SYNTHESIS OF CHLOROTOXIN AND ITS DERIVATIVES AS ENZYME INHIBITORS

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SYNTHESIS OF CHLOROTOXIN AND ITS DERIVATIVES AS ENZYME INHIBITORS

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ABSTRACT

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Over the last decades, peptides have been heavily studied and proven beneficial in drug industry due to their high selectivity and potency compared to small organic molecules. A biologically and pharmaceutically significant peptide, chlorotoxin (CLTX), is one of the important peptide used in cancer studies. It is a neurotoxin, isolated from the venom matrix of Israeli Scorpion, Leirius quinquestriatus and it contains 36 amino acids and four disulfide bridges at a molecular mass of about 4 kDa. CLTX is mostly known for having strong interaction and inhibition with the matrix metalloproteinase-2 (MMP-2) enzyme, which is a zinc dependent enzyme and able to cleave all constituents in extracellular matrix.

In this thesis, CLTX and its derivatives were synthesized with the microwave assisted solid phase peptide synthesis (SPPS) based on 9-fluorenylmethyloxy-carbonyl (Fmoc) chemistry strategy in order to explain and improve inhibition of CLTX on MMP-2. Based on the preliminary docking results, lysine-15 position has a close proximity to catalytic zinc ion of MMP-2. In the light of this finding, the

strategy for inhibitor design in this study is based on the coordination of lysine-15 positions to the zinc ion after elongation with various carbon chain containing amino acids. Two more positions (lysine-23 and histidine-10) were also included to amino acid residues that will be modified because they were amenable to chemical modifications and located in protein interaction loop regions. In the modifications of lysine residues, elongation reactions were performed with three different amino acids with different number of carbons (glycine, methionine and cysteine). Methyltrityl (Mtt) protected lysine was used in the synthesis of CLTX in order to make the modifications on the solid support by the selective deprotection of Mtt group. In the modification of histidine-10 residue, Fmoc-Dap(N_3)-OH were used instead of histidine and modified by using copper(I) catalyzed azide-alkyne cycloaddition on solid phase (CuAAC-SP) with propargyl alcohol and propargyl amine. At the end of this reaction triazole ring was formed as a histidine analogue with an extra binding site. Biologic activity assays were applied on finalized modified CLTXs.

As a result, it was planned to synthesize eight different CLTX modifications (three for two of lysines and two for histidine). Synthesis and activity tests of two of lysine-15 modifications were completed. Unexpectedly, lysine modified CLTX derivatives increased MMP-2 activity, 2 fold for **P8F** and 1.5 fold for **P7F**, rather than inhibition. Four modified CLTXs at lysine residue (one of them at lysine-15 and three of them at lysine-23) were successfully synthesized and activity assays will be completed. Optimization studies for the histidine-10 modifications were successfully completed on a short test peptide (6 amino acid sequenced). The optimized modification procedures will be applied to CLTX.

Keywords: Chlorotoxin, microwave assisted SPPS, MMP-2, azido amino acids, CuAAC-SP.

ÖZ

KLOROTOKSİN VE TÜREVLERİNİN ENZİM İNHİBİTÖRÜ OLARAK SENTEZLENMESİ

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Son zamanlarda, küçük organik moleküllere kıyasla son derece seçici ve etkili olduğu için peptitler yoğun bir şekilde çalışılmış ve ilaç endüstrisinde faydaları kanıtlanmıştır. Biyolojik ve farmakolojik açıdan önemli bir peptit olan klorotoksin (CLTX) kanser çalışmalarında kullanılan önemli peptitlerden biri olmuştur. 4 kDa civarında moleküler kütlesiyle CLTX, İsrail akrebinin (*Leirius quinquestriatus*) zehir matriksinden çıkartılmış olup, 36 amino asit ve 4 disülfür köprüsü içermektedir. CLTX, çinko içeren ve hücre dışındaki bileşenleri parçalayabilen matriks metalloproteinaz-2 (MMP-2) enzimi ile girdiği güçlü etkileşme ve inhibisyonu dolayısı ile bilinmektedir.

Bu tezde, CLTX ve türevleri 9-florenilmetiloksikarbonil (Fmoc) stratejisi temel alınarak mikrodalga yardımlı katı faz peptit sentezi (KFPS) ile CLTX'in MMP-2 üzerindeki inhibisyonunu açıklamak ve geliştirmek için sentezlenmiştir. Yerleştirme (docking) öncalışmalarının sonuçları temel alındığında, lizin-15 pozisyonu MMP-2'nin katalitik çinko iyonuna çok yakın olduğu görülmüştür. Bu

bulgular ışığında, bu çalışmadaki inhibitör tasarım stratejisi, lizin 15 pozisyonunun farklı uzunluklardaki karbon zincirine sahip amino asitler ile uzatılarak çinko iyonuna koordinasyonuna dayandırılmıştır. Ayrıca kimyasal modifikasyonlara uygun oldukları ve protein etkileşim ilmik (loop) bölgesinde yer aldıkları için, modifiye edilecek amino asit rezidülerine iki pozisyon daha (lizin-23 ve histidin-10) eklenmiştir. Lizin rezidülerinin modifikasyonunda, uzatma tepkimeleri farklı sayılardaki karbonlu üç farklı amino asitle (glisin, metiyonin ve sistein) gerçekleştirilmiştir. CLTX sentezinde, metiltritil (Mtt) grubunun seçici olarak uzaklaştırılmasıyla modifikasyonları katı destekte yapabilmek amacıyla Mtt korumalı lizin kullanılmıştır. MMP-2 inhibisyonu denemesi, doğal CLTX ve lizin-15 modifikasyonlarının ikisi üzerinde uygulanmıştır. Histidin-10 rezidü modifikasyonları, Fmoc-Dap(N_3)-OH'nin histidin yerine kullanılmasıyla, katı fazda bakır(I) katalizörlü azit-alkin halkasal katılma tepkimesi (CuAAC-SP) propargil alkol ve propargil amin kullanılarak modifiye edilmiştir. Bu tepkimenin sonunda triazol halkası fazladan bağlanma mevkili histidin analoğu olarak oluşturulmuştur. Biyolojik aktivite denemeleri modifiye edilmiş tamamlanan CLTX'ler üzerinde uygulanmıştır.

Sonuç olarak, sekiz farklı CLTX modifikasyonlarının (lizinlerin ikisi için üç ve histidin için iki adet) sentezlenmesi planlanmıştır. Lizin-15 modifikasyonlarının ikisinin sentezi ve aktivite testleri tamamlanmıştır. Beklenenin aksine, lizinden modifiye edilmiş CLTX türevleri inhibisyonu artırmaktan ziyade MMP-2 aktivitesini **P8F** 2 kat ve **P7F** 1,5 kat artırmıştır. Lizin rezidüsünden (biri lizin-15'te ve üçü lizin-23'te olmak üzere) modifiye edilen dört CLTX başarıyla sentezlenmiş ve aktivite denemeleri yapılacaktır. Histidin-10 modifikasyonları için optimizasyon çalışmaları kısa bir test peptitinde (6 amino asit sıralı) başarıyla tamamlanmıştır. Optimize edilmiş modifikasyon prosedürlerini CLTX'in üzerinde uygulanacaktır.

Anahtar Kelimeler: Klorotoksin, mikrodalga yardımlı KFPS, MMP-2, azitli amino acid, CuAAC-SP.

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LIST OF ABBREVIATIONS

Boc	: Butyloxycarbonyl
CLTX	: Chlorotoxin
CuAAC	: Cu(I) catalyzed azide-alkyne cycloaddition
CuAAC-SP	: Cu(I) catalyzed azide-alkyne cycloaddition on solid phase
DIEA	: N,N-Diisopropylethylamine
DMSO	: Dimethylsulfoxide
EDTA	: Ethylenediaminetetraacetic acid
Fmoc	: Fluorenylmethyloxycarbonyl
HBTU	: N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate
HEPES	: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOBt	: 1-Hydroxybenzotriazole
MMP-2	: Matrix Metalloproteinase-2
Mtt	: Methyltrityl
SPAAC	: Strain promoted azide-alkyne cycloaddition
SPPS	: Solid Phase Peptide Synthesis
TFA	: Trifluoroacetic acid
TIPS	: Triisopropylsilane

CHAPTER 1

INTRODUCTION

1.1. Peptides

The peptides are described as compounds that are forming less than 50 amino acids. After 50 amino acids, it is called protein. Amino acids in peptides and proteins are bonded each other with peptide bonds also called amide bonds. There are different functional groups on different amino acids. These functional groups change many properties for amino acids, such as solubility, reactivity, acidity and basicity, also wavelength absorptions in UV. With combination of these amino acids and three-dimensional structures of peptides, infinite numbers of different peptides with different characteristics are possible.

Over the last three decades, using peptides as drugs has showed accelerated growth, and it is certain that it will continue to grow. First peptide used as drug, in the literature, was insulin and it is still being used as its modified version, insulin lispro.[1] Many different peptide drugs have followed insulin and many of them are being investigated.[2]

Compared to small molecule drugs, peptides have some advantages and disadvantages. These advantages and disadvantages are shown in the Table 1. In other words, small molecules have less targeting properties than peptides as a consequence of their small molecular sizes and it leads to various side-effects.[3]

Table 1. Advantages and disadvantages of peptide drugs.[3]

Advantages	Disadvantages
High potency	Poor metabolic stability
High selectivity	Poor membrane permeability
Broad range of targets	Poor oral bioavailability
Potentially lower toxicity than small molecules	High production costs
Low accumulation in tissues	Rapid clearance
High chemical and biological diversity	Sometimes poor solubility
Discoverable at peptide and nucleic acid levels	

1.2. Disulfide-rich Peptides

Since cysteine has an active thiol part, it is a quite unusual amino acid compare to other amino acids. With the help of this thiol part, between different cysteine residues of the same peptide or between cysteine residues of two separate peptide chains can make a disulfide bond in order to form a cystine.

Disulfide-poor peptides are highly inclined to enzymatic hydrolysis.[4] However, disulfide-rich peptides could reasonably decrease conformational flexibility and secure fixed structures of peptides. Fixed structure brings peptides more thermodynamic stability to enzymatic hydrolysis. Also fixing structures of peptides into their bioactive states provides an increased binding specificity and efficiency because binding cite of peptides will be more open to interact to their areas of interest.[5]

Unfortunately, in the literature, synthesis and activation of disulfide-rich peptides are thought to be difficult on account of the fact that it is highly probable to encounter different isomers, which are formed because of the plentiness of disulfide bridge possibilities, during the oxidative folding procedures.[6,7]

1.3. Cyclotides

Cyclotides are a very special group of cyclic proteins in which the main principle of usual topological simpleness is not followed. These peptides, extracted from plants such as Rubiaceae, Violaceae and Cucurbitaceae families, are not very long peptides and they have three disulfide bridges.[8] In Figure 1, cystine knot motif can be seen.

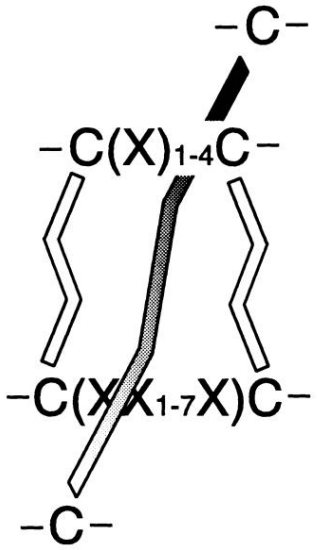


Figure 1. The cystine knot motif.[9]

Cyclotides includes both exceptional amide head to tail cyclized peptide backbone and have cystine knot structural design which consists of an embedded ring constructed by two disulfide bridges and their linking backbone parts penetrated by a third disulfide bridge. With the combination of these two factors, the cyclic cystine knot (CCK) yields a special three dimensional structure which is very complex and it has extremely higher biological and chemical stability than linear peptides. This stability is so extreme that even after boiling, cyclotides could show biological activity including uterotonic action, neurotensin antagonism, anti-HIV activity, and cytotoxicity.[10] In Figure 2, two members of cyclotides can be seen which are MCoTI-II and kalata B1.

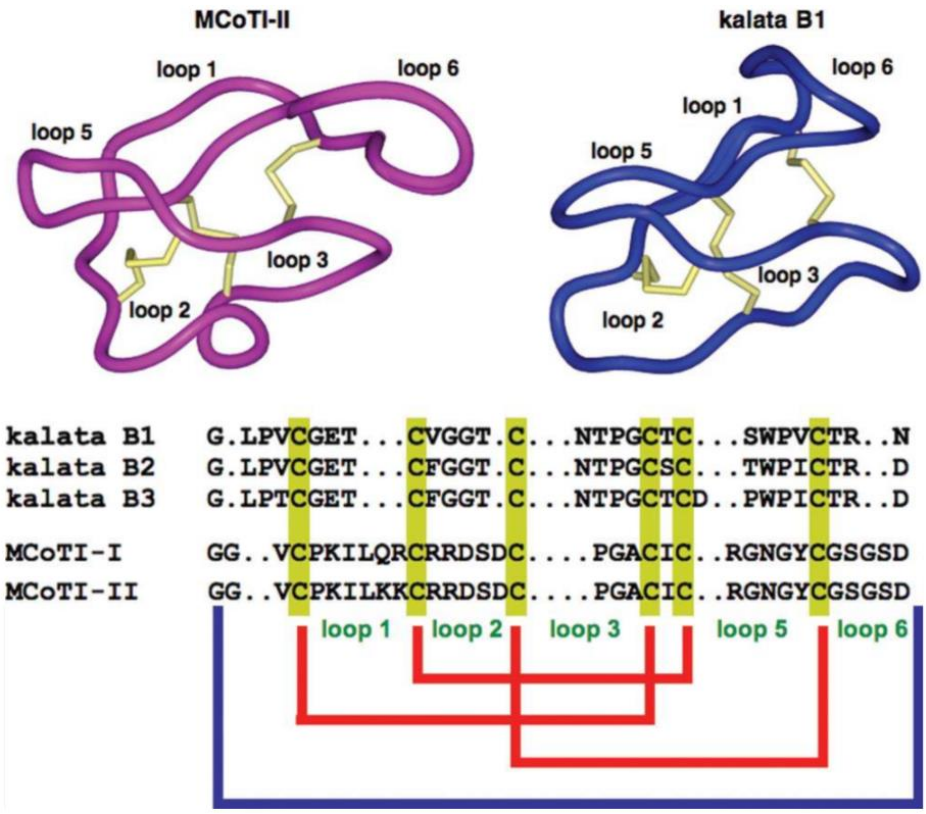


Figure 2. Primary and tertiary structure of cyclotides from the plants Momordica cochinchinensis (MCoTI-II) and Oldenlandia affinis (kalata B1). Red and blue connectors indicate backbone and disulfide bonds, respectively.[11]

1.4. Venom Peptides

A wide range of peptide toxins produced by venomous animals such as snails, spiders, scorpions, and snakes have been developed to catch prey and protect themselves. These animals store these peptide toxins in their specialized glands and when necessary, these toxins can be injected through biting or stinging the soft tissues of target.[12]

Majority of venoms contain a very complex variety of peptides and most of the time they have diverse and selective pharmacologies. In spite of their diverse features, venom peptides have been considered to evolve from a small number of structural templates which are very appropriate to address the important issues of potency and stability and it is exactly for their biodiversity that venom peptides are a very

beneficial source to develop new therapeutics due to their exclusive structural frameworks.[13]

Most of these venom peptides contain disulfide bridges. These bridges bring high resistance to various solvents, different pH's and wide range of temperatures and most importantly degradation by proteases in living organisms. Some examples of venom peptides with disulfide bridge connectivities can be seen in Table 2.[14]

Table 2. Some examples of venom peptides with disulfide bridge formations.[14]

Sequence, disulfide connectivity and pharmacology of selected venom peptides

Source	Name	Sequence	Connectivity	Action of class
Cone snail	α-GI	ECCNPACGRHYSC‡	1–3, 2–4	Muscle nAChR antagonist
	α-GID	IRγCCSNPACRVNNOHVC		Neuronal nAChR antagonist
Cone snail	μ-PIIIA	PRLCCGFOKSCRSRQCKOHRCC‡	1–4, 2–5, 3–6	TTX-sensitive VSSC inhibitor
Cone snail	ω-MVIIA	CKGKGAKCSRLMYDCCTGSCRSGKC‡	1–4, 2–5, 3–6	$Ca_v2.2$ inhibitors
	ω-CVID	CKSKGAKCSKLMYDCCSGSCSGTVGRC‡		
Cone snail	χ-MrIA	NGVCCGYKLCHOC	1–4, 2–3	NET inhibitor
Cone snail	ρ-TIA	FNWRCCLIPACRRNHKKFC‡	1–3, 2–4	α_1-Adrenoceptor inhibitor
Cone snail	Conantokin G	GEγγLQγNQγLIRγKSN	None	NMDA receptor inhibitor
Cone snail	Contulakin G	PSEEGGSNAT$_g$KKPYIIL	None	neurotensin receptor agonist
Spider	GsMTx4	GCLEFWWKCNPNDDKCCRPKLKCSKLFKLCNFSSG	1–4, 2–5, 3–6	Mechanosensitive channel inhibitor
Scorpion	Chlorotoxin	MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR	1–4, 2–6, 3–7, 5–8	Chloride channel inhibitor

1.5. Chlorotoxin

Chlorotoxin (CLTX) is a neurotoxin, extracted from the venom matrix of Israeli Scorpion, *Leirius quinquestriatus* also known as deathstalker. The venom, which is considered as one of the deadliest venoms found in scorpions, is fatal for humans even with a low lethal dose.[15] CLTX consists of 36 amino acids and four disulfide bridges at a molecular mass of 3,995.71 g/mol.[16]

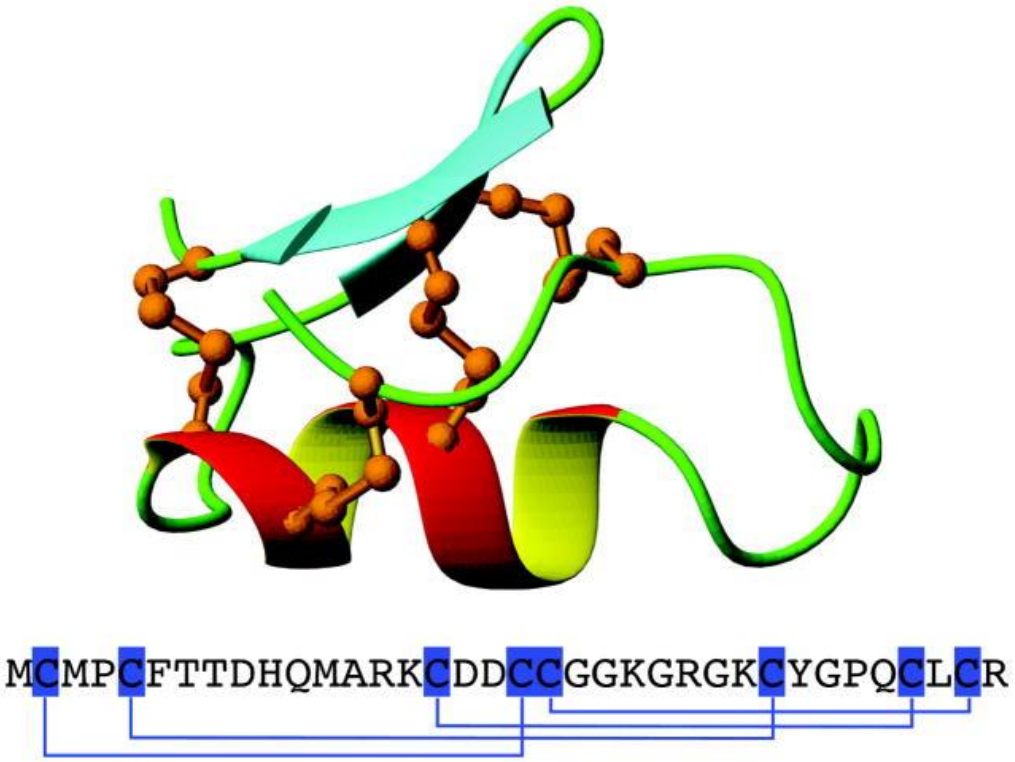


Figure 3. The tertiary structure, sequence and disulfide connectivities of CLTX.

It has been reported that CLTX reduces the membrane permeability to chloride ions in only troubled glioma cells, and consequently, prevents cell volume changes which are necessary to permit cancer cells to replace the healthy ones. CLTX takes its action most likely on a protein or receptor that manipulates a chloride channel found in troubled glioma cells.[17]

As a results of these properties of CLTX, when it is composed with a fluorescent molecular beacon, Cy5.5 NHS ester (Figure 4), CLTX:Cy5.5 bioconjugate can be used for imaging cancer tissues which provides surgeons to ability to demarcate

tumors from the healthy tissues.[18] In a research done by Seibel and his team in 2007, imaging properties of CLTX:Cy5.5 bioconjugate on mousses can be observed very clearly (Figure 5).[19]

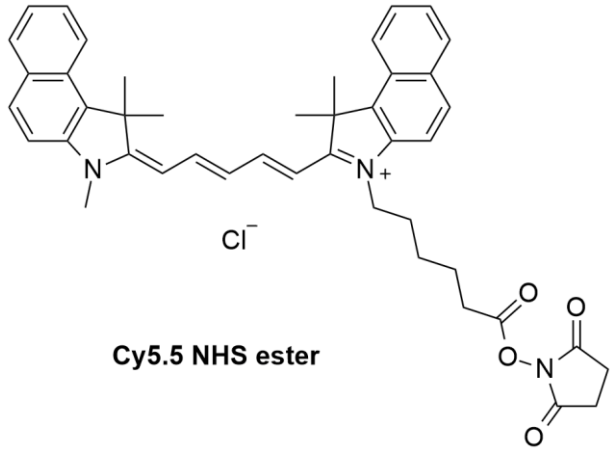


Figure 4. Fluorescent molecular beacon, Cy5.5 NHS ester.

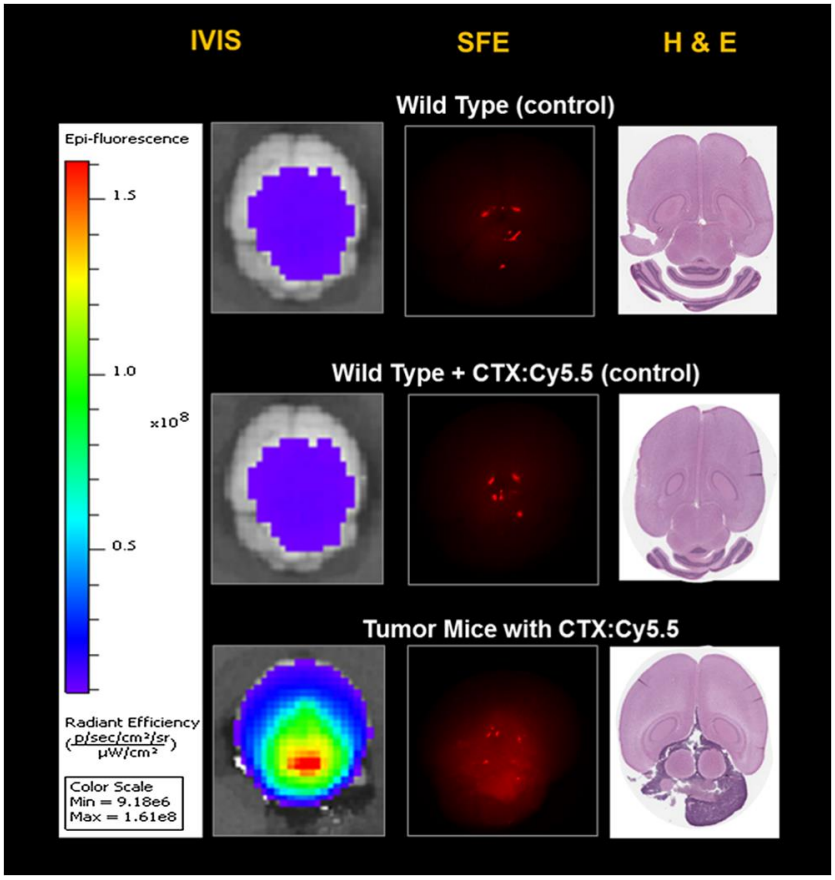


Figure 5. Tumor paint (CLTX:Cy5.5) imaging of mouse xenograft tumor.[19]

In an another research done by Sontheimer and in team in 2003, findings show that interactions between CLTX and matrix metalloproteinase-2 enzyme (MMP-2) explain the anti-invasive effect of CLTX on troubled glioma cells. According to these findings, CLTX has two effects on MMP-2: it lowers the enzymatic activity of MMP-2 and leads to a decreasing in the surface expression of MMP-2. Under the light of these two effects, it could be said that CLTX can be used as a specific inhibitor of MMP-2 with a promising therapeutic potential for glioma cells.[20]

The CLTX and its derivatives are generally synthesized by using solid phase peptide synthesis (SPPS) method.[21]

1.6. Matrix Metalloproteinase-2 (MMP-2)

MMP-2 is a 72 kDa type IV collagenase, quaternary structure can be seen in Figure 6, and it is a member of the matrix metalloproteinase enzyme family which is reliant to zinc and able to dismember all constituents in extracellular matrix and molecules that take part in signal transduction. In this way, MMP-2 helps tissue growth.[22]

Studies show that improved MMP-2 activity could be directly linked in different kinds of cancer such as colorectal, melanoma, breast, lungs, brain, ovarian and prostate.[23] The reason behind this is the ratio of MMP-2 concentration in cancer tissue being denser than the healthy tissues. Thus, the growth of cancer tissue becomes much higher than the normal tissues.[24]

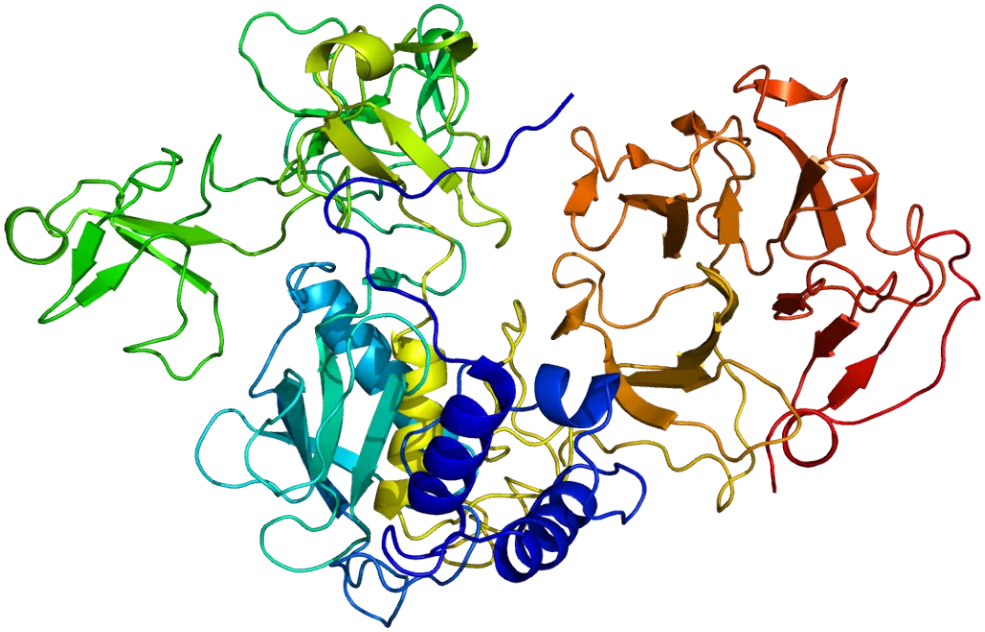


Figure 6. Quaternary structure of MMP-2.

1.7. Microwave Assisted Solid Phase Peptide Synthesis

In 1963, Robert Bruce Merrifield pioneered SPPS method.[25] Schematic representation of SPPS method can be seen in Figure 5. The idea behind SPPS is that a peptide chain could be constructed in a gradual way. The one side of the elongated peptide is bounded to a solid support. By this means, the peptide is bounded to an insoluble solid polymer. Therefore, there is no need for complex purification steps between each coupling. Thus, just by washing, unreacted and unwanted compounds could be removed from the system. Contrary to peptide/protein synthesis in living organisms, in SPPS, peptide synthesis goes from C-terminal to N-terminal of the peptide. In addition to peptide synthesis, solid phase synthesis could also be used for organic and polymeric synthesis.[26]

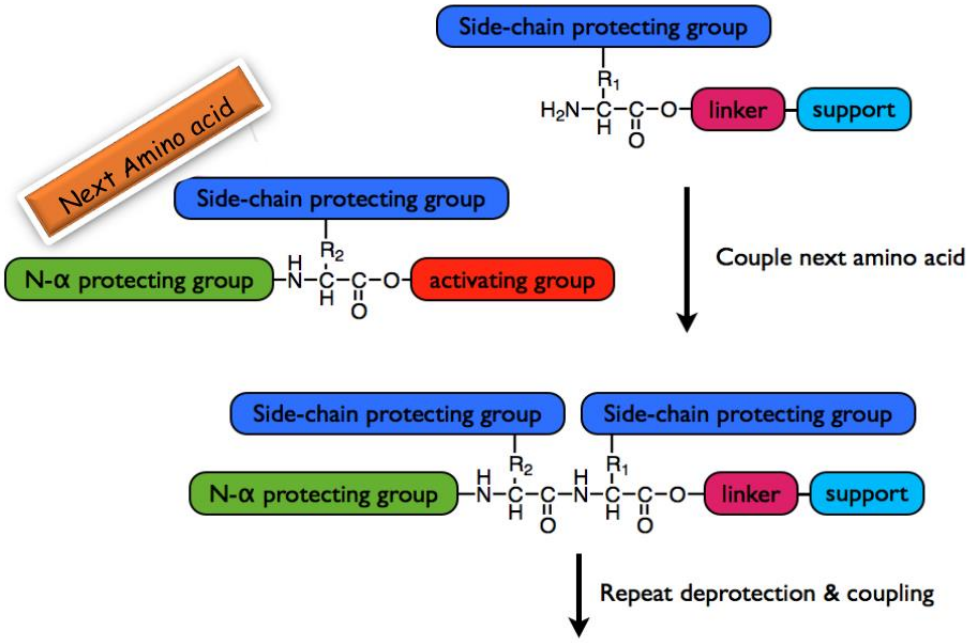
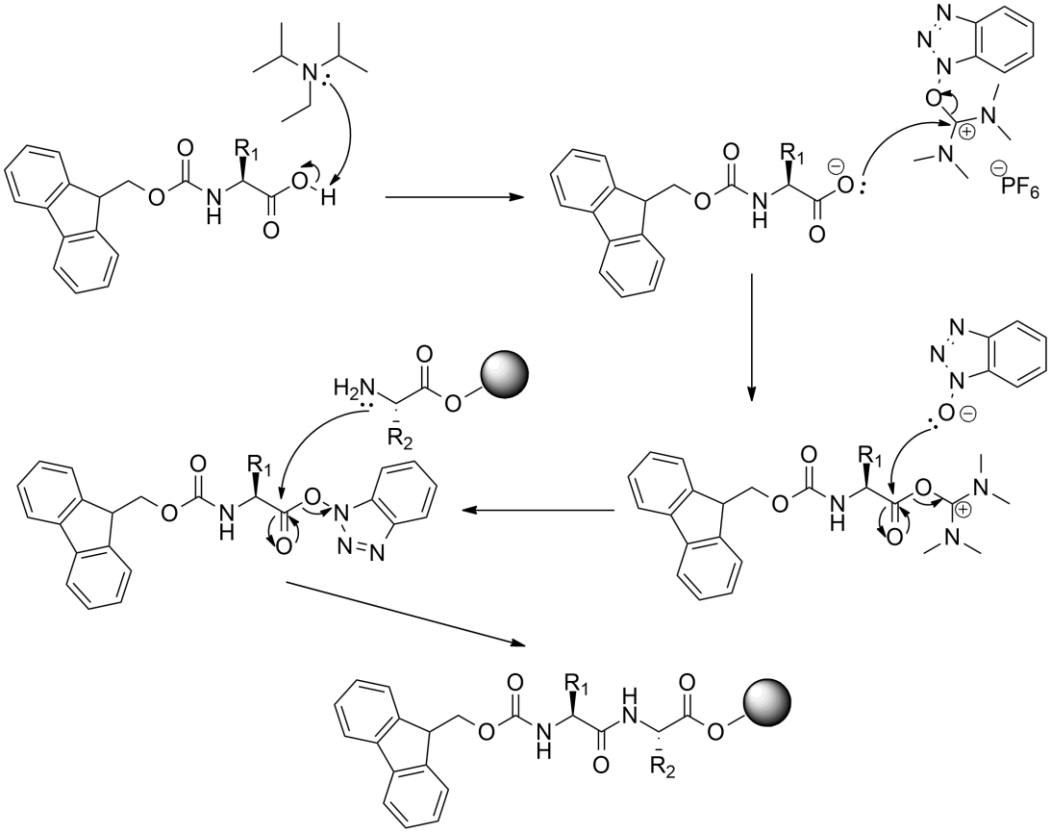


Figure 7. Schematic representation of SPPS method.

Recently, microwave use in peptide synthesis has made a breakthrough in SPPS because it increases the efficiency of coupling reactions, decreases solvent and reagent usage, shortens the coupling time, and enables coupling reactions which are very difficult to occur. Due to polar and ionic species, microwave energy leads to rapid increase the temperature of system during SPPS and this increase helps disturb chain aggregation caused from inter- and intrachain interactions. The resulting disturbance provides more accessible growing end of the backbone. [27]

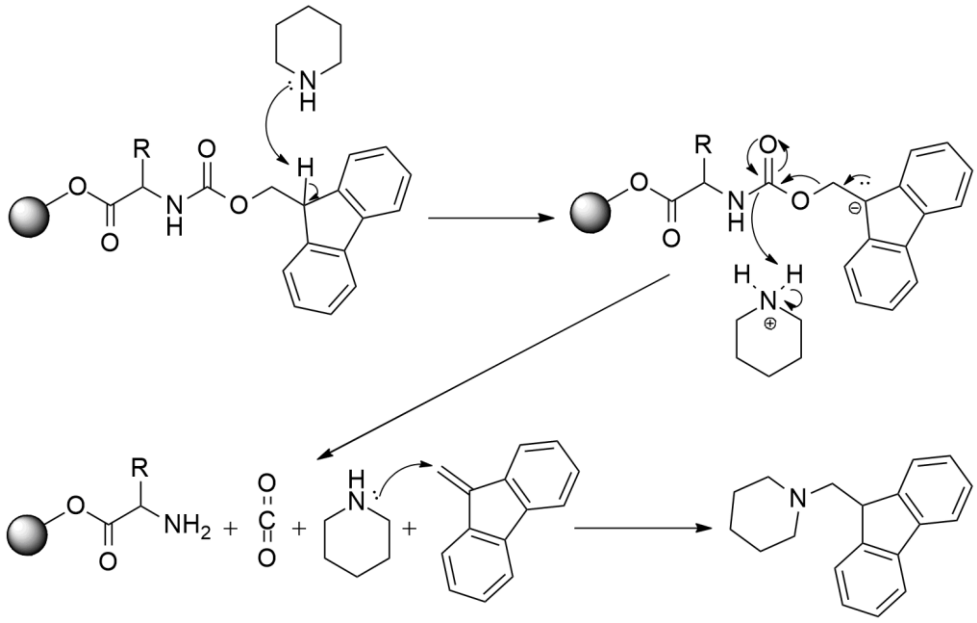
In addition to advantages of micro-wave use in peptide synthesis, it also has some disadvantages. Microwave use leads to aspartimide formation in the deprotection step if aspartic acid is present in the sequence. In order to prevent aspartimide formation a special deprotection solvent, which is 0.1 M 1-Hydroxybenzotriazole (HOBt) and 5% piperazine in dimethylformamide (DMF), could be used.[28] Besides aspartimide formation, the temperature caused by microwave results in racemization of cysteine and histidine residues. This problem could be overcome by adjusting temperature of microwave compartment not more than 50 °C.[28]

Fmoc and Boc strategies are the two main SPPS methods. They both have similar coupling reactions (Scheme 1). The differences between Fmoc and Boc methods are final cleavage and the protecting groups on α-amine and side chains on amino acids.



Scheme 1. C-Terminal activation via HBTU/DIEA mechanism.

In Fmoc strategy, Fmoc protects α-amine groups. Deprotection of Fmoc can be achieved through base catalyzed removal in the presence of piperazine and piperidine as secondary amine. The secondary amine deactivates the dibenzofulvene obtained in Fmoc cleavage. Fmoc deprotection mechanism can be seen in Scheme 2. Following the peptide chain growth, acid sensitive peptide resin linkers and protecting groups of amino acid side chains could be cleaved with trifluoroacetic acid (TFA).[29] In Boc strategy, Boc protects α-amine groups. Deprotection of Boc can be achieved via acid-catalyzed removal by using TFA. Following the peptide chain growth, both acid sensitive peptide resin linkers and protecting groups of amino acid side chains could be cleaved with hydrogen fluoride (HF) in gas phase.[30]



Scheme 2. Fmoc deprotection mechanism on a peptidyl resin.

1.8. Amino Acid Azides and Copper (I) Catalyzed Azide-Alkyne Cycloaddition (CuAAC) on SPPS

1.8.1. Amino Acid Azides

In peptide chemistry, using unnatural amino acids is a powerful method in order to develop new therapeutics, to understand biological chemistry and to attach different building blocks to peptides. Amino acid azides can be used for all three purposes via azide-alkyne Huisgen cycloaddition[31] and Staudinger[32] ligation reactions.

Recently, amino acid azides are commercially available yet expensive (Figure 8).

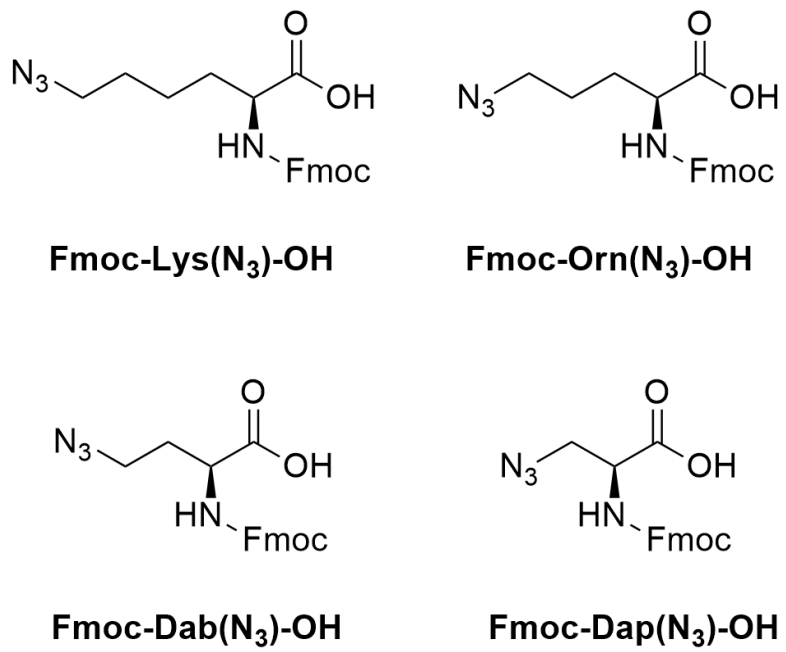


Figure 8. Some commercially available amino acid azides.

1.8.2. Synthesis of Amino Acid Azides

The most common method of introducing azide functionality to amino acids relies on the use of diazotransfer reagents. They are commonly used for conversion of primary amines to azides. Among the diazotransfer reagents, trifyl azide has been favored for a long time. However, recently, many organic sulfonyl-azide derivatives have been found in order to synthesize azides some of which could be seen in Figure 9.[33]

Imidazole-1-sulfonyl azide (ISA) synthesis is relatively easier and inexpensive, also, when precipitated with HCl it is a very shelf-stable compound. However, imidazole-1-sulfonyl azide needs copper or zinc ion to function.[34] Also ISA is explosive when concentrated or precipitated with HCl yet it is safe to handle in solid when precipitated with H_2SO_4.[35]

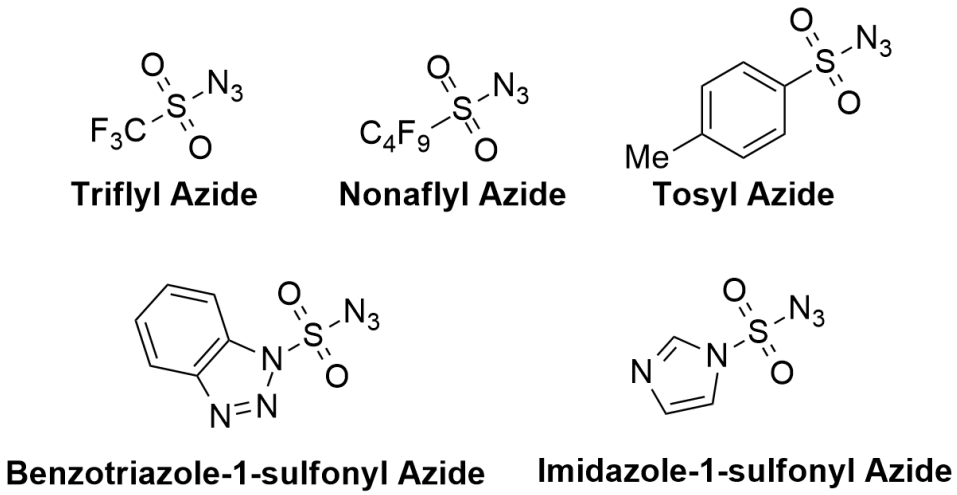


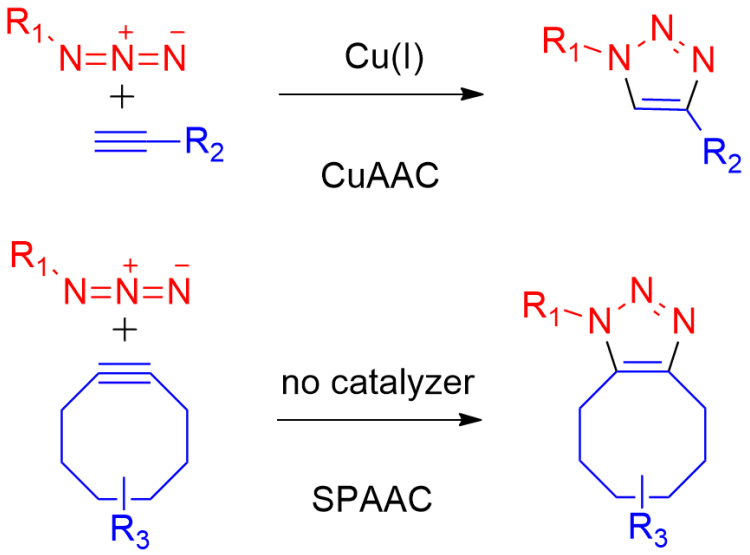
Figure 9. Diazotransfer reagents.

1.8.3. Copper(I) Catalyzed Azide-Alkyne Cycloaddition on Solid Phase (CuAAC-SP)

Click chemistry enables the two molecular building blocks to connect with certain selectivity, high yield and under mild conditions without or few byproducts. Azide-alkyne cycloaddition reactions, both metal catalyzed (mostly Cu(I)) and strain-promoted ones, are included to click chemistry. A general reaction scheme for Cu(I) catalyzed[36] (CuAAC) and strain-promoted azide-alkyne cycloaddition (SPAAC) reactions can be seen in Scheme 3.[37]

In 2002, Meldal and his colleagues reported that they have found a new method for carrying out azide-alkyne cycloaddition using Cu(I) salts as catalyst on solid phase at room temperature by using organic solvents like ACN, THF, DCM, toluene, and DMF.[38]

Mainly, various resins have been used to synthesize compounds like peptides, peptoids, PNA, and nucleotides. The extensive range of reaction possibilities has enabled the synthesis of a considerable diversity of final products via CuAAC-SP (Figure 10). And also reaction could be monitored by infrared spectroscopy, just by following removal of the characteristic azide band at around 2100 cm^{-1} in the infrared spectrum.[39]



Scheme 3. General reaction scheme of CuAAC and SPAAC.

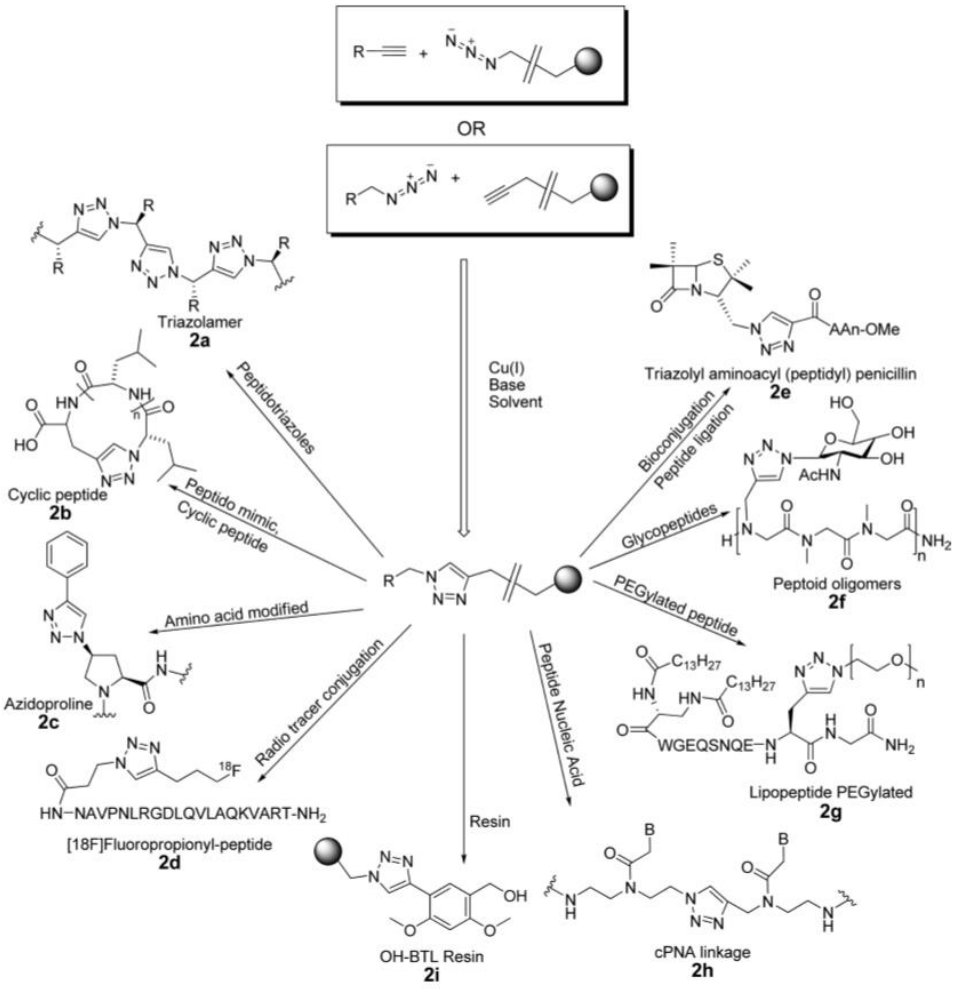


Figure 10. CuAAC on Solid-Phase Peptide Synthesis (SPPS).[39]

CHAPTER 2

EXPERIMENTAL

2.1. Materials and Methods

2.1.1. Materials

All commercial amino acids, coupling reagents and Rink Amide resin were supplied from Chem-Impex International Inc. USA. DIEA, which is activator base, was bought from Fisher Scientific. Piperazine and DMF were supplied from Sigma-Aldrich, and TFA, DCM, ACN and Et_2O from Merck. Other solvents used in synthesis of unnatural amino acids and column chromatography were technical grade. They were purified by distillation and dried with drying agents.

2.1.2. Chromatography

In order to obtain all artificial amino acids, all reactions were monitored by Merck aluminum thin layer chromatography (TLC) plates, silica gel coated with fluorescent indicator F_{254}, and the spots were visualized with 254 nm UV light. Column chromatography purifications were performed by using Merck Silica gel 60 (0.063-0.200 mm) for column chromatography (70-230 mesh ASTM).HPLC purifications of all peptides were done by Dionex Ultimate 3000 Series with four adjustable wavelength absorbance detector by using RP-C18 semi-preparative column (ODC Hypersil, 12 μm, 250 x 10 mm). Flow rate was 3.000 mL/min and mobile phase was gradient elution, Milli Q – 0.1% TFA and ACN – 0.08% TFA. Purities of the peptides were proved with RP-C18 analytical column (Acclaim 120, 3 μm, 4.6 x 150 mm) with a flow rate of 0.500 mL/min with the same mobile phase.

LC-MS, by using RP-C18 analytical column (Acclaim 120, 3 µm, 4.6 x 150 mm) with a flow rate of 0.500 mL/min and HRMS analysis was done with Agilent 6224 LC-MS-TOF with Milli Q – 0.1% Formic acid and ACN – 0.08% Formic acid at UNAM, İhsan Doğramacı Bilkent University.

2.1.3. Nuclear Magnetic Resonance (NMR)

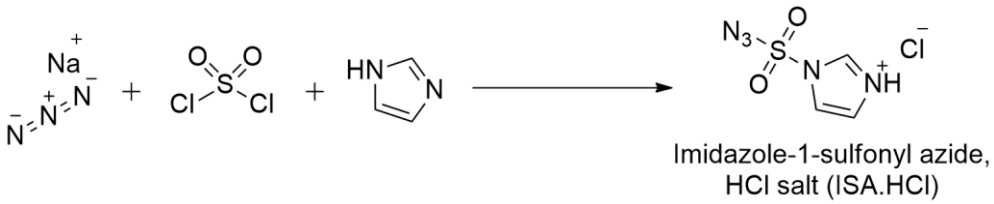
^{1}H-NMR data were obtain from Bruker Spectro Spin Advance 400 and as solvent, D_2O, d_6-DMSO and $CDCl_3$ were used in order to solve our samples. Chemical shifts were given in parts per million (ppm) with TMS as internal reference.

2.1.4. Microwave Synthesizer

All peptides synthesized with CEM Discover Bio manual microwave peptide synthesizer. Nitrogen gas was supplied from Linde, and bubbled during all coupling and deprotection steps.

2.2. The Reaction Procedures for Unnatural Amino Acids Synthesis

2.2.1. Synthesis of imidazole-1-sulfonyl azide, HCl salt (ISA.HCl)



Imidazole-1-sulfonyl azide, HCl salt (ISA.HCl)

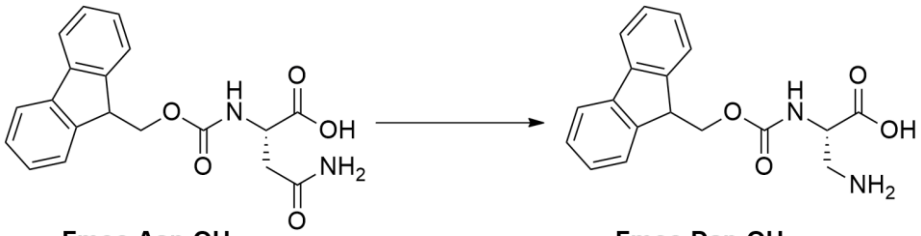
Based on literature procedure[40], 50 mL ACN was dried overnight with 3Å molecular sieve. Sulfuryl chloride (4.00 mL, 50.0 mmol) was added drop-wise to an ice-cold suspension of sodium azide (3.20 g, 50.0 mmol) in 50 mL dry ACN and stirred at room temperature overnight. Then imidazole (6.80 g, 0.10 mol) was added into the

mixture in an ice bath slowly with portions and resulting suspension was stirred at room temperature for 4 h. The resulting mixture was diluted with 100 mL ethyl acetate (EtOAc), and then washed with 100 mL distilled water and saturated aqueous sodium bicarbonate ($NaHCO_3$) solution two times for each. The organic phase was dried over $MgSO_4$ and filtered. After removal of solvent under vacuum imidazole-1-sulfonyl azide was obtained as a yellow oil.

Acetyl chloride (5.7 mL, 80 mmol) was added slowly to 20 mL dry ethanol in ice bath while stirring. Then resulting mixture was cooled to 0 °C and dropped slowly to imidazole-1-sulfonyl azide in an ice bath with stirring. Obtained suspension was filtered and the filter cake was washed with 100 mL cold EtOAc three times to give ISA.HCl as white powder. Yield 5.9 g (56%).

^{1}H NMR (400 MHz, D_2O): δ (ppm) 7.70 (1H, dd, *J* = 0.8, 1.7 Hz), 8.10 (1H, dd, *J* = 1.4, 1.7 Hz), 9.55 (1H, dd, J = 0.8, 1.4 Hz)

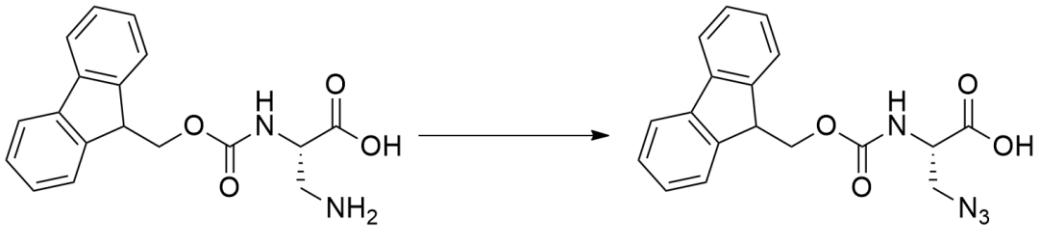
2.2.2. Synthesis of Fmoc-Dap-OH



Based on literature procedure[41], Fmoc-Asn-OH (5.55 g, 15.6 mmol) was added to a biphasic mixture of 24 mL of ACN, 24 mL of EtOAc and 12 mL of distilled water. Diacetoxyiodobenzene (6.10 g, 18.8 mmol) was added in portions to resulting suspension with strenuous stirring and then mixture was stirred overnight. Resulting mixture chilled in an ice bath and the crystals were filtered off with a vacuum filter and washed with 50 mL ice cold EtOAc. Then the solid was dissolved in 75 mL of isopropyl alcohol at 70 °C. Mixture was cooled and the crystals were filtered off and residual solvent removed under vacuum to obtain Fmoc-Dap-OH as white solid. Yield 2.9 g (57%).

^{1}H NMR (400 MHz, d_6-DMSO): δ (ppm) 7.80 (2H, d, *J* = 7.4 Hz), 7.70 (2H, d, *J* = 5.5 Hz), 7.40 (2H, t, *J* = 7.2 Hz), 7.30 (2H, t, *J* = 7.0 Hz), 6.80 (1H, d, J = 4.6 Hz), 4.40–4.20 (3H, m), 3.70–3.85 (1H, m), 3.00 (1H, dd, *J* = 10.6, 4.1 Hz), 2.80 (1H, t, *J* = 10.5 Hz).

2.2.3. Synthesis of Fmoc-Dap(N_3)-OH



Fmoc-Dap-OH **Fmoc-Dap(N_3)-OH**

Based on literature procedure[42], Fmoc-Dap-OH (2.02 g, 6.22 mmol) was added to a biphasic mixture of 30 mL of distilled water, 60 mL of methanol (MeOH) and 50 mL of dichloromethane (DCM). $CuSO_4 \cdot 5H_2O$ (10.0 mg, 40 μmol) and ISA.HCl (4.04 g, 19.2 mmol) were added to mixture and pH was adjusted to 9 very carefully with aqueous Na_2CO_3 solution and left overnight under vigorous stirring. Then 60 mL DCM was added to the mixture and the aqueous phase was taken. The organic phase was extracted with 100 mL saturated $NaHCO_3$ solution two times. Then all aqueous phases were combined and washed with 100 mL Et_2O two times. After adjusting the pH to 2 with concentrated hydrochloric acid (HCl), aqueous phase was extracted with 100 mL Et_2O three times. The organic phase was dried over $MgSO_4$ and the solvent was removed under vacuum to obtain Fmoc-Dap(N_3)-OH as A yellow solid. Yield 2.6 g (54%). Final purification was carried by column chromatography with the mobile phase (3:1/EtOAc:MeOH). 1.9 g (yield 39%) white solid was obtained.

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.75 (2H, d, *J* = 7.5 Hz), 7.60 (2H, d, *J* = 6.2 Hz), 7.45 (2H, t, *J* = 7.5 Hz), 7.30 (2H, tt, *J* = 7.5, 1.1 Hz), 5.70 (1H, d, *J* = 7.6 Hz), 4.60–4.75 (1H, m), 4.50–4.35 (2H, m), 4.20 (1H, t, *J* = 6.7 Hz), 3.70–3.85 (2H, m).

2.3. Solid Phase Peptide Synthesis

2.3.1. A general procedure for microwave assisted SPPS

Deprotection of resin and coupling with an Fmoc protected amino acid:[43,44]

Rink Amide Resin (96.2 mg, 0.05 mmol) (Substitution: 0.52 meq/g) was weighed in reactor of microwave peptide synthesizer and it was washed with DMF two times, and the resin was swollen 20-30 min in 10 mL DMF. After swelling part, 10 mL 5% piperazine and 0.1 M HOBt in DMF was poured to reactor and set MW to 20 W, 75 °C and 1 min. After 1 min with N_2 bubbling, this part was repeated second time but 3 min. Then it was washed with DMF many times. After washing part, coupling part was applied. Fmoc protected amino acid (5.5 eq., 0.275 mmol) and HBTU (5.0 eq., 0.250 mmol) were weighed in a clean vial, and then 2.0 mL 0.3 M diisopropylethylamine (DIEA) in DMF was added to the vial. After addition of DIEA solution, resulting mixture must be added to reaction vessel in 1 min at max. MW synthesizer was set to 20 W, 75 °C and 5 min with N_2 bubbling. During the coupling of histidine and cysteine in order to prevent racemization, temperature was set to 50 °C.[28] After the completion of coupling, resin was washed with DMF extensively. When it is necessary, Kaiser Test can be applied in order to see that coupling is completed.

This process can be repeated, until the desired peptide elongation was obtained. Boc protected amino acids were used as last amino acid in case of CuAAC-SP.

Cleavage:

At the end of peptide elongation and the last Fmoc deprotection, resin was washed with DMF and transferred to a glass peptide synthesis reactor. Resin was washed with DCM and left for drying with pump open for 30 min. After resin was dried, cleavage cocktail, 95% TFA, 5% Milli Q and 5% TIPS, was added to the reactor (for each 50 mg resin, 1 mL cleavage cocktail was needed). 1,4-Dithiothreitol (DTT) or 1,2-ethanedithiol (EDT) can be used when peptide contains cysteine residue. After addition of cleavage cocktail, it was incubated for 1-2 hours. The cleaved peptide solution was precipitated with ice cold diethyl ether, the solution was

centrifuged and the peptide was washed twice with ether in order to remove small impurities.

2.3.2. A general procedure for Mtt group removal

Peptidyl resin was weighed to the peptide synthesis reactor and the resin was swollen in DCM for 20 min. 1.8% (v/v) TFA/DCM solution was added to the reactor (10 mL/1 g peptidyl resin), and waited for 3 min at room temperature. After 3 min, yellow solution in reactor was removed. This cleavage step was repeated nine times. At the end of the ninth step, resin was washed with DCM until the yellow color disappeared. Then, resin was washed with DMF.[45]

2.3.3. A general procedure for coupling from lysine residue

Peptidyl resin that Mtt group were removed was weighed to the peptide synthesis reactor and it was washed with DMF, and left the resin swell 20-30 min in 10 mL of DMF. Desired amino acid (3.5 eq.) and HBTU (3.0 eq.) were weighed in a scintillation vial, and then 0.3 M DIEA in DMF was added to the vial. After addition of DIEA solution, resulting mixture must be stirred and added to reaction vessel within 1 min. The solution was allowed to stand for 2-3 hours and agitated with a glass rod in every 15 min. Kaiser test was applied for the resin before washing. If it is positive, the solution must be allowed until the test is negative. When it was negative, resin was washed with DMF extensively. Then, resin was washed with DCM, and left for drying for 30 min.

2.3.4. A general procedure for CuAAC on solid phase

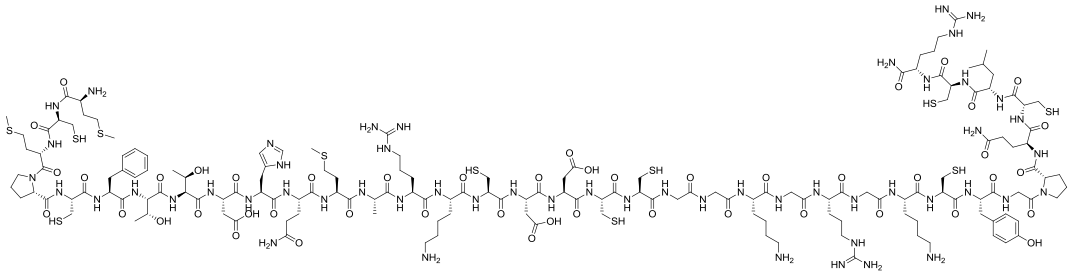
Peptidyl resin that was synthesized with azido amino acid was weighed to the peptide synthesis reactor and it was washed with DMF, and left the resin swell 20-30 min in 10 mL of DMF. 5 eq. of alkyne derivative, 5 eq. CuI and 5 eq. sodium ascorbate were weighed in a vial and mixed with 20% (v/v) piperidine/DMF solution. Final concentration of each reagent should be 61.25 mM. Resulting

mixture was added to reactor and the reaction was monitored with IR spectroscopy. After disappearance of azide peak (~2100 cm^{-1}) in IR spectrum, the resin was washed with 20% (v/v) piperidine/DMF solution. Then resin was washed with DMF and DCM subsequently and left for drying for 30 min.[46]

2.3.5. A general procedure for folding of CLTX and its derivatives

Glutathione redox buffer was prepared with the final concentrations of 0.1 M Tris.HCl, 1.0 mM EDTA, 10 mM reduced and 1.0 mM oxidized glutathione at pH 8.3. Peptide was added to glutathione redox buffer (10 mL buffer for every 1.0 mg peptide). The solution was allowed to stand 96 hours with slow stirring. The reaction was monitored with HPLC. After 96 hours, dialysis was performed in order to exchange out salts. Further purification was applied with RP-C18 semi-preparative column with HPLC.[47]

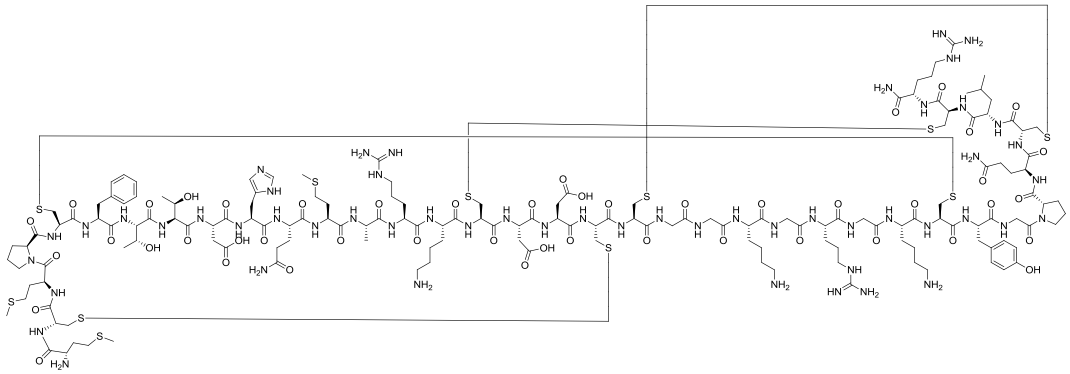
2.3.6. Synthesis of P1



General procedure for microwave assisted SPPS was applied in the synthesis of **P1**. Crude peptide was purified by RP-HPLC using C18 semi-preparative column.

HRMS $C_{158}H_{257}N_{53}O_{47}S_{11}$ $[M+3H]^{3+}$: Calculated 1334.5504, found 1334.5496.

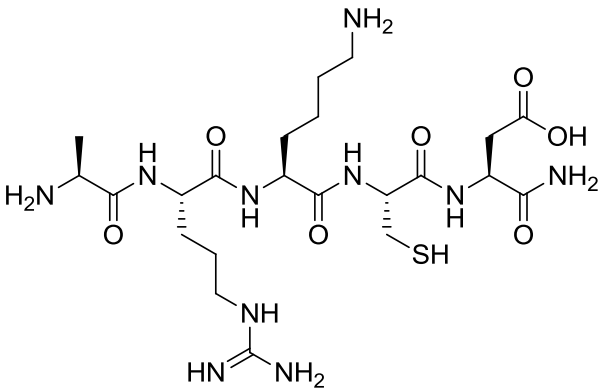
2.3.7. Synthesis of P1F



General procedure for folding of CLTX and its derivatives was applied in synthesis of **P1F**. Dialysis was performed in order to exchange out salts. Further purification was applied with RP-HPLC using C18 semi-preparative column.

HRMS $C_{158}H_{249}N_{53}O_{47}S_{11}$ $[M+2H]^{2+}$: Calculated 1997.2904, found 1997.2913.

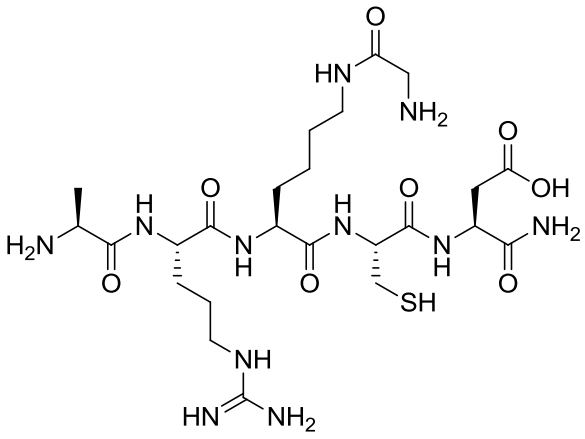
2.3.8. Synthesis of P2



General procedure for microwave assisted SPPS was applied in the synthesis of **P2**. Crude peptide is used for HRMS measurements.

HRMS $C_{22}H_{42}N_{10}O_7S$ $[M+H]^{+}$: Calculated 591.3032, found 591.3129.

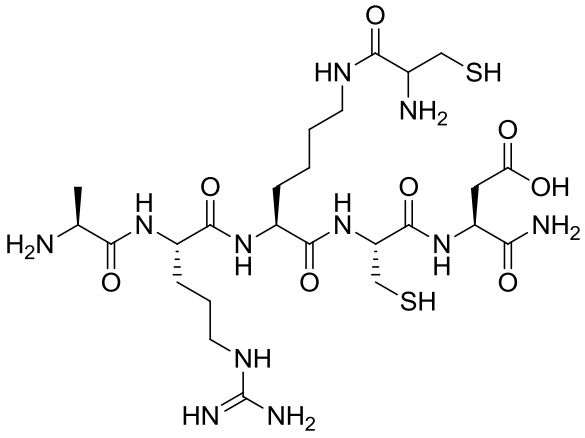
2.3.9. Synthesis of P3



General procedure for coupling from lysine residue was applied in the synthesis of **P3** from **P2** with glycine. Crude peptide is used for HRMS measurements.

HRMS $C_{24}H_{45}N_{11}O_8S$ $[M+H]^+$: Calculated 648.3247, found 648.3260.

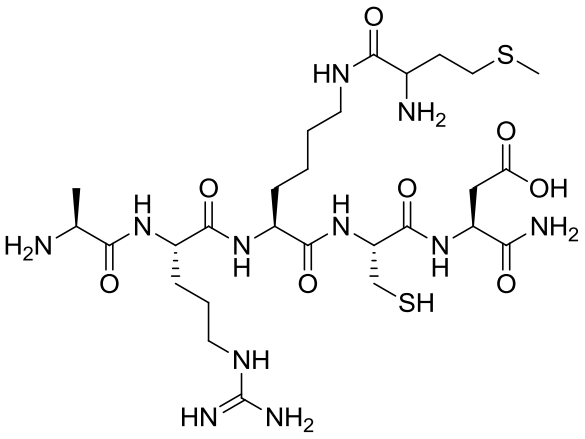
2.3.10. Synthesis of P4



General procedure for coupling from lysine residue was applied in the synthesis of **P4** from **P2** with L-cysteine. Crude peptide is used for HRMS measurements.

HRMS $C_{25}H_{47}N_{11}O_8S_2$ $[M+H]^+$: Calculated 694.3124, found 694.3117.

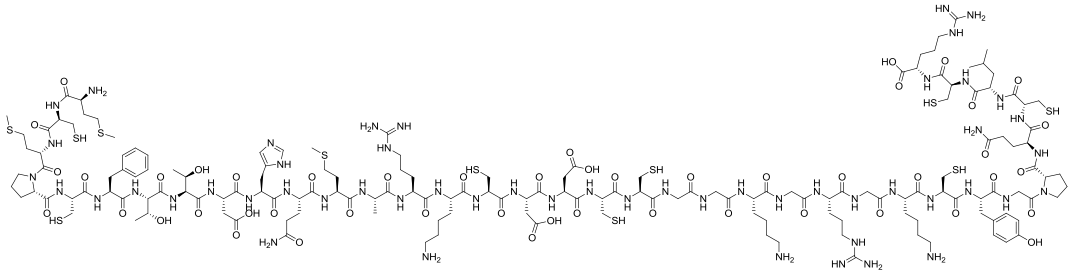
2.3.11. Synthesis of P5



General procedure for coupling from lysine residue was applied in the synthesis of **P5** from P2 with L-methionine. Crude peptide is used for HRMS measurements.

HRMS $C_{27}H_{51}N_{11}O_8S_2$ $[M+H]^+$: Calculated 722.3437, found 722.3464.

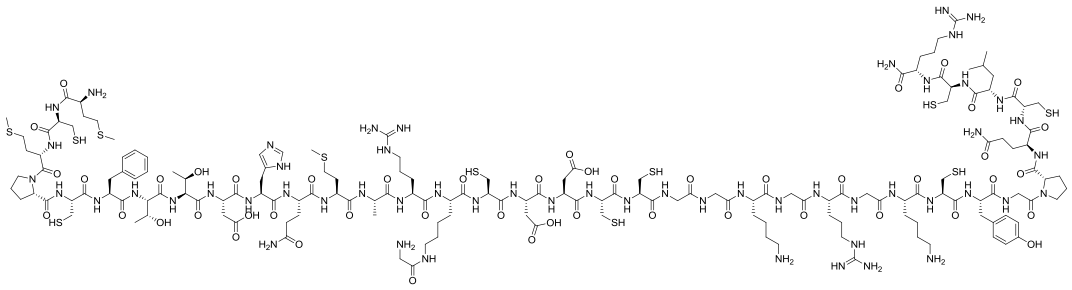
2.3.12. Synthesis of P6



General procedure for microwave assisted SPPS was applied in the synthesis of **P6**. Crude peptide was purified by RP-HPLC using C18 semi-preparative column.

HRMS $C_{158}H_{257}N_{53}O_{47}S_{11}$ $[M+3H]^{3+}$: Calculated 1334.5504, found 1334.5476.

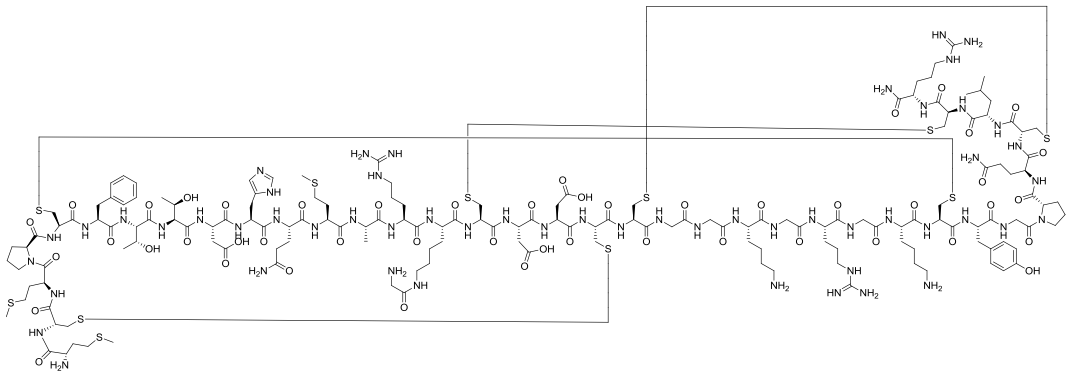
2.3.13. Synthesis of P7



General procedure for coupling from lysine residue was applied in the synthesis of **P7** from **P6** with glycine. Crude peptide was purified by RP-HPLC using C18 semi-preparative column.

HRMS $C_{160}H_{260}N_{54}O_{48}S_{11}$ $[M+3H]^{3+}$: Calculated 1353.5575, found 1353.5469.

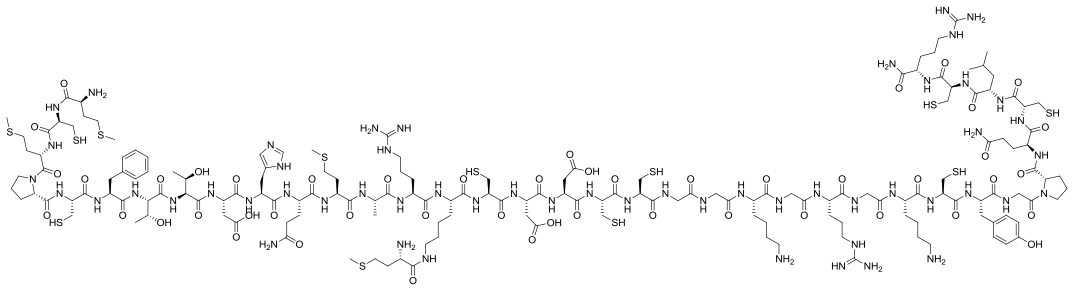
2.3.14. Synthesis of P7F



General procedure for folding of CLTX and its derivatives was applied in synthesis of **P7F**. Dialysis was performed in order to exchange out salts. Further purification was applied with RP-HPLC using C18 semi-preparative column.

HRMS $C_{160}H_{252}N_{54}O_{48}S_{11}$ $[M+3H]^{3+}$: Calculated 1350.8700, found 1350.8700.

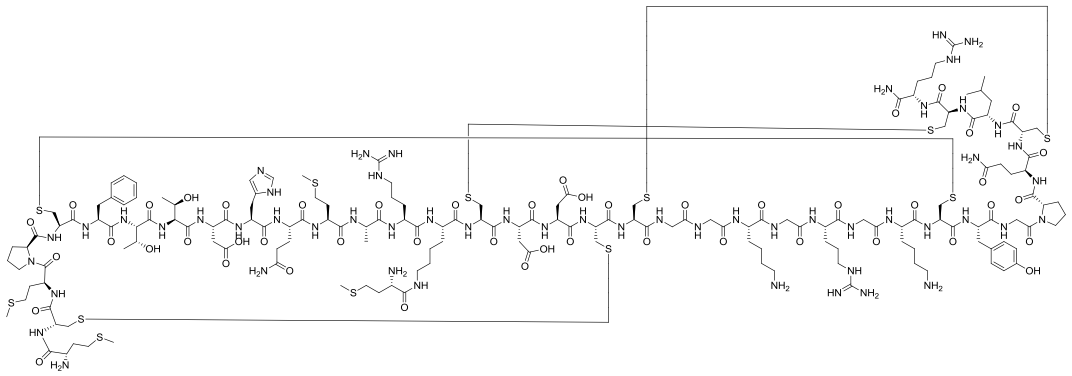
2.3.15. Synthesis of P8



General procedure for coupling from lysine residue was applied in the synthesis of **P8** from **P6** with L-methionine. Crude peptide was purified by RP-HPLC using C18 semi-preparative column.

HRMS $C_{163}H_{266}N_{54}O_{48}S_{12}$ $[M+3H]^{3+}$: Calculated 1378.2305, found 1378.2208.

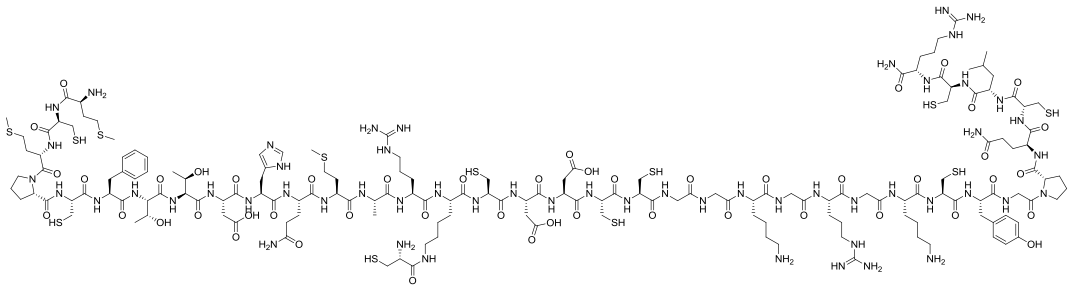
2.3.16. Synthesis of P8F



General procedure for folding of CLTX and its derivatives was applied in synthesis of **P8F**. Dialysis was performed in order to exchange out salts. Further purification was applied with RP-HPLC using C18 semi-preparative column.

HRMS $C_{163}H_{258}N_{54}O_{48}S_{12}$ $[M+4H]^{4+}$: Calculated 1032.1610, found 1032.1438.

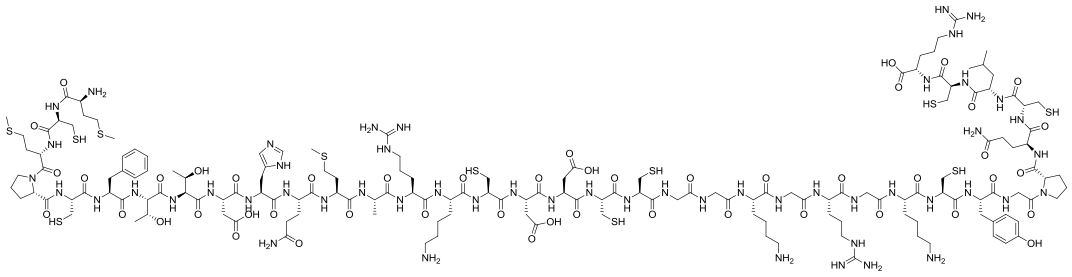
2.3.17. Synthesis of P9



General procedure for coupling from lysine residue was applied in the synthesis of **P9** from **P6** with L-cysteine. Crude peptide was purified by RP-HPLC using C18 semi-preparative column.

HRMS $C_{161}H_{262}N_{54}O_{48}S_{12}$ $[M+3H]^{3+}$: Calculated 1368.8868, found 1368.8823.

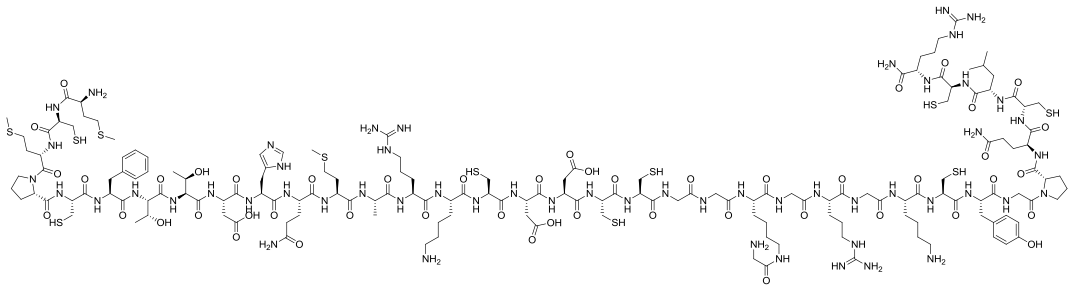
2.3.18. Synthesis of P10



General procedure for microwave assisted SPPS was applied in the synthesis of **P10**. Crude peptide was purified by RP-HPLC using C18 semi-preparative column.

HRMS $C_{158}H_{257}N_{53}O_{47}S_{11}$ $[M+3H]^{3+}$: Calculated 1334.5504, found 1334.5577.

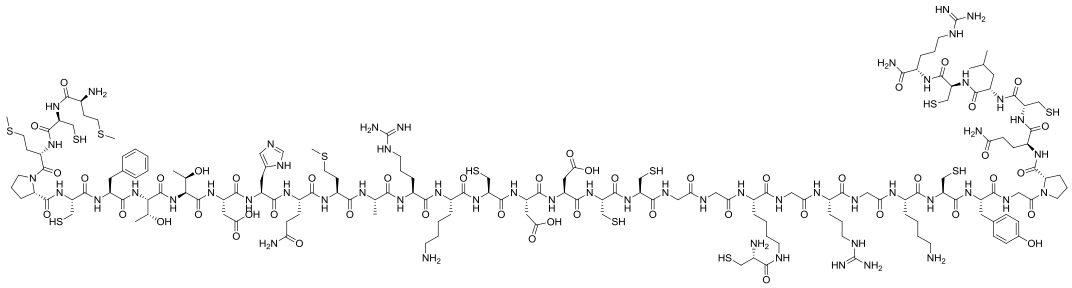
2.3.19. Synthesis of P11



General procedure for coupling from lysine residue was applied in the synthesis of **P11** from **P10** with glycine. Crude peptide was purified by RP-HPLC using C18 semi-preparative column.

HRMS $C_{160}H_{260}N_{54}O_{48}S_{11}$ $[M+3H]^{3+}$: Calculated 1334.5575, found 1334.5615.

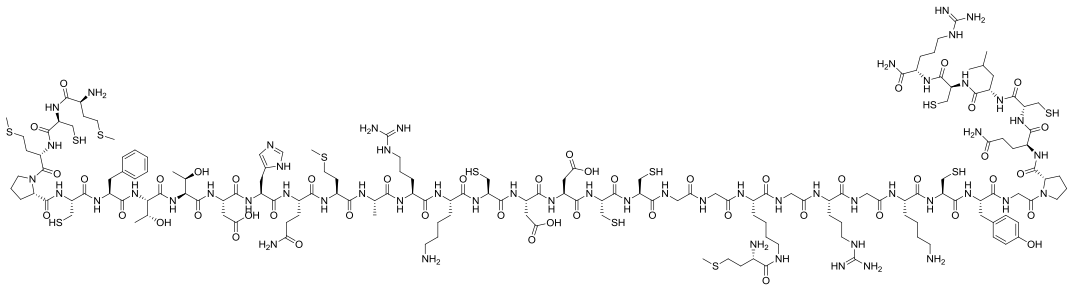
2.3.20. Synthesis of P12



General procedure for coupling from lysine residue was applied in the synthesis of **P12** from **P10** with L-cysteine. Crude peptide was purified by RP-HPLC using C18 semi-preparative column.

HRMS $C_{161}H_{262}N_{54}O_{48}S_{12}$ $[M+3H]^{3+}$: Calculated 1368.8868, found 1368.9327.

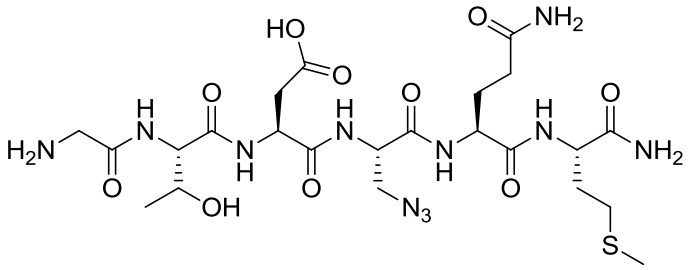
2.3.21. Synthesis of P13



General procedure for coupling from lysine residue was applied in the synthesis of **P13** from **P10** with L-methionine. Crude peptide was purified by RP-HPLC using C18 semi-preparative column.

HRMS $C_{163}H_{266}N_{54}O_{48}S_{12}$ $[M+3H]^{3+}$: Calculated 1378.2305, found 1378.2374.

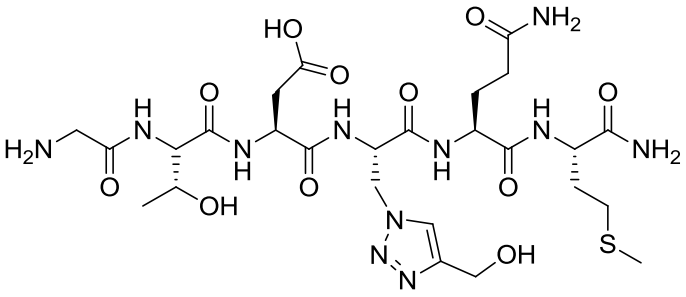
2.3.22. Synthesis of P14



General procedure for microwave assisted SPPS without introducing microwave was applied in the synthesis of **P14** at room temperature, coupling and Fmoc deprotection time were 30 min and 3-10 min respectively. Crude peptide is used for HRMS measurements.

HRMS $C_{23}H_{39}N_{11}O_{10}S$ $[M+H]^{+}$: Calculated 622.2675, found 622.2700.

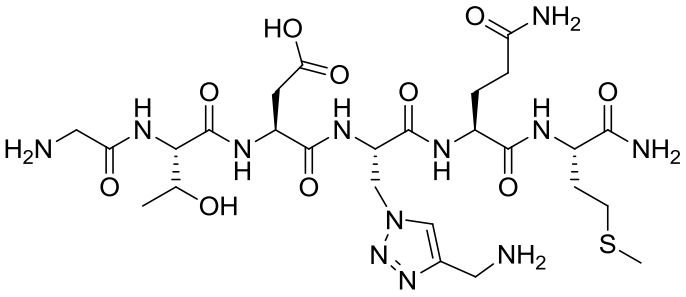
2.3.23. Synthesis of P15



General procedure for CuAAC on solid phase was applied in the synthesis of **P15** from **P14** with propargyl alcohol as alkyne derivative. Crude peptide is used for HRMS measurements.

HRMS $C_{26}H_{43}N_{11}O_{11}S$ $[M+H]^+$: Calculated 718.2937, found 718.2996.

2.3.24. Synthesis of P16



General procedure for CuAAC on solid phase was applied in the synthesis of **P16** from **P14** with propargyl amine as alkyne derivative. Crude peptide is used for HRMS measurements.

HRMS $C_{26}H_{44}N_{12}O_{10}S$ $[M+H]^+$: Calculated 717.3097, found 717.3148.

2.3.25. MMP-2 Inhibitor Screening Protocol

Preparing Stock Solutions:

MMP-2 Enzyme Stock: Enzyme was stored at -70 °C and used by aliquoting in liquid form after thawing with the final activity of 3.28 U/µL x 46 µL = 150 U (total).

MMP-2 Substrate Stock Solution: 40 µL of stock solution in DMSO was prepared with the final concentration of 250 µM substrate and stored at -70 °C.

Inhibitor Stock Solution: 50 µL of stock solution in DMSO was prepared with the final concentration of 1.3 mM inhibitor and stored at -70 °C.

Assay Buffer Solution: 20 mL of buffer solution was prepared using Milli-Q with the final concentrations of 50 mM HEPES, 10 mM $CaCl_2$ and 0.05% Brij® 35 at pH 7.5 and stored at room temperature.

CLTX Stock Solution: 80 µL of stock solutions was prepared using Milli-Q with the final concentration of 5 mM CLTX and stored at -20 °C.

Preparing Diluted Solutions:

Diluted Inhibitor Solution: 1 µL of inhibitor stock solution was diluted with 199 µL assay buffer to achieve the final concentration of 6.5 µM inhibitor and resulting mixture was warmed up to 37 °C.

Diluted CLTX Solution: 2 µL of CLTX stock solution was diluted with 18 µL of Milli-Q to achieve the final concentration of 500 µM CLTX and resulting mixture was warmed up to 37 °C.

Diluted MMP-2 Substrate Solution: 6 µL of substrate stock solution was diluted with 294 µL assay buffer to achieve the final concentration of 5 µM substrate and resulting mixture was warmed up to 37 °C.

Diluted MMP-2 Enzyme Solution: 11 µL of substrate stock solution was diluted with 611 µL assay buffer to achieve the final activity of 58 mU/µL MMP-2 and resulting mixture was warmed up to 37 °C shortly before assay.

Procedure:

Control: 70 µL Buffer + 20 µL MMP-2

Inhibitor: 50 µL Buffer + 20 µL MMP-2 + 20 µL Inhibitor

CLTX: 50 µL Buffer + 20 µL MMP-2 + 20 µL CLTX

Then wells were incubated 60 min at 37 °C to allow enzyme-inhibitor interaction and in order to start reactions, 10 µL of diluted substrate solution was added to each well. Plates in the fluorescent microplate reader was continuously read, using Ex/Em=540/590 nm, with cut-off set at 570 nm, data was recorded at 1 minute time intervals for 116 min.

Data Analysis:

Data as RFU (minus Blank RFU from Calibration) vs time was plotted for each sample. The range of initial time points which reaction was linear was determined. Initial reaction velocity (V) in RFU/min which is the slope of a line fit to initial linear portion of curve was recorded. Then, average of each calculated velocity was calculated.

CHAPTER 3

RESULT AND DISCUSSION

3.1. Synthesis of Native Chlorotoxin

Before starting the synthesis of CLTX derivatives, native CLTX (**P1**) was synthesized by using microwave assisted solid phase peptide synthesis based on Fmoc chemistry strategy with Rink amide resin. Crude HPLC Chromatogram of **P1** (Figure 11) was recorded by using RP-C18 analytical HPLC column.

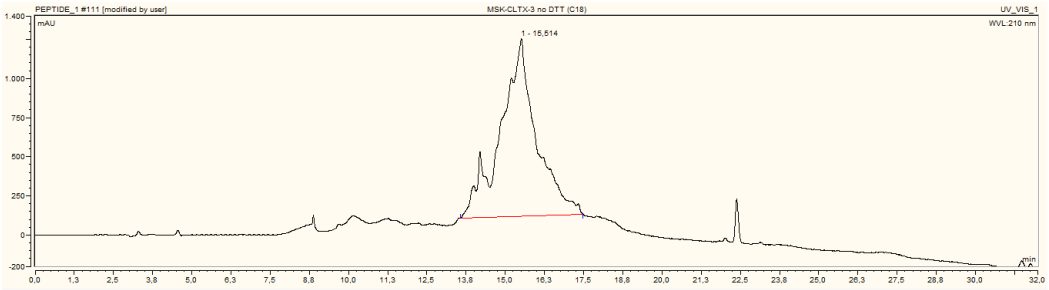


Figure 11. HPLC chromatogram of crude **P1**. Absorbance was monitored at 210 nm.

After the purification of **P1** by using RP-C18 semi-preparative column, more than 90% purity was achieved according to HPLC chromatogram and HRMS spectrum of **P1** (Figure 12).

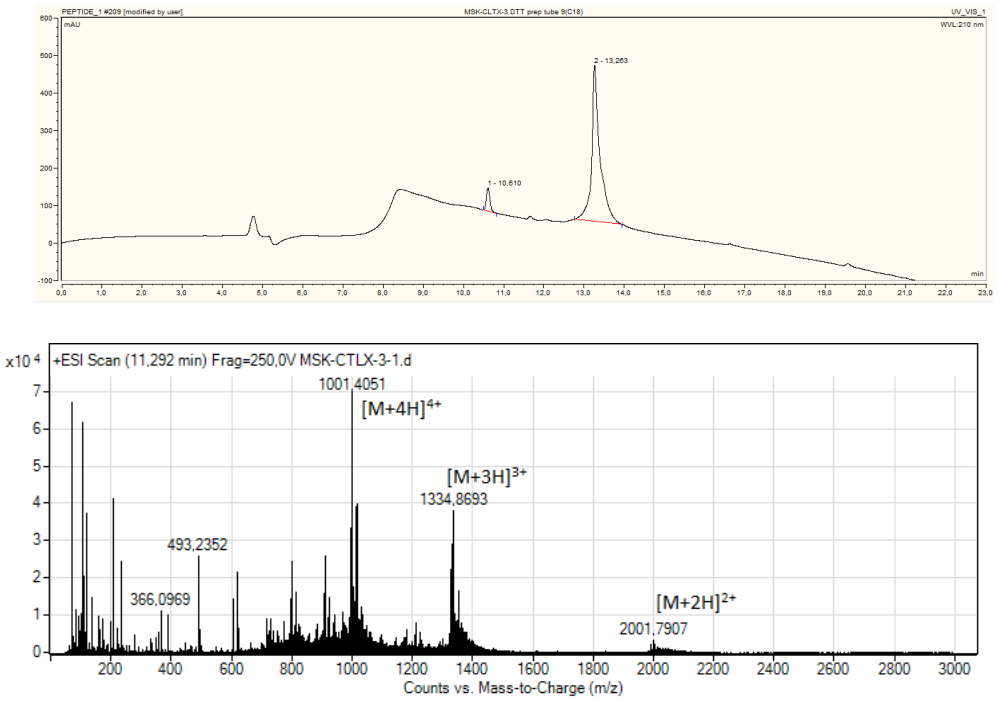


Figure 12. HPLC chromatogram and HRMS spectrum of purified **P1**. Absorbance was monitored at 210 nm.

After successful purification of **P1**, folding procedure was applied. For this purpose, certain amount of peptide is dissolved in folding buffer containing reduced and oxidized glutathione, EDTA, Tris at pH 8.3. After 96 hours of slow stirring, folded peptide was obtained by dialysis and semi-preparative HPLC.

HRMS spectrum of **P1F** (Figure 13) showed that folding procedure was succeeded. When CLTX is folded, it forms four disulfide bridges, which results the elimination of eight hydrogens. The mass difference in unfolded (**P1**) and folded CLTX (**P1F**) clearly indicated that there are four disulfide bridges.

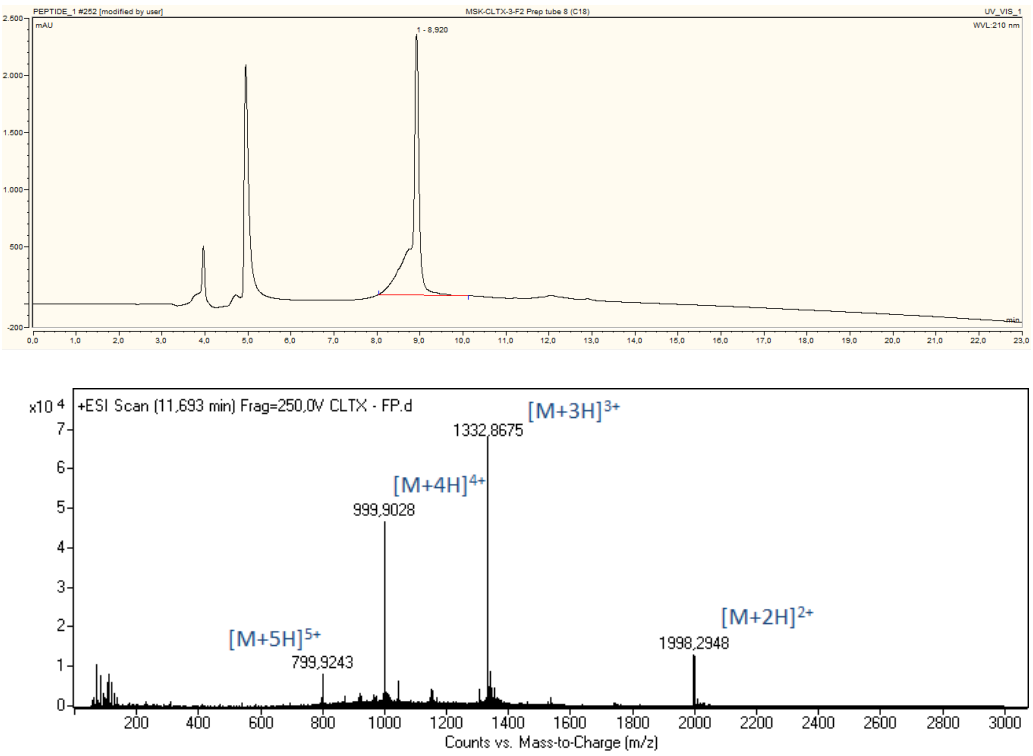


Figure 13. HPLC chromatogram and HRMS spectrum of purified **P1F**. Absorbance was monitored at 210 nm.

3.2. Synthesis of CLTX Derivatives at Lysine Residues

Rigid-body docking simulations were performed using the BioLuminate module of the "Schrödinger Biologics Suite" program in order to estimate the binding site of CLTX to MMP-2 and to determine amino acid positions close to the zinc ion. Since the 3D structure of the active form of MMP-2 has not yet been determined, the pro-domain of the inactive form Pro-MMP2 (PDB: 1CK7) was cut and energy minimization was performed. As the CLTX structure, 1CHL PDB encoded NMR structure was used. The BioLuminate software generated 30 possible complex structures; the first 10 of them were selected. A significant proportion of high-scoring structures indicated that CLTX bound to the active site of MMP-2. It has also been found that the lysine-15 residue of CLTX is at a position which is capable of coordinating to the zinc ion in the active site of MMP-2 and the distance between them was found 8.4 Å (Figure14).

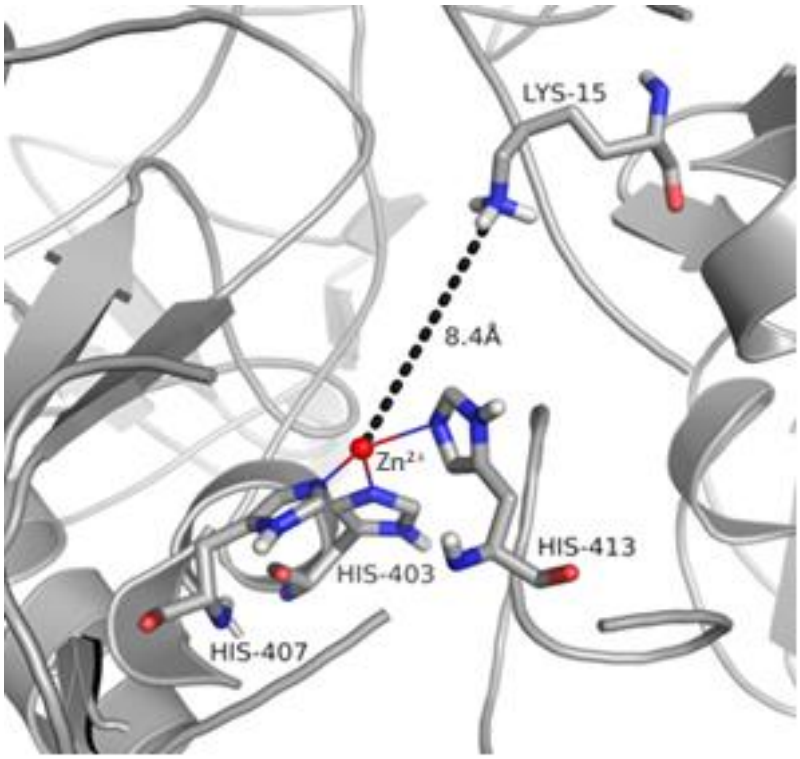


Figure 14. One of the lowest energetic CLTX-MMP-2 complex based on docking.

In the light of these findings, the strategy for inhibitor design in this study is based on the coordination of four positions (His10, Lys15, Lys23 and Lys27) on CLTX to zinc ion in MMP-2 active site via several modifications with different carbon chain lengths which are glycine, methionine and cysteine.

A previous report by Akcan *et. al*. showed that bulky fluorescent dye, attached to lysine-27 position of CLTX, did not change the activity of CLTX on MMP-2.[21] This indicates that CLTX is not interacting with MMP-2 through the site that lysine-27 is located. Thus, lysine-27 position was eliminated from our studies.

Based on the different positions of the targeted amino acids which include protein interaction loop regions, at least one of them is expected to be near the active site for zinc coordination. With these elongated parts on CLTX, we aimed to increase the inhibition effect (K_i) of CLTX on MMP-2.

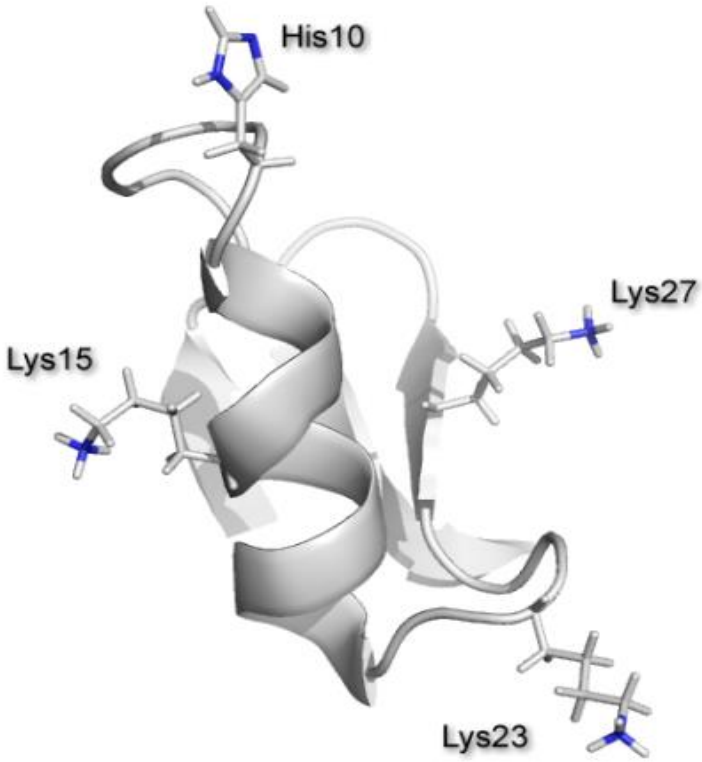


Figure 15. Possible critical positions on CLTX.

Three different amino acids with varying number of carbons were chosen in order to elongate lysine residues (Figure 16).

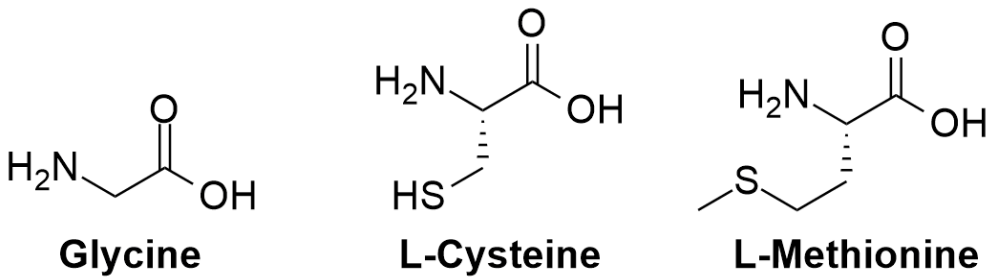


Figure 16. Amino acids that will be used in lysine modifications.

To perform elongation reactions on the solid phase, methyl trityl (Mtt) protected lysine was used in the synthesis of CLTX (Figure 17). Mtt protection group of lysine can be cleaved selectively without touching other protection groups, such as *tert*-butyloxycarbonyl (Boc) group, by using special deprotection procedure.

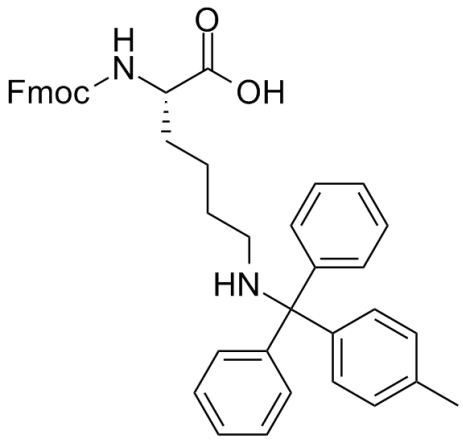


Figure 17. Molecular structure of Fmoc-Lys(Mtt)-OH.

3.2.1. Synthesis of fragment peptides modified from lysine residues

A fragment of CLTX was synthesized to see the feasibility of elongation reactions before synthesizing full sequence of CLTX.

Firstly, unmodified fragment peptide (**P2**) was synthesized with the sequence of ARKCD by using Mtt protected lysine unlike Boc protected one. Then, small amount of resin was taken and fully cleaved by TFA. HRMS spectrum of crude sample confirmed the synthesis of **P2** (Figure 18).

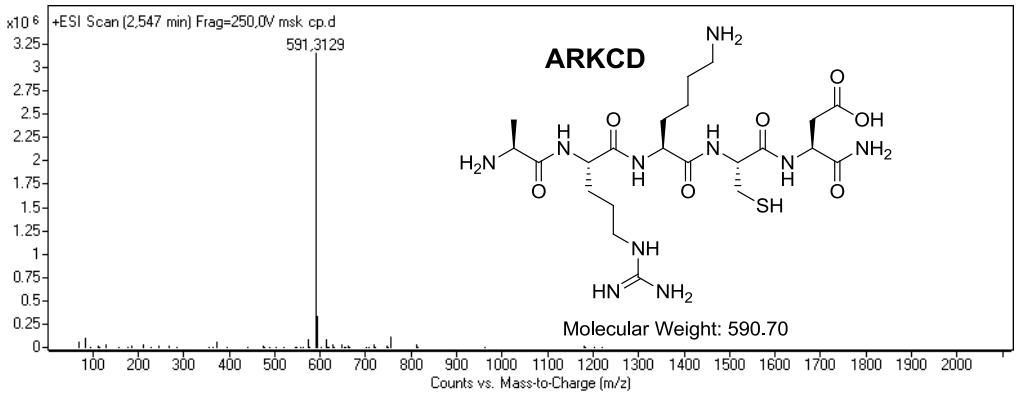


Figure 18. HRMS spectrum of crude **P2**.

Then, in solid phase, Mtt protection was cleaved by using 1.8% TFA in DCM, and Kaiser test was applied in order to monitor the reaction. Free amine group of lysine residue was coupled with Boc-Gly-OH. Small sample of obtained peptidyl resin was fully cleaved by TFA and glycine coupled fragment peptide (**P3**) was achieved according to HRMS spectrum of crude **P3** (Figure 19).

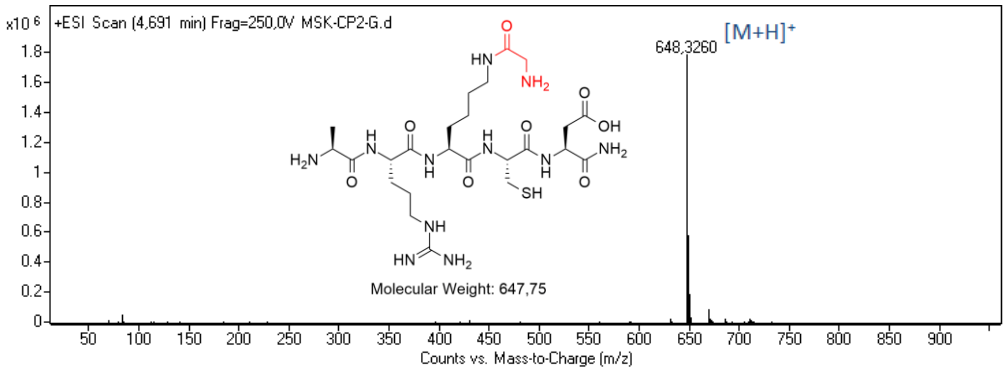


Figure 19. HRMS spectrum of crude **P3**.

P4 and **P5** were synthesized with the same method that **P3** synthesized by using $(Boc\text{-}Cys\text{-}OH)_2$ and Boc-Met-OH respectively. HRMS results of crude **P4** and **P5** showed that synthesis of **P4** and **P5** were succeeded (Figure 20 and 21).

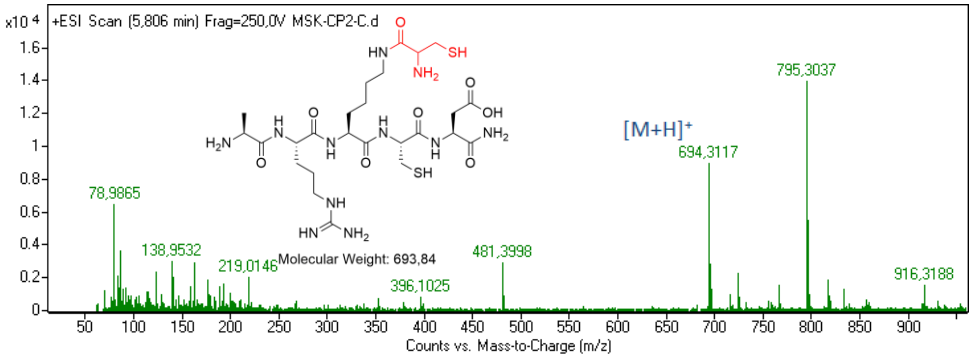


Figure 20. HRMS spectrum of crude **P4**.

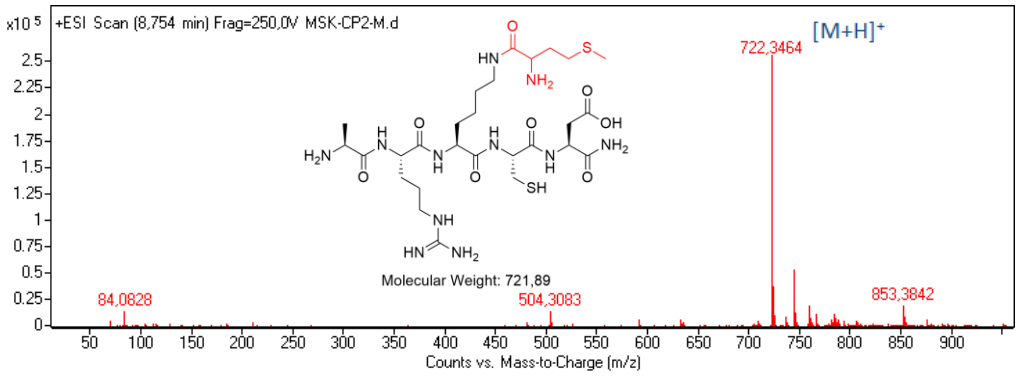


Figure 21. HRMS spectrum of crude **P5**.

3.2.2. Synthesis of CLTX derivatives at lysine-15 position

The synthesis of modified fragment peptides showed that elongation reactions were suitable in SPPS. As a next step, CLTX with Mtt protected lysine in 15th position (**P6**) was synthesized by using microwave assisted SPPS with Rink amide resin. Then, small amount of resin was taken and cleaved by TFA. HPLC chromatogram and HRMS spectrum of crude **P6** showed that synthesis of **P6** was succeeded (Figure 22).

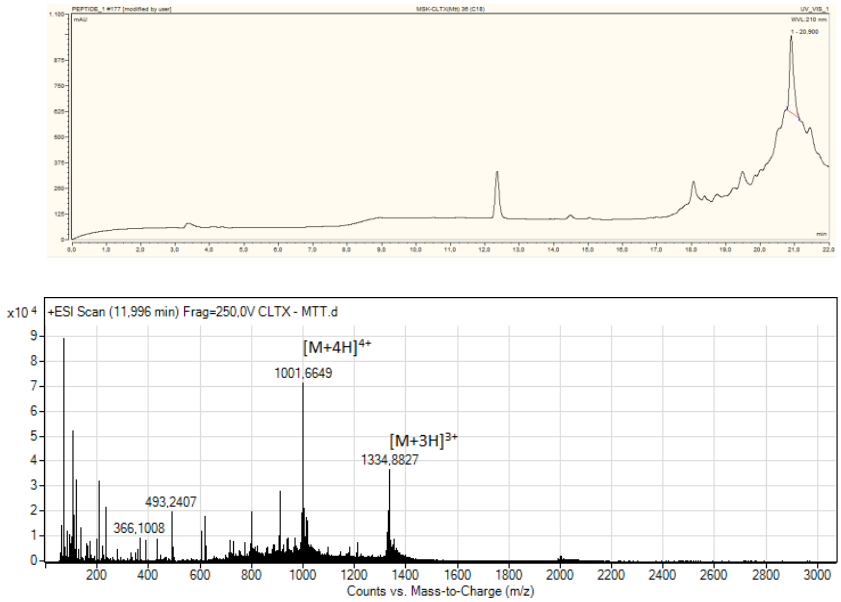


Figure 22. HPLC chromatogram and HRMS spectrum of crude **P6**. Absorbance was monitored at 210 nm.

Then, in solid phase, Mtt protection was cleaved by using 1.8% TFA in DCM, and Kaiser test was applied in order to monitor the reaction. Free amine group of lysine residue was coupled with Boc-Gly-OH. Small sample of obtained peptidyl resin was fully cleaved by TFA and glycine coupled CLTX (**P7**) was achieved according to HPLC chromatogram and HRMS spectrum of crude **P7** (Figure 23).

P8 and **P9** were synthesized from **P6**, with the same method that **P7** were synthesized, by using Boc-Met-OH and $(Boc\text{-}Cys\text{-}OH)_2$ respectively. HPLC chromatogram and HRMS spectrum for **P8** and **P9** showed that synthesis of **P8** and **P9** were succeeded (Figure 24 and 25).

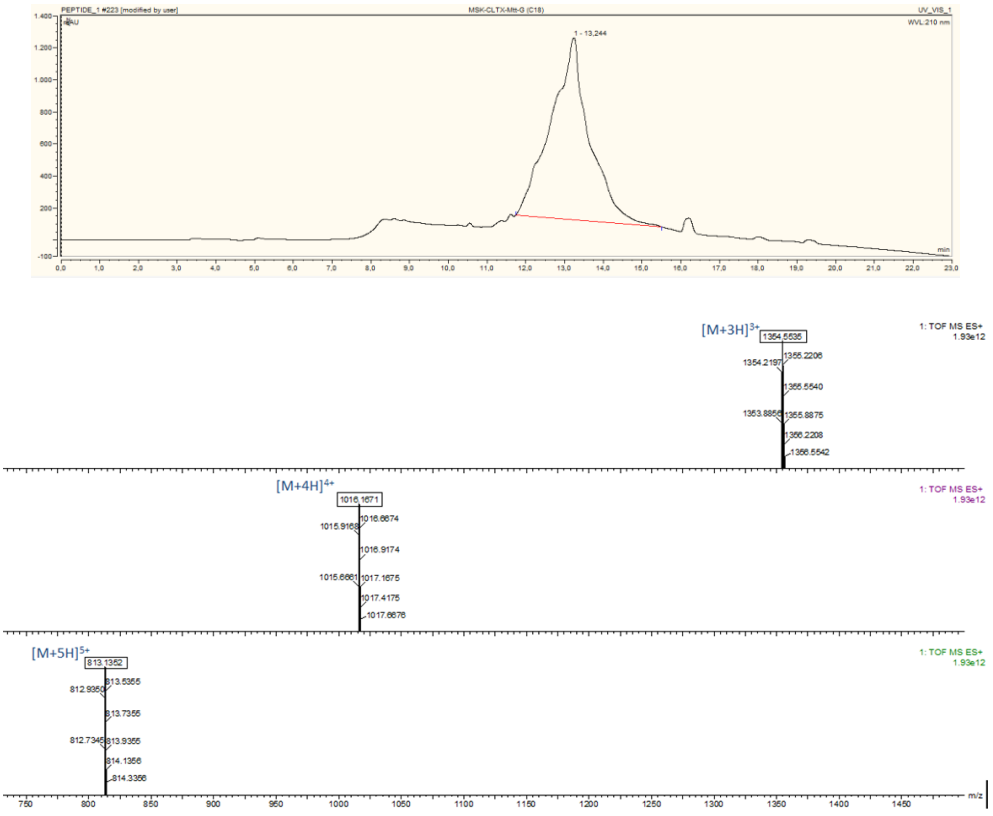


Figure 23. HPLC chromatogram and HRMS spectrum of crude **P7**. Absorbance was monitored at 210 nm.

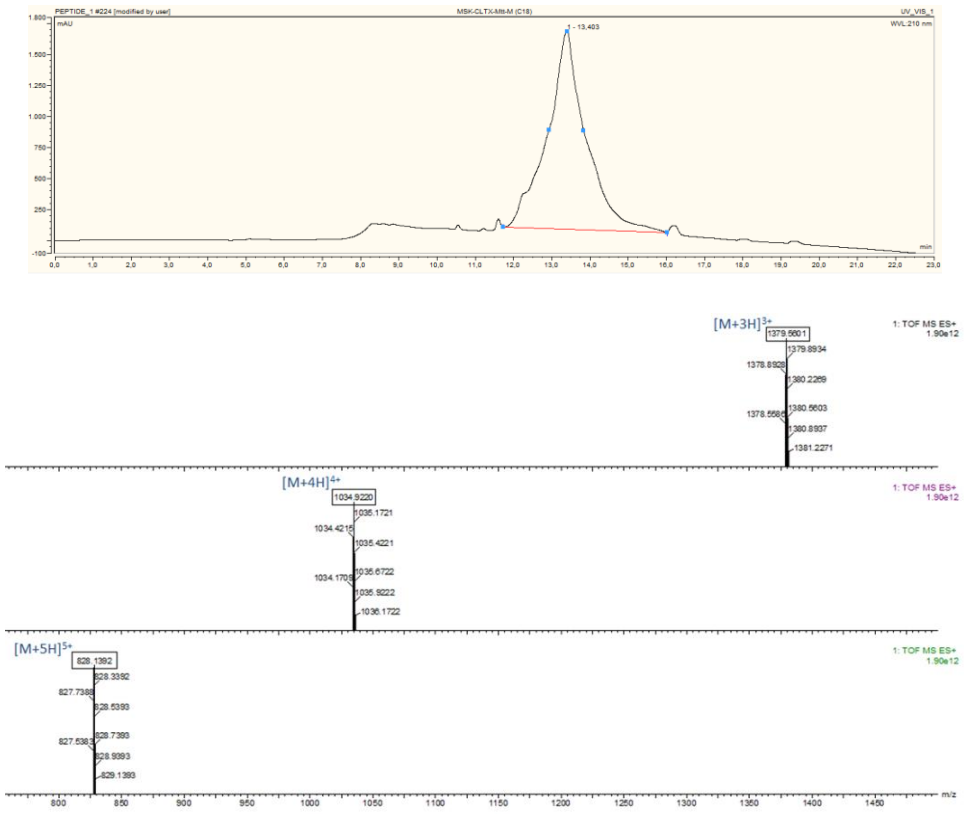


Figure 24. HPLC chromatogram and HRMS spectrum of crude **P8**. Absorbance was monitored at 210 nm.

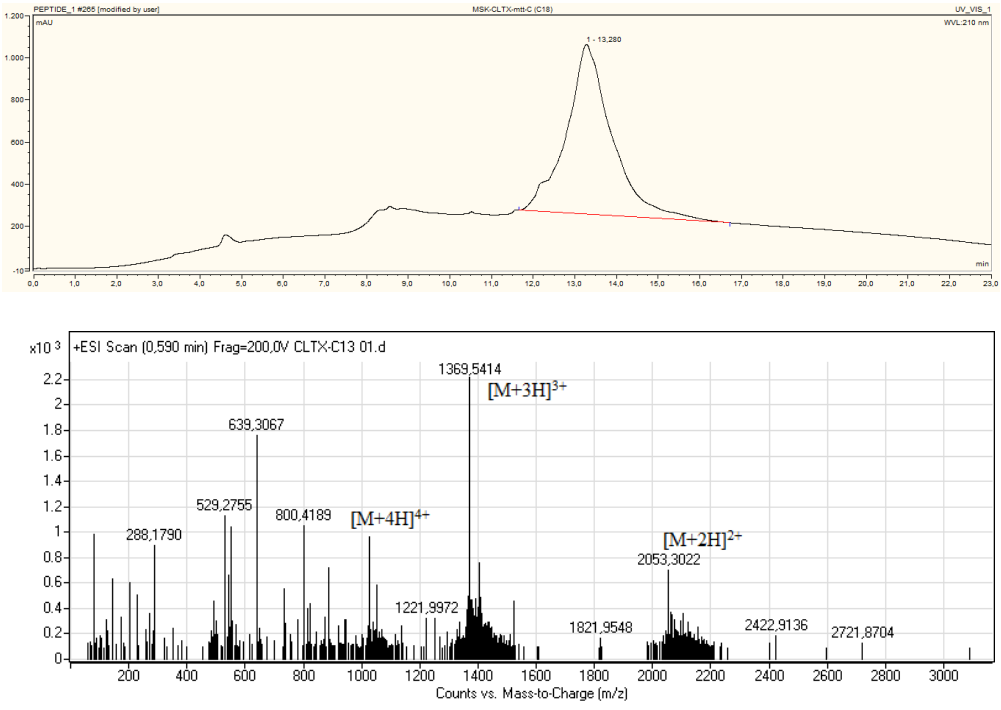


Figure 25. HPLC chromatogram and HRMS spectrum of crude **P9**. Absorbance was monitored at 210 nm.

After purification of **P7** and **P8**, folding procedure was applied to both of them. HRMS spectra of **P7F** (Figure 26) and **P8F** (Figure 27), showed that folding procedure were succeeded.

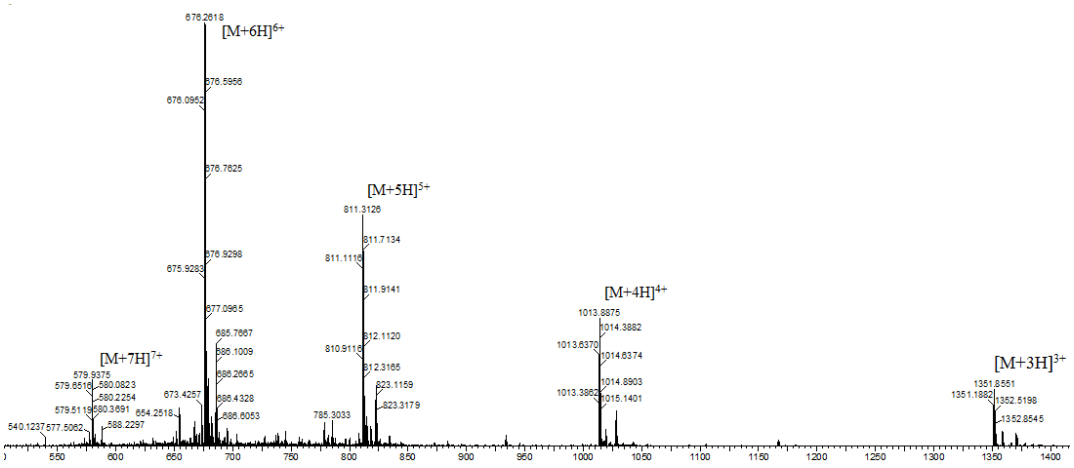


Figure 26. HRMS spectrum of purified **P7F**.

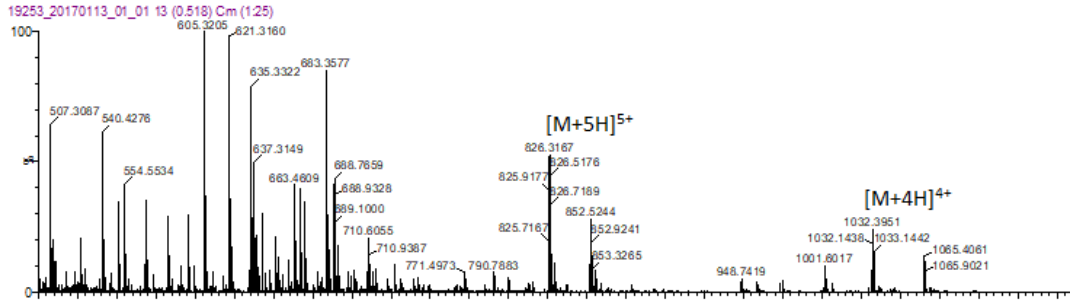
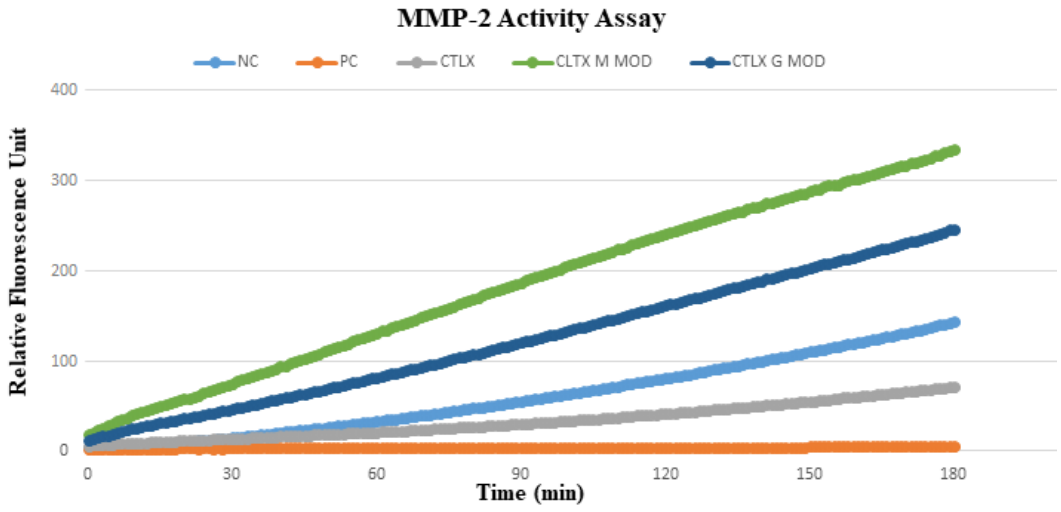


Figure 27. HRMS spectrum of purified **P8F**.

3.2.3. MMP-2 activity assay of CLTX derivatives at lysine-15 position

MMP2-Activity assay was performed using a commercial kit called Flourometric MMP-2 Inhibitor Screening Assay Kit. It is a complete assay system produced for screening inhibitors of MMP-2, using a quenched fluorogenic peptide which is called MMP-2 Fluorogenic Substrate, with a sequence of Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2. Mca fluorescence ((7-methoxycoumarin-4-yl)acetyl) is quenched by the Dpa group (*N*-3-(2,4-dinitrophenyl)-L-α,β-diaminopropionyl) until cleavage by MMP-2 between the Gly-Leu bond separates the two moieties. The

assays are operated in a proper 96-well microplate format. The MMP inhibitor NNGH (*N*-Isobutyl-*N*-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid) is used as a prototypic control inhibitor.



Samples	Activity of MMP-2 (pmol/min)	Inhibition of Activity %
Negative Control	812.6	
Positive Control (NNGH*)	14.5	98.2
P1F	367.2	54.8
P7F	1278.0	-57.3
P8F	1682.5	-107.1

* N-Isobutyl-N-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid

Figure 28. Biological activity assay results of **P1F**, **P7F** and **P8F**.

Biological activity assay results were unexpected (Figure 28). Negative control gave us the activity of MMP-2 which is about 800 pmol/min. As expected, positive control peptide (NNGH) and native CLTX (**P1F**) decreased the activity of MMP-2 around 98% and 50%, respectively. However, instead of inhibition, modified CLTXs, **P7F** and **P8F**, increased the activity of MMP-2 by 57.3% and 107.1%, respectively.

These results might be explained by the change of interaction of chlorotoxin with another site of MMP-2. This small change might cause activation of MMP-2 instead of inhibition.

A similar result was observed in a previous report by Voelter *et. al.* They showed that a scorpion venom peptide called Bs-Tx7 having 66% sequence identity with CLTX increased activity of MMP-2.[48] Although 3D structure of CLTX and Bs-Tx7 are quite similar, they showed opposite activity on MMP-2 (Figure 29).

These results suggested that even a small change in the structure of CLTX, there might have a huge difference in biological activity.

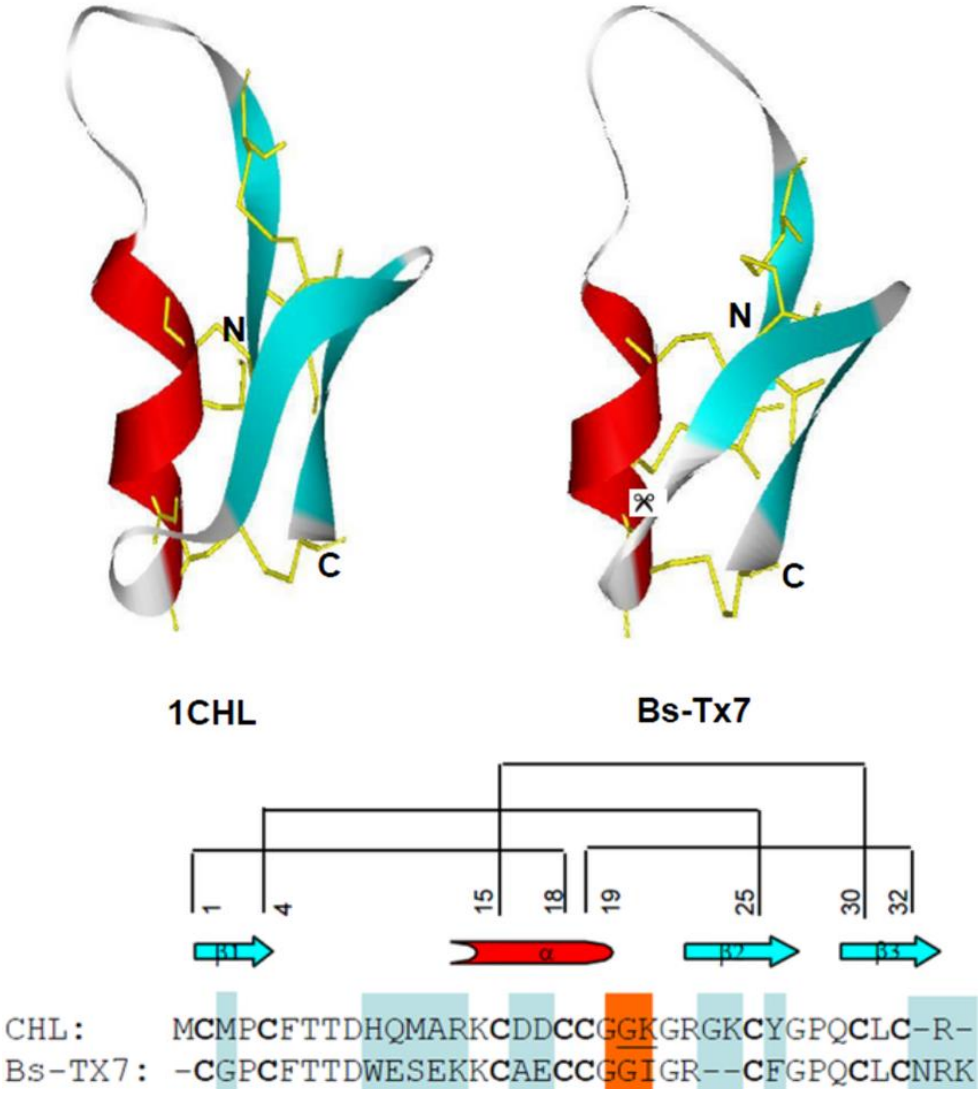


Figure 29. Structural comparison of CLTX and Bs-Tx7.[48]

3.2.4. Synthesis of CLTX derivatives at lysine-23 position

CLTX with Mtt protected lysine at 23rd position (**P10**) was synthesized by using microwave assisted SPPS with Rink amide resin. Small amount of resin was taken and fully cleaved by TFA. **P10** was achieved according to HPLC chromatogram and HRMS spectrum of crude **P10** (Figure 30).

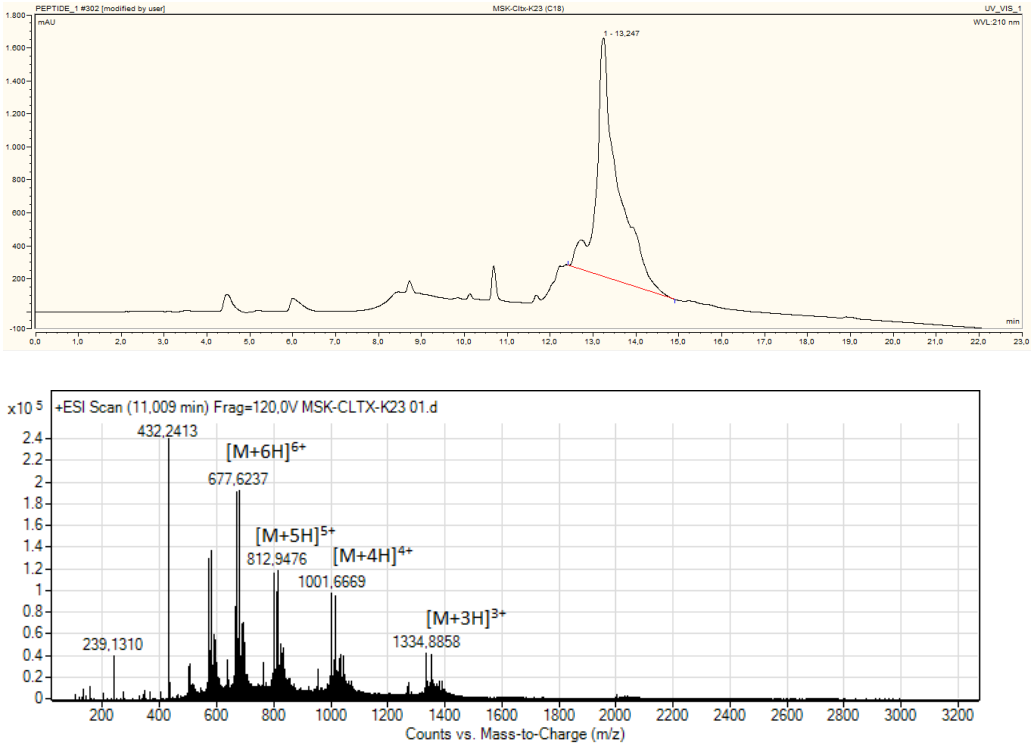


Figure 30. HPLC chromatogram and HRMS spectrum of crude **P10**. Absorbance was monitored at 210 nm.

Then, in solid phase, Mtt protection was cleaved by using 1.8% TFA in DCM, and Kaiser test was applied in order to monitor the reaction. Free amine group of lysine residue was coupled with Boc-Gly-OH (**P11**), $(Boc\text{-}Cys\text{-}OH)_2$ (**P12**) and Boc-Met-OH (**P13**).. Small sample of obtained peptidyl resin was fully cleaved by TFA and **P11**, **P12** and **P13** were achieved based on the HPLC chromatograms and HRMS spectra of crude samples (Figure 31, 32 and 33).

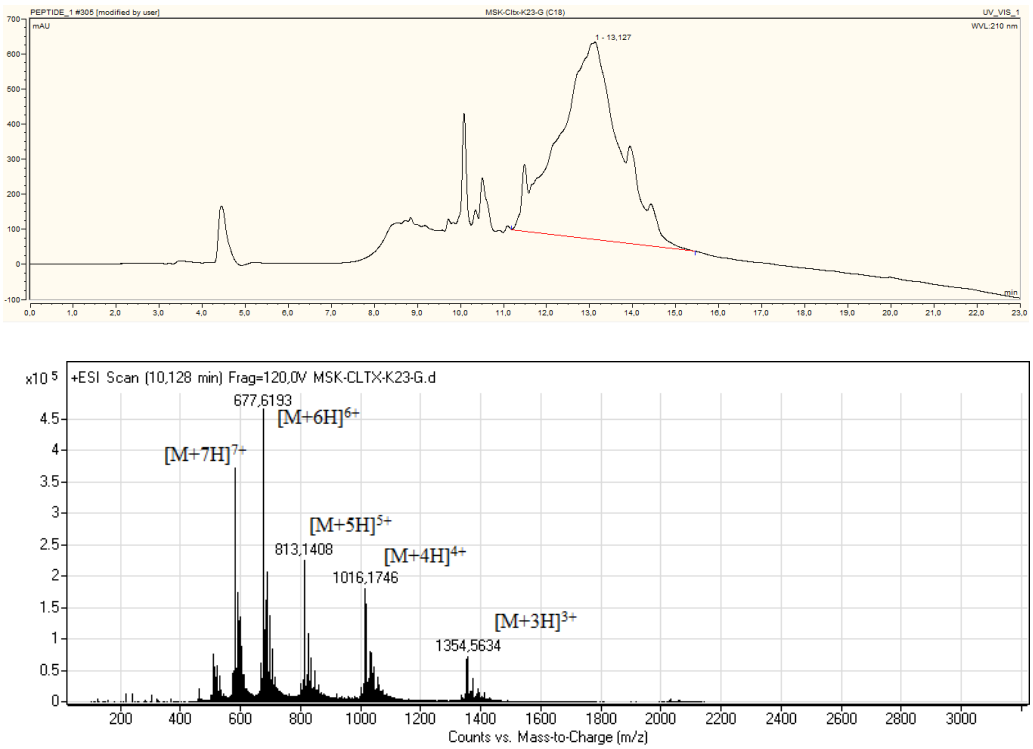


Figure 31. HPLC chromatogram and HRMS spectrum of crude **P11**. Absorbance was monitored at 210 nm.

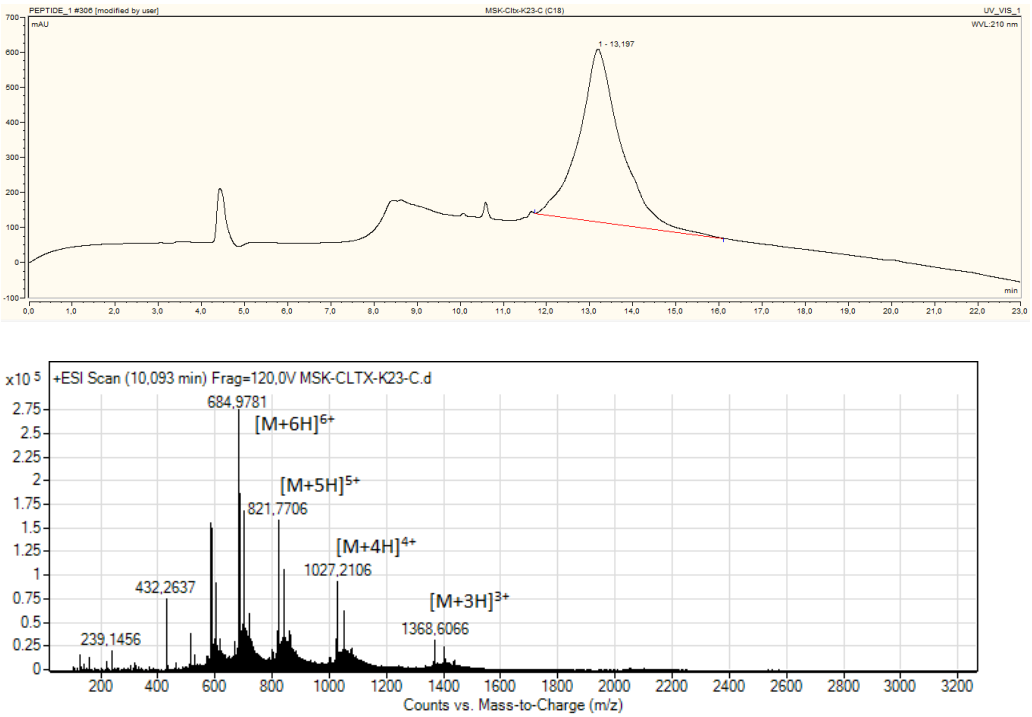


Figure 32. HPLC chromatogram and HRMS spectrum of crude **P12**. Absorbance was monitored at 210 nm.

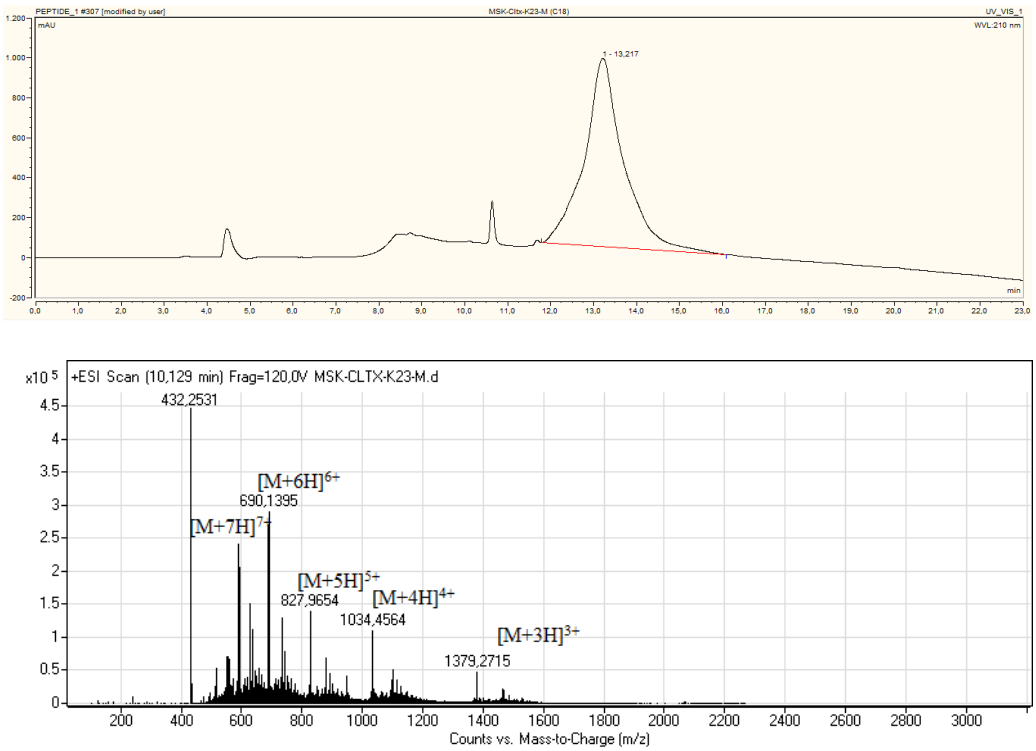
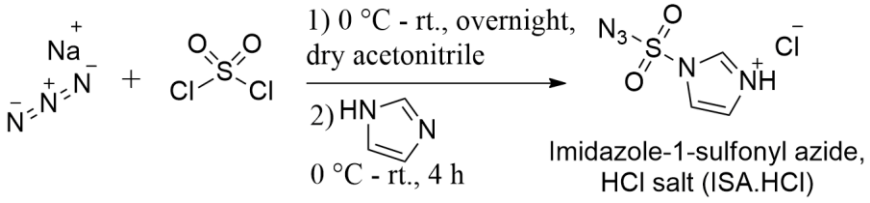


Figure 33. HPLC chromatogram and HRMS spectrum of crude **P13**. Absorbance was monitored at 210 nm.

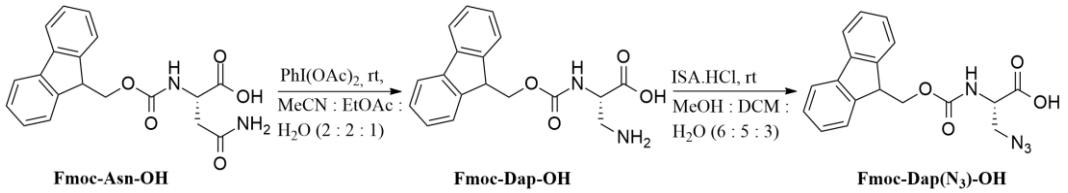
3.3. Synthesis of CLTX Derivatives at Histidine Residue

3.3.1. Synthesis of Fmoc-Dap(N_3)-OH



Scheme 4. The synthesis of **ISA.HCl** using sodium azide and sulfuryl chloride.

Based on literature procedure[40], by using sodium azide, sulfuryl chloride and imidazole as starting materials, **ISA.HCl** was synthesized as diazotranfer reagent. It was isolated with 56% yield. ^{1}H NMR spectrum data were consistent with literature.



Scheme 5. The reaction scheme of Fmoc-Dap(N_3)-OH

Starting from commercial Fmoc-Asn-OH, Fmoc-Dap-OH was synthesized in the presence of diacetoxyiodobenzene in ACN, ethylacetate and water mixture at room temperature.[41] It was purified by flash column chromatography, and isolated with 57% yield. ^{1}H NMR spectrum data were consistent with literature. By following the literature procedure,[42] Fmoc-Dap(N_3)-OH was synthesized from Fmoc-Dap-OH in the presence of ISA.HCl as diazotranfer reagent in methanol, DCM and water mixture. It was purified by flash column chromatography and yield was 54%. ^{1}H NMR spectrum data were consistent with literature.

3.3.2. Synthesis of fragment peptides modified from histidine-10 position

Instead of synthesizing the full sequence of CLTX, a fragment peptide (**P14**) was chosen as a model peptide in order to optimize the reaction conditions. This fragment (GTDXaaQM) was taken from the certain part of the sequence of CLTX involving histidine. However, instead of histidine, Fmoc-Dap(N_3)-OH (shown as Xaa) was used in by microwave assisted SPPS. After the completion of the synthesis, small amount of resin was taken and fully cleaved by TFA. However, HRMS spectrum and HPLC chromatogram showed that synthesis of **P14** was not succeeded (Figure 34).

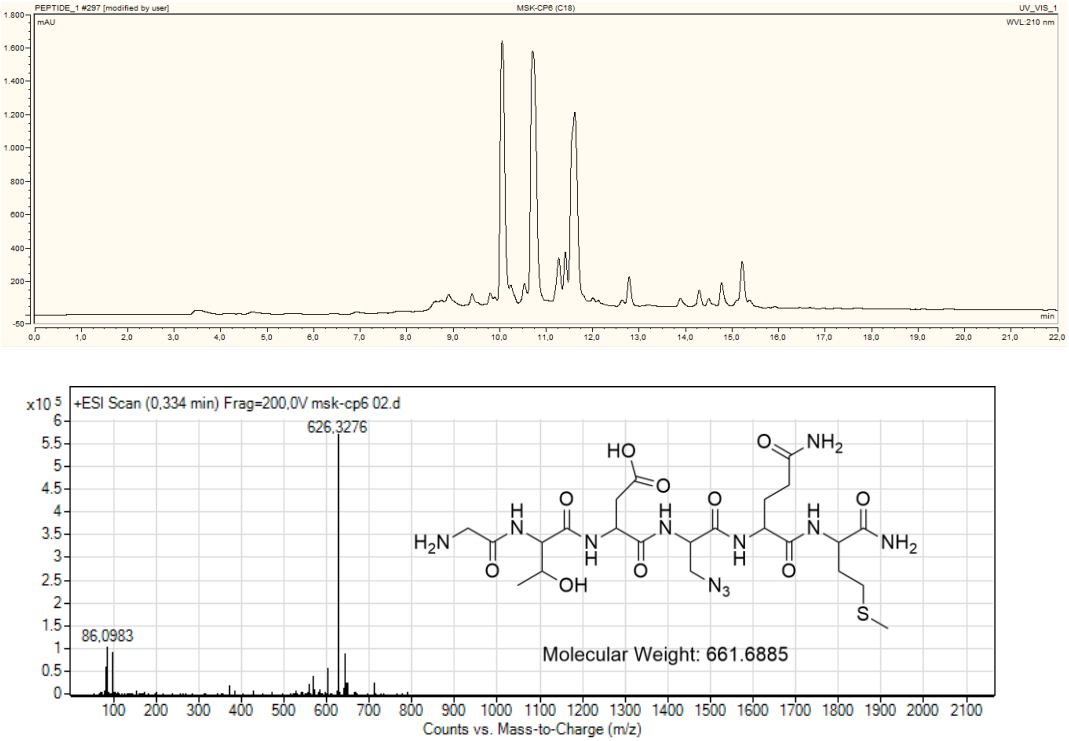


Figure 34. HPLC chromatogram and HRMS spectrum of crude **P14**. Absorbance was monitored at 210 nm.

The reason why the synthesis of azide containing peptide was failed might be the use of microwave heating in the synthesis. To check this idea, **P14** was synthesized without using microwave. After the completion of the synthesis, a small amount of resin was taken and fully cleaved by TFA. HPLC chromatogram and HRMS spectrum of crude **P14** showed that the synthesis was succeeded (Figure 35). This result showed that azide containing peptides should not be synthesized by microwave assisted SPPS.

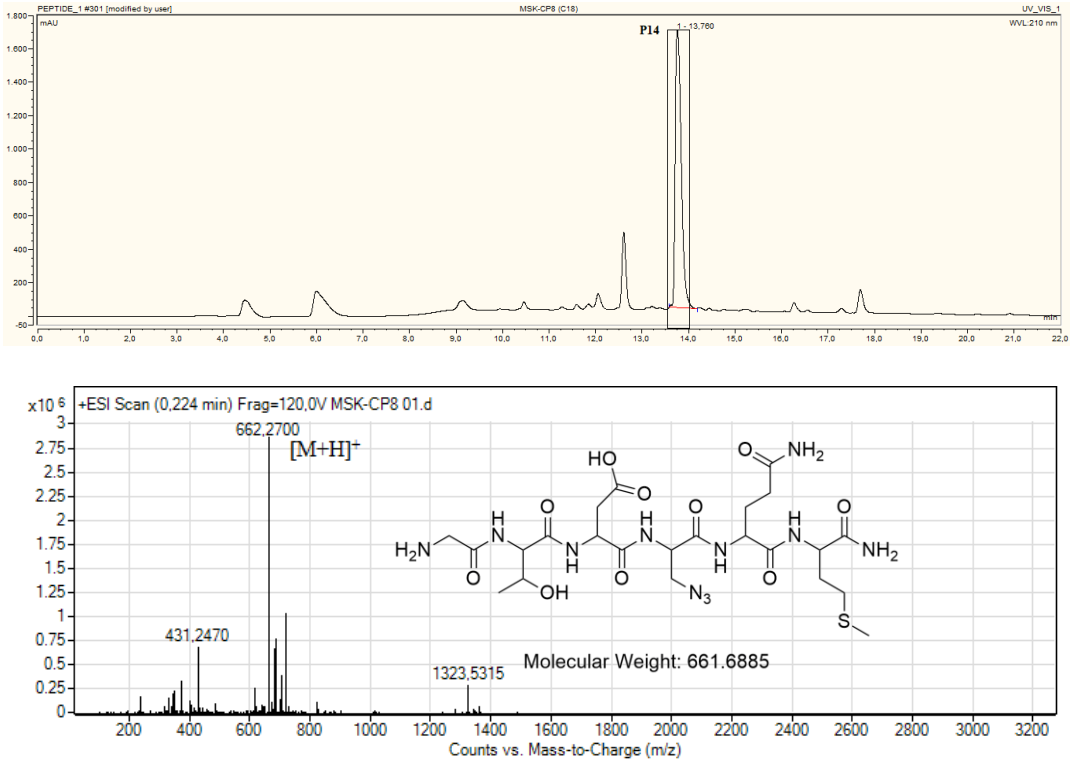


Figure 35. HPLC chromatogram and HRMS spectrum of crude **P14**. Absorbance was monitored at 210 nm.

After synthesis of **P14**, copper(I) catalyzed azide-alkyne cyclo-addition on solid phase (CuAAC-SP) was applied. For this purpose, alkyne derivative, CuI, sodium ascorbate and piperidine:DMF mixture was added on the resin. **P15** and **P16** were obtained using propargyl alcohol and propargyl amine, respectively. After fully cleavage of small amount of resins by TFA, HPLC chromatograms and HRMS spectra of crude **P15** and **P16** showed that the synthesis was succeeded (Figure 36 and 37).

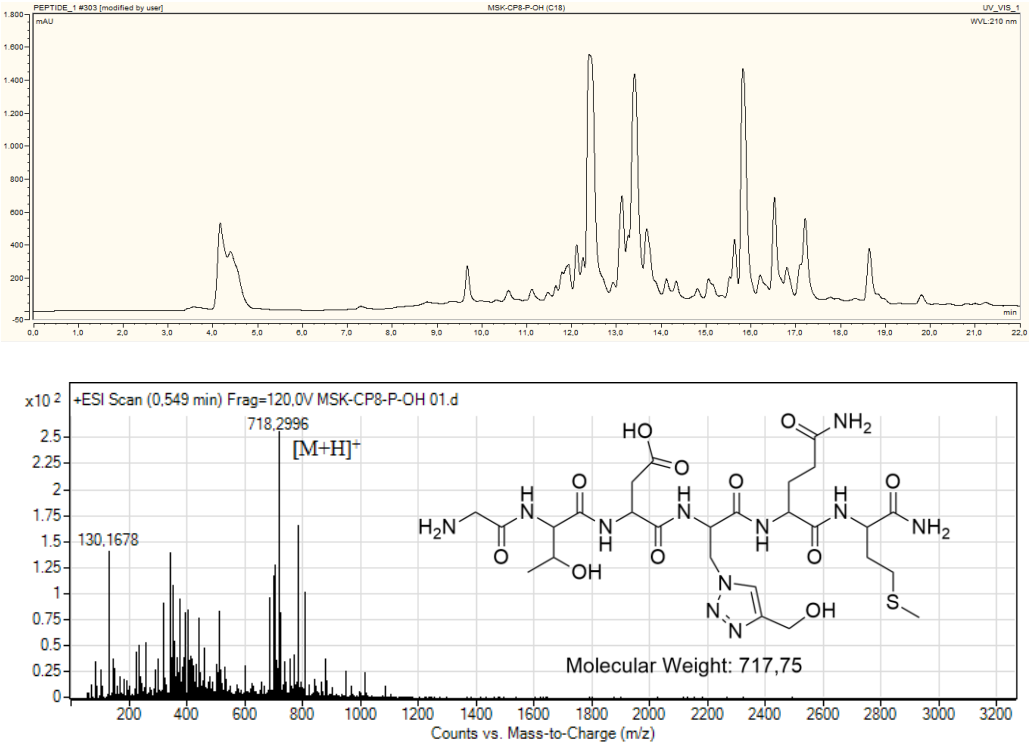


Figure 36. HPLC chromatogram and HRMS spectrum of crude **P15**. Absorbance was monitored at 210 nm.

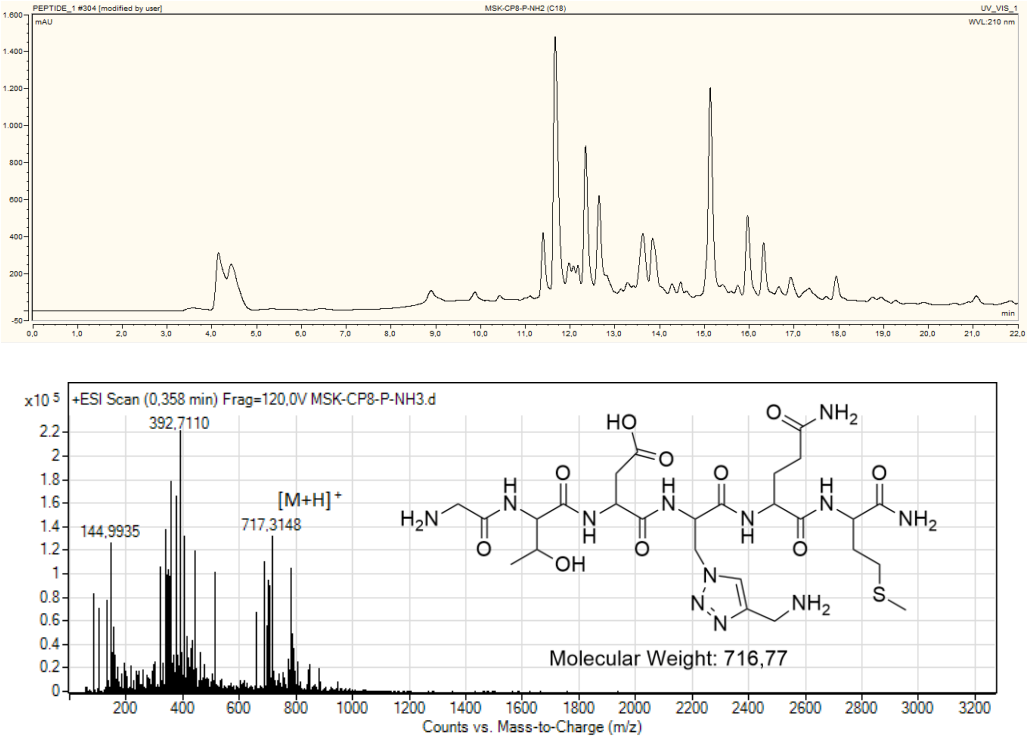


Figure 37. HPLC chromatogram and HRMS spectrum of crude **P16**. Absorbance was monitored at 210 nm.

3.4. Conclusion

In this study, we synthesized CLTX and its derivatives from different positions by using microwave assisted solid phase synthesis based on Fmoc chemistry strategy in order to develop CLTX peptide-based matrix metalloproteinase-2 inhibitors.

Firstly, in order to optimize synthesis of modified CLTX's, fragment peptides were synthesized and all modifications were applied on them.

In addition to SPPS, in this study, an unnatural amino acid, Fmoc-Dap(N_3)-OH, was synthesized. Then Fmoc-Dap(N_3)-OH was used in the synthesis of fragment peptide as a modification of histidine residue.

In summary, during this thesis, CLTX derivatives shown in Table 3 were synthesized and folded and obtained in 2-4 mg quantity and 90-95% purity.

Table 3. List of the CLTX and its derivatives that were synthesized and folded.

Code	Position	Modification Type	Inhibition of MMP-2 Activity %
P1F	-	-	54.8
P7F	K15	Glycine	-57.3
P8F	K15	L-Methionine	-107.1
P9	K15	L-Cysteine	N.D.*
P11	K23	Glycine	N.D.*
P12	K23	L-Cysteine	N.D.*
P13	K23	L-Methionine	N.D.*

*Not determined yet

As a future work, folding and MMP-2 activity assay will be applied to **P9**, **P11**, **P12** and **P13**. CLTX derivatives at histidine-10 position that the preliminary studies were done will be synthesized and MMP-2 activity assay will be performed.

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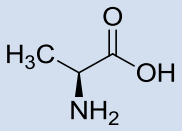
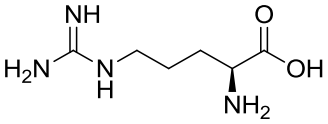
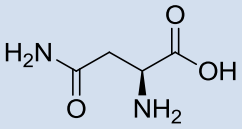
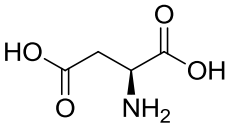
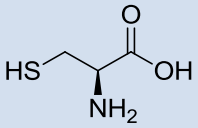
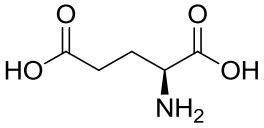
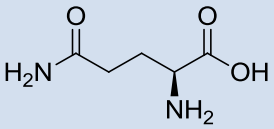
APPENDIX A

LIST OF 21 AMINO ACIDS FOUND IN NATURE

Table A.1. List of 21 amino acids found in nature.

Name	3-Letter code	1-Letter code	Structure
L-Alanine	Ala	A	
L-Arginine	Arg	R	
L-Asparagine	Asn	N	
L-Aspartic acid	Asp	D	
L-Cysteine	Cys	C	
L-Glutamic acid	Glu	E	
L-Glutamine	Gln	Q	

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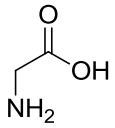
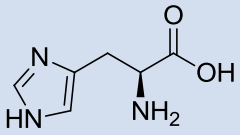
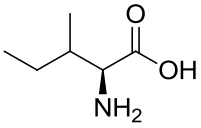
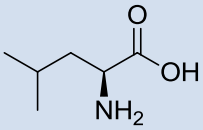
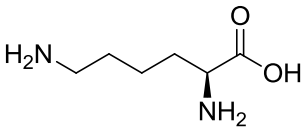
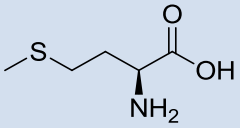
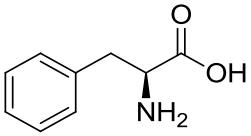
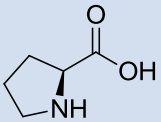
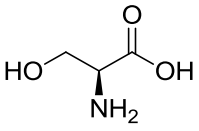
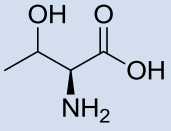
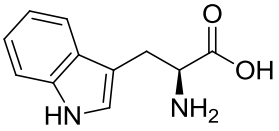
Glycine	Gly	G	
L-Histidine	His	H	
L-Isoleucine	Ile	I	
L-Leucine	Leu	L	
L-Lysine	Lys	K	
L-Methionine	Met	M	
L-Phenylalanine	Phe	F	
L-Proline	Pro	P	
L-Serine	Ser	S	
L-Threonine	Thr	T	
L-Tryptophan	Trp	W	

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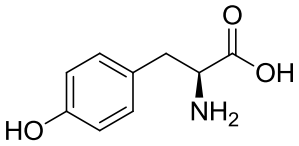
L-Tyrosine	Tyr	Y	
L-Valine	Val	V