Au cycloaddition to form the intermediate digold complex which would then dimerize to produce the unique tetragold complex. It could also be obtained by the *in situ* synthesis of the intermediate compound. A crystal X-ray would be used to

measure the solid state structure of the two dinuclear gold units held together by aurophilic interactions which create a pseudo C_2-symmetric distorted tetrahedral geometry of Au ions. The distortion from a pure tetrahedron would be because of the triazolate bridges which reduce the contact between both Au atoms. Triazolate bridges are widely used to build well-defined heterotrimetallic complexes from multiple ions through an iClick reaction. [21]

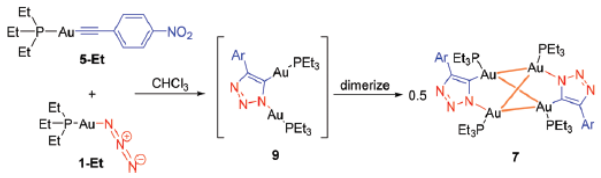


Figure 9: iClick reaction between PPh_3AuN_3 (5-Et) and PEt_3AuN_3 (1-Et) [21]

It is known that an Au-complex is the most developed in iClick reactions, although there are other complexes with Rh, Pt or Ir that are also becoming important. Metallopolymers of transition metals are provided in polymer science through covalent bonds, having the metal ion many new properties. To have a successful iClick reaction with metal ions in group 9 the involved of Au(I)-acetylide is necessary because it plays an important role of the *in situ* generated Cu(I)-acetylide in copper-catalyzed azide-alkyne cycloadditions (CuAAC). The first reaction performed was between the Au(I)-acetylide, $PPh_3Au(C{\equiv}CC_6H_4NO_2)$ and the rhodium-azide, $Rh(CO)(PPh_3)_2N_3$ in chloroform solvent at room temperature (Figure 10). The product 3 would be the kinetic product, which would later form the hetero-tetranuclear complex (product second reaction). The formation of one of the products on top of the other would be temperature dependent; at 0ºC the reaction would lead to the monomer 3, whereas heating the reaction at 55ºC would form the dimer 4, which would have a crystal structure. In the case of Ir(I) the complex form would be a monomer and not a dimer like in Rh(I), even when the reaction was heated. This would be because the Ir-PPh_3 bond is favoured over the Ir-N bond formed in the dimerization. [20]

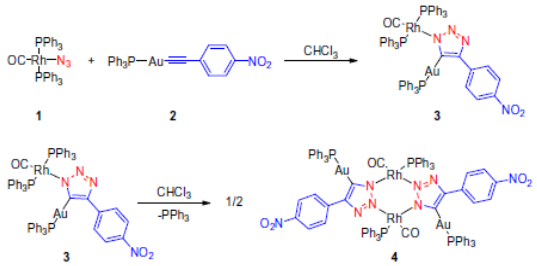


Figure 10: iClick reaction of gold(I)-acetylide and rhodium-azide [20]

Click reactions can be performed for a wide range of reactions and coupling types that could go from polymer conjugation to surface modification to polymerization to polymer functionalization, among others. Some important examples can be: azide-alkyne cycloaddition, Diels-Alder addition, oxime/hydrazine formation and thiolvinyl addition. Many

click reactions have also been performed as a useful tool to modify polymers or materials with a variety of functional components, producing the appearance of unique properties and functions to the previous materials.

The implementation, either simultaneously or sequentially of a combination of two or more click reactions is becoming more important. Click reactions are used as orthogonal to other reactions, which orthogonal´s extent depends mostly on the nature of the substrate; the reaction conditions including reactants, catalysts, and other species; and on the possibility of having other reactions. If two reactions are totally orthogonal, they are produced simultaneously without any interference under a broad range of conditions.

The rise of chemical biology in the past years produces an increase of the development of click reactions in the conjugation and functionalization of biorelated polymers such as proteins, polysaccharides and oligonucleotides, whose reactions are highly chemical selective, have fast reactivity under physiological conditions at low concentrations and are not toxic. Bioconjugation and biofunctionalization click approaches normally use the naturally occurring, highly reactive functional groups in biopolymers.

1.9. Metal bioconjugates

A metal bioconjugate is a biomolecule, for example a peptide coupled to a metal complex. The organometallic complex could be detected by atomic absorption spectroscopy and this could result on valuable insight into cellular processes modulated by bioactive metal compounds or metal bioconjugates. It could also be detected with infrared (IR) spectroscopy. [10]

A chemical synthesis of a peptide to an organometallic compound is performed, through mostly a covalent bond. These organometallic peptide conjugates are used as new antibiotics, to investigate the mechanism of action of anti-cancer drugs candidates or to look the uptake and intra-cellular localization of the attached metal complexes. [10]

Various ferrocenyl chalcone derivates and one ferrocenyl non-chalcone derivate were studied for their interaction with DNA and bovine serum albumin (BSA), which is a very active area of bioorganic chemistry. A chalcone is an aromatic ketone and an enone whose product acts as the main core for many important biological compounds, known as chalcones or chalconoids. It could be very useful to obtain data for developing potential therapeutic applications and to investigate the mechanism of action for drugs. The interactions and bindings of complexes with DNA and BSA were studied with UV-Vis, CD and fluorescence spectroscopy. In addition, human topoisomerase I was inhibited depending to the concentration of some ferrocenyl chalcone derivates whereas it did not happen with ferrocenyl non-chalcone derivate and one ferrocenyl chalcone derivate. Therefore, the results of the inhibition indicate that these compounds can be named as topoisomerase II suppressors but not as poisons. [14]

Whereas metal salts of organic medicinal compounds and also biologically active coordination complexes are lately used in the clinic in high amounts, it does not also happen for organometallic (OM) complexes. Although organometallic medicinal chemistry is growing very fast where some organometallic complexes have been biocompatible or turned into bioconjugates that target specific disease-related biomolecules, but this is still being studied, being proceed in vitro or in small living systems. [15]

The major goal of medicinal chemistry is to obtain molecules which can modify disease-related undesired biomolecular interactions that can cause a disease to emerge. A variety of OM fragments are used to be attached to proteins, saccharides and DNA, but mostly to peptides acting as radiolabels or chemotherapeutic agents when being attached to bioactive compounds. [15]

The modification of DNA is very important for biotechnology and cancer chemotherapy. In genomic engineering a cleavage of DNA strands is needed to remove or introduce oligonucleotide sequences. In addition, the elimination of DNA malignant cells may be beneficial in the chemotherapy of cancer. The drug Cisplatin is one of the most commonly used anti-cancer drugs. [10]

Basically an OM group can be coupled to the N- or C-terminus of a peptide, to its side-chain functionalities, or to the backbone amino groups. This last option can be done replacing the typical C-C(α)-N peptide backbone. The N-terminus can be modified into another functional group, which in return can react in a specific way with an appropriate function in the organic-ligand side of the OM fragment. In addition another method can be performed to convert the N-terminus into another functional group that can react directly with the metal of an OM or coordination complex through the formation of an OM-peptide conjugate. Therefore a small difference can be seen, in the first one the OM fragment is attached to the N-terminus through the organic ligand whereas in the second one it is attached through the metal ion. An example of the first method can be a click reaction and to the second one it can be the reaction of an N-terminally positioned acetylene with $Co_2(CO)_8$. [15]

1.10. Indole conjugation

Bioconjugation reactions have become very important for the study of proteins and for the synthesis of novel biomaterials and functionalized therapeutics. What´s more, reactions which are site-specifically including chemical tags into proteins are also becoming vital to give ways of proving and/or chemically modifying specific residues that could be proved to be key aspects of function and/or structure. [11]

Acetylation (acylations), methylation (alkylations) and succinimide- and maleimide-based conjugations are important techniques which target lysine or cysteine residues to obtain new properties or to incorporate labels like isotopes, radicals or complexes into the macromolecule of interest. In fact, lysine residues are used as appropriate labelling sites because of its abundance of 6-7% in proteins, because of being solvent-exposed most of the times and due to its structurally and functionally importance in intra-, interdomain and interprotein interactions, e.g. cation-π, hydrogen bonds and salt-bridges. Lysine side chain and N-terminal amine tagging are produced by reagents which would invert the positive charge into a neutral one, obtaining a neutral conjugate, therefore it is disturbing for its structure and functionality, and it is normally avoid when possible. As a result, other techniques that do not disturb the charge are used under mild, nondenaturing and aqueous conditions. [11]

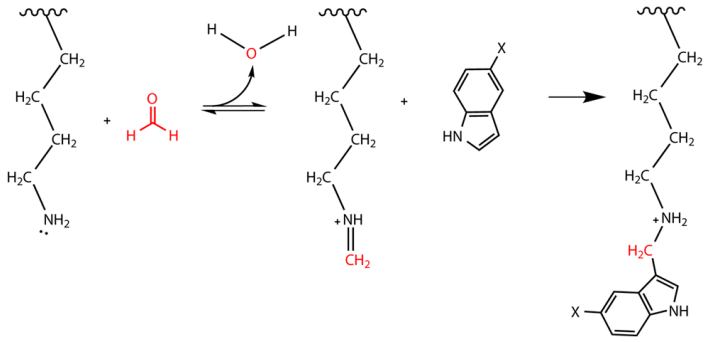


Figure 11: Indole conjugation to an amino acid with formaldehyde as reagent [11]

Indole rings are very reactive with electrophilic species, mostly the C3 position of the indole ring, which is 10^{13} times more reactive than benzene toward aromatic substitution. When formaldehyde is in the solution, protein side chain and N-terminal amino groups react with it forming electrophilic imine intermediates, which are very reactive with the C3 of the indole ring (Figure 11). This method would be performed under nondenaturing temperatures and pH. [11]

This method shows high specificity for lysine side chain and N-terminal amino groups, being proceed in buffered (pH 6-9) or unbuffered solution. What is compulsory is the absence of primary or secondary amines, reducing agents and compounds with highly acidic CH protons as buffer components through the reaction because the presence of any of these can produce undesired reactions. Indole-based conjugates have the advantage that they maintain the protein charge and are smaller than other common lysine- or cysteine-specific tags, e.g. most fluorophores and succinimide-based (NHS-) tags. In addition, aromatics can be better functionalized than saturated aliphatics, that would lead to produce a large library of indole-based probes and cross-linkers which are specific for lysines and N-terminal amino groups. [11]

1.11. Analysis

One selected method for separation and purification of peptides and proteins could be mass spectrometry, which has being a very useful tool in the last decades for the analysis of peptides and proteins. Amino acids or indoles coupled to amino acids have unique mass therefore it is an appropriate method for the characterization of a peptide. Also ionization techniques like MALDI or ESI are in growth. MALDI works best when a considering number of peptide peaks are detected. MALDI-MS and ESI-MS are able to transfer high-molecular mass polypeptide ions into the gas phase without fragmentation.

Tandem mass spectrometry (MS/MS) appeared for the analysis of molecular structure of single ions. The ions are selected by mass, fragmented through collision-induced dissociation (CID) and further analysis of the resulting fragments. [2]

Another very important mechanism can be HPLC, were the components are distributed between a liquid stationary and a liquid mobile phase. The retention of the compounds in the column would be related to the difference of polarity between the compound and the column. For example, with a non-polar column the non-polar compound would elute last because more interactions would be between the non-polar column and the non-polar compound, having longer retention time. [4]

A specific type of chromatography could be thin-layer chromatography. Being a simple technique different solvents were used for different detection systems, sometimes with further electrophoresis. Free peptides can be detected in acid solvent or under basic conditions. In case of big amount of impurities, the product must be purified before the final evaluation. [2]

Another useful type of chromatography is ion-exchange chromatography (IEC), being used for protein purification because of its high scale-up potential. Its separation is related to the interaction of the protein´s net charge with the charged groups on the surface of the packing materials. Polystyrene, cellulose and acrylamide can be support materials for the ion exchanger, quaternary amines and polyethylenimine for anion exchange and sulfonate and carboxylate groups for cation exchange. [2]

Capillary electrophoresis (CE) can not only be helpful for peptide´s and protein´s separation but also for recombinant proteins. Its method is based on the different migration in solution in an electric field due to the charge properties of the compound being separated. All charged peptides are cationic, migrating to the cathode. [2]

The last but not the least important method is the ultrafiltration, a method based on pressure separation of the molecular size using a membrane. Suspended solids and solutes of high molecular mass are retained. This method is performed for the protein solution´s concentration and for the separation of very different mass molecules. The membrane selects the compounds being permeated or retailed. Instead of applying pressure, centrifugation could be done.

2. Motivation

The reason for the peptide conjugation to metal or other ions is that peptides have many important functions for biological mechanisms.

One of the most important functions is the cell penetration where the peptide would act as a chaperone for the transport of other molecules, like for example metal ions, into the cell nucleus. This has a high impact in diseases or sickness as the needed molecule for the cure could be conjugated to the peptide and inside the cell it would detach and perform its function. Many peptides act as hormones with different biological activities, as the oxytocin. In the same way it can act as drug delivery. On the other hand, peptides could work as the attachment of other molecules that would be transported around the cell.

Small peptides are promising for the treatment and diagnosis of cancer as they can have a tumour-homing behaviour inside the cells. Many short bioactive peptides with linear structure tend to have α-helical structures which can be directly attached to DNA, helping an easy and unharmed insertion. The structural character can be used for the formation of pore membranes or the reparation of them.

The aim of this research was to synthesize different peptide sequences and modify them in order to try different conjugation methods. The first goal is to prepare the peptides TSFDL and VLAKVAA via the solid phase peptide synthesis and with a Wang resin as a support for this mechanism. One of the peptides should be conjugated to an alkyne (in this case the first peptide) to be able to perform an iClick reaction with for example a metal azide complex. The second peptide has a lysine sidechain to be used for an indole conjugation and for a later metal indole conjugation.

3. Results and discussion

3. 1. Synthesis of TFSDL

A peptide with the sequence of TFSDL was synthesized through standard-SPPS-Fmoc-synthesis starting with a Fmoc-Leu-Wang resin beginning with the deprotection of the resin with a piperidine/dimethylformamide mixture (30%v/v) for 15 min and the coupling of the amino acid for 40 minutes which was prepared dissolving the solid amino acid in HBTU/HOBt mixture ((2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate / hydroxybenzotriazol) and N,N-Diisopropylethylamine (DIPEA). This was repeated for each amino acid and the resin with the complete sequence was deprotected with piperidine/ *N,N*-dimethylformamide.

Afterwards it was dried under vacuum and detached with trifluoroacetic acid (TFA), triisoprophylsilane (TIS) and water for 90 min at room temperature.

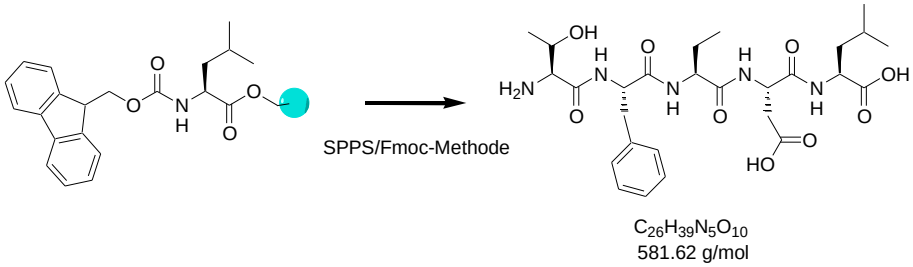


Figure 12: Reaction for the synthesis of TFSDL

The product was then precipitated with diethylether, centrifuged (2000 rpm/ 15 min) and the solid decanted was dried in the vacuum. The product was measured through mass spectrometry and HPLC.

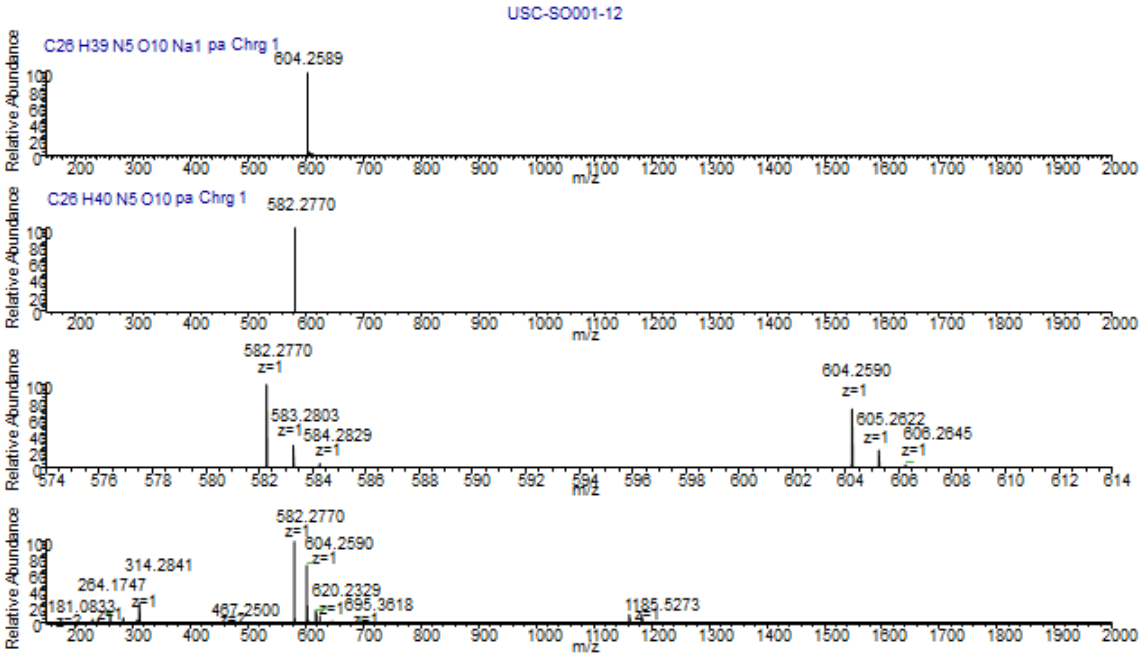


Figure 13: ESI-mass spectrum of the product TFSDL dissolved in water

In mass spectrometry a peak at 582.2770 Da and another at 604.2590 Da were obtained. The first was the peak from the peptide formed with one more proton. In this case positive mass spectrometry was performed therefore it would mean one positive charge form one extra proton ($[M+H]^{1+}$ where M stands for the product molecule). The peak at 604.2590 Da would be from the product with sodium ($[M+Na]^{1+}$) instead of with one proton charged.

The two first mass spectra are the calculated values of the peptide with one proton and the other with one sodium. Therefore it could be seen that the mass spectrum from the product would be correct and only product would be obtained, there are not any byproducts.

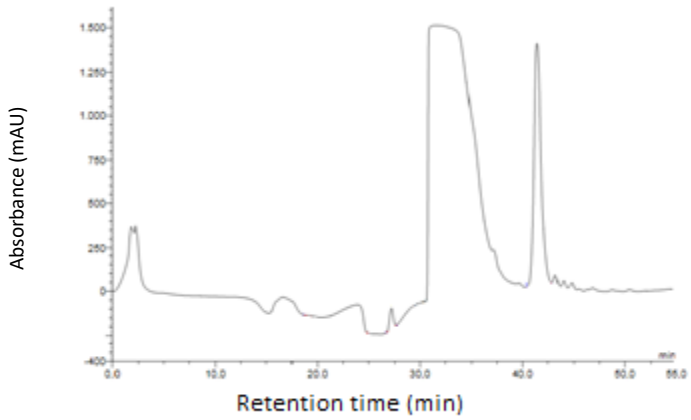


Figure 14: HPLC spectrum of the product TFSDL with used column: Jasco, ReproSil 100 C18 as packing material, particle size of 5 µm and inner diameter of 250 x 10 mm

Through the HPLC also two important peaks were obtained. The peak showing at 2 minutes is from the injection, it is not from a product.

The appearing peak from 31 to 38 min is from the sodium salt and the other would be the product with a proton because the column is nonpolar so the nonpolar compound would elute last. The sodium is more polar therefore it has a smaller retention time thus it appears first and the last peak is from the peptide. A 3D acquisition was done for all the spectra of all the wave lengths of all the peaks.

3. 2. Coupling of TFSDL with a masked alkyne

The attachment of the diene (2-carboxylic-3-trifluoromethyl-oxanorbornadiene) to the peptide was performed firstly by mixing 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate / hydroxybenzotriazol mixture solved in N,N-dimethylformamide and diene in *N,N*-dimethylformamide, then Diisopropylethylamine and lastly the peptide was added to couple for 2 hours at room temperature. Afterwards it was deprotected with trifluoroacetic acid, triisopropylsilane and water and shaken for 90 minutes. With diethylether precipitate should have been obtained although it did not occur therefore the solution with the solvent was removed under vacuum.

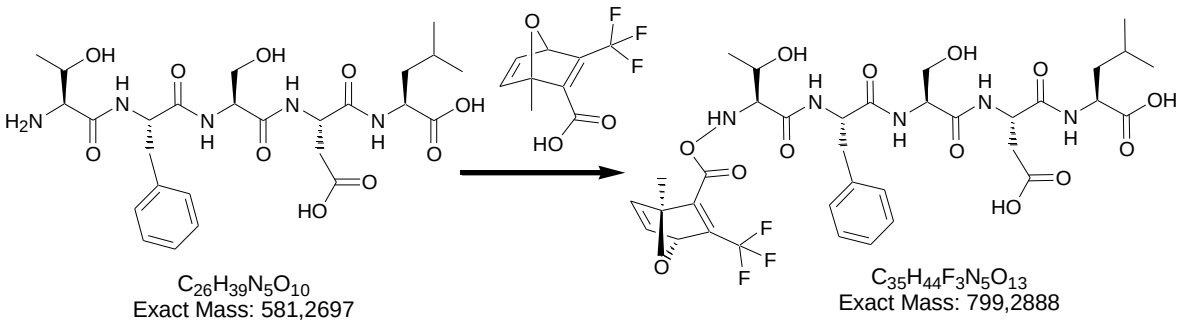


Figure 15: Coupling of the peptide TFSDL with diene

A HPLC and mass spectrum were obtained and the appearing peaks at 24, 26, 43 and 52 min do not belong to the product, just the initial peptide, which shows a peak at 47 min. Therefore it would not be a good method for the attachment of the diene.

As the coupling did not complete, the same reaction was done with different reagents: benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and Diisoprophylethylamine (DIPEA), O- [(Ethoxycarbonyl)cyanomethylenamino]-N,N,N´,N´-tetramethyluronium tetrafluoroborate (TOTU) and N-Methylmorpholine (NMM), (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) and DIPEA, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 4-dimethylaminopyridine (DMAP). Mass spectrometry was used to verify the product.

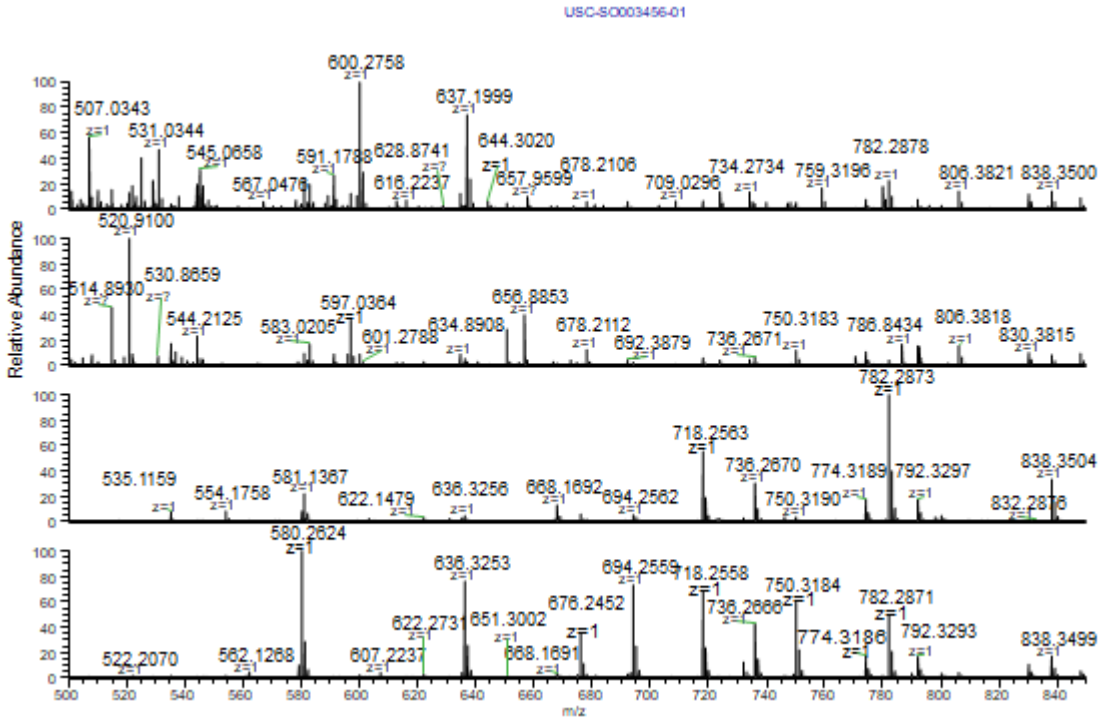


Figure 16: ESI-mass spectrum of the coupling of TFSDL with diene through four different mixtures of reagents

The first mass spectrum would be for the PyBOP/DIPEA, the second for TOTU/NMM, third COMU/DIPEA and last one for EDC/DMAP. All were measured in negative mode.

In the third and fourth spectra a peak at 782.2876 Da is seen, which belonged to the peptide with diene attached minus a proton. In the first spectrum it can be seen but in less quantity. Therefore the coupling worked better with COMU/DIPEA, in which spectrum a peak at 581.1367 Da can also be seen from the initial peptide. In the latter spectrum the 580.2624 Da peak meant most of the peptide does not react, which belongs to the peptide with one less proton. In all spectra except for the third one many different peaks are obtained from different side reactions.

In the positive mass spectra with TOTU/NMM, COMU/DIPEA and EDC/DMAP a peak was seen at 638.3390 Da from a side reaction. It would not appear in the negative mass spectra. In COMU/DIPEA and EDC/DMAP a peak at 582.2767 Da would be seen which belonged to the initial peptide that did not react. It was not seen for PyBOP/DIPEA and TOTU/NMM meaning all the initial peptide reacts but not with the norbornadiene producing side products. The peak from the product can only be seen for COMU/DIPEA at 784.3009 Da therefore it would be the only coupling that did work properly.

In conclusion, the best reagents for the coupling were COMU/DIPEA as seen in the spectra above (Figure 16); therefore it would be the reactant chosen for the following reactions. A HPLC was measured for the product obtained with the chosen reactant (COMU/DIPEA) to verify the purity of the product as in the mass spectrum other compounds were seen.

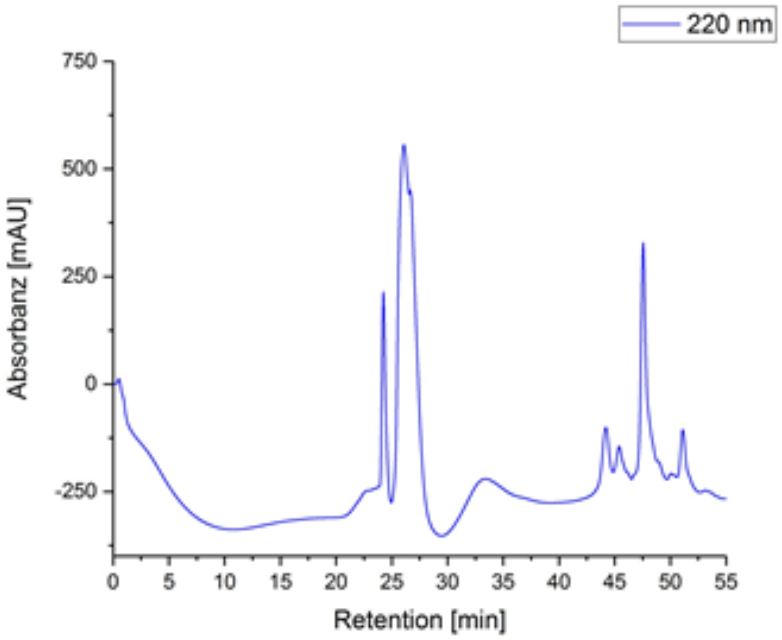


Figure 17: HPLC spectrum of the coupling of TFSDL with the diene. Column used: Jasco, ReproSil 100 C18 as packing material, particle size of 5 µm and inner diameter of 250 x 10 mm

In HPLC six peaks are seen, meaning the product was not completely pure. Another run was done and each peak was collected as a fraction and analysed to know which one contains the product. Initially with the HPLC it looked like the product would be the peaks with the retention time of 43 min, 47 min or 51 min as they were retained longer and its peaks were longer than the one at 45 min.

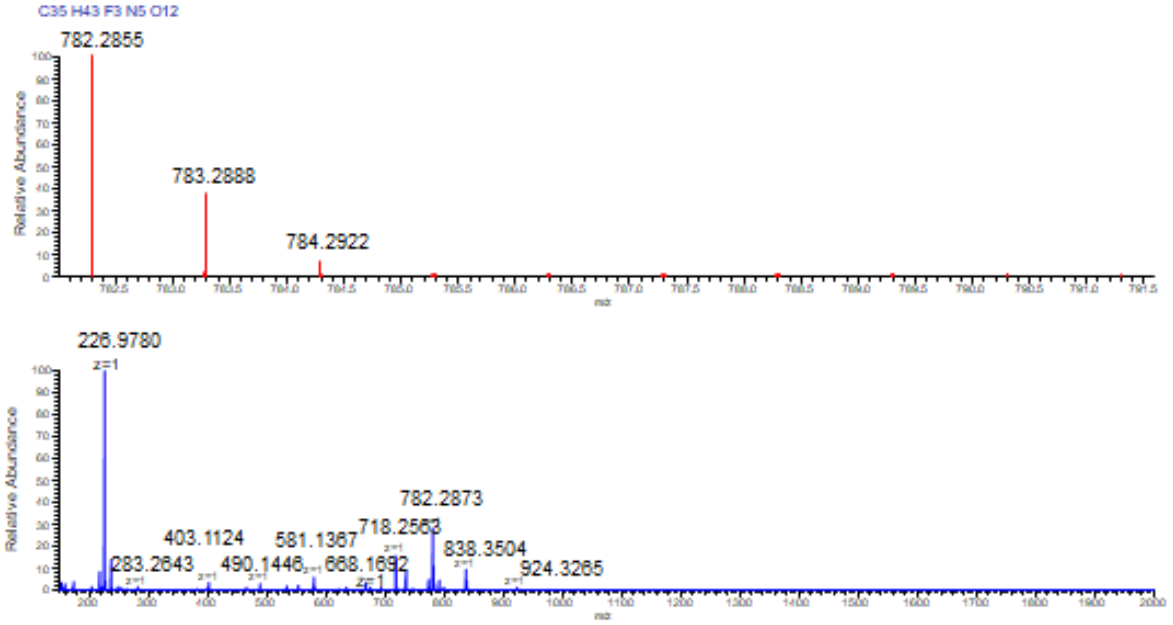


Figure 18: ESI-mass spectrum of the solution from the product peak from the HPLC, dissolved in water

After having all peaks have been measured in mass spectrometer, it was seen that only peak number 5 (47.157-49.987 min) contained the final product, as seen in the mass spectrum above (Figure 18). The HPLC peak number 2 had a peak in the mass spectrometer at 580.2631 Da belonging to the initial peptide.

In the mass spectrum from our product (blue spectrum of Figure 18) a pic at 782.2873 Da can be seen and another one at 226.2780 Da belonging to a TFA dimer. The upper spectrum (red) would be calculated with the product´s formula, showing the peak from the product with one proton less at 782.2855 Da and the following peaks each with a different possible isotope.

To sum up, the HPLC and mass spectrometry confirmed that the product was not pure, therefore purification was needed. Many HPLC runs were done and the peak from the product was collected in a falcon tube which later on was dried on the lyophilisator. The solid was then analysed through mass spectrometry and HPLC.

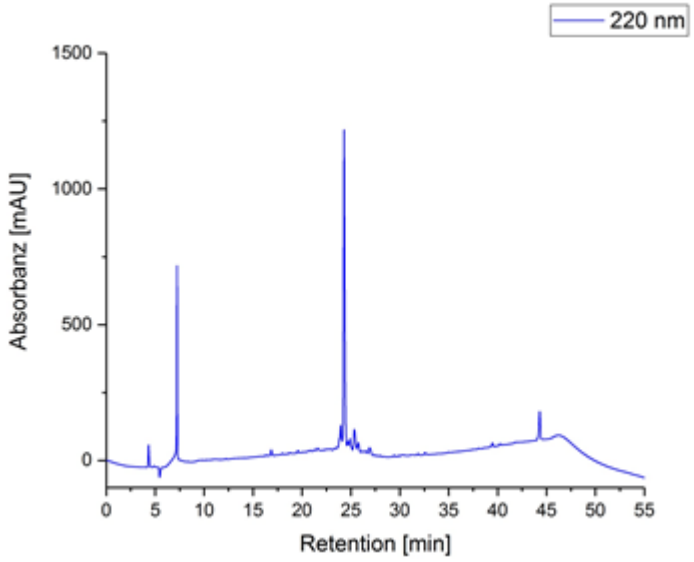


Figure 19: Analytical column: Jasco, ReproSil 100 C18 as packing material, 5 µm particle size, inner diameter of 250 x 4.6 mm

Measuring the pure product through a HPLC with an analytical column, two main peaks are seen, being the first from a secondary product and the second for the peptide with the diene. The small peaks at 5 min which go a bit up and down are from the injection. In comparison to the previous HPLC (Figure 17) it is a fact that the retention time was reduced from 47 to 25 minutes and the peak had sharpened. Next to the product small peaks had appeared that were not seen before. The reason of this result is that analytical columns due to their smaller diameter, all product´s peaks were separated more than producing an appearance on non-existing peaks before because the same flowrate is used therefore it produces a higher pressure and shorter retention times. It would show that the product is still not completely pure.

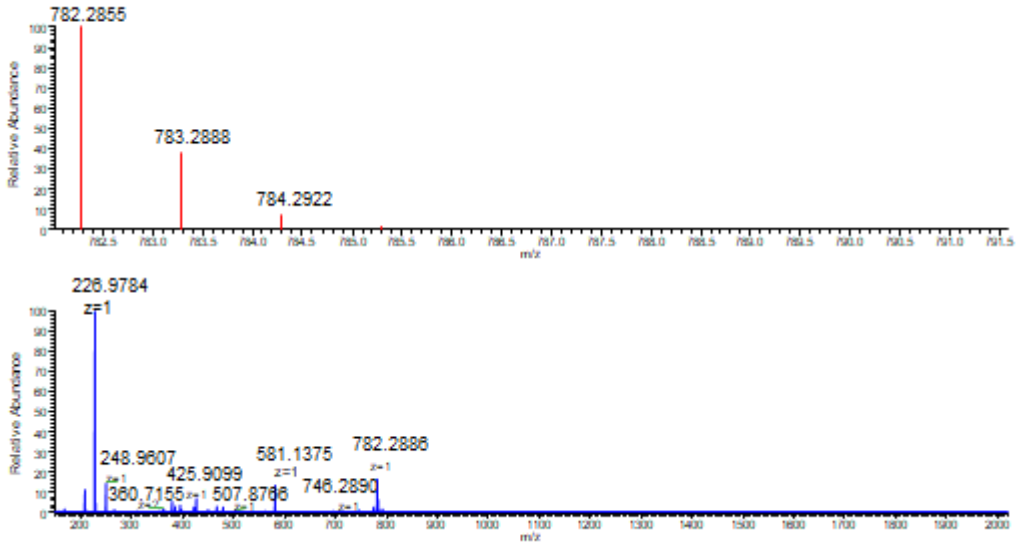


Figure 20: ESI-mass spectrum of the purified TFSDL dissolved in water

In the mass spectrum of the purified product (blue spectrum of Figure 20) the pic at 782.2886 Da from the product was found and also the trifluoroacetic acid (TFA) dimer at 226.9784 Da. The difference can be seen in the peaks in the previous spectrum at 718.2563 Da and 838.3504 Da which do not appear in this new spectrum because they were eliminated through the purification. The red spectrum (Figure 20) was the calculated mass spectrum with the product´s formula which would be the same to the previous mass spectrum before the purification (Figure 18).

A 3D acquisition was done for all the spectra of all the wave lengths of all the peaks.

3. 3. Loading of the resin and synthesis of VLAKVAA

A Wang resin was used as support for this reaction. The resin, *N,N*-dimethylformamide (DMF) and dichloromethane (DCM) were shaken for 10 minutes at room temperature. Alanine and hydroxybenzotriazol (HOBt) after being dissolved both in DMF, are added to the resin. Afterwards Diisopropylcarbodiimide (DIC) and later 4-dimethylaminopyridine (DMAP) were introduced to the mixture and shaken all together for 4 hours. Then acetic acid anhydride and pyridine were added and shaken at room temperature again for 1 hour to cap the remaining hydroxyl groups of the resin. The product was washed several times with DMF, DCM and methanol (MeOH) and lastly dried under vacuum.

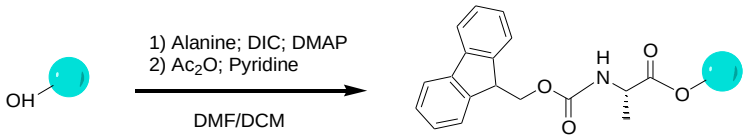


Figure 21: Synthesis of the resine with alanine

To the product of the reaction above, *N,N*-dimethylformamide (DMF) was added and shaken at room temperature for 45 minutes. After that the same procedure to the first peptide synthesis (3.1.) was done: deprotection, washing, coupling and washing which was repeated for each amino acid. The product was dried under vacuum overnight and detached with trifluoroacetic acid (TFA), triisopropylsilane (TIS) and water.

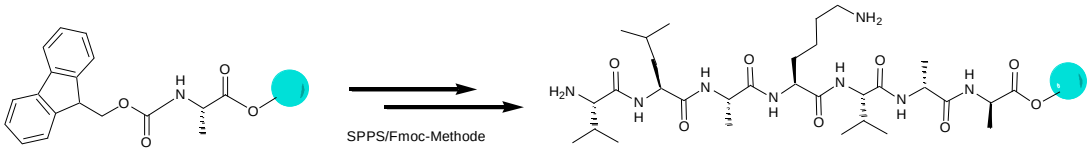


Figure 22: Synthesis of the VLAKVAA peptide

A precipitate was obtained with diethylether, which was then dried in the lyophilisator overnight. The solid obtained was measured with mass spectrometry and HPLC. In the first various peaks were observed from which three were related to the product, at 300.7064 Da, 600.4061 Da and 671.4450 Da.

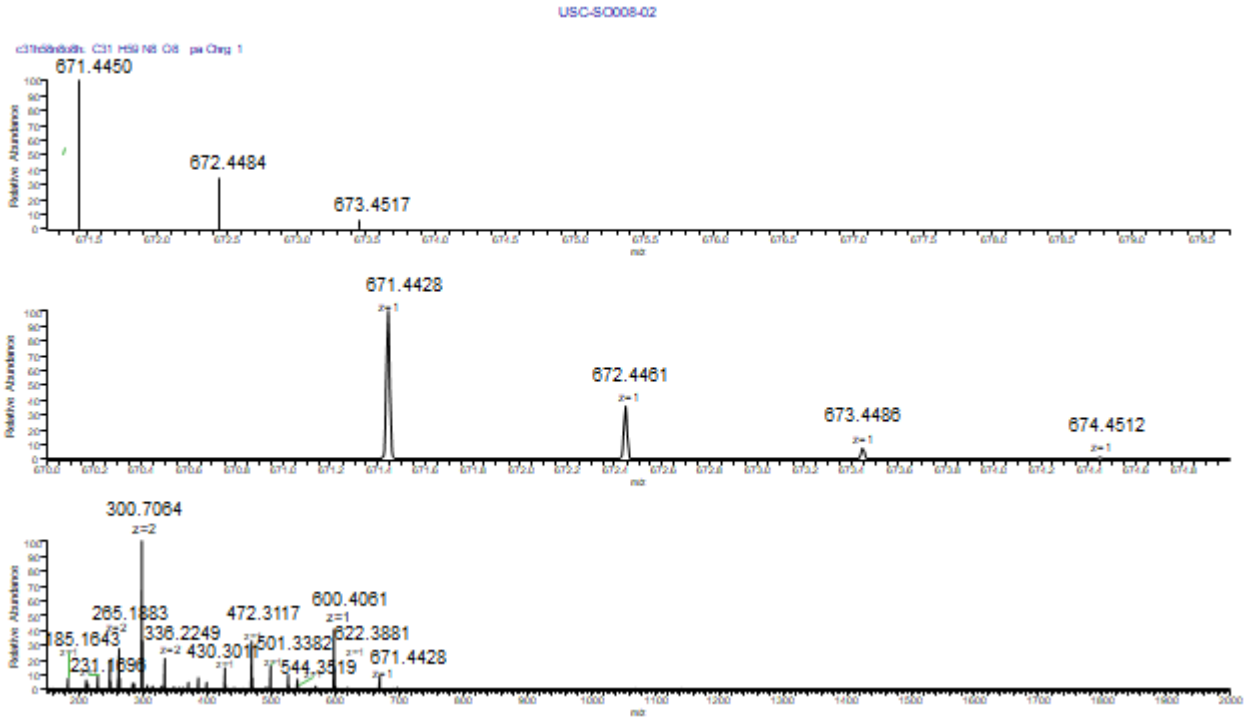
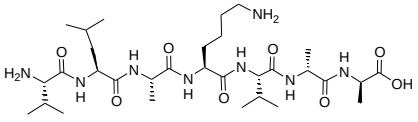


Figure 23: ESI-mass spectrum of VLAKVAA dissolved in water

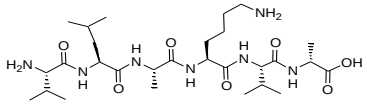
In mass spectrometry three important peaks can be seen at 300.7064 Da, 600.4061 Da and 671.4428 Da. The latter was corresponded to the peptide product (Figure 24) with one proton extra. The spectrum on the top was the calculated spectra with the formula which had also a peak at 671.4450 Da from the product with different isotopes.



Exact Mass: 670,44
Molecular Weight: 670,84

Figure 24: Peptide without the resin

Looking at the 600.4061 Da peak it was found it belonged to the peptide without the alanine (Figure 25), being the amino acid at the c-terminal end. It is fragmented during the mass analysis because the resin was capped after attaching, therefore the alanine was not detached with the resin.



Exact Mass: 599,40
Molecular Weight: 599,76

Figure 25: Peptide without the last alanine

Lastly the 300.7064 Da peak belonged to the half of the molecule above (Figure 26) with a positive charge. Both sides of the molecule have the same mass. It means it is more stable without the last alanine and even more stable the half of it.

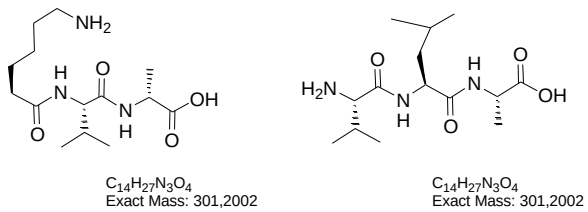


Figure 26: Fractions of the peptide without the alanine

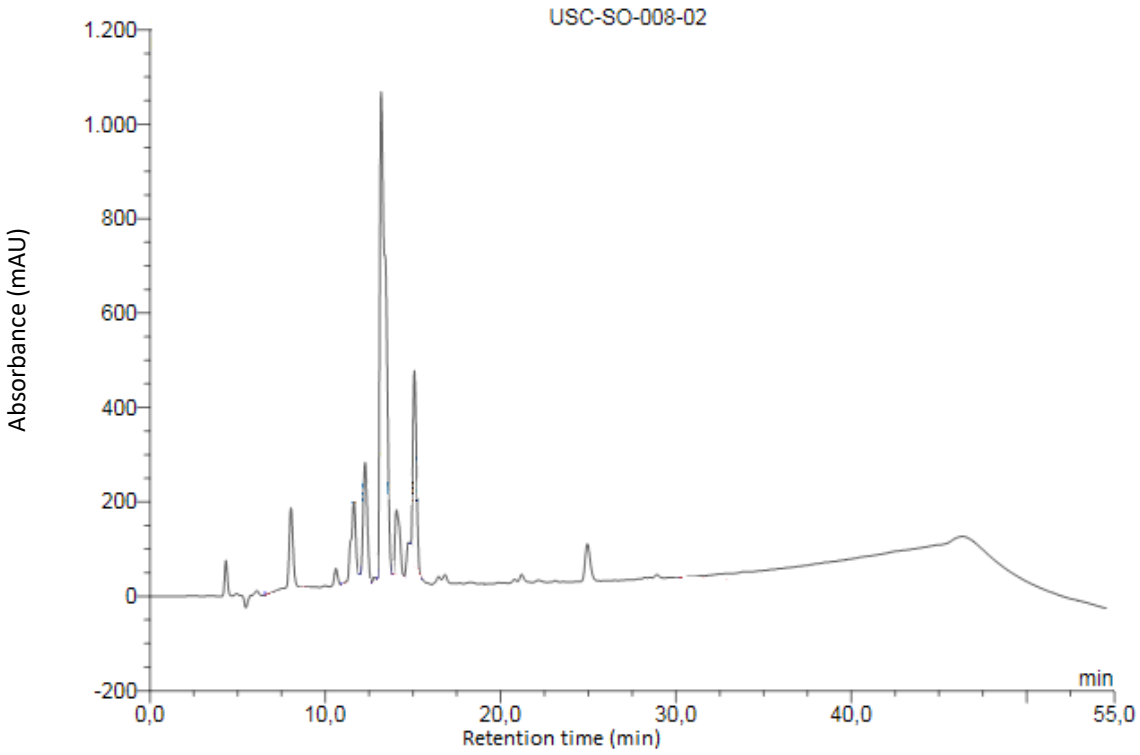


Figure 27: HPLC spectrum of VLAKVAA. Column used: Jasco, ReproSil 100 C18 as packing material, particle size of 5 µm and inner diameter of 250 x 10 mm

From the HPLC spectrum above, it can be seen that the product is not completely pure but it might be pure enough to use it for the indole conjugation in the next reaction, therefore it is performed. A 3D acquisition was done for all the spectra of all the wave lengths of all the peaks.

3. 4. Synthesis of the indole conjugate

The synthesis of the indole conjugate was started with the addition of the product from the previous reaction (Figure 22) to the indole and formaldehyde and was shaken for 5 days in a solution of water/dimethyl sulfoxide (DMSO).

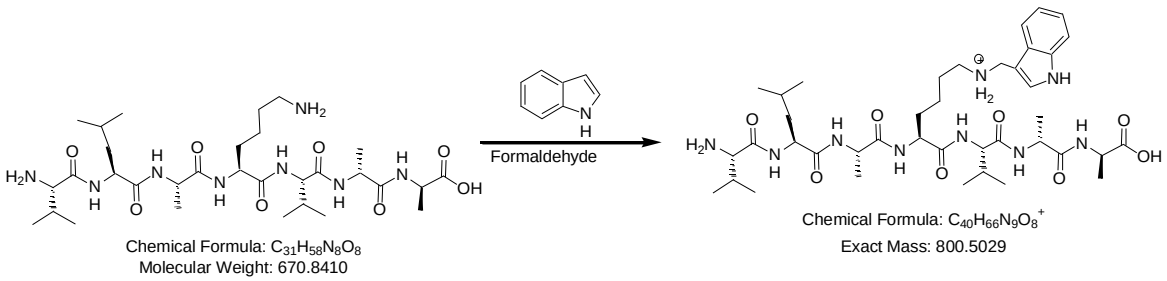


Figure 28: Synthesis of the indole conjugate

When the reaction was completed, the solvent and remaining formaldehyde were removed under vacuum. The dried solid was analysed with mass spectrometry where like in the mass spectrum before (Figure 23), many peaks can be seen, being five of them important.

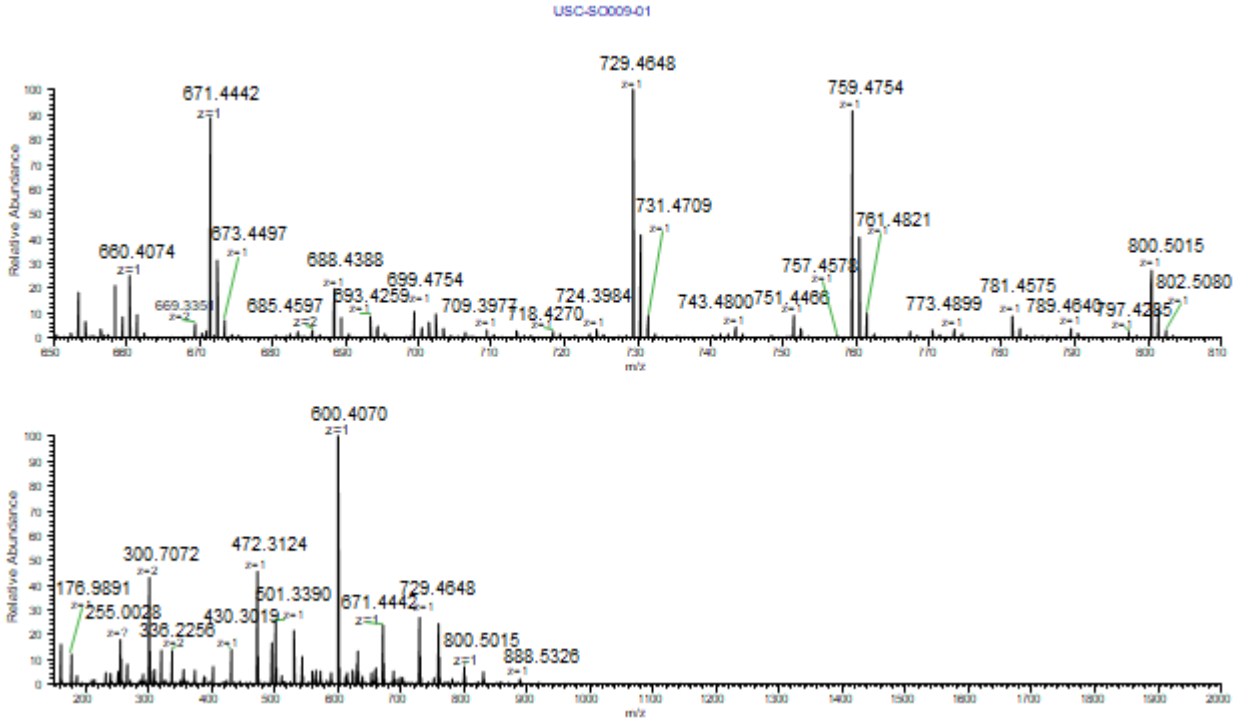


Figure 29: ESI-mass spectrum of the indole conjugate of VLAKVAA

The mass spectra above showed many peaks, being the most important ones at 300.7072 Da, 600.4070 Da, 671.4442 Da, 729.4648 Da and 800.5015 Da. The latter belonged to the indole conjugated to the peptide.

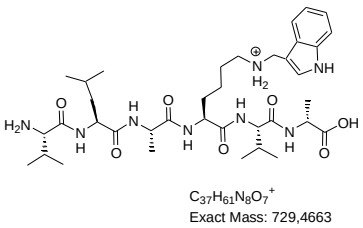


Figure 30: Indole conjugate without an alanine

The one before the last was known to be from the product without the alanine (Figure 30), which as it has seen that it is not very stable with it. Firstly it can be thought that the alanine is separated during the detachment of the resin but it is not possible because the resin was capped, therefore it is a fragmentation reaction during the analysis in the mass spectrometer. The first three peaks were the same to the reaction before as they were referred to the peptide without the indole at 671.4442 Da (Figure 24), to the peptide without the indole and alanine at 600.4070 Da (Figure 25) and to the half of this last one at 300.7072 Da (Figure 26). A 3D acquisition was done for all the spectra of all the wave lengths of all the peaks.

To conclude, it was a good method to obtain an indole conjugate as all the peaks were related to it, but for the indole fragments first leaving the initial peptide and its fragmentations. Or it can be said that the conversion is not completed and there is a peptide and indole conjugate as a mixture.

Before being able to repeat the same reaction as the one just explained (3. 4.), the detachment from the resin was needed as all pure peptide was used in the reaction before. Then a new reaction was preceded the same way.

The mass spectrometry results obtained were exactly the same as the synthesis of the indole conjugate above, showing the same peaks and proving that this method performed was a good method for indole conjugation as in both cases good results were obtained.

3. 5. Synthesis of a metal-indole conjugate

It was proceeded in the same way as the reaction before; the peptide VLAKVAA, the indole (*N*-Cymantrenylmethyl-6-aminomethylindole) and formaldehyde were dissolved in water and dimethyl sulfoxide.

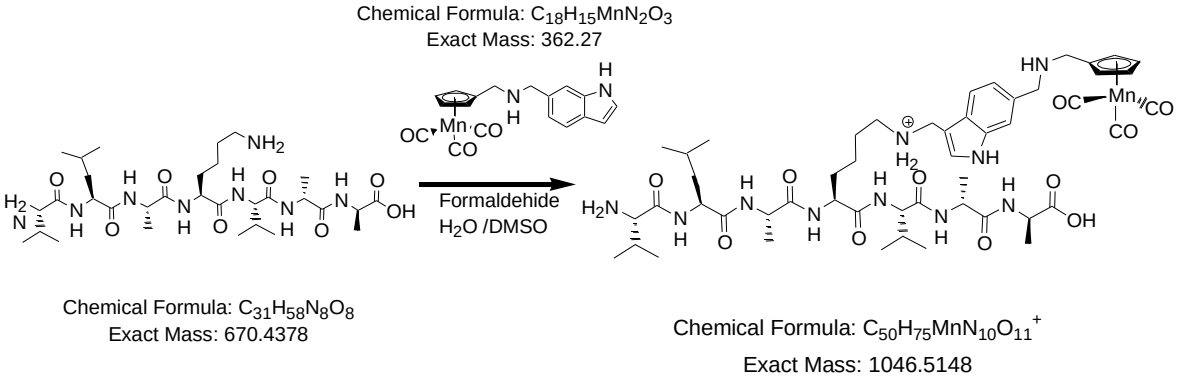


Figure 31: Synthesis of a metal-indole conjugate

After heating the product at 50ºC overnight, a mass spectrum was measured every day for 5 days and a peak for the product was not shown. Therefore it can be said that the cymantrene (*N*-Cymantrenylmethyl-6-aminomethylindole) does not attach to the peptide because it is not as reactive as the indole itself, leading to a research of another indole that would react better.

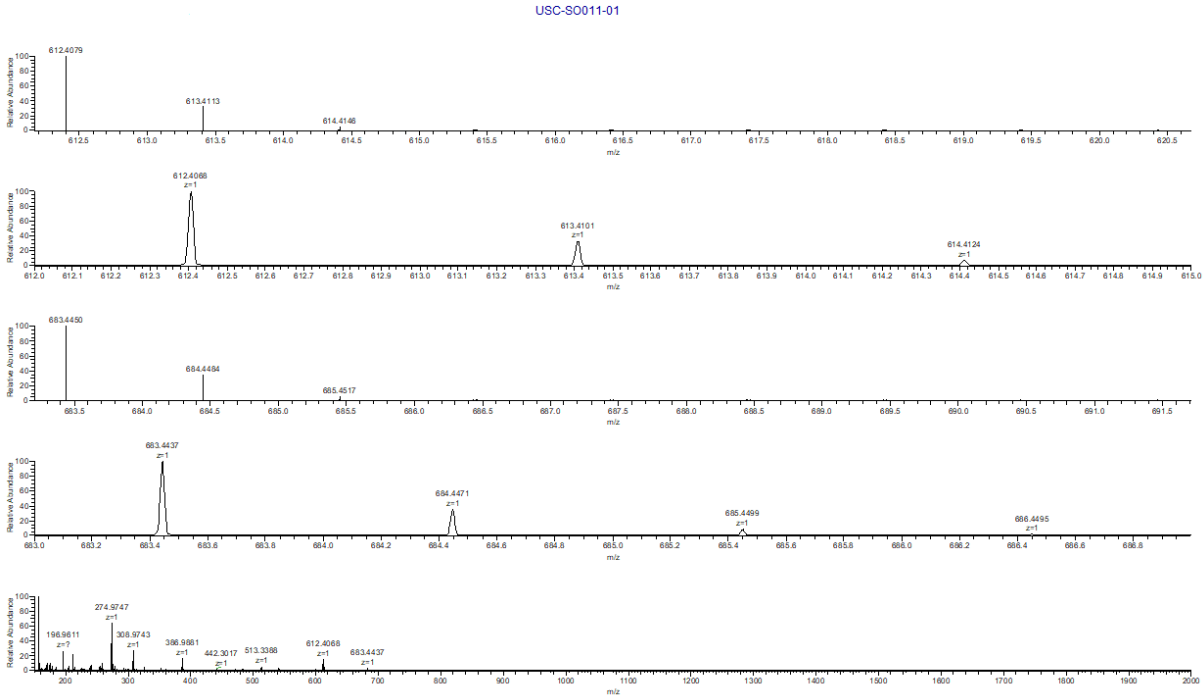


Figure 32: ESI-mass spectrum of the metal-indole conjugate at room temperature

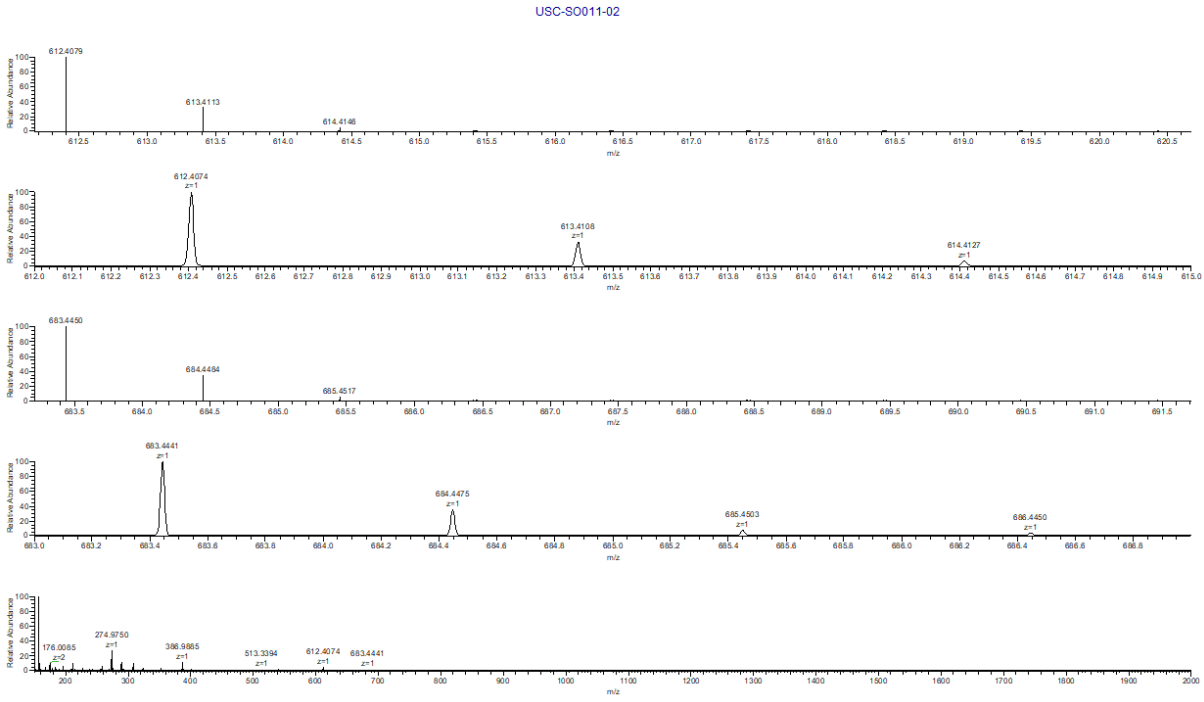
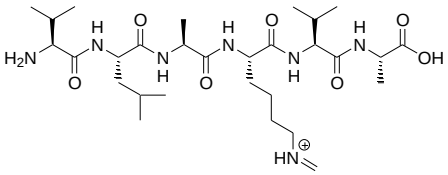


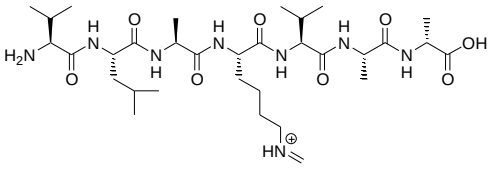
Figure 33: ESI-mass spectrum of the metal-indole conjugate at 50ºC

A peak at 683.4437 Da could be seen from the intermediate of the conjugation, the formaldehyde attached to the amine, forming an imine, is bonded to the peptide (Figure 35). Another peak appeared in the mass spectrum at 386.9881 Da belonging to the indole attached to the product of the formaldehyde and the amine. At 612.4068 Da an important peak can be seen from the peptide without the alanine and with an extra CH from the formaldehyde (Figure 34).



Chemical Formula: $C_{29}H_{54}N_7O_7^+$
Molecular Weight: 612,7925

Figure 34: Peptide without alanine with formaldehyde



$C_{32}H_{59}N_8O_8^+$
Exact Mass: 683,45

Figure 35: Peptide with formaldehyde

Both spectrum, at room temperature and the one heated at 50ºC look very similar. The small differences are that in the first one (Figure 32) the peaks at 274.9747 Da and 386.9881 Da are bigger than in the second spectrum (Figure 33) and the peak at 308.9743 Da in the first does not appear in the latter. A 3D acquisition was done for all the spectra of all the wave lengths of all the peaks.

In conclusion, a more reactive indole would be needed to conjugate to the peptide because the indole did not conjugate at all with the peptide.

3. 6. Synthesis of another metal-indole conjugate

The coupling of the indole to the peptide VLAKVAA was done with formaldehyde and being dissolved in water and dimethylsulfoxide. This reaction was performed at two different temperatures, at 50ºC and at room temperature, both being overnight.

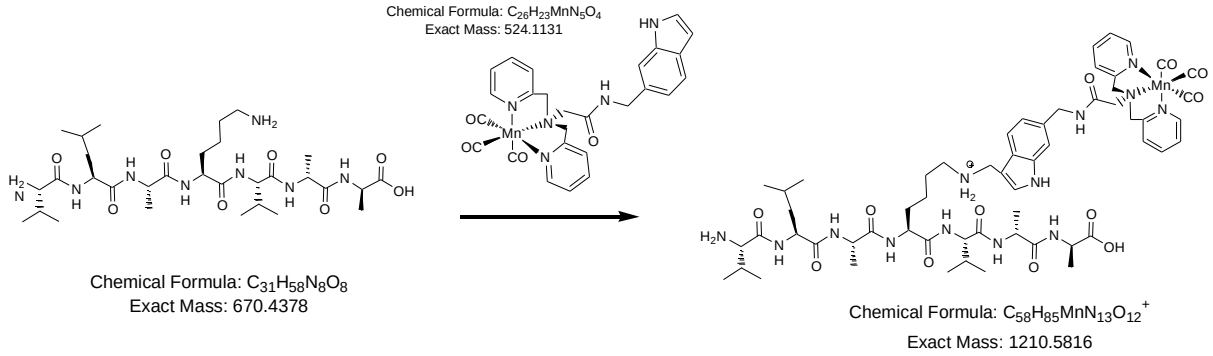


Figure 36: Synthesis of a metal-indole conjugate

The indole (Mn(bpg$^{6\text{-aminomethyl)indol}}$-k^{3}N)($CO_3$)) is too reactive, much more reactive than the cymantrene (*N*-Cymantrenylmethyl-6-aminomethylindole), and it reacted with itself. The mass spectra at room temperature and at 50ºC are a little bit different. At room temperature a peak at 524.1125 Da can be seen whereas at 50ºC that peak does not appear but at 530.1127 Da a peak is observed.

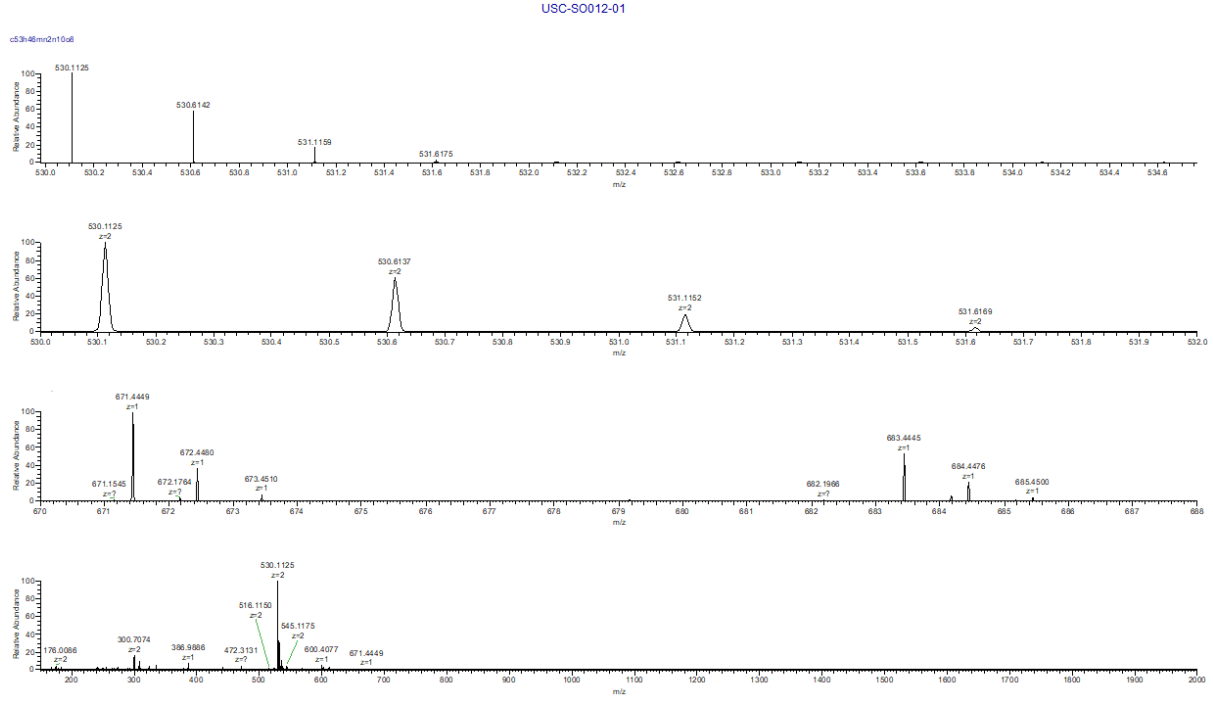


Figure 37: ESI-mass spectrum of the product at 50ºC

When the product is heated, a peak at 530.1127 Da with a charge of 2+ can be seen which would be the monomer from a dimer united through a CH_2 as a bridge. The dimer (Figure 38) has a mass of 1060.2230, whose half would be the peak obtained .Three other peaks are obtained at 671.4449 Da from the initial peptide (Figure 24), at 600.4077 Da from the peptide without alanine (Figure 25) and at 300.7074 Da from the half of the peptide without alanine (Figure 26).There not a peak at 1210 Da from the product, therefore it is also not a good indole to synthesize a metal-indole conjugate.

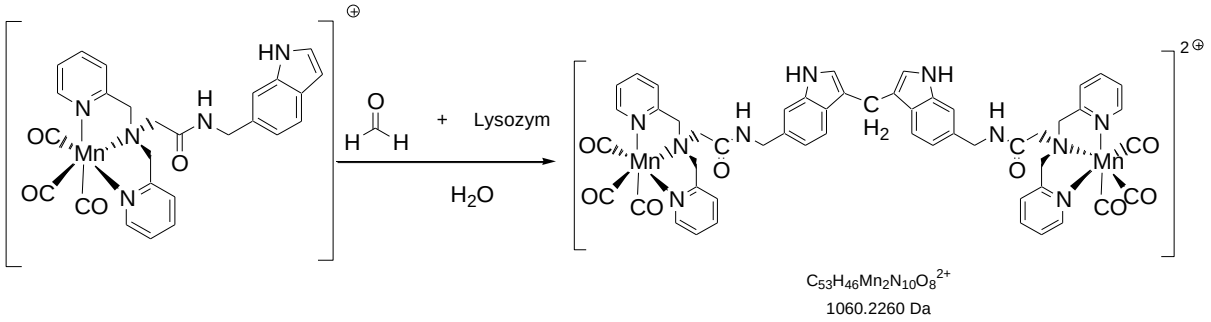


Figure 38: Formation of the dimer of the indole

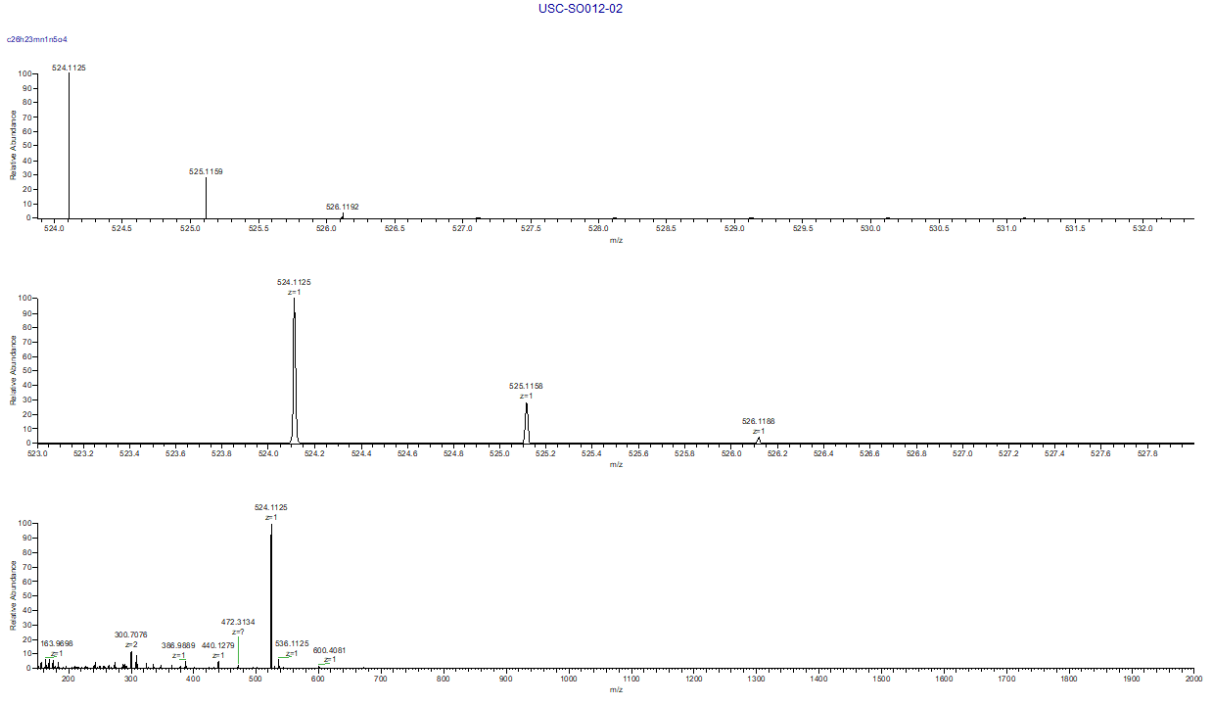


Figure 39: ESI-mass spectrum of the product at room temperature

When the reaction is done at room temperature we obtain a peak at 524.1125 Da which belongs to the indole that did not react at all, and also the peaks at 600.4081 Da and 300.7076 Da that were also obtained before when heating the reaction.

To sum up, an indole would be needed that is between both reactivities, more reactive to the first indole (Figure 31) and less reactive than the last indole (Figure 36).

3. 7. iClick reaction of the TFSDL masked alkyne with a Ruthenium complex

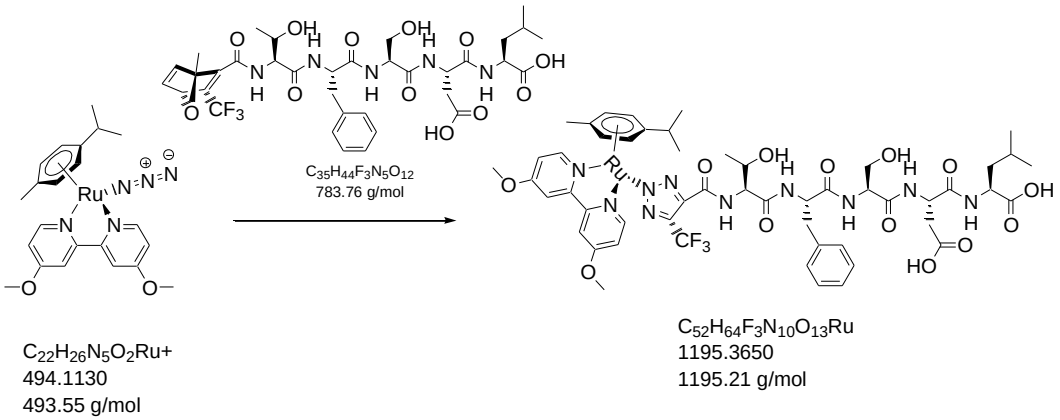


Figure 40: iClick reaction of the TFSDL peptide with a ruthenium complex

The TFSDL peptide with the diene as masked alkyne and the ruthenium complex ($Ru(N_3)(bpym)(p\text{-}cym)$) are dissolved in water and shake during 6 days while HPLC was measured firstly every hour and after the second day twice or once a day to be able to see the formation of the product, being a slow reaction.

Both the ruthenium (Figure 43) and the peptide (Figure 41) have a retention time of 24-25 minutes, therefore only one peak could be seen in the spectrum from the reaction (Figure 44). In this last spectrum also another small peak appeared at around 18-19 minutes that would be from the product, although in the mass spectrometer done for this peak not big concentrations of the product were found. In the mass spectrometer done for the bigger peak, both the ruthenium complex and the peptide were seen (Figure 45).

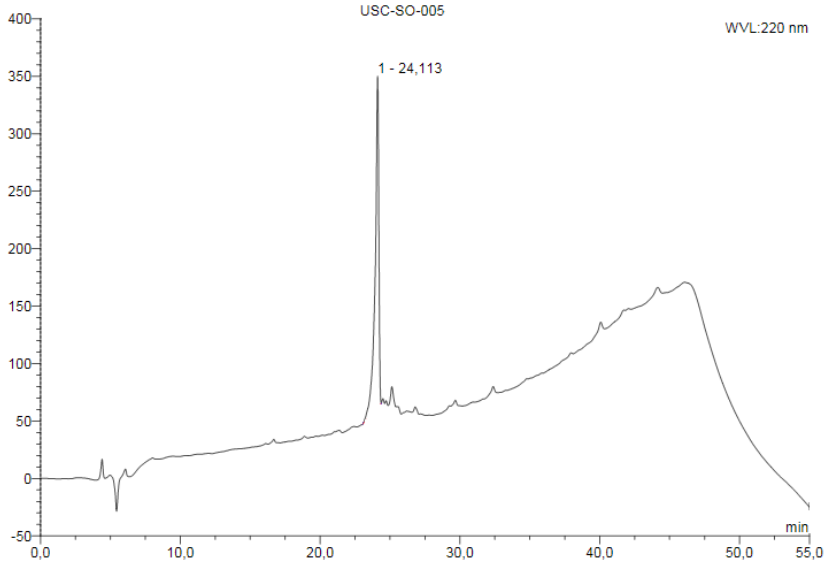
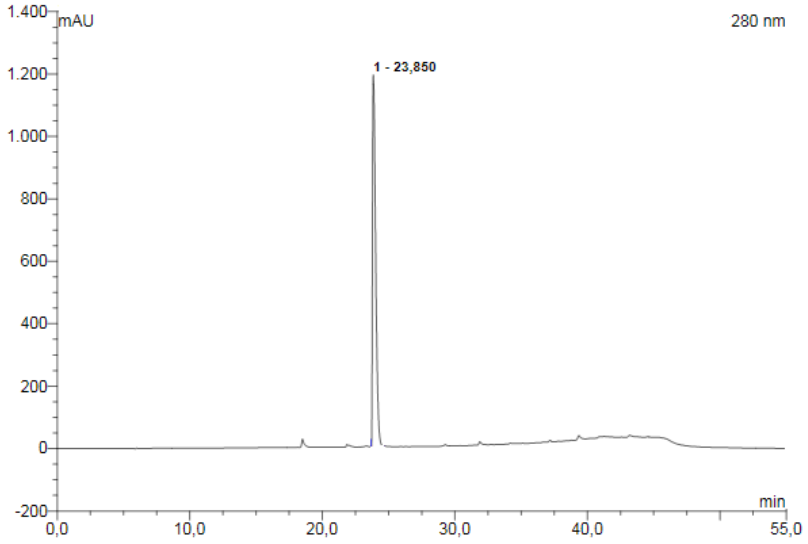


Figure 41: HPLC spectrum from the initial peptide. Column used: Jasco, ReproSil 100 C18 as packing material, particle size of 5 µm and inner diameter of 250 x 10 mm

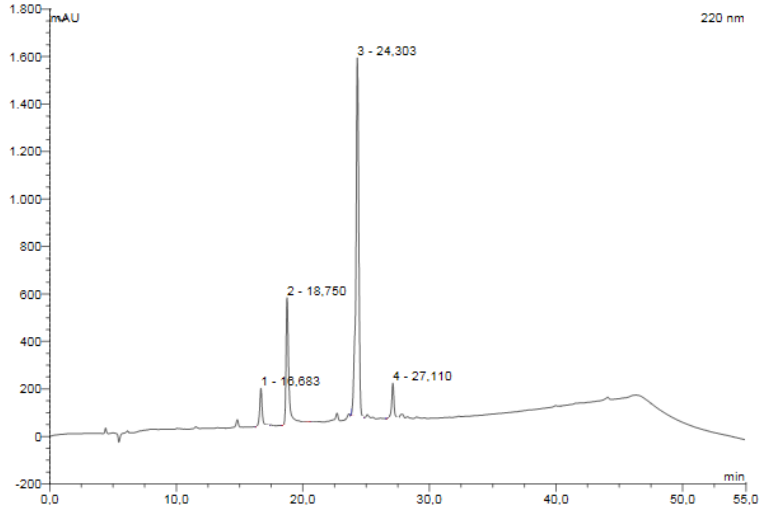
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	24,11	n.a.	288,107	86,559	100,00	n.a.	BMB
Total:			288,107	86,559	100,00	0,000	

Figure 42: Data from the HPLC spectrum above



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	23,85	n.a.	1191,473	330,529	100,00	n.a.	BMB
Total:			1191,473	330,529	100,00	0,000	

Figure 43: HPLC spectrum from the ruthenium complex. Column used: Jasco, ReproSil 100 C18 as packing material, particle size of 5 µm and inner diameter of 250 x 10 mm



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	16,68	n.a.	159,865	35,989	5,92	n.a.	BMB
2	18,75	n.a.	535,492	137,978	22,68	n.a.	BMB
3	24,30	n.a.	1513,071	402,657	66,20	n.a.	BMB
4	27,11	n.a.	148,217	31,612	5,20	n.a.	BMB
Total:			2356,644	608,236	100,00	0,000	

Figure 44: HPLC spectrum of the reaction after 3 days. Column used: Jasco, ReproSil 100 C18 as packing material, particle size of 5 µm and inner diameter of 250 x 10 mm

In the HPLC spectra of the reaction over time a slight change in the retention time of both the ruthenium complex and the peptide can be seen as well as a slow appearance of side products with time like seen in Figure 44 of the peaks at 16.68 min and 27.11 min. The peak at 18.75 min would be from the reaction´s product.

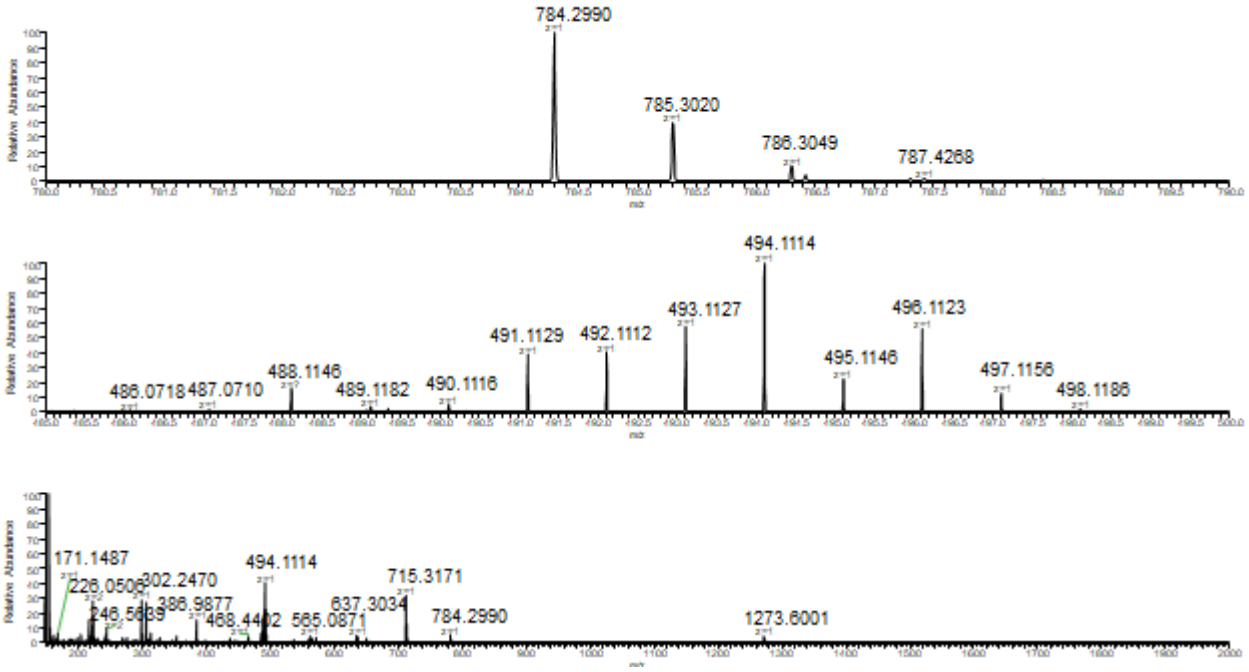


Figure 45: ESI-mass spectrum of the peak at 24 minutes from the HPLC

In the mass spectrum above which belonged to the collected peak from the HPLC from the peptide and ruthenium together (Figure 44), it is seen that this assumption is correct as the peak at 784.2290 Da belonged to the peptide and the one at 494.1114 Da to the ruthenium complex. The product should appear at 1195 Da, therefore it doesn´t appear also in that peak. A mass spectrum was obtained for the HPLC peak at 44 minutes (Figure 44) but the product is not observed either.

A kinetic study was performed for the formation of the peak at 18 minutes of the HPLC spectrum from Figure 44, that it is thought to be the product, where an increase of this peak´s height could be seen, which meant that the initial compound reacted to form the product.

Moreover, different HPLC methods were tried and the best one was the following: at a flow of 0.600 ml/min it was started with 5% B (acetonitrile with 0.1% TFA) and increase to 31 % until 37 minutes were it was maintained until 55 minutes. Then it was increase to 95 % until 60 minutes, decrease to 5 % until 65 minutes and finally maintain that concentration until the end of the measurement, 70 minutes.

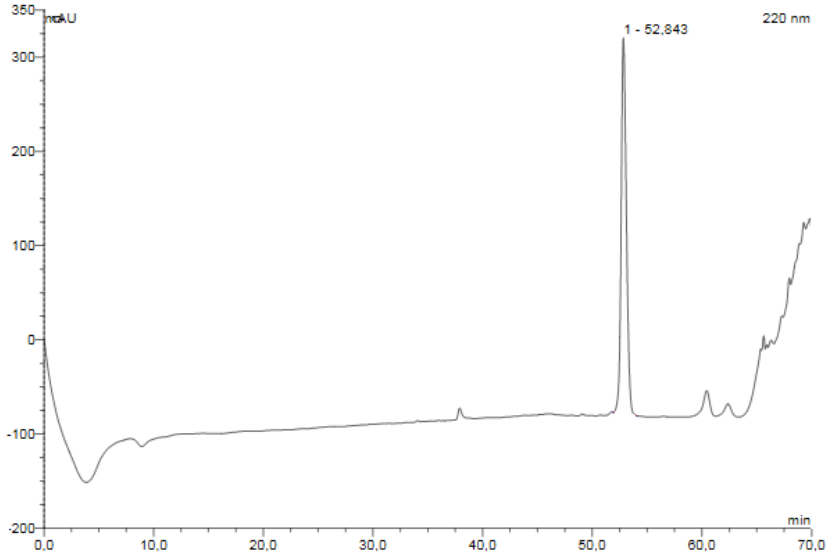
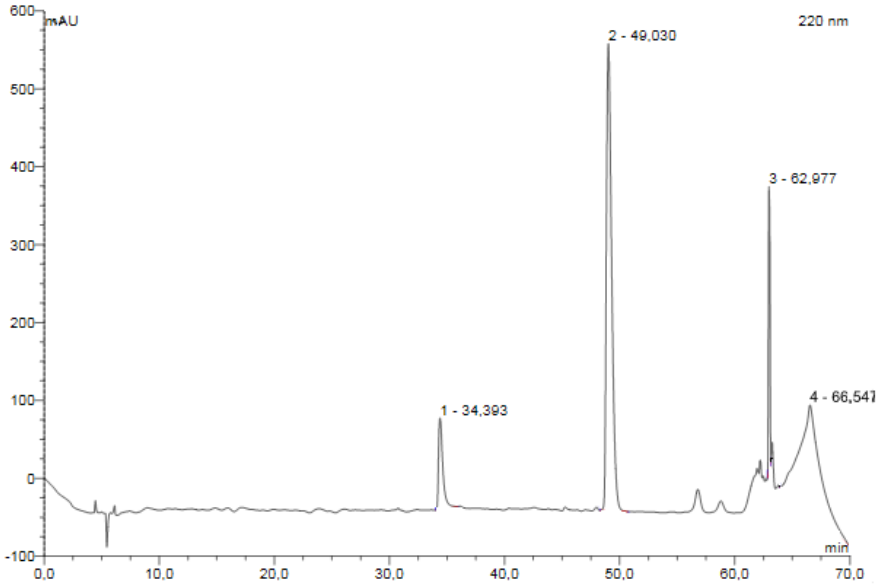


Figure 46: HPLC spectrum of the reaction after 10 minutes. Column used: Jasco, ReproSil 100 C18 as packing material, particle size of 5 µm and inner diameter of 250 x 10 mm

With this new method the goal was reached as the peaks from the peptide and ruthenium complex are separated 8 minutes, because both peaks were shifted in comparison to the HPLC spectrum of the method before (Figure 44). In the HPLC spectrum (Figure 46) big peak at 52 minutes could be seen which belonged to the Ruthenium complex, this is because the concentration is higher and also because the column is nonpolar, therefore the nonpolar compound would elute last. The peptide is more nonpolar than the ruthenium, so the first peak would be from the ruthenium and the last one from the peptide. That is the reason of which the peak at 60 minutes would be from the initial peptide and a small peak would be seen at 39 minutes from the product. It was measured for 5 days, in which a growth of the small peak can be seen and also a slight decrease on the ruthenium complex.

In the HPLC spectrum from Figure 46 from 65 minutes to the end an increase could be observed which belonged to the solvent. Firstly it was thought that was an impurity that was stack on the column and it was cleaned for a whole day with different solvents and it still had an increase at the same point, therefore it was said that belonged to the solvent and not to an impurity.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	34,39	n.a.	117,146	51,164	7,11	n.a.	BMB
2	49,03	n.a.	599,835	322,010	44,78	n.a.	BMB
3	62,98	n.a.	363,059	55,564	7,73	n.a.	BMB
4	66,55	n.a.	138,543	290,388	40,38	n.a.	BMB
Total:			1218,583	719,127	100,00	0,000	

Figure 47: HPLC spectrum of the reaction after 6 days. Column used: Jasco, ReproSil 100 C18 as packing material, particle size of 5 µm and inner diameter of 250 x 10 mm

Due to the masking alkye the reaction time appears to be slow as a probable product peak forms only after several hours, in the HPLC spectra a peak after the peptide peak appears like the third peak at 62.977 min from the Figure 47. A 3D acquisition was done for all the spectra of all the wave lengths of all the peaks.

A mass spectrum was performed to analyse which peak if either this one or the one at 38 minutes is from the product but in both cases the concentration is not enough to see it, therefore it cannot be determined exactly which peak would belong to the product. Moreover, a mass spectrometer was done to the reaction solution and a small peak from the product could be seen (Figure 48).

As it can be seen, the peaks from the last HPLC spectrum where shifted in comparison to the HPLC spectrum before (Figure 46) which is resulted from a pressure problem which was solved but the spectrum peaks afterwards were shifted.

A kinetic study of the peak at 38 minutes of the HPLC spectra was performed, but because of the inexact knowledge of which peak is the final product, it was not further studied.

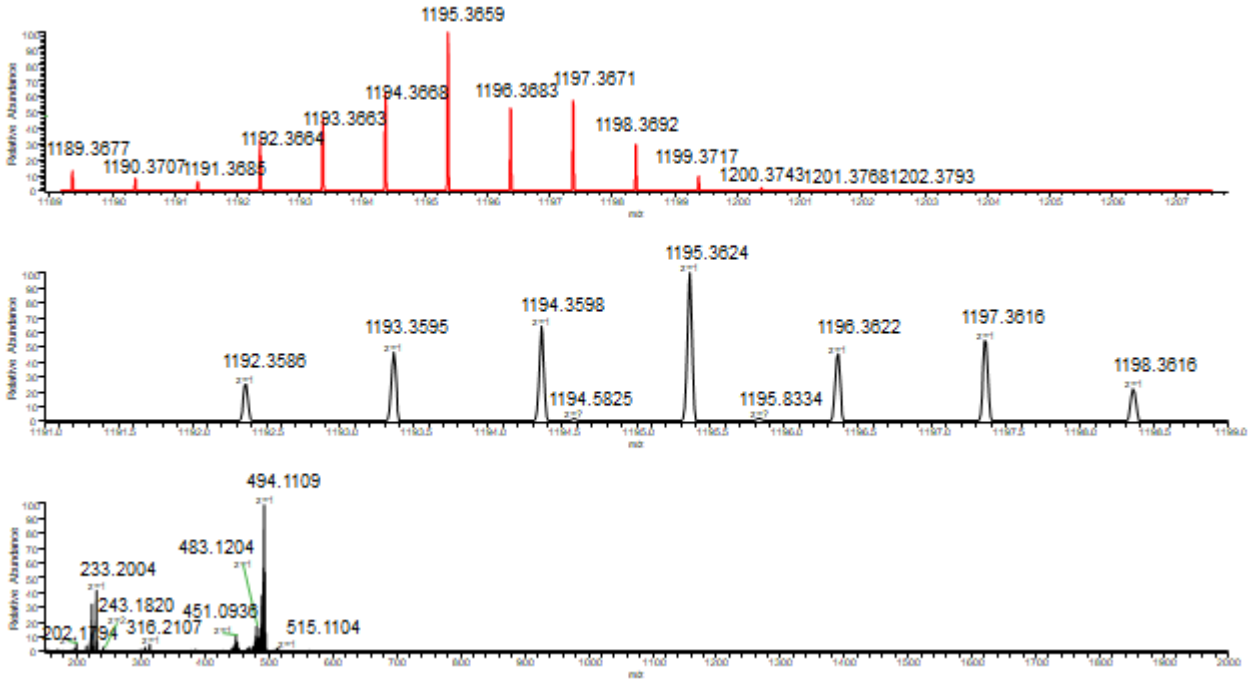


Figure 48: ESI-mass spectrum of the reaction

In the spectrum above which belonged directly to the reaction´s solution, a peak at 1195.3659 Da was observed but it is in very small concentration as it cannot be seen in the normal sized spectrum (bottom spectrum), only when it is zoom in. The red spectrum would be from the calculated spectrum of the product. In conclusion, the product would need to be concentrated.

4. Summary

Two different peptides were successfully synthesized, the sequences TFSDL and VLAKVAA via solid phase synthesis using in the first case a Fmoc-Leu-Wang resin and in the second case the attachment of the first amino acid to the Wang resin was done ourselves. The characterisation of both was done through mass spectrometry and HPLC, were good results were obtained.

The first peptide obtained was coupled to a diene (2-carboxylic-3-trifluoromethyl-oxanorbornadiene) trying with 4 different coupling reactants which lead to the COMU/DIPEA ((1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate / Diisoprophylethylamine) as the chosen reagents for this reaction. In addition purification was needed, which was done by separating the different peaks that appeared in the HPLC measurement. A mass spectrum was obtained from each peak to characterise the product.

The peptide with the masked alkyne attached onto it was used for iClick reaction with a ruthenium complex, ($Ru(N_3)(bpym)(p\text{-}cym)$), which was measured for 6 days by HPLC, and a positive result from the coupling was obtained. It was performed two times because the first method could not separate the peaks of the ruthenium and the peptide.

The second peptide synthesized (VLAKVAA) was used for the indole conjugation where all the peaks obtained in the mass spectrometer are related to the product, therefore it was a good method for the conjugation. Using the same procedure a metal indole conjugation was performed with the cymantrene (*N*-Cymantrenylmethyl-6-aminomethylindole) both at 50ºC and room temperature, but unfortunately the product was not formed because the cymantrene was not reactive enough. Another try was done with another metal indole ($Mn(bpg^{6\text{-}aminomethyl)indol}\text{-}k^3N)(CO_3)$) but also did not work because it was too reactive that it formed a dimer with itself.

In conclusion the goal of this research was archived, which is to obtain a metal peptide, although the metal indole conjugation did not work with both the metal indole used.

5. Experimental part

5.1. Synthesis of TFSDL

USC-SO001-10

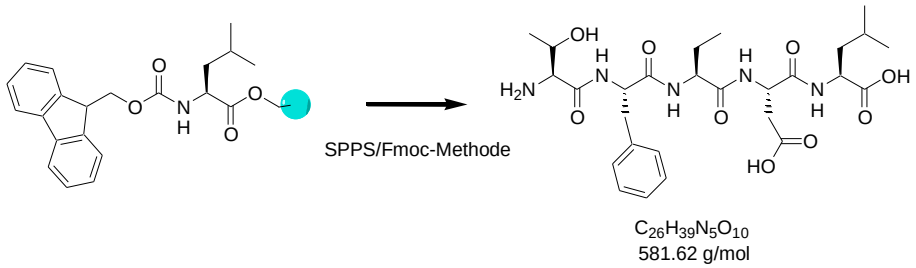


Figure 49: Synthesis of TFSDL

Nr	Monomer	MM [g/mol]	m [mg]	DIPEA	coupling	washing	deprotection	washing
1	Fmoc-Leu-Wang	x	x	x	x	x	13:10-13:26	5
2	Fmoc-Asp(tBu)-OH	411.5	823	+	13:45-14:22	5	14:45-15:05	5
3	Fmoc-Ser(tBu)-OH	383.4	766.8	+	15:14-15:56	5	16:27-16:43	5
4	Fmoc-Phe-OH	387.4	774.8	+	16:55-17:36	5	9:59-10:14	5
5	Fmoc-Thr(tBu)-OH	397.47	794.94	+	10:29-11:09	5	13:09-13:26	5
-	HOBt pro Stufe	135.12	270.24					
-	HBTU pro Stufe	379.24	758.48					

Fmoc-Leucine-Wang resin was used as solid support for this reaction. When the syringe was prepared with 626 mg resin and 4 ml *N,N*-dimethylformamide (DMF), it was shaken for 1 hour. The deprotection of the resin was done with 4 ml piperidine/DMF. After shaking for 15 minutes it was washed with 2 ml DMF 5 times.

The amino acid´s solution was prepared adding 4 ml 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate / hydroxybenzotriazol(HBTU/HOBt) mixture to the amino acid and 10 eq. Diisopropylethylamine (DIPEA) (2 ml). The coupling was done absorbing the first amino acid solution (Fmoc-Asp(tBu)-OH) with the syringe.

After that it was washed several times with 2 ml DMF. All this process starting with the deprotection with piperidine/DMF was produced with each of the amino acids.

When all the amino acids had been added, it was washed 5 times with 2 mL DMF and deprotected with 4 ml piperidine/DMF, shaken for 15 minutes and then washed 5 times with DMF. The product was dried in the pump; being attached directly the syringe to the Slenck-line. After drying for 30-35 minutes it was deprotected/detached with 3.80 ml trifluoroacetic acid (TFA), 0.10 ml triisoprophylsilane (TIS) and 0.10 ml water, shaking for 90 minutes.

The syringe was then washed with 1 mL TFA and the product was precipitated with diethyl ether. It was left in the freezer for 1 hour to obtain more brown precipitate.

The centrifuge was used to separate the solid for 15 minutes at 2000 rpm. The solid part was dried again with liquid nitrogen for 30 minutes.

A Kaiser-test was done to confirm that the coupling was completed, being done after the second attach of the amino acid, after the third and after all were attached. It was performed with phenol in ethanol 80% w/v (4 drops), ninydrin in ethanol 5% w/v (2 drops) and potassium cyanine (KCN) (1 mM) in Pyridin/H_20 2% v/v (4 drops). It was heated at 90ºC for 5 minutes. The resin would turn from red to yellow and finally to white. Red would show that the coupling is not complete whereas white shows it is complete.

5.2. Coupling of TFSDL with a masked alkyne

USC-SO002-01

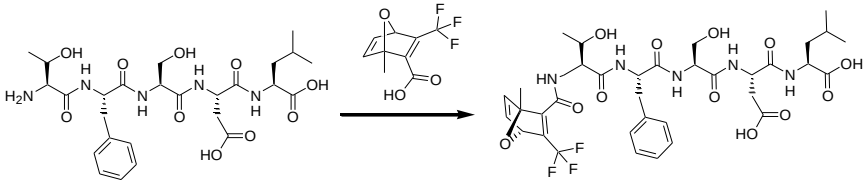


Figure 50: Coupling of TFSDL with diene

Reactants	MM [g/mol]	Eq	m [mg]	mmol
Peptide	581.62	1	308.56	0.2464
HBTU	379.24	4	373.78	0.9856
HOBt	135.12	4	133.17	0.9856
Norbornadiene	220.15	4	216.98	0.9856
DIPEA	129.25	10	318.47 --> 419µl	2.46

As seen in the previous table 379.24 mg 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 135.12 mg hydroxybenzotriazol (HOBt) were added to 2 mL N,N-dimethylformamide (DMF) and the same to the 220.15 mg 2-carboxylic-3-trifluoromethyl-oxanorbornadiene. After mixing both solutions together and with 0.5 mL Diisoprophylethylamine (DIPEA), a change in colour from yellow-orange to red-black was seen. The peptide and the solutions were coupled for 2 hours with shaking at room temperature and then washed 5 times with DMF.

Afterwards it was deprotected with 1.90 mL trifluoroacetic acid (TFA), 50 µL triisoprophylsilane (TIS) and 50 µL water and shaken for 90 minutes. The product in the syringe was washed with 1 mL TFA and transferred to a plastic flask where diethyl ether was added to precipitate it. It didn´t precipitate directly with diethyl ether therefore drying under vacuum was needed to obtain the solid.

The same method is repeated but with different reactants to couple.

USC-SO003-01

Reactants	MM [g/mol]	Eq	m [mg]	mmol
Peptide	581.62	1	206.44	0.1651
PyBOP	520.39	1.5	128.90	0.2477
DIPEA	129.25	3	64.01 --> 86.27µl	0.4953
Norbornadiene	220.15	1.5	54.53	0.2477

It was proceeded the same way dissolving the norbornadiene and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) in DMF and adding it with the DIPEA and peptide to the resin. In the detachment 850 µL TFA, 25 µL TIS and 25 µL H_2O were used and shaken for 2 hours at room temperature. A precipitate would be obtained then.

The whole exact process would be done for the coupling with O- [(Ethoxycarbonyl)cyanomethylenamino]-*N,N,N´,N´*-tetramethyluronium tetrafluoroborate *N*-Methylmorpholine(TOTU) and *N*-Methylmorpholine (NMM):

USC-SO004-01

Reactants	**MM [g/mol]**	**Eq**	**m [mg]**	**Density[g/cm3]**	**V[µl]**	**mmol**
Peptide	581.62	1	206.38	-	-	0.1651
TOTU	328.07	1.5	81.26	-	-	0.2477
NMM	101.15	3	64.01	0.92	69.57	0.4953
Norbornadiene	220.15	15	54.53	-	-	0.2477

With the addition of diethyl ether, a precipitate was obtained.

And for the reaction with (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) and Diisopropylethylamine (DIPEA) (USC-SO005-01) :

Reactants	**MM [g/mol]**	**Eq**	**m [mg]**	**Density[g/cm3]**	**V[µl]**	**mmol**
Peptide	581.62	1	242.70	-	-	0.1942
COMU	428.27	1.5	124.71	-	-	0.2912
DIPEA	129.25	3	75.29	0.742	101.47	0.5825
Norbornadiene	220.15	1.5	64.11	-	-	0.2912

In this case the reaction was shaken for 4 hours at room temperature and deprotected with 1.90 µL TFA, 50 µL H_2O and 50 µL TIS. Diethylether was added for precipitation but it didn´t happen, therefore it was the dried under vacuum.

This exact method with the 4 hours shaking would be realised for 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 4-dimethylaminopyridine (DMAP):

USC-SO006-01

Reactants	**MM [g/mol]**	**Eq**	**m [mg]**	**mmol**
Peptide	581.62	1	202	0.161
EDC	155.25	1.5	37.57	0.242
DMAP	122.17	3	75.29	0.485
Norbornadiene	220.15	1.5	53.28	0.242

With diethyl ether precipitate was seen. After cooling down in the freezer for 30 minutes it was centrifuged using program 3: 2000 rpm for 15 minutes. The solid would be decanted from the liquid and dried it under vacuum.

5.3. Loading of the resin

USC-SO007-01

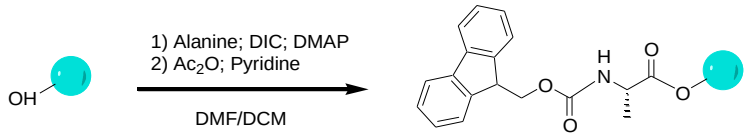


Figure 51: Synthesis of resine with alanine

Reactants	MM [g/mol]	Eq	density [g/cm3]	m [mg]	v [mL]	mmol
Resin	1.1 mmol/g	1	-	500	-	0.55
DMF	73.09	2	0.944	80.399	85.17	1.1
HOBt	135.12	2	-	297.264	-	1.1
DMAP	122.17	0.1	-	6.17	-	0.055
DIC	126.2	2	0.815	138.82	0.17	1.1
Ac2O	102.1	2	1.08	112.31	0.104	1.1
Pyridine	79.1	2	0.982	87.01	0.089	1.1

A Wang resin was used as support for this reaction. The 500 mg resin, 22 mL *N,N*-dimethylformamide (DMF) and 4 mL dichloromethane (DCM) were shaken for 10 minutes at room temperature. Firstly 342.46 mg Alanine and 80.40 mL hydroxybenzotriazol (HOBt), both dissolved with DMF were shaken for a few minutes and added to the resin. After that 0.2 mL Diisoprophylcarbodiimide (DIC) was introduced and a few minutes later the 122.17 mg 4-dimethylaminopyridine (DMAP) dissolved in DMF. The whole reaction is then shaken for 4 hours.

After shaking at room temperature for 4 hours the Ac_2O and pyridine were introduced and shaken for 1 hour. The resin was then washed with 10 mL DMF 3 times, with 10 mL dichloromethane (DCM) for 3 times and with 10 mL methanol 3 times. Finally the product was dried in the vacuum.

5.4. Synthesis of VLAKVAA

USC-SO008-01

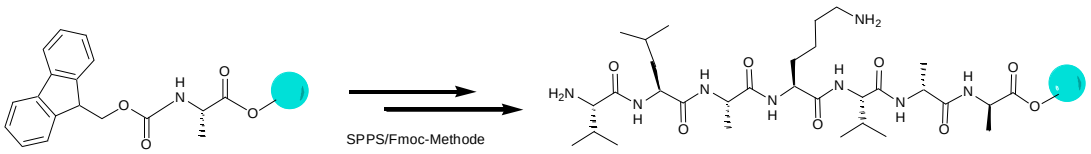


Figure 52: Synthesis of VLAKVAA

Reactants	MM [g/mol]	Eq	density [g/cm3]	m [mg]	v [µl]	mmol
Resine	1.1 mmol/g	1	-	500	-	0.55
HBTU	379.24	4	-	1668.67	-	2.2
HOBt	135.12	4	-	594.53	-	2.2
DIPEA	129.25	10	0.742	710.86	958.05	5.5

Nr	Monomer	MM [g/mol]	Eq	mmol	m [mg]	DIPEA	coupling	washing	deprotection	washing
1	Resin-Alanine	x	x	x	342.46	x	x	x	14.15-14.57	5
2	Fmoc-Alanine-OH	311.33	4	2.2	684.926	+	15.29-16.15	5	10.59-11.19	5
3	Fmoc-Valine-OH	339.39	4	2.2	746.658	+	11.39-12.29	5	12.39-12.59	5
4	Fmoc-Lysine-OH	368,43	4	2,2	810,546	+	13.13-14.00	5	14.10-14.30	5
5	Fmoc-Alanine-OH	311,33	4	2,2	684,926	+	14.45-15.32	5	9.07-9.27	5
6	Fmoc-Leucine-OH	353,41	4	2,2	777,502	+	9.39-11.00	5	9.19-9.42	5
7	Fmoc-Valine-OH	339,39	4	2,2	746,658	+	9.58-11.20	5	11.36-12.10	5

Using the product from the previous reaction, 2 mL DMF was added and shaken for 45 minutes at room temperature. Then the same procedure was performed as in the first peptide synthesis: deprotection, washing, coupling, washing repeated for each amino acid. After being dried under vacuum overnight and detached with 1.90 mL trifluoroacetic acid, 50 µL triisopropylsilane and 50 µL water, the product was cleaned with TFA and poured into a plastic flask where diethylether was added obtaining precipitate. Afterwards the solid was dried in the lyophilisator.

5.5. Synthesis of the indole conjugate

USC-SO009-01

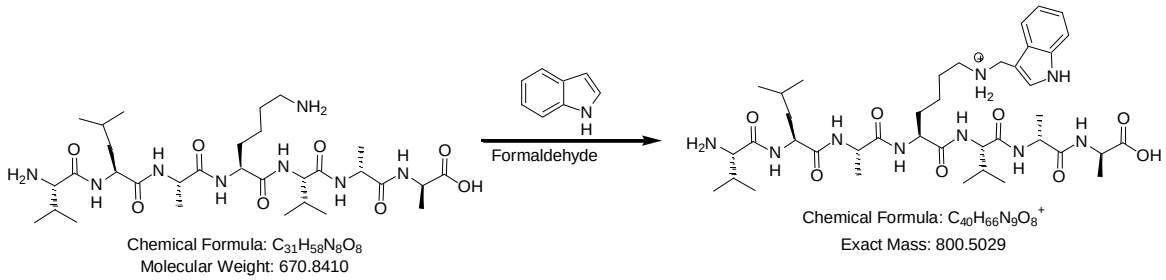


Figure 53: Synthesis of the índole conjugate

Reactants	**MM [g/mol]**	**m [mg]**	**Eq**	**Density [g/ml]**	**V [µl]**	**mmol**
Peptide	670.8	3.7	1	-	-	0.0055
Formaldehyd 40%	30.03	0.495	1.2	1.09	0.54	0.0066
Indole	117.15	0.644	1	-	-	0.0055

The peptide from the last reaction was added to the indole and a drop of formaldehyde. It was then dissolved with water/dimethyl sulfoxide 10% v/v (2 mL water and 0.5 mL DMSO), shaken for 5 days and dried under vacuum.

The same reaction would be performed again although a detachment from the resin would be needed as all the pure peptide was used in the reaction above, before being able to start with the reaction itself.

USC-SO010-01

Reactants	**MM[g/mol]**	**m [mg]**	**Eq**	**Density [g/ml]**	**V [µl]**	**mmol**
Peptide	670.8	2.24	1	-	-	0.0033
Formaldehyd 40%	30.03	0.30	1.2	1.09	0.276	0.0040
Indole	117.15	0.387	1	-	-	0.0033

To start with this reaction 201.19 mg were taken from the peptide VLAKVAA with the resin and it was detached with 1.90 mL of trifluoroacetic acid, 50 µL of water and 50 µL of triisopropylsilane is needed. Before the deprotection 2 mL of *N,N*-dimethylformamide were added to the peptide and shaken for a few minutes. The syringe was cleaned with TFA and poured into a plastic flask where with diethyl ether precipitation was seen. After half an hour in the freezer it was centrifuged for 15 minutes at 2000 rpm (program 3). Through decantation the solid was obtained and dried under vacuum.

The 117.15 mg indole was added to the peptide with a drop of formaldehyde. It was then dissolved with 1 mL H_2O and 0.2 mL DMSO. For the reaction to take place it was introduced in an Eppendorf tube and heated at 50ºC overnight.

5.6. Synthesis of a metal-indole conjugate

USC-SO011-01

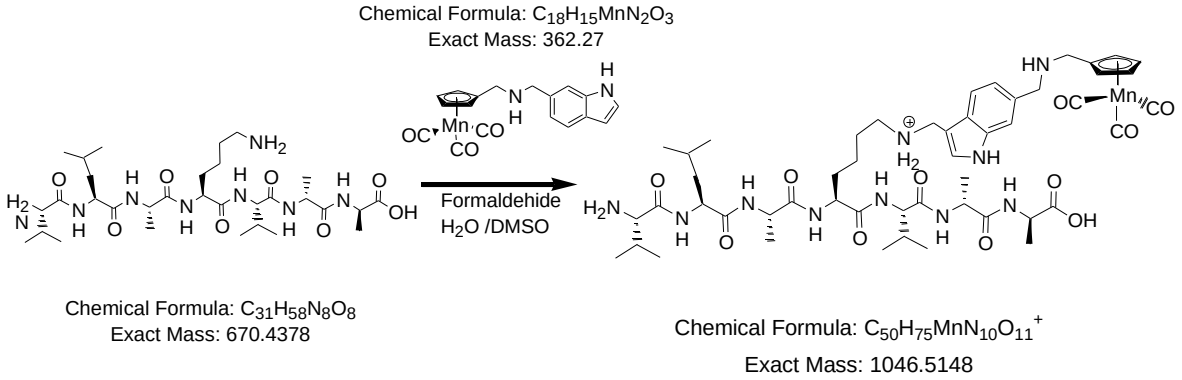


Figure 54: Synthesis of the metal-indole conjugate

Reactants	MM [g/mol]	m [mg]	Eq	Density [g/ml]	V [µl]	mmol
Peptide	670.8	2	1	-	-	0.00298
Formaldehyd 40%	30.03	0.268	1.2	1.09	0.246	0.00358
Indole	362.27	1.0796	1	-	-	0.00298

The peptide VLAKVAA detached from the resin was mixed with the 362.27 mg indole (*N*-Cymantrenylmethyl-6-aminomethylindole) and a drop of the formaldehyde. After being dissolved in 1 mL water and 0.2 mL dimethyl sulfoxide, it was heated to 50ºC overnight in an Eppendorf tube. The same reaction would be performed at room temperature.

5.7. Synthesis of another metal-indole conjugate

USC-SO012-01

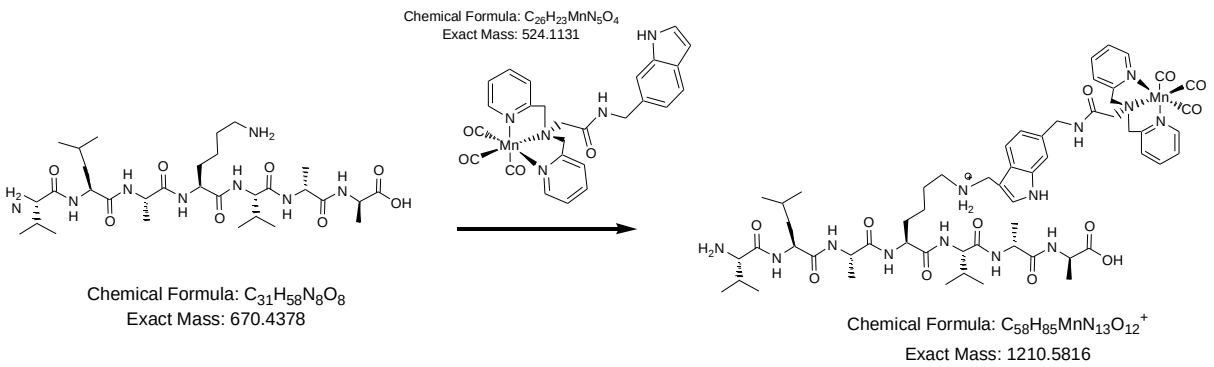


Figure 55: Synthesis of the metal-indole conjugate

Reactants	MM[g/mol]	m [mg]	Eq	Density [g/ml]	V [µl]	mmol
Peptide	670.8	2	1	-	-	0.00298
Formaldehyd 40%	30.03	0.268	1.2	1.09	0.246	0.00358
Indole	524.11	1.562	1	-	-	0.00298

The peptide VLAKVAA was used in this reaction being added to the indole (Mn(bpg$^{6\text{-aminomethyl)indol}}$-k^{3}N)($CO_3$)) with a drop of formaldehyde. To conclude it was dissolved in 1 mL water and 0.2 mL dimethyl sulfoxide.

It was done twice, one reaction was heated to 50 ºC overnight and the other one was left at room temperature in the shaker overnight.

5.8. iClick reaction of TFSDL marked alkyne with a Ruthenium complex

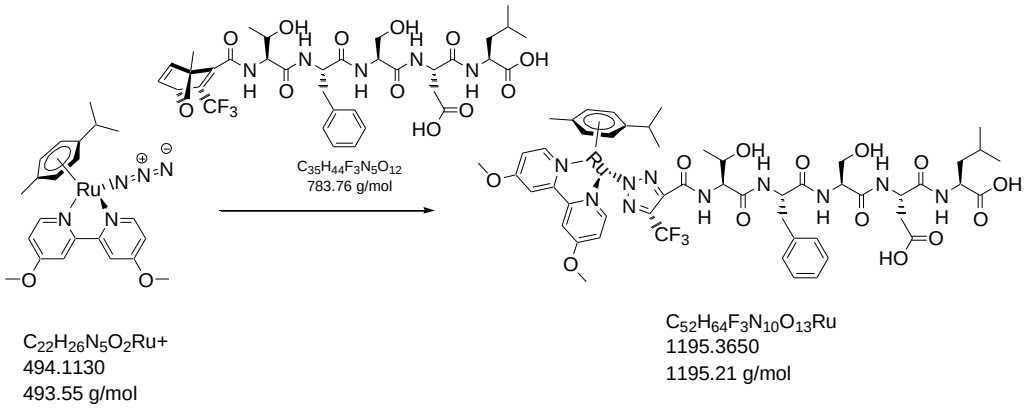


Figure 56: iClick reaction of TFSDL peptide with a ruthenium complex

The ruthenium complex ($Ru(N_3)(bpym)(p\text{-}cym)$), 0.5 mg, and 0.5 mg of the TFSDL already synthesized peptide with the diene as marked alkyne were dissolved in 1 mL of water. It was shaken and measured in the HPLC for 6 days.

6. References

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Declaration

I declare that this thesis (HPLC analysis of the organometallic modification of synthetic peptides: An evaluation of the indole and iClick conjugations) has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

Würzburg, the ……

…… (Sign)

Sofia Chueca Azlor