SYNTHESIS OF PEPTIDE INHIBITORS FOR MATRIX
METALLOPROTEINASE-2 AND ANGIOTENSIN CONVERTING ENZYME

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ABSTRACT

SYNTHESIS OF PEPTIDE INHIBITORS FOR MATRIX METALLOPROTEINASE-2 AND ANGIOTENSIN CONVERTING ENZYME

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Matrix Metalloproteinases (MMPs) are one of the enzyme families of proteases that have zinc atom in the active site and they are involved in the degradation and regeneration of extracellular matrix. Specifically, they play an important role in tumor formation, tissue invasion, angiogenesis, and tumor metastasis. Matrix metalloprotease-2 (MMP-2) is a member of gelatinase class of MMP family and it has high activity in progression of skin, prostate, bladder, breast, lung, and ovary cancer. To control this increase in the activity, many MMP inhibitors were developed. However, these inhibitors considered to be not successful due to the specificity problem. On the other hand, chlorotoxin (Cltx), originally isolated from the scorpion venom, is a peptide consisting of 36 amino acids and it shows high stability and selective for inhibition of the MMP-2, due to the cysteine knot motif in its structure. Cyclic-chlorotoxin is known to be more stable than linear chlorotoxin in the plasma, since its proteolytic cleavage is significantly lower due to cyclic structure. Aim of this study is to synthesize cyclic and linear chlorotoxin derivatives, selective and highly potent inhibitors that targets MMP-2 which is an important molecular component in cancer therapy. Consequently, only linear

chlorotoxin derivative that has 7 additional amino acids could be synthesized. Result of MMP-2 activity assay performed with this derivative showed no inhibitory effect on the enzyme activity.

Angiotensin-converting enzyme (ACE), another enzyme that belongs to zinc metallopeptidase family as MMP-2, is a major component of renin-angiotensin system that controls blood pressure and fluid and electrolyte homeostasis. Its best known function is the in vivo conversion of angiotensin I (Ang I) into the potent vasopressor angiotensin II (Ang II). Therefore, ACE inhibitors have a broad range of application in cardiovascular disease, ranging from mild hypertension to post-myocardial infarction. Kojic acid is used as inhibitor of enzyme tyrosinase to treat melanin hyperpigmentation disorders. This inhibitory effect is due to the ability of kojic acid to chelate copper ion at the active site of the enzyme. Kojic acid shows this chelating property with other metal ions such as zinc, nickel, iron and gold. Since ACE has zinc ion in the active site, kojic acid can chelate with zinc ion for the inhibition of this enzyme. Objective of this study is to synthesize conjugates of kojic acid with various small peptides that are expected to show greater selectivity as an ACE inhibitor. As a result, two derivatives of kojic acid-phenylalanine conjugate were synthesized. However, due to the activity problems of commercial ACE, interactions of these conjugates with the enzyme could not be studied.

ÖZ

MATRİKS METALLOPROTEİNAZ-2 VE ANJİYOTENSİN DÖNÜŞTÜRÜCÜ ENZİM İÇİN PEPTİT İNHİBİTÖRLERİ SENTEZİ

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Matriks Metalloproteinazlar (MMPler) aktif bölgelerinde çinko bulunan ve ekstraselüler matriksin parçalanması ve yeniden oluşumunda görev alan bir proteaz enzim grubudur. MMP'ler özellikle tümör oluşumu, doku invazyonu, anjiyogenez ve tümör metastazlarında önemli rol oynamaktadırlar. Matriks Metalloproteinaz-2 (MMP-2), MMP ailesinin jelatinaz sınıfına üye bir enzimdir ve pankreas, cilt, prostat, mesane, göğüs, kolon, akciğer ve yumurtalık kanserlerinde önemli miktarlarda aktivite artışı gösterdiği belirlenmiştir. Bu aktivite artışını kontrol altına almak amacı ile pek çok MMP inhibitörü geliştirilmiş, ancak geliştirilen inhibitörlerde seçicilik problemi ortaya çıkmıştır. Diğer taraftan, 36 aminoasitten oluşan klorotoksin (Cltx), akrep zehiri kökenli bir peptittir. Yapısında bulunan inhibitor sistein düğümü motifi sayesinde yüksek kararlılığa sahiptir ve seçici olarak MMP-2'yi hedefleyerek inhibe etmektedir. Halkalı klorotoksin peptitinin plazma içerisindeki kararlılığının lineer klorotoksine göre daha kararlı olduğu bilinmektedir. Bu durumun proteolitik bölünmeyi azalttığı düşünülmektedir. Bu çalışmanın amacı, kanser çalışmalarında önemli rolü olan MMP-2 enzimini yüksek seçicilikte inhibe edebilecek halkalı ve

düz klorotoksin peptit türevleri sentezlemektir. Sonuç olarak, sadece 7 aminoasit fazlası olan düz klorotoksin türevi sentezlenmiştir. Fakat, MMP-2 enzimi aktivite çalışmaları sonucu, sentezlenen bu türevin enzim üzerinde inhibisyon etkisinin olmadığını gösermiştir.

Bir diğer çinko metallopeptidaz olarak bilinen anjiyotensin dönüştürücü enzim (ACE), kan basıncı, sıvı ve elektrolit dengesinin kontrolü için önemli bir bileşendir. En iyi bilinen fonksiyonu, canlı dokularda anjiyotensin I (Ang I) maddesini güçlü bir vazopressör olan anjiyotensin II (Ang II) maddesine dönüştürmesidir. Bu nedenle, ACE inhibitörleri, yüksek tansiyondan damar tıkanıklığına kadar pek çok kalp ve damar hastalığı alanında kullanılabilir. Diğer taraftan kojik asit, melanin hiperpigmentasyon hastalığına neden olan tirozinaz enziminin inhibitörü olarak kullanılır. Bu inhibitör etki kojik asitin, enzimin aktif bölgesindeki bakır iyonunu kıskaçlama yeteneğinden kaynaklanmaktadır. Ayrıca kojik asit çinko, nikel, demir ve altın gibi iyonlara karşı da kıskaçlama özelliğine sahiptir. Bunun yanında, ACE aktif merkezinde çinko iyonu bulunduğu için, kojik asit çinko ile kıskaç oluşturarak bu enzimi de inhibe edebilir. Bu çalışmanın amacı, yüksek ölçüde ACE inhibitörü etkisi gösterebilecek kojik asit-peptid konjugatlarının sentezlenmesidir. Sonuç olarak, iki adet kojik asit-fenilalanin konjugatı sentezlenmiştir. Fakat, hazır alınan ACE ile yapılan aktivite çalışmaları sonucunda enzimde herhangi bir aktivite gözlemlenmediği için sentezlenen konjugatların enzim üzerindeki etkileri çalışılamamıştır.

Anahtar Kelimeler: Amino asit, klorotoksin, katı faz peptit sentezi, matriks metalloproteinaz-2, anjiyotensin dönüştürücü enzim, kojik asit

To my beloved family

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ACE   : Angiotensin Converting Enzyme

ACN   : Acetonitrile

Boc   : Butyloxycarbonyl

DCM   : Dichloromethane

DIPEA   : N,N-Diisopropylethylamine

DMF   : N,N-Dimethylformamide

Et_3N : Triethylamine

Fmoc   : Fluorenylmethyloxycarbonyl

HBTU   :2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HF   : Hydrogen fluoride

HOBt   : Hydroxybenzotriazole

HPLC   : High Performance Liquid Chromatography

LC-MS   : Liquid Chromatography–Mass Spectrometry

MeOH   : Methanol

MMP-2   : Matrix metalloproteinase-2

NCL   : Native chemical Ligation

NMP   : N-Methyl-2-pyrrolidone

NMR   : Nuclear Magnetic Resonance Spectroscopy

SPPS   : Solid Phase Peptide Synthesis

TFA   : Trifluoroacetic acid

THF   : Tetrahydrofuran

TIPS   : Triisopropylsilane

TLC   : Thin-layer chromatography

CHAPTER 1

SYNTHESIS OF PEPTIDE INHIBITORS FOR MATRIX METALLOPROTEINASE-2

1.1. Introduction

1.1.1. Matrix Metalloproteinases

Matrix Metalloproteinases (MMPs) are one of the members of protease family. Proteases are the enzymes that break the peptide bond between the amino acids that form the polypeptide chain. MMPs belong to the family of zinc-dependent endopeptidases, due to the zinc ion at the active site and breaking the non-terminal peptide bond.

1.1.1a. Structure, Function and Types of Matrix Metalloproteinases

Matrix Metalloproteinases are also called as matrixins, which are subfamily of zinc metalloprotease family M10.[1] In 1962, MMP was firstly analyzed by Gross and Lapiere, which was named as interstitial collagenase due to the function of degrading the fibrillar collagen in tadpole tails during metamorphosis.[2] MMPs are known as extracellular proteins that are excreted into the extracellular space or attached to the cell membrane as inherent proteases. There are 25 members of MMPs; however the last member is MMP-28. The members MMP-4, MMP-5, MMP-6 and MMP-22 are missing in the list due to their identical structure with other members.[3] (Table-1)

Table 1. Matrix Metalloproteinases and their common names (adapted from[4]).

MMP	Alternative name	MMP	Alternative name
1	Collagenase-1	16	Membrane type-3 MMP
	Interstitial collagenase		
2	Gelatinase A	17	Membrane type-4 MMP
3	Stromelysin-1	19	None
	Transin-1		Human ortholog of *Xenopus* MMP-18
7	Matrilysin	20	Enamelysin
	Pump-1		
8	Collagenase-2	21	None
	Neutrophil collagenase		Human ortholog of *Xenopus* xMMP
9	Gelatinase B	23	Cysteine array MMP
10	Stromelysin-2	24	Membrane type-5 MMP
11	Stromelysin-3	25	Membrane type-6 MMP
			Leukolysin
12	Macrophage metallo-elastase	26	Matrilysin-2/endometase
13	Collagenase-3	27	None
14	Membrane type-1 MMP	28	Epilysin
15	Membrane type-2 MMP		

MMPs are multidomain enzymes, that the most of the members contain a similar domain organization consisting of; propeptide, the catalytic domain (CAT), and hemopexin-like domain (except MMP-7, MMP-23 and MMP-26).[5] However, there are some differences in the domain organization of certain types of MMPs. MMP-23 does not have hemopexin-like domain, it rather contains a special cysteine-rich domain and an immunoglobulin-like domain following the catalytic domain. MMP-2 and MMP-9 include three repeats of fibronectin type-II-like domain added in the catalytic domain. MMPs have a Zn^{2+} ion and a zinc-binding HEXXHXXGXXH motif in the CAT. After this zinc-binding motif, there is a preserved methionine containing structure, called as 'Met-turn' that forms a hydrophobic area to hold up the structure around the catalytic zinc.[6] In addition, the propeptide domain has a motif PRCGXPD, called as 'cysteine-switch' that makes coordination with the catalytic zinc ion via the cysteine residue it has. The coordination between Cys-Zn^{2+} prevents the coordination of water molecule with the catalytic Zn^{2+} ion, so keeps the enzyme in inactive form.[1,7]
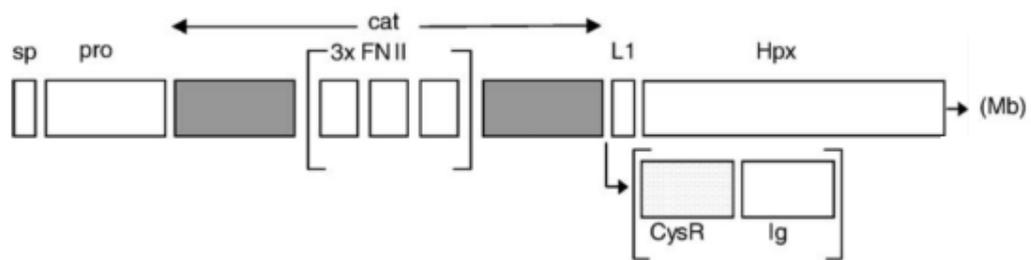
Figure 1. General domain structure of MMPs (adapted from[1]) (sp; signal sequence, pro; propeptide domain, cat; catalytic domain, FNII; fibronectin type-II domain, L1; linker, CysR; cysteine-rich, Ig; immunoglobulin-like, Hpx; hemopexin-like domain , Mb; plasma membrane).

MMPs play a major role in the degradation of extracellular matrix (ECM).[8] Degradation of ECM changes the cell-matrix and cell-cell interactions, which affects the relationship between cell and receptor. So, MMPs are implicated in the embryonic development, tissue remodeling and cell migration during morphogenesis. In addition, unrestrained ECM reconstruction of the myocardium causes some cardiovascular disorders such as stenosis, atherosclerosis, aneurysm and heart failure. MMPs also are involved in the liberation of latent growth factors, growth-factor binding proteins, and cytokines from the cell (so-called shedding).[9]

According to organization of domains and substrate priority, MMPs are sub grouped into six classes: the collagenases (MMP-1, MMP-8, MMP-13 and MMP-18 in *Xenopus*), the gelatinases (MMP-2 and MMP-9), the stromelysins (MMP-3, MMP-10 and MMP-11), matrilysin (MMP-7 and -26), membrane-type (MT)-MMPs (MMP-14, -15, -16, and -24) and others (seven MMPs are not gathered in the categories above; MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27 and MMP-28).[1]

1.1.1b. Matrix Metalloproteinase-2 (MMP-2)

Matrix Metalloproteinase-2 (gelatinase A) belongs to the gelatinase class of the MMP subfamily which also includes MMP-9 (gelatinase B). They are called as gelatinases due to the ability of digesting the gelatin (denatured collagen). MMP-2 also plays a role in the degradation of collagen I, II and III.[10] Dissimilar to other MMPs, MMP-2 include three repeats of type II fibronectin-like domains or FN2 domains which form cysteine-rich catalytic domains also known as collagen-binding regions. The rest domain structures of MMP-2 are certainly similar as the other type of MMPs that is composed of propeptide domain, N-terminal catalytic domain and C-terminal hemopexin-like domain (Figure 2). MMP-2 is expressed as a 72-kDa proenzyme and activated with Membrane type-1 matrix metalloproteinase (MT1-MMP) by cleaving propeptide domain of proMMP-2, resulting a 64-kDa active enzyme.[11]
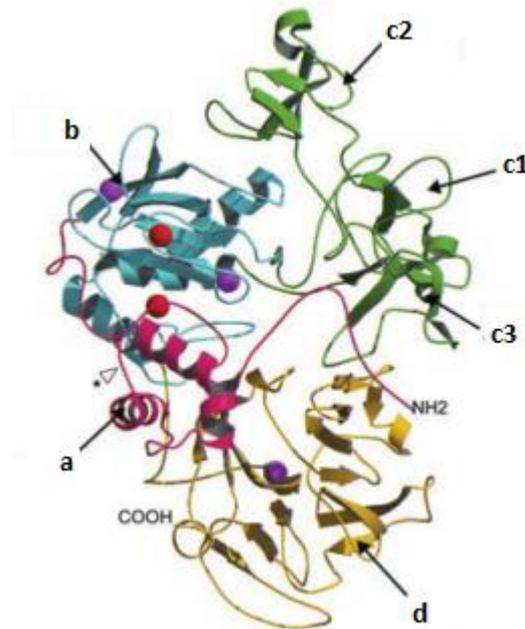
Figure 2. Domain structure of proMMP-2. a) propeptide domain b) catalytic domain c1, c2, c3) fibronectin type II domains 1, 2 and 3 d) hemopexin domain (adapted from[12]).

The gelatinases are participated in the formation of new blood vessels (angiogenesis) by degrading the collagen in vascular basal membranes and leading the way for movement of endothelial cells to form new blood vessels.[13] Angiogenesis appears in several diseases such as cancer and arthritis. As a result of this, MMP-2 plays an important role in tumor growth, metastasis and also in several fatal diseases such as; breast cancer, brain tumors, lung cancer, skin cancer, urogenital cancers, and colorectal cancer.

1.1.1c. Matrix Metalloproteinase Inhibitors (MMPI)

Since the gelatinases and other MMPs play an important role in cancer and other diseases, proteolytic activity of these enzymes should be controlled. MMPs can be inhibited by natural inhibitors or synthetic inhibitors. Tissue inhibitors of metalloproteinases (TIMPs) are one of the naturally existing MMP inhibitors. TIMPs contain N-terminal domain that interacts with catalytic site of the enzyme and C-terminal domain that have divided enzyme binding sites for different types of MMPs.[14] Disadvantage of these naturally occurring MMP inhibitors is that they are not specific for one type of MMPs and it is difficult to produce and use such large molecules. On the other hand, synthetic inhibitors are commonly designed for chelating the active site Zn^{2+} ion of the MMPs and to displace with the water molecules that are bounded to that ion. There are peptidomimetic inhibitors that mimic the cutting region of the substrates. The favored synthetic Zn^{2+} binding group (ZBG) for MMPs is the hydroxamates. Hydroxamates chelates to the Zn^{2+} ion and also they make hydrogen bonds with the backbone residues of the enzyme.[15] In addition to hydroxamates, there are also non-hydroxamate ZBG inhibitors which are thiols, carboxylic acids and phosphorus-based groups. However, all of these peptidomimetic inhibitors have specificity problems. They may also inhibit different types of enzymes in the cell that have Zn^{2+} ion in their structure by chelating. Unsuccessful clinical trials of some type of hydroxamates (batimastat and marimastat) prove this problem by showing some serious side effects.[16]
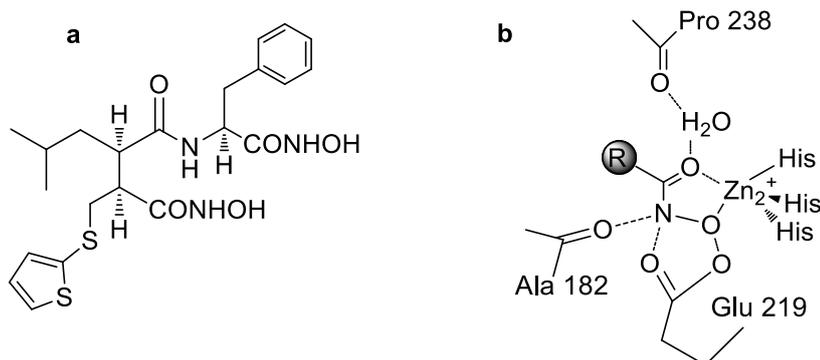
Figure 3. a) Structure of batimastat,[14] **b)** Interaction between hydroxamate inhibitor and Zn^{2+} ion in the active site of the enzyme.[17]

1.1.2. Venom Peptides

Venoms are peptide toxins that some animals have developed to catch their prey and to use them as defense mechanism. Most venom contains a diverse arrangement of different peptides with selective pharmacology. Due to their diverse bioactivity they are involve in the development of new therapeutic agents.[18]

The stability of venom peptides against chemical and enzymatic degradations, either in the venom itself or in the tissues of prey species is crucial for peptide to survive. The use of post-translational modifications or disulfide bonds that give a stabilized structure to the peptide by folding helps to overcome this stability problem.[19]

1.1.3. Disulfide-rich Peptides

Disulfide bonds play crucial role for the biological function of polypeptides and are crucial to the stabilization of 3D structure of these entities. Peptides with disulfide-rich structure can be found in extensive functionalities like toxins, growth factors and enzyme inhibitors. They are also the main component of animal venoms.

6

Disulfide-rich peptides have got much more attention since they contribute for the development of novel therapeutic agents and bio-insecticides by targeting neuronal receptors and ion channels with a high degree of selectivity and potency. Structural and functional characterization of these disulfide-rich peptides is necessary for the development of a reliable expression system that controls their native disulfide structure.[20]

1.1.3a. Cyclotides

Cyclotides are small disulfide-rich polypeptides that have unusual characteristic of a cyclic backbone. They have cystine knot topology which is formed by 3 disulfide bonds, with two of them and their connecting backbone sections form an enclosed ring in the structure that is penetrated by the third disulfide bond. With this circular and knotted structure they are more stable to chemical, thermal and enzymatic degradation than other polypeptides.[21]
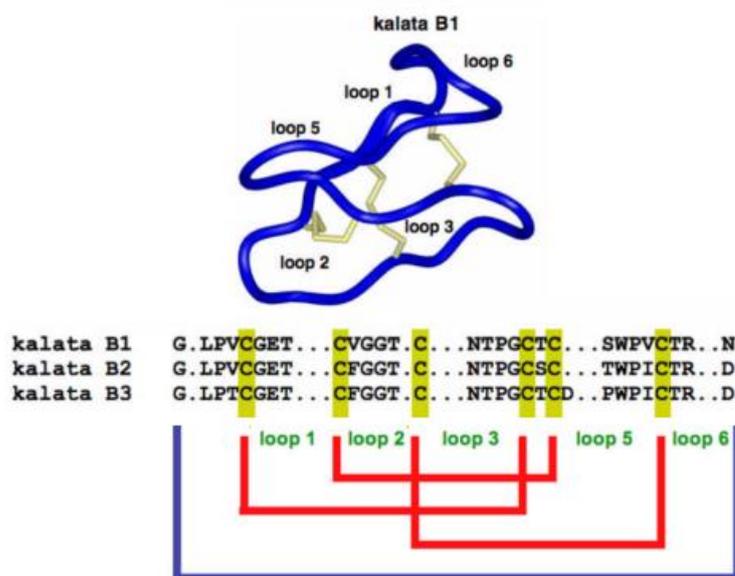
Figure 4. Primary and tertiary structure of a cyclotide, kalata B1.[21]

Cyclotides can be used to promote new peptide-base drugs due to their biological activities like anti-HIV, protease inhibitory, insecticidal and hormone like activity, their abilities to pass over the cell membrane and also due to their stability. In addition, their small size gives them an advantage of being synthesized chemically. They are also open to substitutions in their structure that some properties can be changed (like hydrophobic residues) without any change in activity.[21]

1.1.3b. Cystine Knot Motif

Cystine knots have typical structural motifs that are composed of three antiparallel β-strands which are connected through short loop, and the knotted disulfide connections. These knotted disulfides occupying the molecular core in the structure of the proteins and forcing some hydrophobic residues to be allocated on the surface. This motif is highly effective for stabilization of the structure that makes it an ideal framework for molecular engineering applications.[22] Peptides containing this cystine knot are 26-48 residues long and include ion channel blockers, hemolytic agents as well as molecules having antiviral and antibacterial activities.
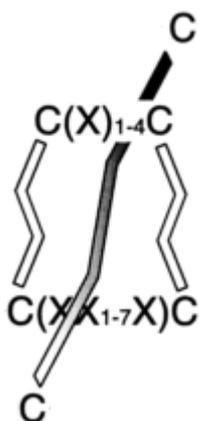
Figure 5. Structure of cysteine knot motif.[23]

There are three classes of cystine knots: Growth Factor Cystine Knot (GFCK), Inhibitor Cystine Knot (ICK) and the Cyclic Cystine Knot (CCK). In the GFCK model, the Cys(I-IV) disulfide bond penetrates the enclosed ring formed by the other two disulfide bonds, on the other hand Cys(III-VI) is the penetrating disulfide bond in the ICK and CCK models (Figure 6).
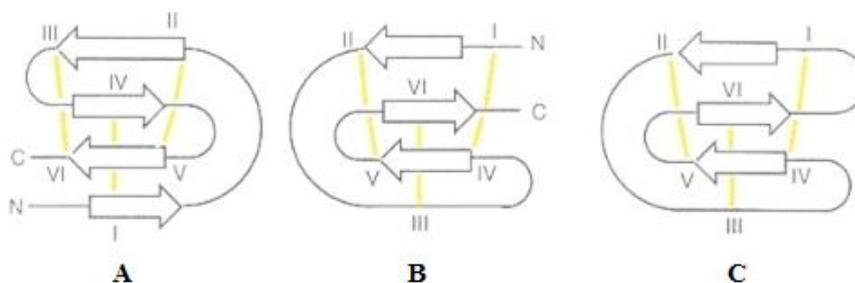
Figure 6. Schematic diagram of **A)** GFCK, **B)** ICK, **C)** CCK classes of cysteine knots.[24]

Cysteine knot peptides can be synthesized by either recombinant or chemical synthesis. In chemical synthesis, solid phase peptide synthesis (SPPS) has advantages over the recombinant synthesis with the new methodologies during chain assembly, backbone cyclization and oxidative folding towards the regioselective formation of multiple disulfide bonds. In addition, it has advantage on the synthesis of derivatives of the peptides by making changes in the structure of peptides at any desired position with non-natural elements.

1.1.4. Chlorotoxin

Chlorotoxin (Cltx) is a 36 amino acid peptide with four disulfide bridges and isolated from the Israeli scorpion *Leiurus quinquestriatus*. Its name comes from the ability of inhibiting chloride channels in embryonic rat brain and epithelia. After discovery of the fact that it binds to glioma cells the interest in the molecule is increased rapidly. Its entering pathway and localization in the glioma cells is different from the normal cells. This difference gives an advantage for the development of glioma therapeutics. For instance, a derivative of synthetic

chlorotoxin (TM-601) was conjugated with iodine-131 (131I) and the resulting product (131I-TM-601) was given to patients who have repetitive glioma with a result, showed anti-tumor effect without dose-limiting toxicity.[25]

The tumor binding of chlorotoxin is supported by its interaction with MMP-2, which is receptor on the surface of glioma cells. MMP-2 is not normally expressed in brain, but they are upregulated in gliomas and some other cancers. Interactions between Cltx and gliomas are explained by the inhibitory effects of Cltx on the MMP-2 enzyme activity. It is also indicated that Cltx has a specific and selective inhibitory effect on MMP-2 that it is not interacting with other types of MMPs (MMP-1, MMP-3 and MMP-9) which are also expressed in glioma cells.[26]
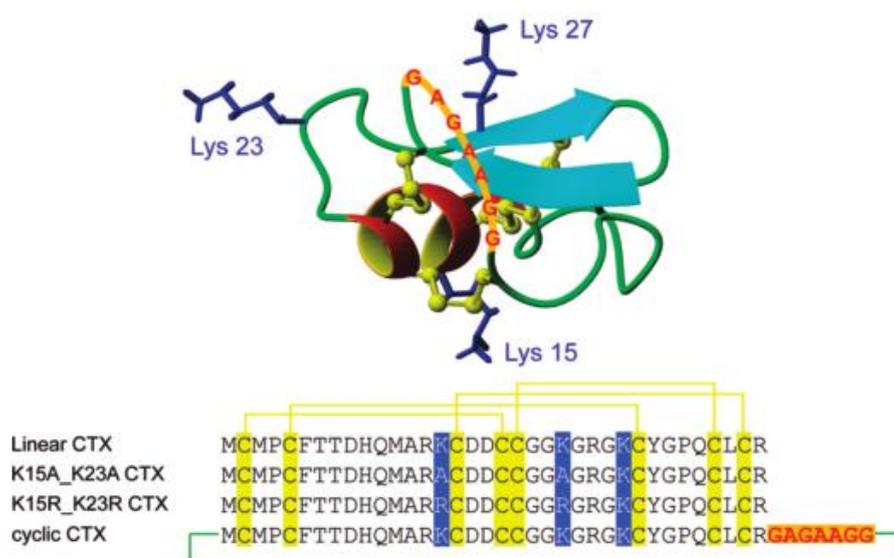
Figure 7. Structure of linear, substituted and cyclic chlorotoxin.[27]

On the other hand, when Cltx is bio conjugated with the fluorescent molecular beacon Cy5.5, it can be used as an optical imaging contrast agent that provides to surgeons to distinguish cancer foci from adjacent normal tissue during surgical operation. Cltx has three Lys residues in its structure (Lys15, 23, 27) and the dye conjugates with these Lys residues. The resulting peptide is 75-85% mono-labeled

at Lys27 with di- and tri-labeled side products. However with the substitution of Lys15 and Lys23 with different residues, fully mono-labeled product is obtained. On the other hand, without any substitution, only by using 7-amino acid linker cyclic chlorotoxin is synthesized as shown above (Figure 7) and it only gave the mono-labeled product when conjugated with dye. Cyclization also resulted with in enhanced serum stability compared to the linear form of chlorotoxin when conjugated with Cy5.5.[27]

1.1.5. Solid Phase Peptide Synthesis (SPPS)

The concept of peptide synthesis on a solid support was first developed by Robert Bruce Merrifield in 1963.[28] It is based on the idea that a peptide chain can be assembled in a stepwise manner while it is attached at one end to a solid support. Unlike ribosome protein synthesis, SPPS starts at a C-terminal of peptide and completed at an N-terminal of peptide. Today, the concept has been extended not only to peptides but has been used in organic synthesis on polymeric supports as well.
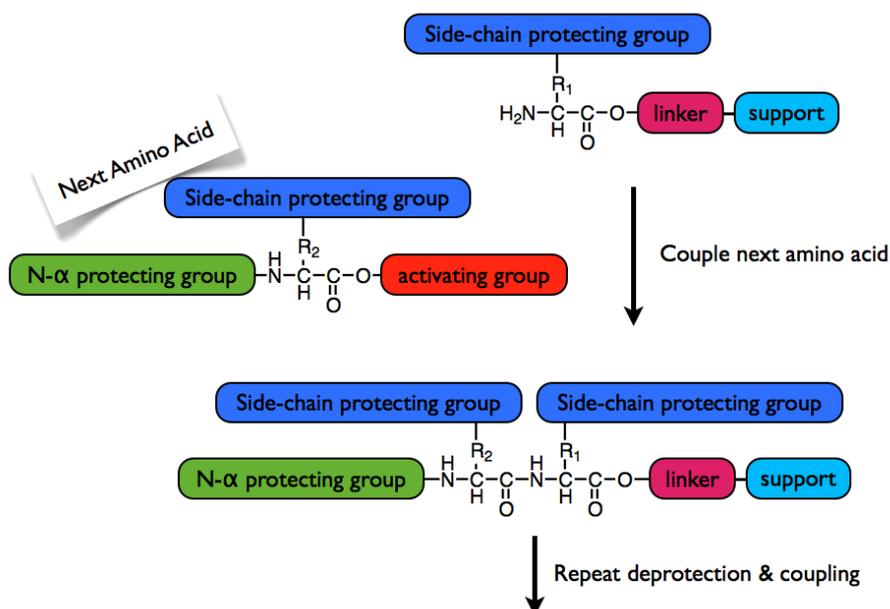
Figure 8. Schematic representation of general solid phase peptide synthesis.

There are two types of SPPS according to the protecting group of α-amine and side chains at the amino acids. In the first type, the *tert*-butyloxycarbonyl (Boc) group is used for temporary N_α-protection which requires selective cleavage at every cycle of amino acid addition. Usually, Boc can be cleaved by acid-catalyzed elimination in the presence of TFA. After the completion of peptide chain growth, peptide-resin linkers and side chain protecting groups can be cleaved by using liquid hydrogen fluoride (HF).

On the other hand, second type depends on the protection of α-amine group with fluorenylmethyloxycarbonyl (Fmoc) group. In this strategy, different from Boc method, Fmoc can be cleaved by base-catalyzed elimination in which the secondary amine (Piperidine) also traps the dibenzofulvene formed in the reaction. After the peptide chain growth is completed, acid labile peptide-resin linkers and mostly Boc type side chain protecting groups can be cleaved at the same time under mild reaction conditions with TFA.

Boc strategy has some advantages over Fmoc strategy as shown in Table 2. However, peptides with acid-sensitive functional groups are not achievable by the Boc strategy due to the strongly acidic conditions employed at final cleavage stage. In addition, highly toxic HF is needed for cleavage of peptides and special equipment is required to handle anhydrous HF. On the other hand, in Fmoc strategy no special equipment is required for the use of TFA.

Table 2. Advantages and disadvantages for Boc and Fmoc strategies.[29]

	Boc Strategy	Fmoc Strategy
Requires special equipment	Yes	No
Cost of reagents	Lower	Higher
Solubility of peptides	Higher	Lower
Deprotection efficiency	High	May be lower
Purity of hydrophobic peptides	High	May be lower
Problems with aggregation	Less frequently	More frequently
Synthesis time	~20 min/amino acid	~20–60 min/amino acid
Final deprotection	HF	TFA
Safety	Potentially dangerous	Relatively safe
Orthogonal	No	Yes

1.1.5a. Reactions & Mechanisms in SPPS

In Fmoc strategy for SPPS, the C-terminus of the residue is attached to a TFA-labile linker and the functional groups on side chains are protected with TFA-labile linkage agents as well. The temporary N-Fmoc protecting groups can be removed with 20% (v/v) piperidine in DMF. The deprotection mechanism is shown in Figure 9. Initial deprotonation of the fluorenyl ring is the key step to produce aromatic cyclopentadiene type intermediate. It rapidly eliminates to form dibenzofulvene which is then scavenged by piperidine. The product of deprotection has a distinctive UV absorbance so that reaction can be monitored by this way.
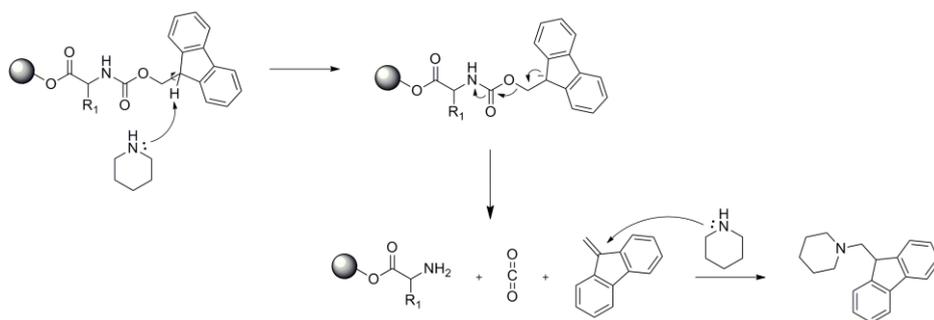
Figure 9. N-Fmoc deprotection mechanism.

Following the deprotection step, the stepwise introduction of N_α-protected amino acids is achieved via in situ carboxy activation of the incoming amino acid. Hence, coupling is generally carried out in DMF or NMP with the help of activation agents. In order to drive the acylation to completion, an excess of activated amino acid is used, usually the 2– 6 times the resin functionality. Figure 10 shows the activation of an amino acid by using HBTU as an activator.
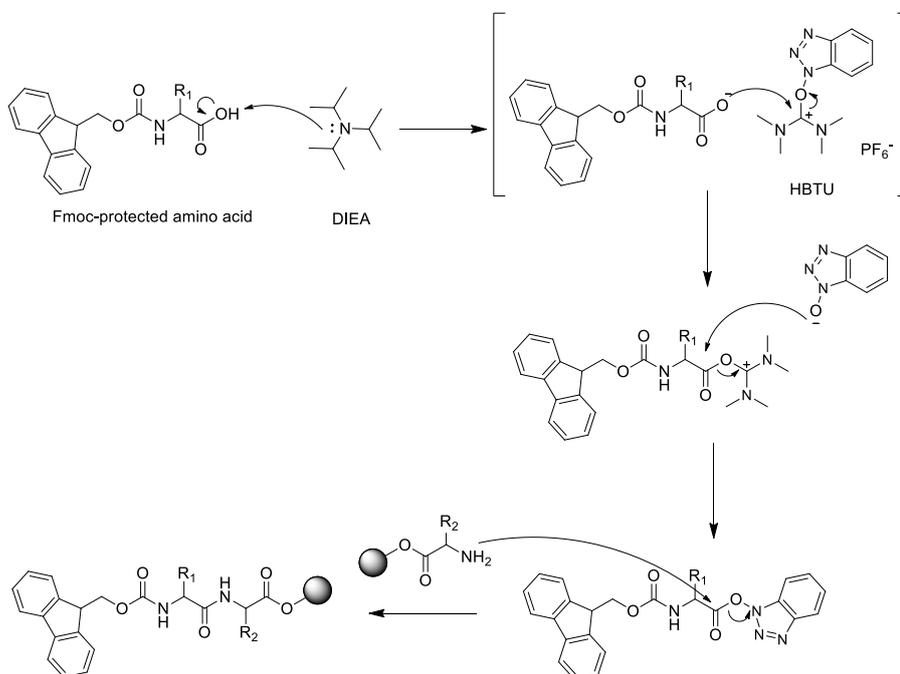
Figure 10. C-terminus activation mechanism via HBTU-DIPEA.

1.2. Results and Discussion

1.2.1. Synthesis of Chlorotoxin

Chlorotoxin is a 36 amino acid containing disulfide-rich peptide and it was tried to be synthesized by SPPS. Actually, this peptide can be synthesized more easily by Boc-SPPS method, because, the length of peptide in Fmoc-SPPS method is limited due to the lower yields of the peptides while synthesizing peptides that have more than 20 amino acids. However, Boc-SPPS method needs special equipment due to the usage of corrosive and highly toxic HF. So, more environmental method, Fmoc-SPPS is preferred for the peptide synthesis in our laboratory.

The aim of this study is to synthesize a cyclic derivative of chlorotoxin by adding 7-amino acid linker and to check its inhibitory effect on MMP-2 activity. To do this, firstly linear chlorotoxin synthesis was attempted by using Rink Amid resin. At the end of coupling 16 amino acids the small amount of resin was taken from the reactor and the peptide (pep-1) bound to the resin was cleaved and deprotected by using cleavage cocktail. The HPLC and LC-MS analysis were done and mass of the desired peptide was observed.
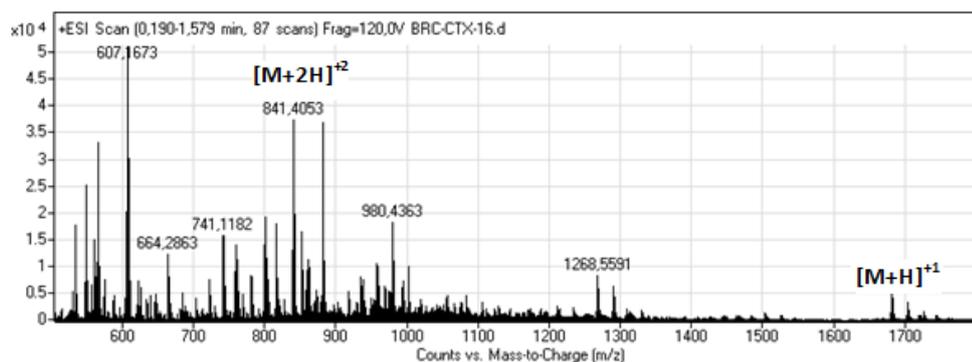
Figure 11. Mass spectrum of pep-1.

Then the synthesis was completed by coupling all 36 amino acids, but the mass of the chlorotoxin (pep-2) was not observed according to LC-MS results.

1.2.2. Synthesis of Cyclic Peptide by Native Chemical Ligation

In the synthesis of cyclic disulfide rich peptides, native intramolecular native chemical ligation (NCL) is the most commonly used method. In intramolecular NCL a reaction occurs between the thioester group at the C-terminus and a cysteine residue at the N-terminus. However, thioester group is unstable for the basic conditions used in Fmoc-SPPS, so Boc-SPPS strategy is required for this method. On the other hand, by using a C-terminal N-acylurea group and an N-terminal cysteine residue, intramolecular NCL can be applicable for Fmoc-SPPS strategy. In this method, *o*-aminoanilides are stable synthetic intermediates that can be efficiently transformed into an aromatic N-acylurea moiety and after the chain elongation and, the resin-bound acylurea peptide can be classically deprotected and cleaved from the resin by using TFA.[30]
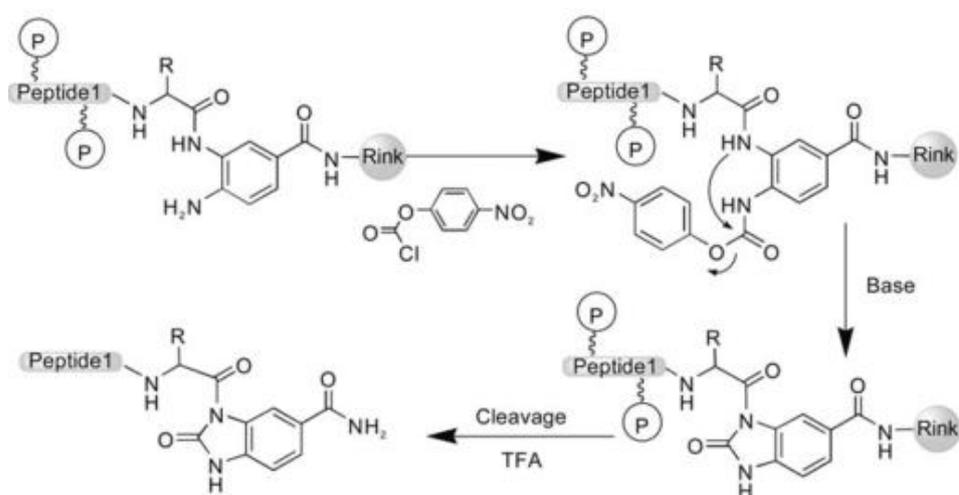
Figure 12. Formation of N-acylurea moiety and cleavage form the resin in NCL.

As a result, it was thought that cyclic chlorotoxin could be synthesized by intramolecular NCL method. Before starting to synthesize the cyclic Cltx, a small cyclic peptide (pep-4) that contains 9 amino acids was synthesized to test this method. The first amino acid of pep-2 for coupling was chosen as Gly, because the sequence of cyclic Cltx starts with this amino acid and also coupling of Gly to the C-terminal linker is easier. The last amino acid for coupling should be a Boc-protected one, because during the acylation of the linker, basic conditions are used which may cleave Fmoc protecting group. Other amino acids in the sequence of pep-4 were also chosen according to sequence of cyclic Cltx. As a result, the sequence of pep-4 was designed as CMDCKYRCG.

cyc(Cys-Met-Asp-Cys-Lys-Tyr-Arg-Cys-Gly)
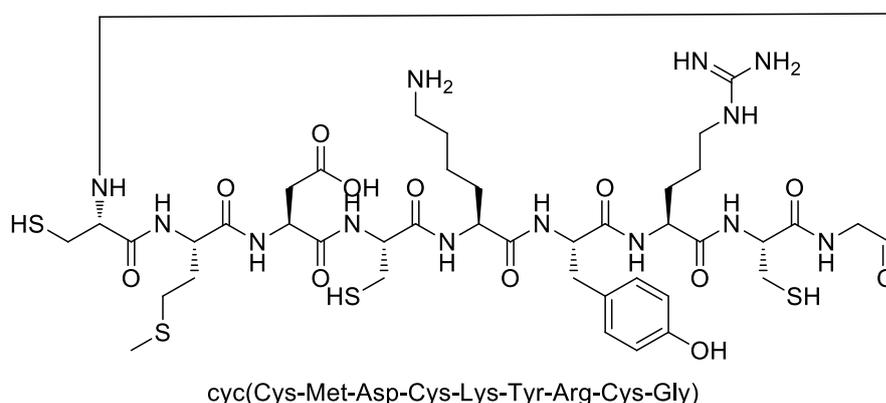
Figure 13. The molecular structure of pep-2.

Firstly, the linker 3, 4-diaminobenzoic acid (Dbz) was protected with Fmoc group and coupled to the Rink Amid resin. Starting from Gly all 9 amino acids were coupled one-by-one ending with Boc-Cys(Trt)-OH. Then, Dbz group was acylated and the acylurea peptide cleaved from the resin. After the cleavage, in order to prevent the disulfide formation of three cysteine residue that exist in the peptide sequence DTT was added as a reducing agent. The mass of desired peptide (pep-3) (CMDCKYRCG-Nbz) was observed according to LC-MS analysis.
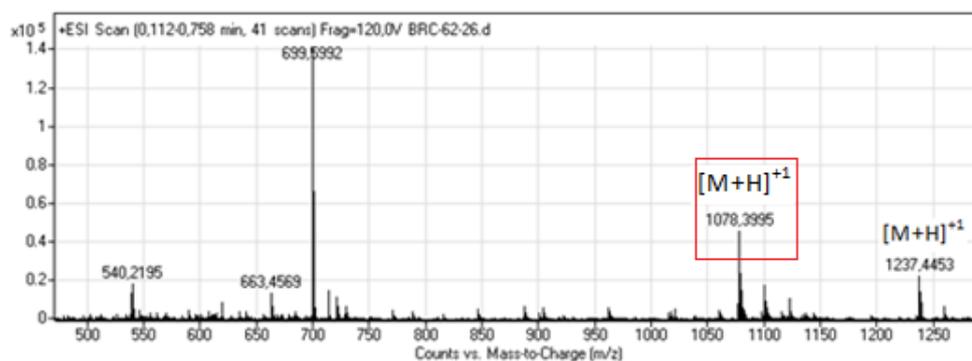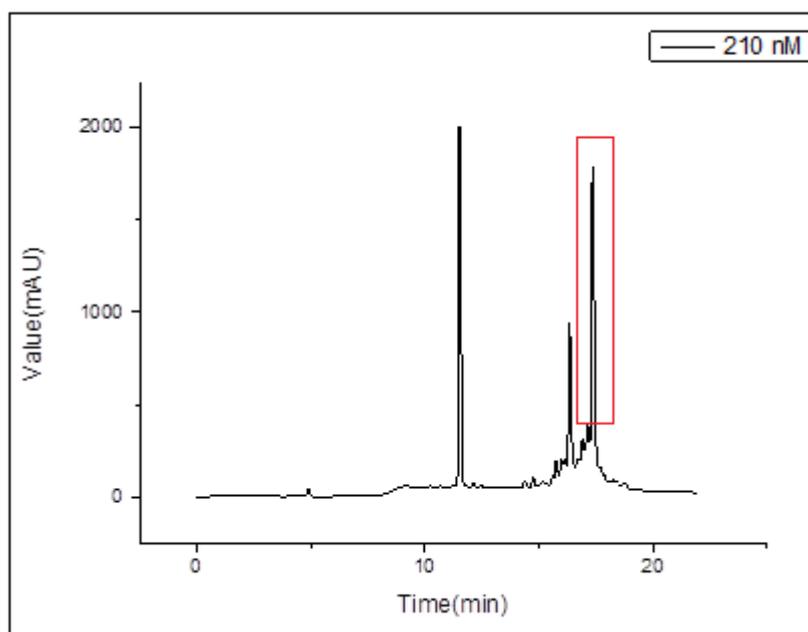
Figure 14. HPLC chromatogram and Mass spectrum of pep-3. Peak labeled with red line belongs to the mass of linear peptide without Nbz group.

After the purification of the peptide with semi-prep HPLC, cyclization reaction was done with pure peptide. At the end of the cyclization reaction LC-MS analysis show us both mass of the desired cyclic (pep-4) peptide and linear peptide without Nbz group.
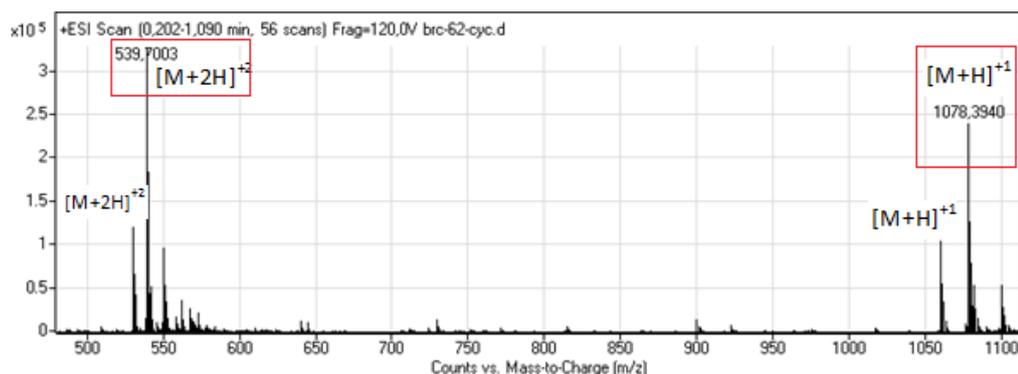
Figure 15. Mass spectrum of pep-3 and pep-4. Peaks labeled with red lines are belong to linear peptide without Nbz group.

The reason of this observation was attributed the fact that DTT which was used in cyclization reaction, may cleaved the linker from the peptide, so some peptide could not give ligation reaction and they stayed in linear form. Because of these side reactions and low yields, synthesis of cyclic Cltx was not pursued via NCL method.

1.2.3. Synthesis of Small Disulfide-Rich Cyclic Peptide: Test Reaction

After the synthesis of small disulfide-rich cyclic peptide by NCL method with low yield, the synthesis of the same peptide (pep-4) with simple Fmoc-SPPS method based on literature process was pursued.[31] In this method, synthesis was started by coupling of the first amino acid Gly to the 2-chlorotrityl resin. Then, by starting Fmoc deprotection, all 8 amino acids were coupled one-by-one. At the end of the chain elongation, peptide was cleaved only from the resin without cleaving side chain protecting groups. According to the result of LC-MS analysis, the mass of side chain protected peptide was observed (pep-5).
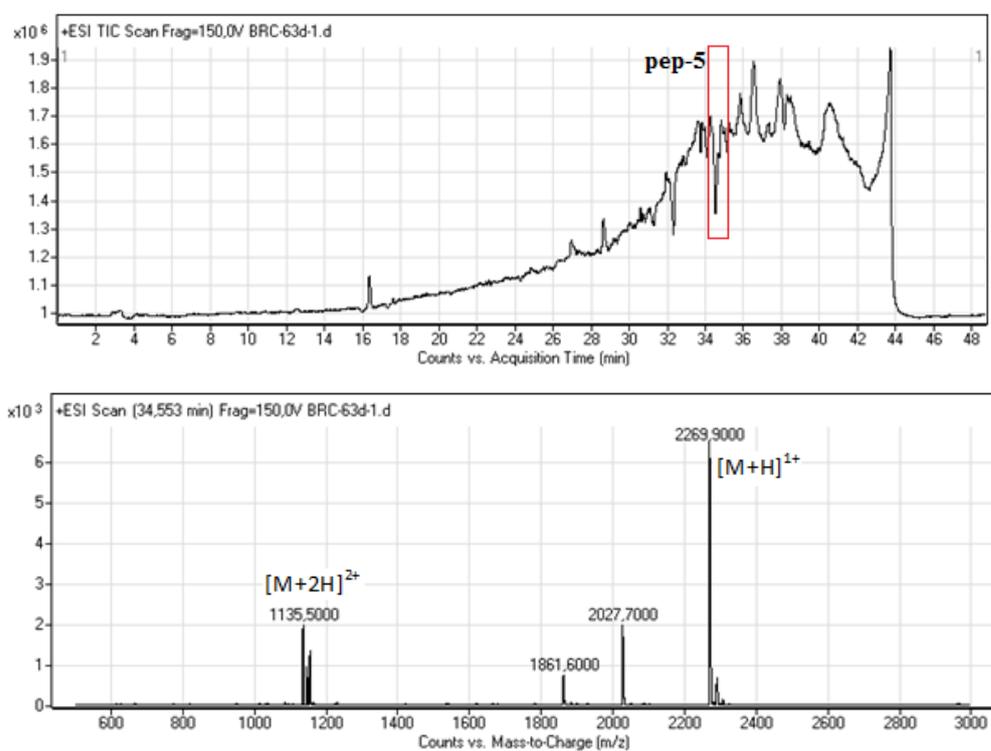
Figure 16. LC-MS chromatogram and spectrum of pep-5.

Cyclization reaction was performed in solution by using this partially protected peptide and HBTU/DIPEA system as coupling reagents. Before the cleavage of side chain protected groups, mass of the protected cyclized peptide (pep-6) was noticed by LC-MS analysis. All the side chains of the cyclized peptide were fully deprotected and peptide was purified to get the desired cyclic peptide (pep-4).
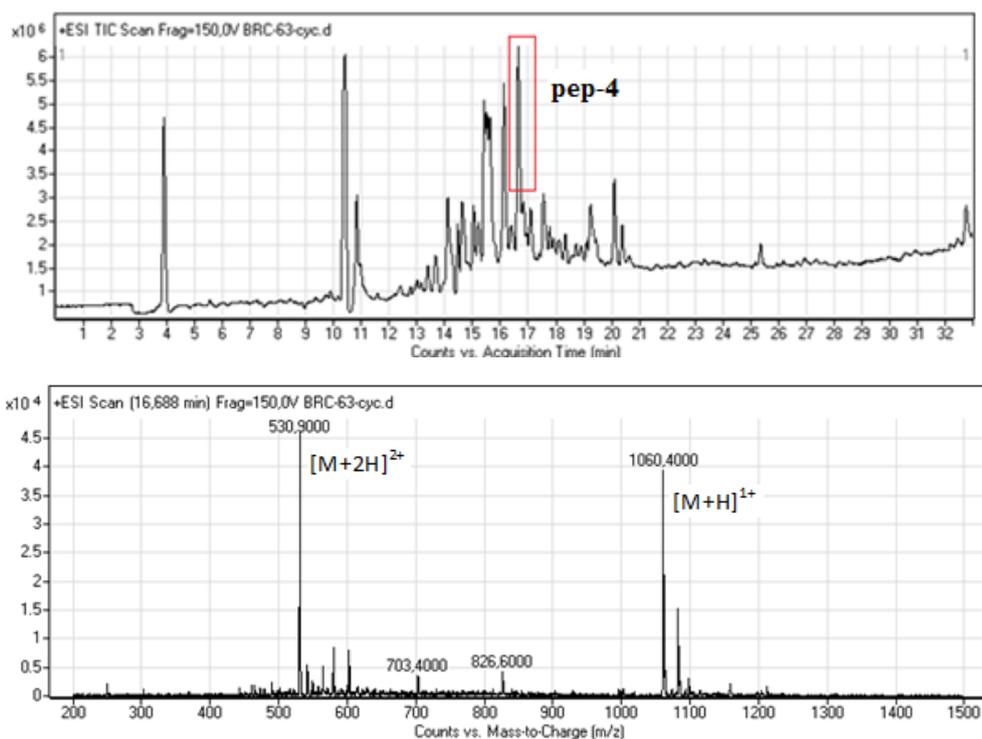
Figure 17. LC-MS chromatogram and spectrum of pep-4.

1.2.4. Synthesis of Chlorotoxin Derivative and Cyclic Form

A derivative of chlorotoxin that has additional 7 amino acids was synthesized by simple Fmoc-SPPS method based on the literature procedure.[31] The goal in the synthesis of this derivative is that it can be converted to cyclic form of chlorotoxin without losing activity using the cyclization method which was applied for smaller cyclotides very recently. The sequence of this chlorotoxin derived peptide (pep-7) is   MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCRGAGAAGG. The peptide synthesis was started with coupling of first amino acid Gly, to the 2-chlorotrityl resin. At the end of coupling 20 amino acids the small amount of resin was taken from the reactor and the peptide (pep-8) bound to the resin was cleaved and deprotected by using cleavage cocktail. According to results of HPLC and LC-MS analysis, all amino acids up to here were successfully coupled to each other.
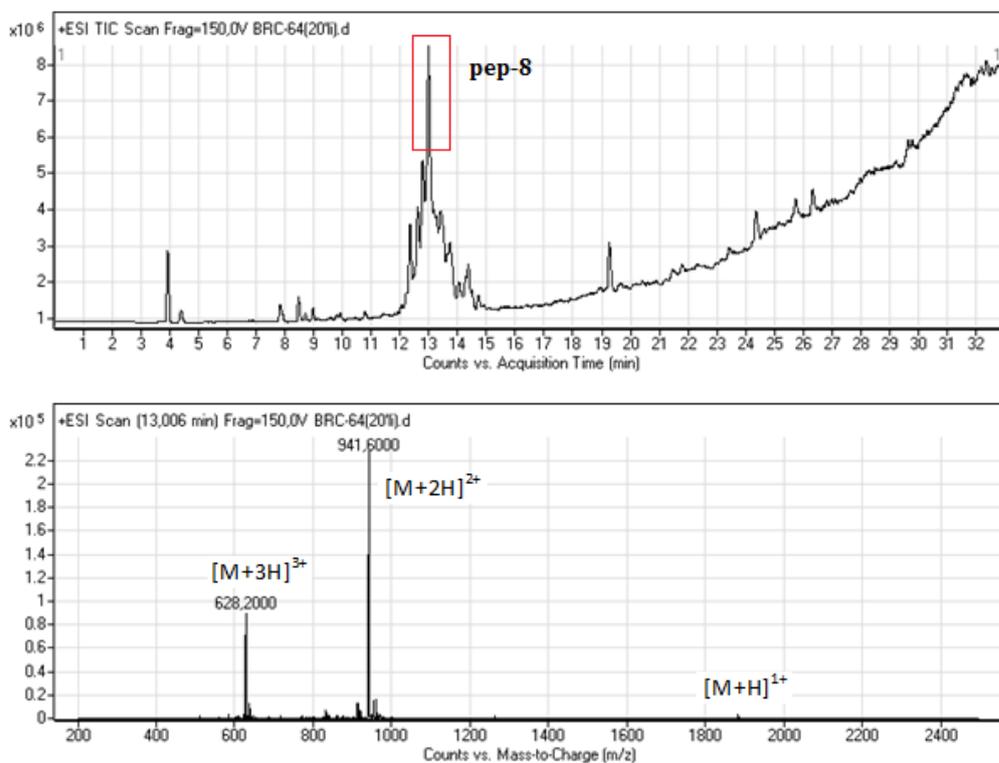
Figure 18. Mass chromatogram and spectrum of pep-8.

When the synthesis was completed by coupling all 43 amino acids, again small amount of resin was taken from the reactor, cleaved from the resin and fully deprotected in acidic conditions by using TFA. Results of the HPLC and LC-MS analysis shown us the desired linear chlorotoxin derivative (pep-7) was successfully synthesized.
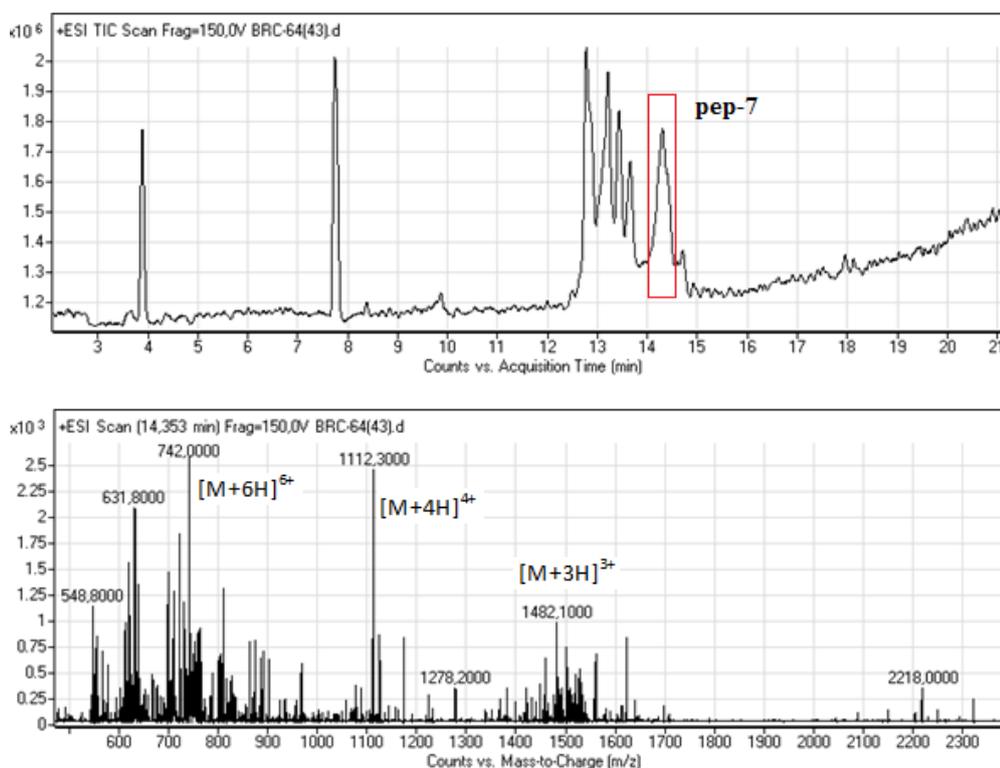
Figure 19. Mass chromatogram and spectrum of pep-7.

At the end of the synthesis, there was 500 mg of peptides bounded to resin. Half of this resin bounded peptide was used for cyclization and the other half was cleaved from the resin to get pure linear chlorotoxin derivative. Firstly, to get linear derivative, 250 mg resin bounded peptide was cleaved from the resin and deprotected from side chain protected groups by using 3 mL cleavage cocktail. However, the mass of desired linear peptide (pep-7) which was seen before was not observed in the LC-MS results at the end of this cleavage step. It was thought that, the amount of cleavage cocktail was not enough to cleave that large amount of peptide from the resin. The amount of cleavage cocktail was increased and cleavage step was repeated by using 10 mL cocktail for 100 mg resin bounded peptide and the resultant mass analysis shown that the linear derivative (pep-7) was successfully deprotected and cleaved from the resin. This Cltx derivative is a disulfide rich peptide and it has 8 cysteine residues in its structure. To get truly folded disulfide bridges, an oxidative folding condition which was successfully

used in native Cltx in literature was used.[27] A buffer with 0.1 M Tris-HCl, 0.2 M NaCl, 5 mM reduced glutathion, 0.5 mM oxidized glutathion (pH 7.8) was used for folding of pep-7. At the end of 24 hours, a small shift was observed in the HPLC chromatogram and the LC-MS analysis showed that the shifted peak was belong to the folded peptide (pep-8). After folding, the peptide was purified using RP-HPLC and pure folded Cltx derivative was collected.
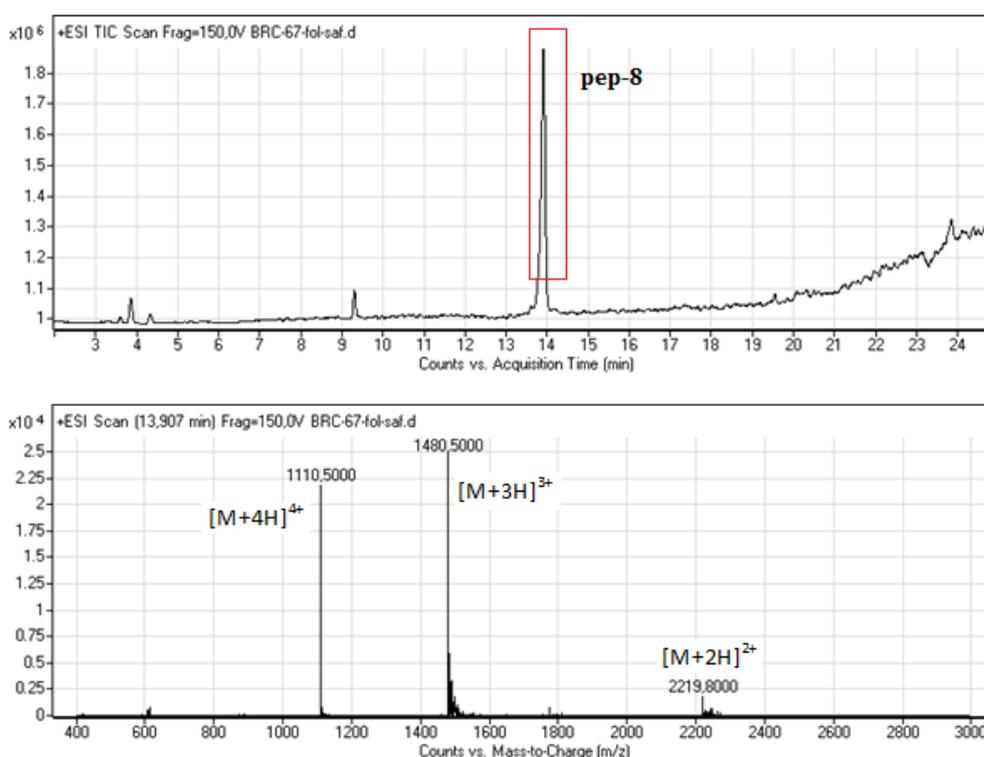
Figure 20. LC-MS chromatogram and spectrum of pep-8.

On the other hand, for the synthesis of cyclic Cltx derivative, based on literature procedure[31] 250 mg of the resin bounded peptide which was synthesized before was taken and it was cleaved only form the resin, without cleaving side chain protecting groups. Cyclization reaction of this partially protected peptide was performed by using HBTU and DIPEA in the solution. At the end of 24 hours, reaction was diluted with a mixture of 50% ACN (0.05% TFA) and H_2O and

lyophilized overnight. Then, all protecting groups of side chains were cleaved by cleavage cocktail. HPLC and LC-MS analysis were performed however the mass of cyclic Cltx derivative was not detected. The same procedure was applied to another small amount of resin bounded peptide by using HATU rather than HBTU but the desired cyclic peptide could not be obtained again.

It was thought that the reason of unsuccessful cyclization reactions was due to use of partially protected peptide. Side chain protecting groups of this peptide which consists of 43 amino acids may cover a lot of space, so the amine group at one end point and the carboxylic acid group at the other end point of the peptide could not get close to make the amide bond. Based on this, it was decided to perform cyclization reaction with fully deprotected and folded linear peptide which was synthesized before (pep-8). Cyclization reaction of pep-8 was performed in DMF by using HATU/DIPEA system. Reaction was monitored by HPLC and a small shift was observed. However, when the LC-MS analysis was done, the mass of the cyclic peptide was not noticed.

1.2.5. Interactions between Chlorotoxin Derivative and MMP-2 Enzyme

MMP-2 activity assay was done by Ayşenur Biber. The assay was performed in 96-well microplate fluorometer. Interaction of pep-8 with MMP-2 was analyzed while using commercial chlorotoxin as positive control. Assay was performed with three different concentrations that are 1X (115 nM) which equals to IC_{50} value of chlorotoxin, 10X (1.15 μM) and 100X (11.5 μM). As a result of activity assay, no inhibition property was observed for pep-8. (Figure 21) Commercial chlorotoxin which was used as positive control also not showed good inhibition effect. So, it was thought that this reason may be due to some problems with the activity assay. Activity assay has not been tried more than once. Another reason of this result might be the misfolding of the chlorotoxin derivative or the additional 7 amino acid residue may affect the activity of the peptide on the MMP-2.
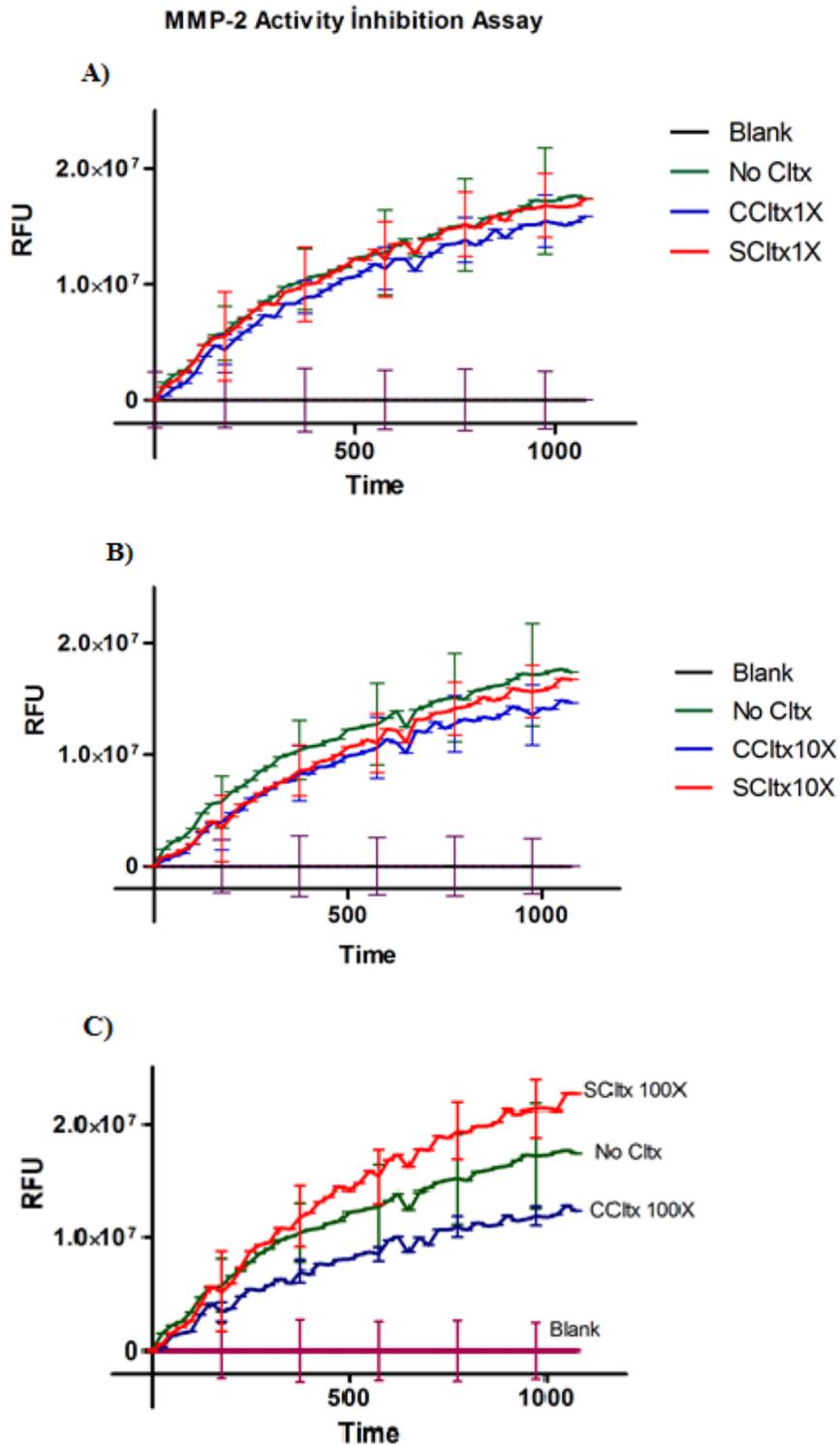
Figure 21. Graphs of MMP-2 activity assay with concentrations of; A) 1X, B) 10X, C) 100X. Commercial chlorotoxin was shown as 'CCltx' and synthetic chlorotoxin derivative pep-8 was shown as 'SCltx'.

CHAPTER 2

SYNTHESIS OF PEPTIDE INHIBITORS FOR ANGIOTENSIN CONVERTING ENZYME

2.1. Introduction

2.1.1. Angiotensin Converting Enzyme (ACE)

Angiotensin converting enzyme (ACE), is a member of the M2 family of zinc metallopeptidases. It is also known as a dipeptidyl carboxypeptidase due to the catalytic activity on the hydrolytic cleavage of dipeptides from the carboxyl terminus of oligopeptides in vitro. It shows this functionality for the in vivo conversion of Ang I that has a sequence of DRVYIHPFHL and mobilizes in plasma, into Ang II, which shows the potent vasopressor property, by removal of the C-terminal His–Leu dipeptide.[32]

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu   (Ang I)

ACE

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe   (Ang II)
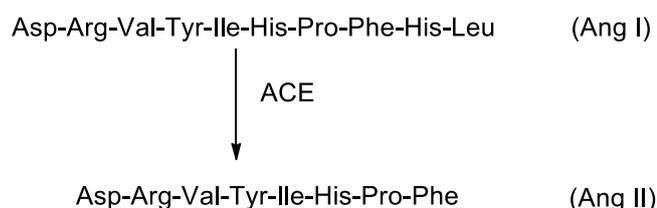
Figure 22. Converting of Ang I to Ang II.

ACE also influences blood pressure by cleaving bradykinin (BK, RPPGFSPFR), thus abolishing its vasodilating activity. Due to this reason, ACE is sometimes referred to as kininase II.

2.1.1a. Crystal Structure of ACE

ACE contains basically two domains, an N-terminal and a C-terminal domain. There are around 612 amino acids at the N-terminal domain, 15 amino acids at the interdomain and around 650 amino acids at the C-terminal domain.[33] Both N-terminal and C-terminal domains have an amino acid sequence of HEMGH, which is typical component of catalytic zinc sites found in neutral endoproteinases.[34] Comprehensive mutational and kinetic analyses have proved that the zinc sites of both domains have catalytic activity. In addition, the chloride concentration highly affects activity of the C-terminal domain, while it does not have much effect on the activity of the N-terminal domain.[35]
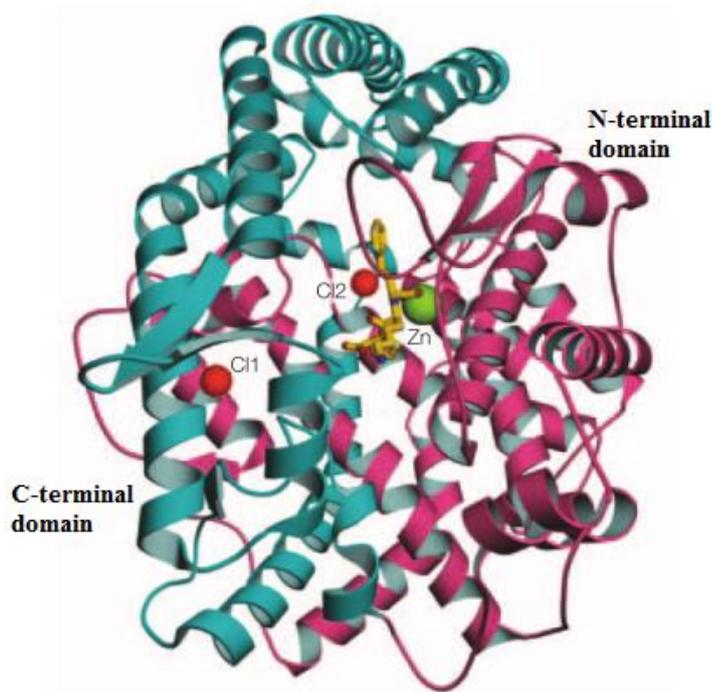
Figure 23. Crystal structure of ACE.

2.1.1b. Inhibitors of ACE

Captopril, which was approved in 1981, is the first clinically used ACE inhibitor. It was authorized immediately as a powerful therapeutic agent in the treatment of heart failure and hypertension.[36] However, due to the sulfhydryl group in its structure, it caused side effects such as skin rush and loss of taste. Hence, lots of N-carboxylalkyl dipeptides were tested on the structure-activity studies and consequently enalaprilat and lisinopril were found that have inhibition constants on nanomolar scale.
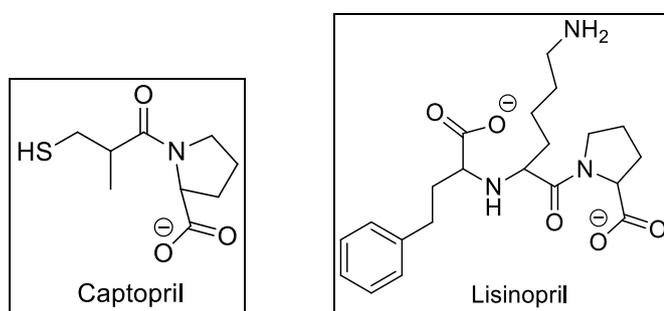
Figure 24. Structures of ACE inhibitors; Captopril and Lisinopril.

Lisinopril and enalaprilat are basically tripeptide analogues. They have a Zn^{2+}-coordinating carboxyl group that substitutes to the substrate scissile amide carbonyl. Lisinopril forms large contacts with the active site of the enzyme. With interaction of a lysine by the C-terminal carboxylate, connection of a hydrogen bond with the (substrate) scissile amide nitrogen, and coordination of the active-site Zn^{2+} by the carboxylalkyl carboxylate lisinopril covers all the S1, S1′and S2′ pockets. It also binds with two fold greater affinity than enalaprilat due to the better contacts of lysine side chain with the S1′ pocket.[33]
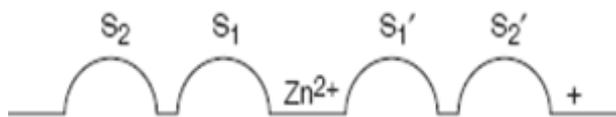
Figure 25. Schematic representation of ACE active site.

2.1.2. Kojic Acid

Kojic acid, 5-Hydroxy-2-(hydroxymethyl)-4-pyrone, was discovered by K. Saito in 1907.[37]
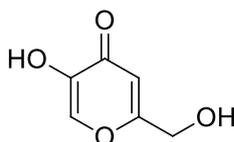
Figure 26. Structure of kojic acid.

It has been known as an additive in food industry to prevent browning of certain foods like shrimp, cramb and fresh vegetables. Numerous bacterial or fungal strains such as *Aspergillus oryzae*, *Penicillium* or *Acetobacter spp.* produce this biologically important natural antibiotic in an aerobic process from a wide range of carbon sources.[37]

Kojic acid reacts with metal acetate salts such as beryllium, tin, zinc, nickel, copper, iron, cobalt, manganese, chromium, gold, palladium, gallium, indium, vanadium, and aluminum and forms stable metal complexes.[38,39]
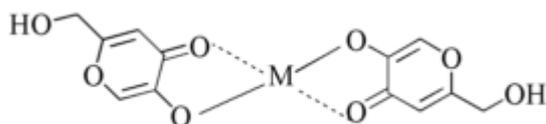
Figure 27. Kojic acid-metal complexes, M= Mn, Co, Ni, Cu, Zn, Cd and Hg.

2.1.2a. Inhibitory Effect of Kojic Acid

The best known function of kojic acid is its inhibitory effect on the enzyme tyrosinase. Tyrosinase is an enzyme that has Cu^{2+} ion at the active site and controls melanin synthesis. Kojic acid prevents formation of melanin with a slow and reversible competitive inhibitory effect on human tyrosinase. So, it was used as a skin-whitening product in cosmetic particularly in Japan.[40]

Kojic acid inhibits tyrosinase activity by chelating copper ion at the active site of the enzyme through the 5-hydroxyl and 4-carbonyl groups. However, the inhibitory activity of kojic acid is not enough at low concentrations by itself due to stability problems. Therefore, conjugates of kojic acid-tripeptide amides were developed that show more stable tyrosinase inhibitory activity.[41]

2.2. Results and Discussion

Kojic acid-tripeptide conjugates are known to be tyrosinase inhibitors. They show inhibitory effect by chelating to Cu^{2+} ion in the active site of the enzyme tyrosinase and peptide side chains contribute to the stability by interacting with the active site pockets. Based on this, kojic acid-tripeptide conjugates are thought to be good inhibitor for ACE, which has Zn^{2+} ion in its active site. Firstly, synthesis of kojic acid-amino acid conjugates was attempted by both SPPS and in solution. Phenylalanine was used as the amino acid that will be conjugated because, from the structure of ACE inhibitors such as lisinopril and enalaprilat, phenyl ring interacts with sub-sites of the enzyme and leads to a better inhibition.

2.2.1. Synthesis of Kojic Acid-Amino acid Conjugates by SPPS

Firstly, kojic acid was chlorinated to make it more reactive.
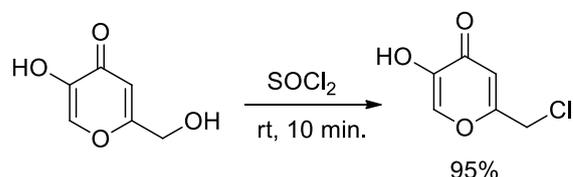
Figure 28. Chlorination of kojic acid.

Synthesis of kojic acid-phenylalanine conjugate was attempted by using general Fmoc-SPPS method. Fmoc protected phenylalanine was coupled to Rink Amid resin. Then, chlorokojic acid was dissolved in DMF and coupled as an amino acid by using DIPEA. Kojic acid-phenylalanine conjugate was cleaved from resin and deprotected. According to the result of NMR and LC-MS analysis, the synthesis of the desired conjugate was not achieved.

Another SPPS method, based on literature[42] was used, which need to synthesize kojic acid-imidazole (Figure 28) first, to be able to activate kojic acid more easily.
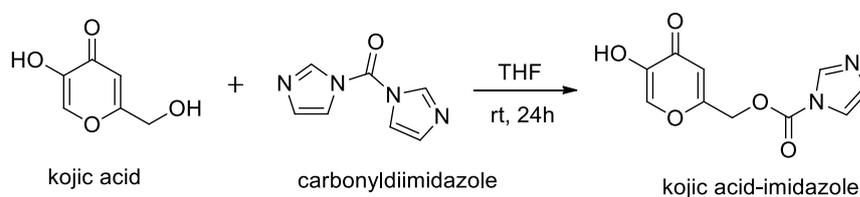
Figure 29. Synthesis of kojic acid-imidazole.

Kojic acid and carbonyldiimidazole was stirred in THF at room temperature. At the end of 24 hours, kojic acid-imidazole was obtained. SPPS was performed by coupling Fmoc-Phenylalanine to the Rink Amid resin as first amino acid. Then, kojic acid-imidazole was dissolved with HOBt in DMF and coupled as next amino acid. Following the cleavage from resin, NMR result gave us the desired kojic acid-phenylalanine conjugate with a yield of 78%.

2.2.2. Synthesis of Kojic Acid-Amino acid Conjugates in Solution

Two different approaches were used for the synthesis of kojic acid-amino acid derivatives in solution. In the first approach, chlorokojic acid and iodokojic acid were synthesized to get more reactive form of kojic acid. Conjugation was expected to be obtained by the attack amine group of phenylalanine to chlorokojic acid.

33
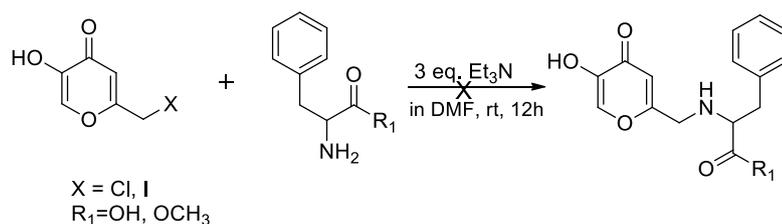
Figure 30. Schematic representation for the synthesis of kojic acid-amino acid conjugates by first approach.

Reaction was firstly performed between chlorokojic acid and unprotected phenylalanine, but the product was not obtained. Then, carboxylic group of the phenylalanine was protected by as a methyl ester group. The reaction of chlorokojic acid and phenylalanine methyl ester was performed in DMF by using Et$_3$N as base, under inert conditions. However, the desired product was not obtained. Based on a different procedure from literature,[43] reaction was performed with a different of solvent in which ACN was used rather than DMF. Additionally, reaction was performed under reflux conditions due to solubility problem of the reactants. However, the product could not be obtained.

In the second approach, amine derivative of kojic acid was synthesized and conjugation was expected to occur by the attack of this amine group to the carbonyl group of the amino acid.
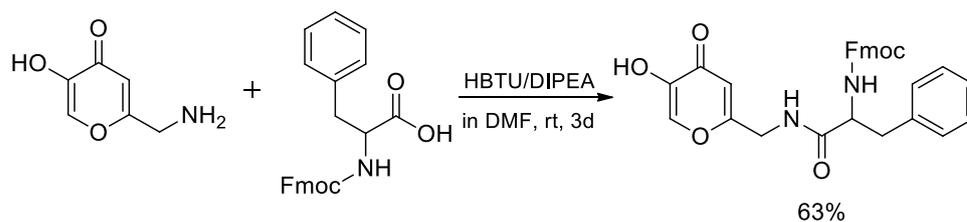
Figure 31. Schematic representation for the synthesis of kojic acid- amino acid conjugates by second approach.

For the synthesis of kojic amine, azide derivative of kojic acid was synthesized from chlorokojic acid with NaN_3 in DMSO. Then, kojic amine was synthesized from kojic azide according to a procedure based on literature.[44] Kojic azide was dissolved in a mixture of THF and H_2O. Triphenylphosphine was added to this mixture and reaction was stirred overnight at room temperature. At the end of the reaction, some precipitate was observed and reaction mixture was centrifuged. According to ^{1}H NMR spectrum, the precipitate was our product. Conjugation reaction was performed in between this kojic amine and Fmoc-phenylalanine. Kojic amine was dissolved in DMF and Fmoc-phenylalanine, activated by HBTU/DIPEA, was added to the solution and stirred at room temperature. After 3 days, reaction was stopped by adding H_2O. Our product was precipitated after addition of water due to its low solubility. These precipitates were collected by centrifuge and their structure was confirmed by result of NMR analysis. To cleave Fmoc group from kojic acid-Phe-Fmoc product, it was dissolved in solution of 20% piperidine in DMF. It was expected to precipitate when cold tert-butyl methyl ether was added to the solution, but it did not. Another method was used to cleave Fmoc group from kojic acid-phenylalanine conjugate. Fmoc protected conjugate was dissolved in the mixture of Piperidine:ACN (1:4) and stirred at room temperature. At the end of 1 hour, reaction was stopped by adding MeOH and H_2O and precipitation was occurred. Precipitates were filtered and the filtrate was analyzed. Unfortunately, NMR result showed that synthesis of the desired product was not achieved.

Deprotection of Fmoc was proved to be difficult, so kojic acid-phenylalanine conjugate was decided to be synthesized starting from Boc protected phenylalanine.
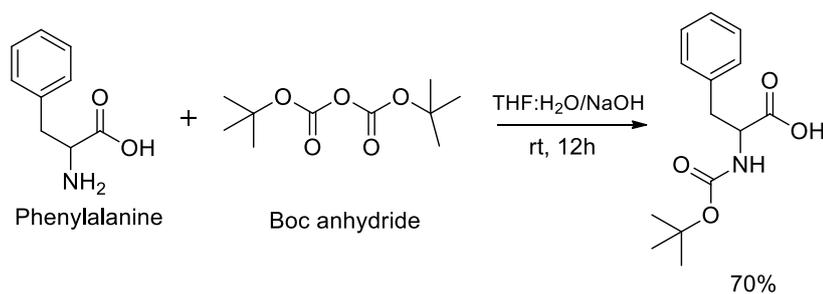
Figure 32. Synthesis of Boc-Phe-OH.

Unprotected phenylalanine was protected by Boc group with a reaction of Boc anhydride (Boc$_2$O). Phenylalanine was dissolved in the mixture of THF:H$_2$O (1:1) and Boc$_2$O and NaOH added to reaction mixture. The reaction was stirred for overnight at room temperature. At the end of reaction, extraction was performed with DCM. According to NMR result, Boc-Phenylalanine was synthesized successfully. Kojic amine-Phe-Boc product was synthesized with the same procedure as previously performed. By cleavage of Boc group with TFA, the desired kojic acid-phenylalanine conjugate was obtained.
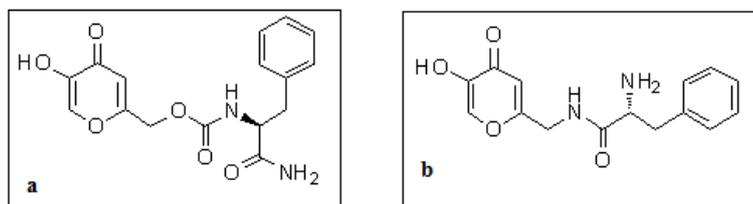
Figure 33. Kojic acid-phenylalanine conjugates synthesized by **a)** SPPS **b)** solution phase.

As a result, two different kojic acid-Phe conjugates were synthesized (Figure 32) and their inhibitory effect on ACE need to be analyzed before the synthesis of dipeptide or tripeptide derivatives. To do these analyses, different activity assays of ACE were applied to the commercial enzyme. However, no activity was observed in these analyses. So, effects of these kojic acid-phenylalanine conjugates on the enzyme could not be measured. Three different batches of

commercially available ACE have been tested but none of these enzymes seem to be active in our hands. That might be due to activity loss of enzymes during the long shipping and waiting period in the custom.

CHAPTER 3

CONCLUSION

To conclude, peptide inhibitors for two enzymes, MMP-2 and ACE, which belong to the family of zinc-metallopeptidases were tried to be synthesized. In the first part of the study, a derivative of chlorotoxin was synthesized for inhibition of MMP-2 enzyme activity, by Fmoc based SPPS method. Chlorotoxin is a disulfide rich peptide and it is known that it has an inhibitory effect on MMP-2 activity. In addition, cyclic form of chlorotoxin, that has additional 7 amino acids, is known as more stable than the linear form. Based on here, cyclic and linear forms of this 7 amino acid residue added chlorotoxin derivatives are tried to be synthesized by SPPS. As a result, linear form of chlorotoxin derivative was obtained successfully while the synthesis of cyclic form was failed. The inhibitory effect of this linear chlorotoxin derivative on the MMP-2 was measured by activity assay, however no inhibitory effect was observed.

In the second part of the study, kojic acid-amino acid conjugates were synthesized. Kojic acid is known as a good metal chelator and it inhibits activity of tyrosinase by chelating Cu^{2+} ion in the active site of the enzyme. On the other hand, ACE is known as a zinc metalloproteinase. It was thought that, kojic acid can inhibit activity of ACE by chelating Zn^{2+} ion in the active site of the enzyme. To increase selectivity and stability of inhibition, kojic acid-amino acid conjugates are tried to be synthesized. As a result, two derivatives of kojic acid-phenylalanine conjugate were synthesized. However, due to the activity problems of commercial ACE, interactions of these conjugates with the enzyme could not be studied.

CHAPTER 4

EXPERIMENTAL

4.1. Materials and Methods

For the organic synthesis, all reactions were monitored by TLC using pre-coated silica gels plates visualized by UV-light. Final column chromatography separations were performed by silica gel purchased from Aldrich. Compounds were named by using ChemDraw Ultra 12.0. For the synthesis of pre-designed peptide, all natural amino acids were purchased from ChemImpex.

4.1.1. Nuclear Magnetic Resonance

Nuclear magnetic (^{1}H-NMR) spectra were recorded in $CDCl_3$ and d_6-DMSO on a Bruker Spectro Spin Advance DPX 400 spectrometer. Chemicals shift are given in parts per million (ppm) with TMS as internal reference.

4.1.2. HPLC

HPLC purification of the cleaved peptides were performed with Dionex Ultimate 3000 Series equipped with a variable wavelengths absorbance detectors using a reverse phase C18 column (Hypersil Gold, 12 μm, 250 x 10 mm). A binary gradient of water (0.1% TFA) and acetonitrile (0.08% TFA) was used with a flow rate of 3 mL min^{-1} and the eluent was monitored by UV absorbance at 210, 280, 330 and 450 nm. Fractions were gathered and lyophilized after their purities were confirmed by analytical HPLC performed using a RP-C18 column (Acclaim 120, 3 μm, 4.6 x 150 mm) with a flow rate of 0.5 mL min^{-1}.

4.1.3. LC-MS-QTOF

Analytical LC-MS-QTOF analyses of synthetic amino acids and peptides were recorded on an Agilent Technologies High Resolution Mass Quadropole Time-of-Flight (TOF) LC/MS 1200 series and Zorbax Eclipse XDB-C18 analyticals 4.6 x 150 mm 5-micron column was employed. *For the peptides synthesized by SPPS, MS analysis is enough for the characterization of the peptides. No other spectrum such as NMR or IR is needed.*

4.2. Reaction Procedures for Synthesis of Chlorotoxin and Its Derivatives

4.2.1. Synthesis of 3-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)-4-aminobenzoic acid
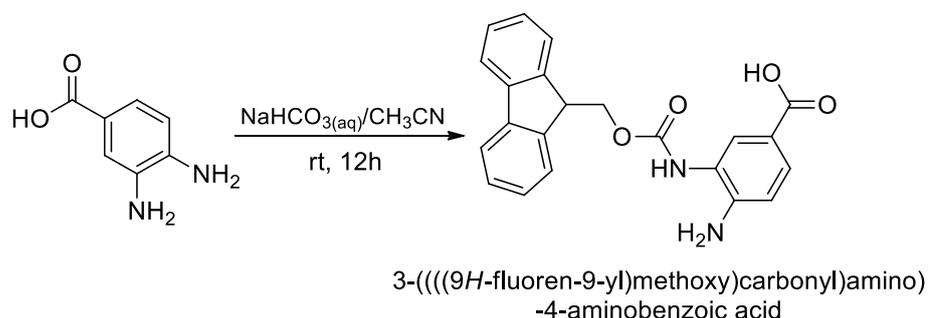
Figure 34. Schematic diagram of 3-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)-4-aminobenzoic acid synthesis.

Based on literature procedure,[30] to a solution of 3,4-diaminobenzoic acid (1.0 g, 6.6 mmol) in $NaHCO_3$(aq) (168 mg,) water/acetonitrile (1:1, 20 mL) was added N-(9-fluorenylmethyloxy carbonyloxy) succinimide (2.2 g, 6.6 mmol) in small amounts and stirred overnight at room temperature. Reaction was stopped by acidification with 1 M HCl (aq) forming a precipitate. Then, precipitate was filtered, washed with methanol, cold ether and hexane. The solid was dried under vacuum affording the expected compound as a pale white solid (1.9 g, 80%).

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^{1}H NMR (400 MHz, d^6-DMSO): δ 8.78 (s, 1H), 7.91 (d, J=7.4 Hz), 7.72-7.80 (m, 3H), 7.51 (d, 1H), 7.44 (t, 2H), 7.33-7.36 (m, 2H), 6.72 (d, 1H), 4.29-4.40 (m, 3H).

4.2.2. Synthesis of Chlorotoxin by Using Rink Amid Resin (Pep-2)

85 mg (0.050 mmol) Rink Amide resin was weighed in reaction vessel in which the desired peptide will be synthesized. The resin was washed with DMF (2x) and swelled in 5-6 mL DMF for 40-45 min. The solution is drained and Fmoc group that attached to the resin was deprotected by adding 5 mL of 20% piperidine in DMF (2x10 min). Then Fmoc-protected first amino acid (0.275 mmol, 5.5 eq.) was dissolved in HBTU (0.5 mL, 0.5 M in DMF). DIPEA (100 μL) was added to this amino acid solution, stirred for 30 seconds and added to the resin in the reactor. The amino acid was coupled to the resin for 1 h. At the end of 1 h, resin was washed with DMF and all 36 amino acids were coupled with the same procedure by starting with the Fmoc deprotection step. When all 36 amino acids were coupled, Fmoc group of last amino acid was deprotected and the resin was washed with DMF (4x5 mL), DCM (4x5 mL) and dried under vacuum. To cleave peptide from the resin, 88% TFA, 5% DTT, 5% H$_2$O and 2% TIPS solution was added to the resin and the mixture was stirred for 2 h (2x). After the treatment of resin with cleavage cocktail, eluent was collected, evaporated in rotary and triturated with cold diethyl ether.

4.2.3. Synthesis of Small Cyclic Peptide by Native Chemical Ligation (Pep-4)

Based on literature procedure,[45] Fmoc-protected 3,4-diaminobenzoic acid (0.275 mmol) was coupled to the Rink Amid (0.050 mmol) as the first amino acid. Then, all 9 amino-acids were coupled with the same procedure described in previous section with a difference of 10 min coupling time instead of 60 minutes. After the

all amino acids are coupled, 1 mL DCM was added to the reactor stirred for 30 seconds and removed (4x). Then 4-nitrophenyl chloroformate (20 mg, 0.1 mmol) was dissolved in DCM and added to the reactor. At the end of 1 h, solution was removed from the reactor and resin was washed with DCM (4x1 mL) and DMF (6x1 mL) respectively by stirring 30 seconds. 1 mL of 10% DIPEA/DMF (v/v) was added to the resin, stirred for 15 minutes and removed from reactor. Resin was washed with DMF and DCM and dried under vacuum. To cleave peptide from the resin, 96% TFA, 2% H_2O and 2% TIPS solution was added to the resin for 2 h. After the treatment of resin with cleavage cocktail, eluent was collected, evaporated in rotary and triturated with cold diethyl ether.

4.2.4. Synthesis of Small Cyclic Peptide by General SPPS (Pep-4)

Based on literature procedure,[31] for coupling of first amino acid onto the resin, 55 mg (0.05 mmol) 2-Cltrt resin was swollen in DCM. 59 mg Gly (0.20 mmol) was dissolved in the mixture of 500 µL DCM:DMF (1:1) and mixture was coupled onto the resin for 1 h after the addition of 70 µL DIPEA. At the end of 1 h, 500 µL DIPEA was added onto resin and mixed for 15 minutes to cap any remaining reactive functionalities on the resin. The resin was washed with DMF, DCM, MeOH and DMF again. Fmoc deprotection was carried out with 20% piperidine in DMF twice for 10 min. Subsequent 4.5 fold excess amino acids were coupled twice for 10 min. using 400 µL HBTU and 70 µL DIPEA per amino acid. After chain assembly was complete, the terminal Fmoc group was removed and the resin washed with DMF and DCM and dried under a flow of nitrogen for 2 h.
To obtain the cyclic reduced peptide, a three-step procedure was followed. In the first step peptide was cleaved from the resin by leaving the side-chain protecting groups intact. The dried peptide was treated 10 times with 2 mL of 1% TFA in DCM (v/v) for 5 min. The eluents were collected and combined into a round-bottomed flask containing 60 mL of 50% ACN, 0.05% TFA in water (v/v). DCM and TFA were removed with rotary, and the resulting solution was lyophilized.

In the second step resulting 50 mg side-chain protected peptide was dissolved in DMF at a concentration of 2 mM in a round-bottom flask. Solid HATU was added to the solution to give a final concentration of 5 mM and mixed for 30 s. DIPEA was added to a final concentration of 10 mM, and the solution was stirred for 3 h at room temperature. The mixture was diluted with twice the volume of 50% ACN (0.05% TFA) in water (v/v) and lyophilized overnight. In the third step, side–chain protecting groups were cleaved from the peptide by adding 3 mL of TFA:TIPS:H_2O (96:2:2, (v/v/v)) and stirring for 2.5 h at room temperature. The mixture was evaporated and the peptide was triturated with ice-cold diethyl ether.

4.2.5. Synthesis of Chlorotoxin Derivative (Pep-8) and Cyclic Form

The same procedure was applied that was described in section 1.4.2d for cyclic peptide. To get linear form of chlorotoxin derivative (pep-8) at the end of chain assembly, the terminal Fmoc group was removed and the resin washed with DMF and DCM and dried under a flow of nitrogen for 2 h. 100 mg of dried, resin bounded peptide was taken and cleaved from resin with all side-chain protecting groups by stirring with 10 mL cleavage cocktail. At the end of 3 hours, cocktail was evaporated and peptide was triturated with ice-cold diethyl ether.

4.2.6. Disulfide Formation and Folding

A buffer solution of 0.1 M Tris-HCl, 0.2 M NaCl, 5 mM reduced glutathione/0.5 mM oxidized glutathione at pH 7.8 was prepared. 10 mg of pep-8 was dissolved in 20 mL of this folding buffer and stirred at room temperature. At the end of 24 hours, the mixture was filtered and RP-HPLC was done to get pure folded peptide.

4.2.7. MMP-2 Activity Assay

MMP-2 activity assay was performed in 96-well microplate fluorometer. For the assay, commercial *SensoLyte® 520 MMP-2 Assay Kit* was used. For the assay measurement, firstly 200 ng/mL enzyme was prepared by dilution of 1.5 µL MMP-2 standard with 73.5 µL assay buffer. Then, 0.75 µL 4-aminophenylmercuric acetate (APMA) was added to the 74.25 µL of this diluted MMP-2 standard and incubated at 37 ºC for 1 h for activation. Secondly, solutions of commercial chlorotoxin and synthesized chlorotoxin derivative were prepared with three different concentrations that are 1X (115 nM) which equals to IC_{50} value of chlorotoxin, 10X (1.15 µM) and 100X (11.5 µM). Activated MMP-2 was added to all these solutions with a final concentration 10 ng/mL. From each concentration of MMP-2/Cltx mixture, 37.5 µL samples was taken and transferred to 3 cells of the microplate. The same thing was done for MMP-2/Cltx derivative containing mixture and also for standard MMP-2 which was used as negative control. Into another 3 cells, 75 µL assay buffer was transferred as blank. Finally, 37.5 µL of 5-FAM/QXL^{TM}520 fluorescence resonance energy transfer (FRET) peptide, which was diluted by assay buffer with a dilution rate of 1:100 was added to each cell, except blank, to start the enzymatic reactions. Fluorescence measurement was started immediately and continued for 18 hours by recording data every 5 min at 490-520 nm.

4.3. Reaction Procedures for Synthesis of Kojic Acid-Amino acid Conjugates

4.3.1. Synthesis of 2-(chloromethyl)-5-hydroxy-4H-pyran-4-one (Chlorokojic acid) (1)
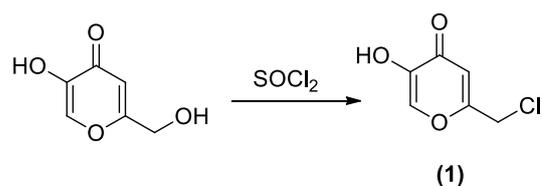
Figure 35. Synthesis of chlorokojic acid.

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Kojic acid (0.7 g, 0.5 mmol) was dissolved in $SOCl_2$ (0.7 mL) and the reaction was stirred at room temperature. After 10 minutes, precipitation was observed. This precipitate was washed with hexane and solid product was obtained in 95% yield.

^{1}H NMR (400 MHz, CDCl$_3$): δ 7.89 (s, 1H), 6.58 (s, 1H), 4.36 (s, 2H).

4.3.2. Synthesis of 2-(((5-hydroxy-4-oxo-4H-pyran-2-yl)methyl)amino)-3-phenylpropanoic acid (2) by SPPS
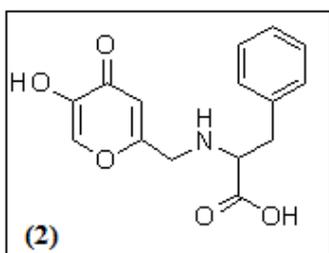
0.1 mmol (169.5 mg) Rink Amide resin was weighed in reaction vessel in which the desired peptide will be synthesized. The resin was washed with DMF (2x) and swelled in 5-6 mL DMF for 40-45 min. The solution is drained and Fmoc group that attached to the resin was deprotected by adding 10 mL of 20% piperidine in DMF (2x10 min). Then Fmoc-Phe (0.55 mmol, 5.5 eq.) was dissolved in HBTU (1 mL, 0.5 M in DMF). DIPEA (200 μL) was added to this amino acid solution, stirred for 30 seconds and added to the resin in the reactor. The amino acid was coupled to the resin for 1 h. At the end of 1h, resin was washed with DMF and Fmoc group of phenylalanine was deprotected by addition of 10 mL of 20% piperidine in DMF (2x10 min). Chlorokojic acid was dissolved in DMF and coupled to phenylalanine as an amino acid by adding DIPEA. At the end of 2 hours, resin was washed with DMF and DCM. Dried resin was cleaved by addition of cleavage cocktail (TFA:TIPS:H$_2$O/95:2.5:2.5). Desired product was not obtained according to NMR and LC-MS results.

4.3.3. Synthesis of (5-hydroxy-4-oxo-4H-pyran-2-yl)methyl 1H-imidazole-1-carboxylate (kojic acid-imidazole) (3)

(3) kojic acid-imidazole
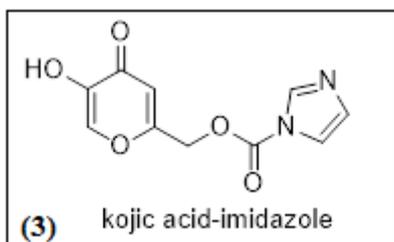
Based on literature procedure[42], kojic acid (250 mg, 1.76 mmol) was dissolved in 4 mL dry THF and 1 mL of DMF. Carbonyldiimidazole (256 mg, 1.58 mmol) was dissolved in 3 mL dry THF and added to the kojic acid solution. Reaction was stirred under inert atmosphere by monitoring TLC. At the end of 48 hours, reaction was centrifuged and white precipitate was obtained as desired product.

^{1}**H NMR** (400 MHz, d^6-DMSO): δ 9.32 (bs, OH), 8.34 (s, 1H), 8.13 (s, 1H), 7.67 (t, J = 1.4 Hz, 1H), 7.10 (dd, J = 1.5, 0.8 Hz, 1H), 6.66 (s, 1H), 5.31 (s, 2H).

4.3.4. Synthesis of (5-hydroxy-4-oxo-4H-pyran-2-yl)methyl (1-amino-1-oxo-3-phenylpropan-2-yl)carbamate (4) by SPPS
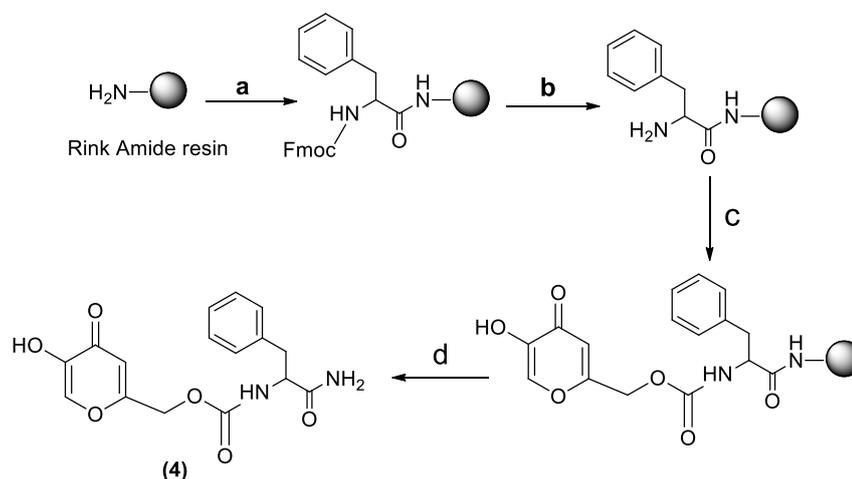
Figure 36. Schematic diagram for synthesis of compound (4) by SPPS. **a)** coupling of Fmoc-Phe on to resin **b)** Fmoc deprotection **c)** coupling of kojic acid-imidazole **d)** cleavage from resin.

0.1 mmol (169.5 mg) Rink Amide resin was weighed in reaction vessel in which the desired peptide will be synthesized. The resin was washed with DMF (2x) and swelled in 5-6 mL DMF for 40-45 min. The solution is drained and Fmoc group that attached to the resin was deprotected by adding 10 mL of 20% piperidine in DMF (2x10 min). Then Fmoc-Phe-OH (0.55 mmol, 5.5 eq.) was dissolved in HBTU solution (1 mL, 0.5 M in DMF). DIPEA (200 µL) was added to this amino acid solution, stirred for 30 seconds and added to the resin in the reactor. The amino acid was coupled to the resin for 1 h. At the end of 1h, resin was washed with DMF and Fmoc group of phenylalanine was deprotected by addition of 10 mL of 20% piperidine in DMF (2x10 min). Kojic acid-imidazole (0.55 mmol, 5.5 eq.) was dissolved in HOBt solution (1.4 mL, 0.5M in DMF) and added to the resin in the reactor. At the end of 2h, resin was washed with DMF and DCM and dried under vacuum. Resin was cleaved with 1 mL cleavage cocktail (2x1 h) and product was triturated by washing cold Et_2O. According to NMR result, desired product was obtained in 78% (26 mg) yield.

^{1}H NMR (400 MHz, d^6-DMSO): δ 8.04 (s, 1H), 7.31 – 7.23 (m, 5H), 6.35 (s, 1H), 4.78 (s, 2H), 4.14 (td, J = 10.5, 4.0 Hz, 1H), 3.00 (dd, J = 13.7, 4.1 Hz, 1H), 2.74 (dd, J = 13.9, 10.5 Hz, 1H).

4.3.5. Synthesis of 5-hydroxy-2-(iodomethyl)-4H-pyran-4-one (Iodokojic acid) (5)
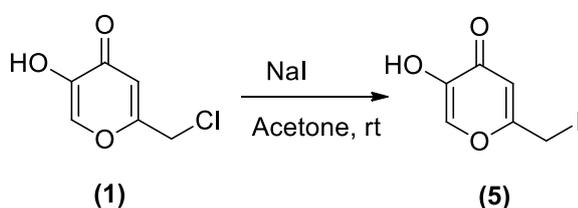
Figure 37. Synthesis of Iodokojic acid (5).

NaI (280 mg, 1.87 mmol) was dissolved in 1.5 mL acetone. Chlorokojic acid (300 mg, 1.87 mmol) and NaI were dissolved in totally 2.5 mL acetone. Reaction was stirred at room temperature and monitored by TLC. At the end of the reaction, the precipitate was filtered off and filtrate was evaporated. Iodokojic acid was isolated by silica gel column chromatography using DCM:MeOH as eluent (93:7).

^{1}H NMR (400 MHz, CDCl$_3$): δ 7.78 (s, 1H), 6.43 (s, 1H), 4.09 (s, 2H).

4.3.6. Synthesis of 2-((((5-hydroxy-4-oxo-4H-pyran-2-yl)methyl)amino)-3-phenylpropanoic acid (2) in Solution
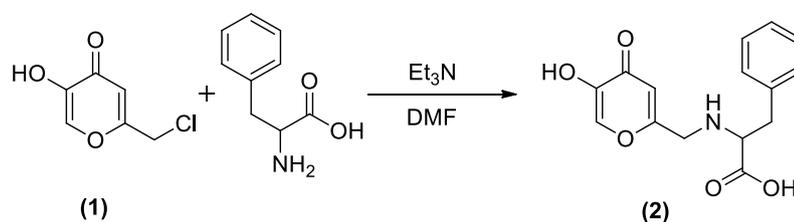
Figure 38. Schematic diagram for synthesis of compound (2) in solution.

Based on literature procedure for a similar compound,[46] chlorokojic acid (300 mg, 1.87 mmol) and phenylalanine (340 mg, 2.06 mmol) were dissolved in 1.5 mL DMF. 780 µL (5.6 mmol) triethylamine was added dropwise and reaction mixture was stirred at room temperature under inert atmosphere. At the end of the reaction, H$_2$O was added and extraction was done with DCM. However, desired product could not be obtained.

4.3.7. Synthesis of methyl 2-amino-3-phenylpropanoate hydrochloride (Phenylalanine protected with methylester) (6)
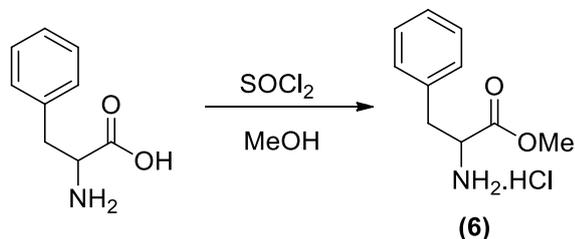
Figure 39. Protection of phenylalanine with methylester group.

To a suspension of phenylalanine (570 mg, 3.43 mmol) in methanol (10 mL) was added $SOCl_2$ (0.4 mL, 5.15 mmol) drop wise at 0 $^\circ$C. The reaction mixture was stirred at room temperature for 24 hours. At the end of the reaction, MeOH was evaporated and white solid product (compound **6**) was obtained with a yield of 70% (800 mg).

^{1}**H NMR** (400 MHz, CD$_3$OD): δ 7.43–7.26 (m, 5H), 4.34 (dd, *J* = 7.5, 6.1 Hz, 1H), 3.83 (s, 3H), 3.28 (dd, *J* = 9.0, 5.5 Hz), 3.18 (dd, *J* = 14.4, 7.5 Hz).

4.3.8. Synthesis of methyl 2-(((5-hydroxy-4-oxo-4H-pyran-2-yl)methyl)amino)-3-phenylpropanoate (7)
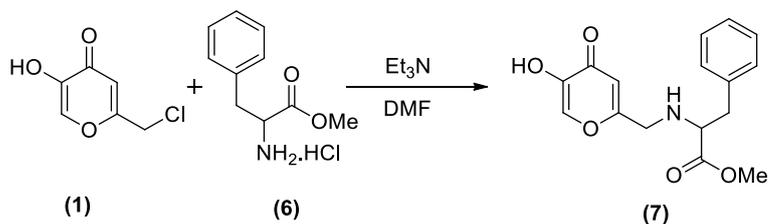
Figure 40. Schematic representation for the synthesis of compound (**7**).

Based on literature procedure for a similar compound[46], chlorokojic acid (300 mg, 1.87 mmol) and methylester protected phenylalanine (340 mg, 2.06 mmol) were dissolved in 1.5 mL DMF. 780 µL (5.6 mmol) triethylamine was added dropwise and reaction mixture was stirred at room temperature under inert atmosphere. At the end of the reaction, H_2O was added and extraction was done with DCM. However, desired product could not be obtained. The same procedure was performed by changing the solvent from DMF to ACN, but the product could not be obtained again.

4.3.9. Synthesis of 2-(azidomethyl)-5-hydroxy-4H-pyran-4-one (Kojic azide) (8)
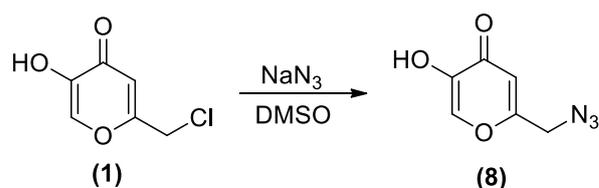
Figure 41. Schematic diagram for the synthesis of compound (**8**).

Chlorokojic acid (300 mg, 3.1 mmol) was added to a solution of NaN_3 (6.8 mL, 0.5M in DMSO) and stirred at room temperature. Reaction was monitored by TLC and after all starting material was consumed and then stopped by adding H_2O. Then the mixture was extracted with Et_2O and organic layer was dried with $MgSO_4$ and evaporated. As a result, kojic azide (**8**) was synthesized in 78% yield (404 mg).

¹H NMR (400 MHz, CDCl₃): δ 7.80 (s, 1H), 6.43 (s, 1H), 4.15 (s, 2H).

4.3.10. Synthesis 2-(aminomethyl)-5-hydroxy-4H-pyran-4-one (9)
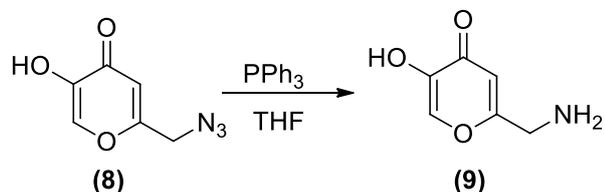
Figure 42. Schematic diagram for the synthesis of compound (**9**).

Based on literature procedure[44], triphenylphosphine (100 mg, 0.38 mmol) was added to a solution of kojic azide (**8**) (64 mg, 0.38 mmol) in THF (5 mL) and water (0.5 mL). The reaction was stirred overnight at room temperature. At the end of the reaction, precipitation was observed and mixture was centrifuged. The precipitate was our desired product with a yield of 76% (300 mg).

¹H NMR (400 MHz, CDCl₃): δ 7.99 (s, 1H), 6.39 (s, 1H), 3.53 (s, 2H).

4.3.11. Synthesis of (9H-fluoren-9-yl)methyl (1-(((5-hydroxy-4-oxo-4H-pyran-2-yl)methyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (10)
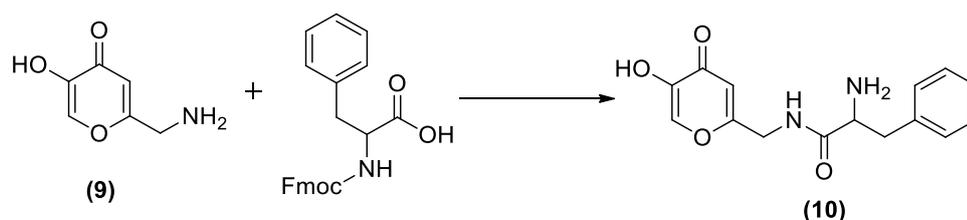
Figure 43. Schematic diagram for the synthesis of compound (**10**).

Kojic amine (176 mg, 1.25 mmol) was dissolved in 1.5 mL DMF. Fmoc-Phe-OH (550 mg, 1.38 mmol) was dissolved with HBTU (2.5 mL, 0.5 M in DMF). 500 μL DIPEA was added to this amino acid solution stirred for 30 seconds and added to the solution of kojic amine. Reaction was stirred at room temperature and monitored by TLC. After 3 days, H₂O was added to reaction mixture and

precipitation occurred. Centrifugation was done and pure Fmoc-protected compound was obtained after column chromatography with DCM:MeOH (95:5) eluent system. To cleave Fmoc group, 130 mg product was dissolved in 10 mL of 20% piperidine in DMF. Cold tert-butylmethyl ester was added to mixture and it was expected to precipitate. However, no precipitation and no product formation were observed.

A different procedure was tried to cleave Fmoc group based on literature.[47] 110 mg Fmoc protected product was dissolved in the mixture of piperidine:ACN (1:4, 1 mL) and stirred for 1h at room temperature. At the end of 1h, all reaction mixture was evaporated under vacuum. However, again deprotected compound **10** was not obtained.

4.3.12. Synthesis of 2-((tert-butoxycarbonyl)amino)-3-phenylpropanoic acid (11) (Protection of Phe with Boc-group)
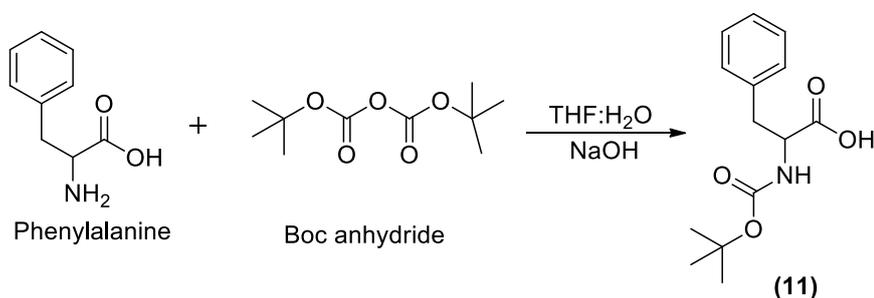
Figure 44. Schematic diagram for the synthesis of Boc-Phe-OH (**11**).

Procedure was based on literature.[48] Phenylalanine (1.7 gr, 10 mmol) was dissolved in the mixture of THF:H_2O (1:1, 50 mL) at room temperature. Boc-anhydride (2.4 gr, 11 mmol) and NaOH (880 mg, 22 mmol) was added to the reaction mixture and stirred overnight. At the end of the reaction extraction was done with DCM. Aqueous layer was collected and 10% HCl was added drop wise to this layer until to get pH value around 4. This acidified layer was extracted with

DCM again and organic layer was collected, dried with MgSO$_4$ and evaporated with vacuum. As a result, Boc-Phe-OH (**11**) was obtained as sticky oil.

^{1}H NMR (400 MHz, d^6-DMSO): δ 7.30–7.17 (m, 5H),) 4.12–4.04 (m, 1H), 3.01 (dd, J = 13.7, 4.4 Hz, 1H), 2.81 (dd, J = 13.7, 10.4 Hz, 1H), 1.32 (s, 9H).

4.3.13. Synthesis of (9H-fluoren-9-yl)methyl (1-(((5-hydroxy-4-oxo-4H-pyran-2-yl)methyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (10) from Compound (11)
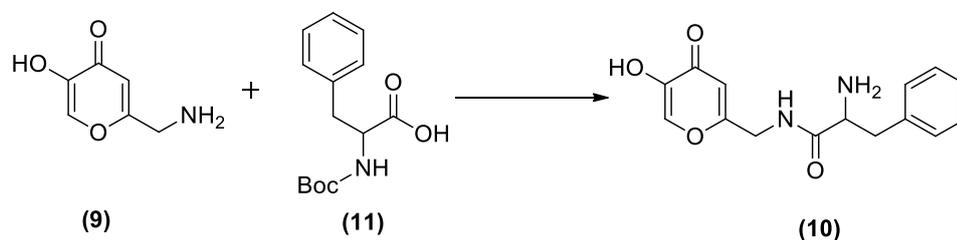
Figure 45. Synthesis of compound (**10**) from Boc-Phe-OH (**11**).

Kojic amine (**9**) (176 mg, 1.25 mmol) was dissolved in 2 mL DMF. In another vessel, Boc-Phe-OH (11) (375 mg, 1.25 mmol) was dissolved in HBTU solution (2.5 mL, 0,5 M in DMF). 500 µL DIPEA was added to this amino acid solution stirred for 30 seconds and added to the solution of kojic amine and stirred at room temperature by monitoring with TLC. At the end of the reaction, H$_2$O was added and extraction was done with DCM. Silica gel column chromatography was done by using eluent system of DCM:MeOH (90:10) and Boc-protected compound **10** was obtained. To cleave Boc group of the product TFA was added to a solution of compound (**10**) in DCM and stirred at room temperature. At the end of 3 hours, reaction was stopped by evaporating all solvent under vacuum and washed with cold Et$_2$O to remove TFA from the compound (**10**) obtained from the reaction.

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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
Alanine	Ala	A	
Arginine	Arg	R	
Asparagine	Asn	N	
Aspartic acid	Asp	D	
Cysteine	Cys	C	
Glutamic acid	Glu	E	
Glutamine	Gln	Q	
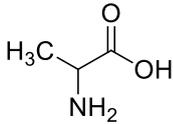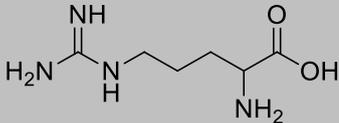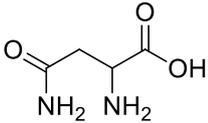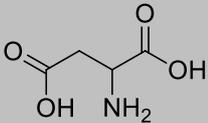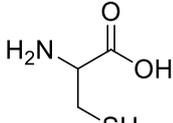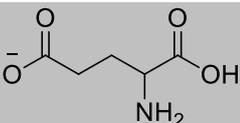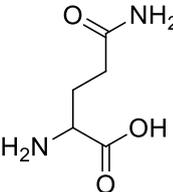
Table A.1. List of 21 Amino acids Found in Nature (Continued)
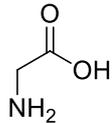
Glycine	Gly	G	
Histidine	His	H	
Isoleucine	Ile	I	
Leucine	Leu	L	
Lysine	Lys	K	
Methionine	Met	M	
Phenylalanine	Phe	F	
Proline	Pro	P	
Serine	Ser	S	
Threonine	Thr	T	
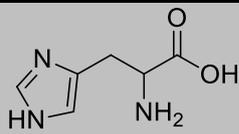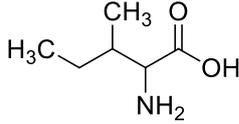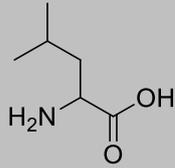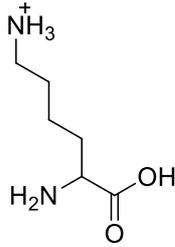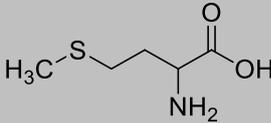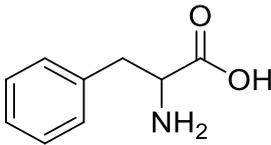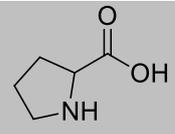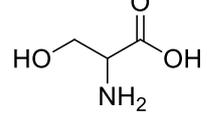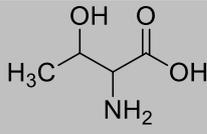
Table A.1. List of 21 Amino acids Found in Nature (Continued)
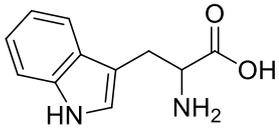
Tryptophan	**Trp**	**W**	
Tyrosine	Tyr	Y	
Valine	Val	V	