THE PREPARATION OF AZIDO AMINO ACIDS AND THE APPLICATION OF NATIVE CHEMICAL LIGATION IN THE SYNTHESIS OF CHLOROTOXIN

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THE PREPARATION OF AZIDO AMINO ACIDS AND THE APPLICATION OF NATIVE CHEMICAL LIGATION IN THE SYNTHESIS OF CHLOROTOXIN

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ABSTRACT

THE PREPARATION OF AZIDO AMINO ACIDS AND THE APPLICATION OF NATIVE CHEMICAL LIGATION IN THE SYNTHESIS OF CHLOROTOXIN

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In this project, the pharmaceutically and biologically important peptide, chlorotoxin (CLTX), and some derivatives of CLTX containing azido amino acids was synthesized by using solid phase peptide synthesis (SPPS) based on 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry strategy and native chemical ligation. The native chemical ligation provides more efficient than only SPPS. Directly synthesis of CLTX by SPPS has very low yield, by using native chemical ligation, the yield is aimed much higher. The CLTX can bind to matrix metalloproteinase-2 (MMP-2), which is involved in the breakdown of extracellular matrix in normal physiological processes and inhibit this enzyme. The important role of MMP-2 for spreading cancer cell is known from literature and the inhibition of this enzyme could potentially be used for treatment of cancer in the future. The inhibition mechanism of CLTX on MMP-2 has not been determined yet. There are imaging methods which involves functionalization of CLTX using fluorescence dyes from lysine residue which is not selective. The synthesis of CLTX derivatives containing azido amino acids could provide the selective functionalization of of CLTX using Huisgen ‘Click

Chemistry' together with propargyl dyes. The azido amino acids that are used for CLTX was synthesized by using new method 2-Azido-1,3-dimethylimidazolinium hexafluorophosphate (ADMP) from that own to free amine and hydroxyl group. ADMP can make di-azo transfer to primary amine group and also replacement between azide and hydroxyl group.

Keywords: Amino acid, chlorotoxin, SPPS, natural chemical ligation, azido amino acid.

ÖZ

AZİDO AMİNO ASİTLERİN HAZIRLANMASI VE DOĞAL KİMYASAL BAĞLANMA TEPKİMESİNİN KLOROTOKSİN SENTEZİNE UYGULANMASI

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Ağustos 2014, 57 sayfa

Bu projede, ilaç endüstrisinde ve biolojik olarak öneme sahip olan Klorotoksin (CLTX), ve bu peptidin türevlerinin Fmoc'a dayalı katı faz peptit sentezi ve doğal kimyasal bağlanma tepkimesi kullanılarak sentezlenmesi hedeflenmiştir. Doğal kimyasal bağlanma methodu, sadece katı faz peptit sentezi method kullanılarak yapılan sentezlerden daha etkili olmaktadır. Peptitlerin sadece katı faz peptit sentezi methodu kullanılarak sentezlenmesinin verimliliği diüşük olurken, doğal kimyasal bağlanma tepkimesi yardımıyla bu verimliliğin artması hedeflenmektedir. Kanserli dokularda, hücreler arasındaki maddeyi yıkan matriks metalloproteinaz-2 (MMP-2), ile Klorotoksinin birbirlerine karşı bir ilişkisi olduğu literatürden biliniyor ve ayrıca klorotoksinin MMP-2'yu inhibe ettiği savunulmaktadır. MMP-2'in inhibe edilmesiyle kanser hücrelerin yayılmasının engellenebilecği düşünülmektedir. Bu inhibisyon reaksiyonun mekanşzması tam olarak bilinememektedir, bunun ana sebeplerinden biri klorotoksini boyayabilen boyaların kesin ve spesifik olarak

boyayamamısıdır. Bu boyalar lizin üzerindeki serbest amin gruplara bağlanarak çalışmaktadır. Sentezleyenecek olan klorotoksin türevleri azit grupları içereceğinden tık reaksiyonu ve proparcil grubuna dayalı boyalar kullanılarak bunlar spesifik olarak boyanbilecektir. Sentezde kullanılacak azitli amino asitlerin sentezi yeni bir method ile 2-Azido-1,3-dimethylimidazolinium hexafluorophosphate (ADMP) kullanılarak yapılacaktır. Bu kimyasal reaktant yapıda bulunan serbest amin gruplarına diazo transfer ederek ve ayrıca serbest alkol grupları ile azit grubunun yer değiştirmesi azit içeren amino asit elde edilmesini sağlamaktadır.

Anahtar Kelimeler: Amino asit, klorotoksin, SPPS, doğal kimyasal bağlama, azido amino asit

To my beloved mother

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LIST OF ABBREVIATIONS

ADMP	: 2-Azido-1,3-dimethylimidazolinium hexafluorophosphate
Boc	: Butyloxycarbonyl
Cbz	: Carboxybenzyl
DBU	: 1,8-Diazabicycloundec-7-ene
DCM	: Dichloromethane
DIPEA	: N,N-Diisopropylethylamine
DMAP	: 4-Dimethylaminopyridine
DMF	: N,N-Dimethylformamide
Et_2O	: Diethyl ether
Et_3N	: Triethylamine
Fmoc	: Fluorenylmethyloxycarbonyl
HBTU	:2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HF	: Hydrogen fluoride
HPLC	: High Performance Liquid Chromatography
KOt-Bu	: Potassium tert-butoxide
LC-MS	: Liquid Chromatography–Mass Spectrometry
MeOH	: Methanol
MMP-2	: Matrix metalloproteinase-2
NCL	: Native chemical Ligation
NMR	: Nuclear Magnetic Resonance Spectroscopy
SPPS	: Solid Phase Peptide Synthesis
TFA	: Trifluoroacetic acid
TIPS	: Triisopropylsilane
TLC	: Thin-layer chromatography

CHAPTER 1

INTRODUCTION

1.1 Peptides as Drugs

The peptides are defined as molecules which are containing fewer than 50 aminoacids, their difference from protein is the size and length of these molecules. In the organisms, the peptides regulate many biological processes and activities; neurotransmitters, growth factors, endocrine or paracrine signals. In the few decades, the utilization of peptides as drugs shows a rapid expansion.[1] The peptide drugs generally mimic the ligands of enzymes or receptors such as insulin lispro[2]. Insulin lispro can be injected to patient immediately after the meal, unlike regular human insulin that should be injected half an hour earlier.

The peptide drugs have potential advantages and disadvantages, compared to small molecule drugs. The comparison between peptide drugs and the other small molecule drugs, are shown in Table 1. For example, because of their small sizes, conventional small molecule drugs have low target selectivity and that causes some side-effects, on the other hand; the peptide drugs have potentially lower toxicity than small molecule drugs.

Table 1: Advantages and disadvantages of peptide drugs[1b]

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

1.2 Venom Peptides

Venomous animals have developed a extensive range of peptide toxins as part of defending or capturing the preys. The peptide toxins are generally stored in very specialized glands which secrets peptides infused via hollow teeth or bites which allows their transportation into the tissues of animals via different routes. [3]

Most of venoms contains a complex mixture of peptides. These venoms have selective and diverse pharmacologies. Despite that, some of the venom peptides in this mixture have evolved to affecting the crucial issues of stability and potency in tissues and cells. The structures of some peptide venoms are shown in Figure 1.[3-4]

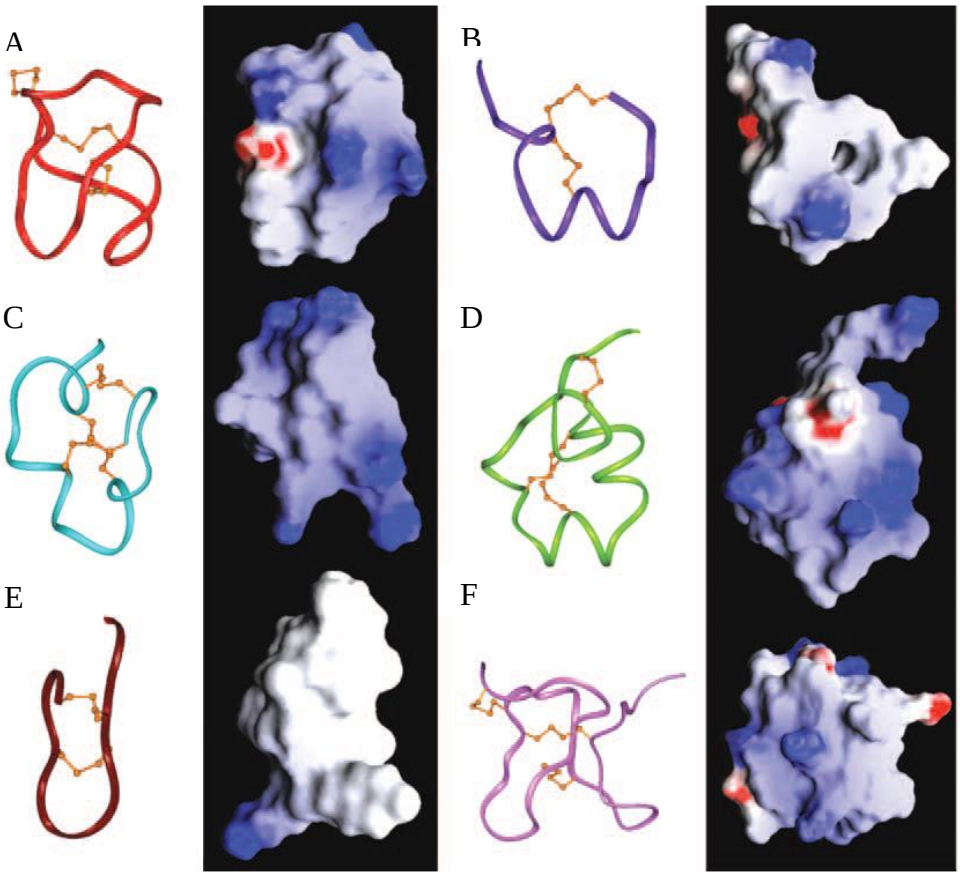


Figure 1. Molecular structures of some venom peptides that have therapeutic potential. A) ω-CVID B) α-GID (1MTQ) C) μ-PIIIA D) ShK (1ROO) E) χ-MrIB (1IEO) F) GsMTx4 (1LQR)[3]

Table 2. The instances of some peptide-based drugs obtained from venoms[1b]

Peptidyl Drug	**Size (aa)**	**Target disease**	**Pharmacological mechanism**
Captopril (Capoten)	3	Hypertension	ACE inhibitor
Tirofiban (Aggrastat)	3	Anticoagulant	Platelet inhibitor
Epifibatide (Integrilin)	7	Acute coronary syndrome	Anticoagulant
Bivalirudinrub (Angiomax)	20	Unstable angina	Anticoagulant
Ziconotide (Prialt)	26	Neuropathic pain	N-type Ca channel blocker
Exenatide (Byetta)	39	Type 2 diabetes	GLP-1 receptor antagonist

1.3 Disulfide-rich Peptides

Cysteine is considered as an unusual amino acid because the occurrence of this amino acid is only around 2.26% of all mammalian proteins, and this ratio is decreasing in protein structures belonging to simpler organisms such as algae and bacteria.[5] However, higher than 10% disulfide-rich peptide sequences consists of cysteine residues[6]. These singular peptides are self-conscious structures composed by few residues, fewer than 100 amino acids, and they have a common deficiency of hydrophobic core and limited and unfamiliar secondary structure features, stabilized mostly by three or more disulfide bonds[5, 7].

The disulfide-rich peptides are usually the main element of animal venoms. These disulfide-rich peptides have got more attention for developing new therapeutic agents and bio-insecticides because these peptides target ion channels and neuronal receptors with a high degree of selectivity and potency. Additionally, their rigid disulfide framework structure results in higher in vivo stability which is of course very crucial issue for performing in vivo experiments. Functional and structural characterization of these disulfide-rich peptides is necessary for the development of a consistent expression system that preserves their native disulfide framework.[8]

1.3.1 Cyclotides

Cyclotides are disulfide-rich peptides which have the unfamiliar characteristic of a cyclic backbone (so called as cyclo-peptide). They include 6 conserved cystine amino acids settled in a cystine knot topology in which two disulfide bonds and their linking backbone divisions form an embedded cycle in the assembly which then penetrated by a third disulfide connection, as presented under.

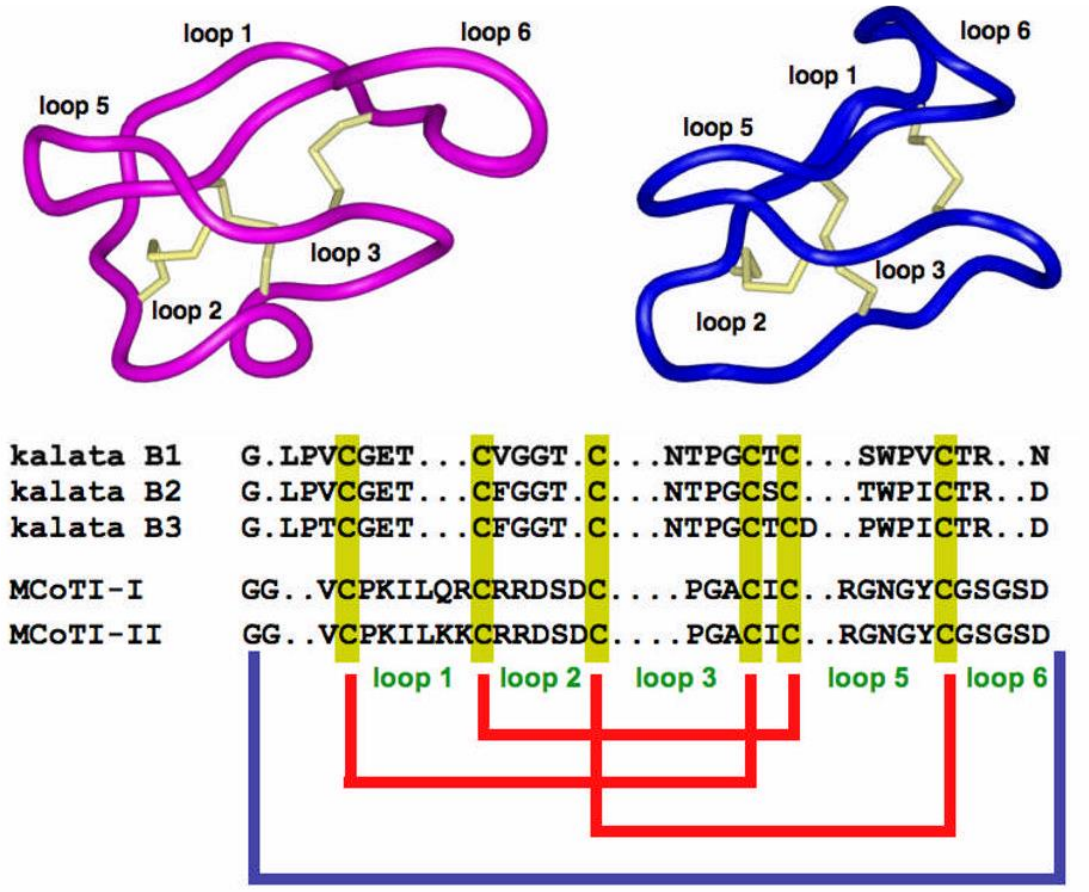


Figure 2. The tertiary and primary structure of two cyclotides that are found in the plants Momordica cochinchinensis (MCoTI-II) and Oldenlandia affinis (kalata B1). Blue and red connectors shows disulfide bonds and backbone, respectively.[9]

Cyclotides have a variety of appealing biological activities comprising neurotensin inhibition and anti-HIV, insecticidal and antimicrobial activity. Cyclotides are established in a diversity of humid florae from the Violaceae and Rubiaceae families.

1.3.2 Cystine Knots

The cystine knot structural design, also called knottins, is seen in proteins and peptides from a diverse species, such as fungi, animals, spiders and insects. It contains an embedded ring made by 2 disulfide bonds and their connecting backbone parts that is penetrated by another disulfide bond.[10]

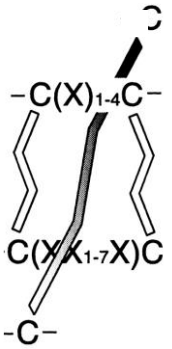


Figure 3. The cystine knot motif [10]

The knotted arrangement of disulfide bonds was associated with several beta-strands in these types of structures. This is a highly efficient motif for structure stabilization. Peptides including this cystine knot which contains between 26 or 48 amino acid residues and comprise ion channel blockers, hemolytic agents in addition to molecules having anti-viral and anti-bacterial activities. The stability of peptide venoms including the cystine knot motif, their range of bioactivities and their unique structural scaffold can be harnessed for molecular engineering applications and in drug design. There are three types of cystine knots: Inhibitor Cystine Knot (ICK), Growth Factor the Cyclic Cystine Knot (CCK) and Cystine Knot (GFCK). Their structures are represented below figure.

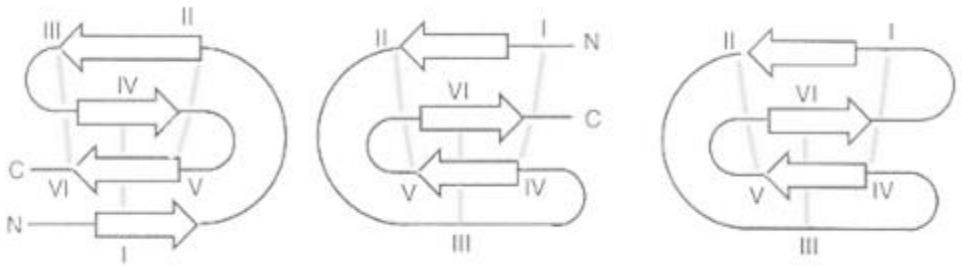


Figure 4. The classes of cystine knots [10]

In the diagram of the types of cystine knots, the beta strands are shown as arrows, the cysteine amino acids are represented I-VI and the disulfide bonds between cysteine residues are drawn as gray lines. The penetrating disulfide linkage for the inhibitor cystine knot and cyclic cystine knot peptidesare Cys(III-VI) whereas it is Cys(I-IV) in the growth factor cystineknot.[10]

1.4 Chlorotoxin

Chlorotoxin (CTLX) is a neurotoxin, which is containing 36-amino acids and four disulfide bonds at a molecular mass of 3996 g/mol, found in the venom of the Israeli scorpion, *Leirius quinquestriatus,* which inhibits low-conductance chloride channels.[11] It has been reported that CTLX can bind to matrix metalloproteinase-2 (MMP-2), which is membrane type-1 metallo proteinase and involved in the breakdown of extracellular matrix in normal physiological processes.

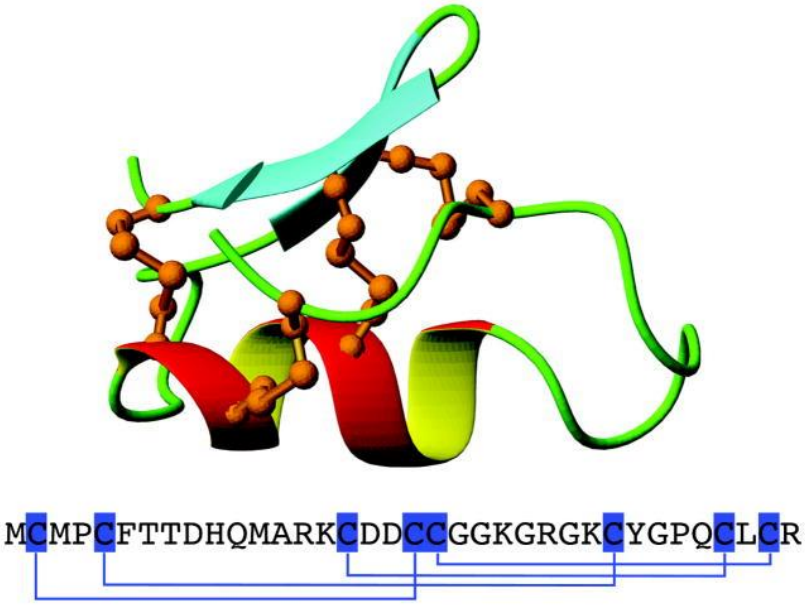


Figure 5. The tertiary structure and sequence of chlorotoxin (CLTX)

The CLTX is classified as a knottin due to the existence of a 'disulfide through disulfide knot' structure. When it is bio conjugated with the fluorescent molecular beacon Cy5.5, it can be used as an optical imaging contrast agent, which provides surgeons the ability to distinguish cancer foci from adjacent normal tissue during surgical operation.[12] It is also in phase II clinical trial under the name TM-601, for crossing blood-brain barrier and binding to malignant brain tumor cells without affecting healthy cells.[13]

The chlorotoxin and derivatives are usually synthesized by using solid phase peptide synthesis method. [14]

1.5 Solid Phase Peptide Synthesis

The solid phase peptide synthesis (SPPS) was developed by Robert Bruce Merrifield in 1963.[15] This method is based on the idea that a peptide chain can be assembled in a stepwise manner while the one end of growing peptide is attached to a solid support. The idea behind this approach is that the peptide is attached to an insoluble solid particle, hence the filtering and washing free of reagents and by-products is more convenient after each step. Unlike ribosome protein synthesis, SPPS starts at a C-terminal of the peptide and completed at a N-terminal of the peptide. This synthetic method has been widely used not only for peptides but also in polymer and organic synthesis.

There are two mainly used forms of SPPS, which are Fmoc and Boc. The difference of these methods based on the protecting group used for the α-amine and side chains at the amino acids and final cleavage. In the first method, α-amine groups are protected with Fmoc, and Fmoc can be cleaved by using base catalyzed elimination in presence of the secondary amine (DMAP or Piperidine). It also catches the dibenzofulvene which is obtained in the deprotection. After the peptide chain growth is completed, acid labile peptide-resin linkers and side chain protecting groups which are generally Boc, trityl and tert butyl can be cleaved at the same time under acidic conditions with trifluoroacetic acid (TFA). In the second method, α-amine groups are protected with Boc, and Boc can be cleaved by acid-catalyzed elimination in presence of the TFA. After the completion of peptide chain growth, peptide-resin linkers and

side chain protecting groups that are different from Fmoc based strategy can be cleaved by using hydrogen fluoride (HF).

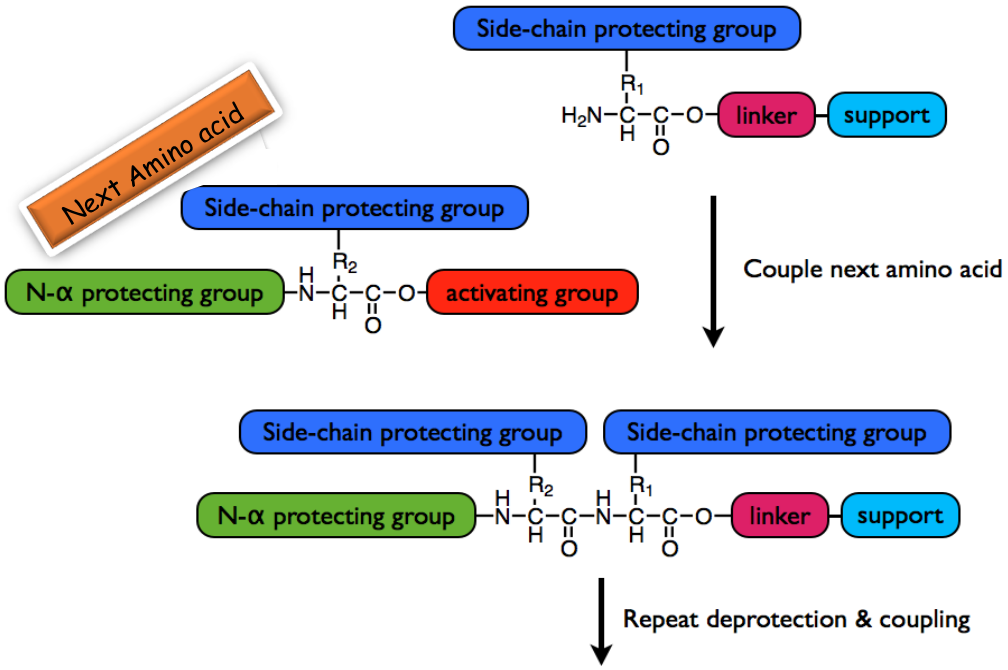


Figure 6. Schematic representation of SPPS

The deprotection mechanism of α-amine group at the amino acids is shown in Figure. Firstly, the fluorenyl ring is deprotonated, the important step which produces the aromatic intermediate. Then, this intermediate rapidly decarboxylates to form dibenzofulvene that is scavenged by piperidine. The product of deprotection reaction has a distinctive UV absorbance at 210 nm so that reaction yield can be monitored by using UV spectrum.

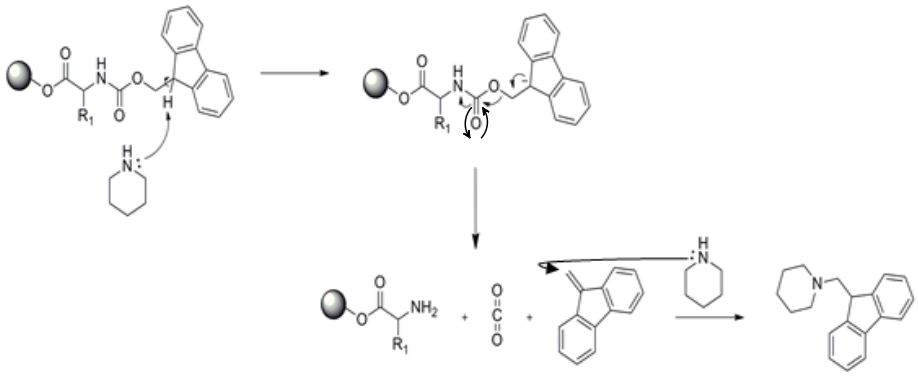


Figure 7. Fmoc deprotection mechanism of N-termini at aminoacid.

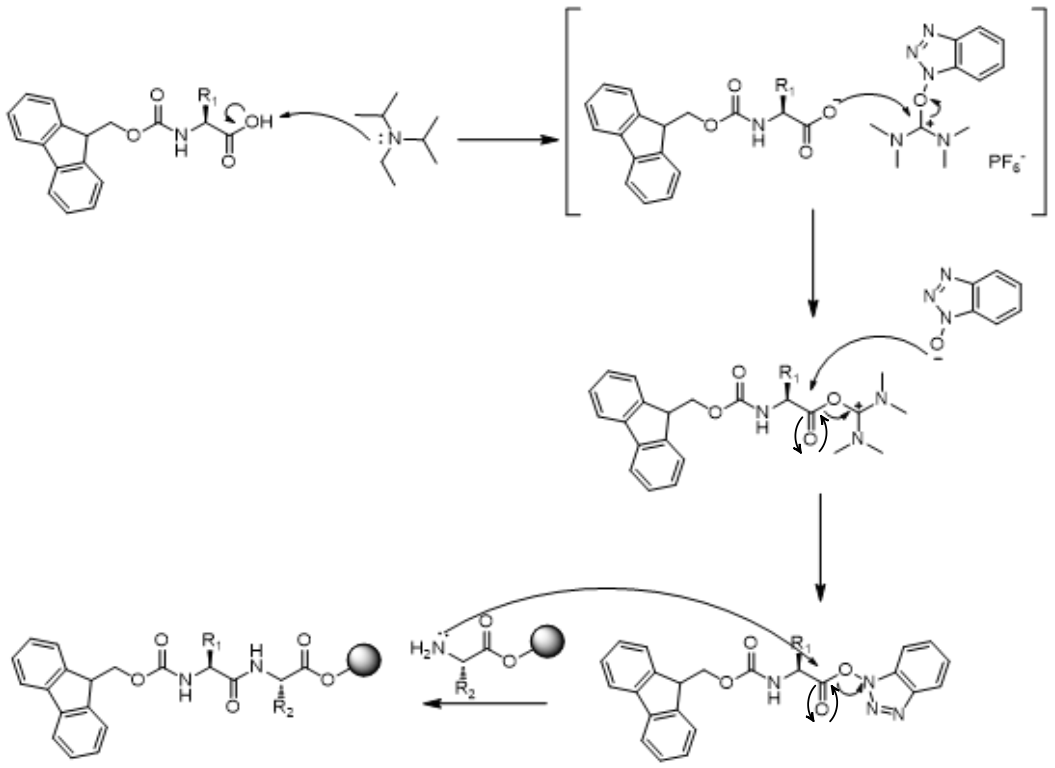


Figure 8. C-terminus activation via HBTU-DIEA mechanism

Following the deprotection of N-termini at peptide bound to resin, the carboxy group of the incoming amino acid is deprotonated in situ by using N,N-diisopropylethyl amine. Then, the coupling reagent reacts with carboxy group and activated amino acid is obtained. The activated amino acid react with amino acid bound resin support to form the new peptide bond. HBTU included activation is represented in Figure .

1.6 Native Chemical Ligation

Native chemical ligation (NCL) is generally used for obtaining proteins by semi or total-synthesis.[16] By using ligation of small peptides, the total syntheses of proteins are enabled, and the native chemical ligation grants researchers the complete bond by bond control over the structure of the protein.

Native chemical ligation is based on the reaction of 2 deprotected peptides in situ to give a single covalently bound ligation artifact as polypeptide. The peptide that has thio-ester at C-termini is reacted with a cysteine residue at another peptide to give a polypeptide product with a native amide linkage at the ligation position. The all aspects of reaction mechanism details of NCL haven't yet been determined exactly. One of the proposed reaction mechanism scheme is represented in below figure.[17]

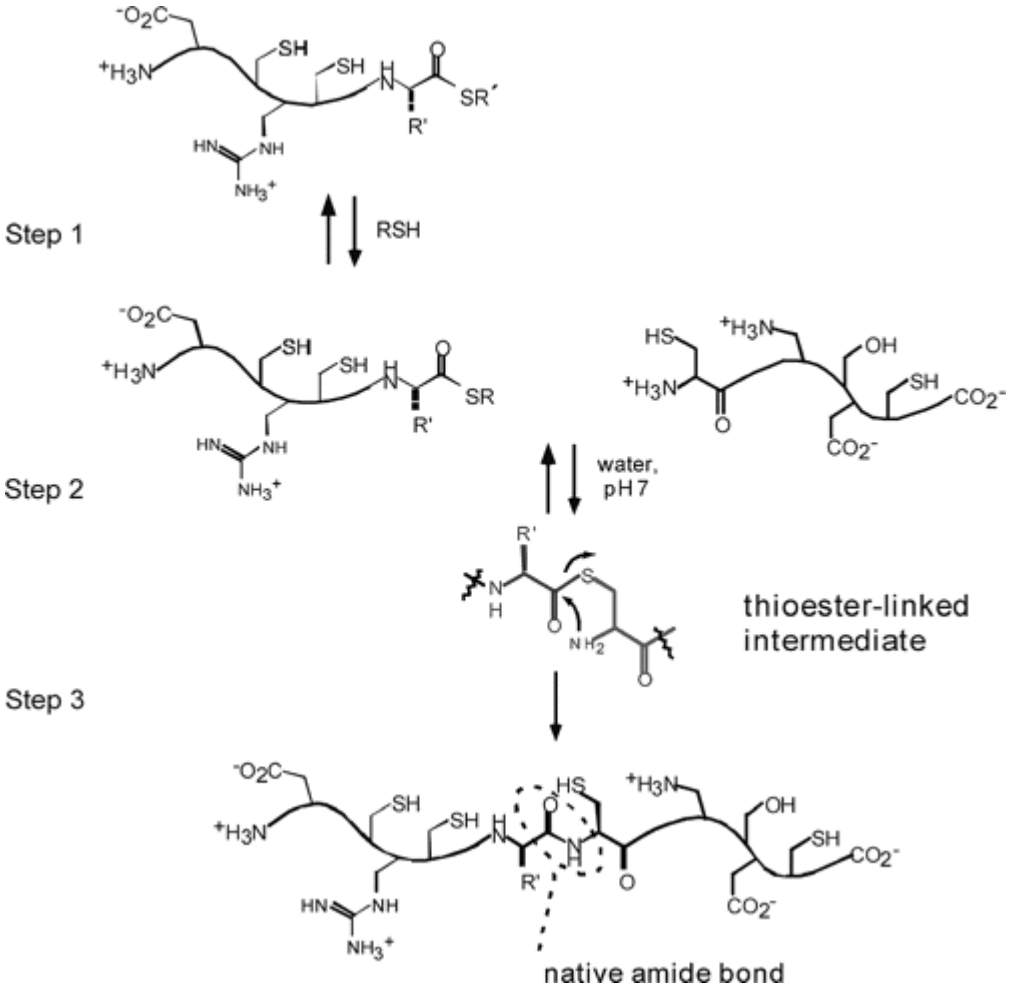


Figure 9. The suggested reaction mechanism of native chemical ligation process [17]

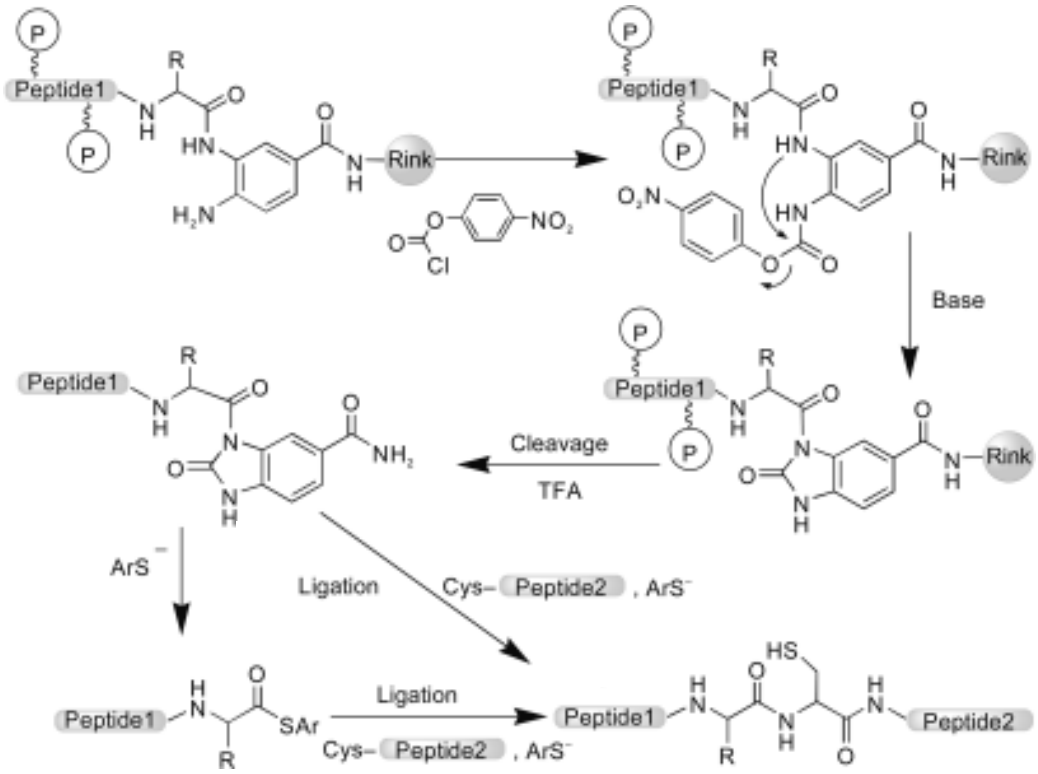


Figure 10. The reaction mechanism and scheme of chemical ligation assisting with o-aminoanilides

Currently, the most effective method for the synthesis of peptides containing thio-esters is the in situ neutralization protocol for Boc-SPPS strategy using thioester linkers. The thioester linkers used for Boc-SPPS could not be used for Fmoc-SPPS because of the requirement for Fmoc deprotection under basic conditions. However, Dawson and his colleagues found a new method for the synthesis of peptidyl thio-esters with Fmoc-SPPS strategy.[18] In this protocol, o-aminoanilides are stable intermediates which can be effectively converted into an aromatic N-acyl urea moiety and after the chain elongation, the resin-bound acyl urea peptide can be clasically unprotected and cleaved from the solid support by using TFA. The cleaved product is stable under the acidic conditions. However, in neutral conditions, this acyl urea peptide product can undergo rapid thiolysis reaction, and forms thioester peptide to be used in the native chemical ligation.

1.7 Azido Amino Acids

Unnatural amino acids that contains azide functional group which is useful building parts for artificial peptides and engineered proteins, since the azide group provides a bio-orthogonal handle to which additional functionality can be set up via azide–alkyne cycloaddition reactions [19]. There are many applications which have been established for azido amino acids constructed on these bioconjugation methods. For instance, Prof. Benjamin G. Davis and his colleagues have used L-azido-homo alanine as a position for connecting glycans onto a LacZ reporter enzyme skeleton, reaching site-selective glycosylation in a related approach to post-translational modification of proteins.[20] L-azido alanine is used as an attaching point for the galactose group.

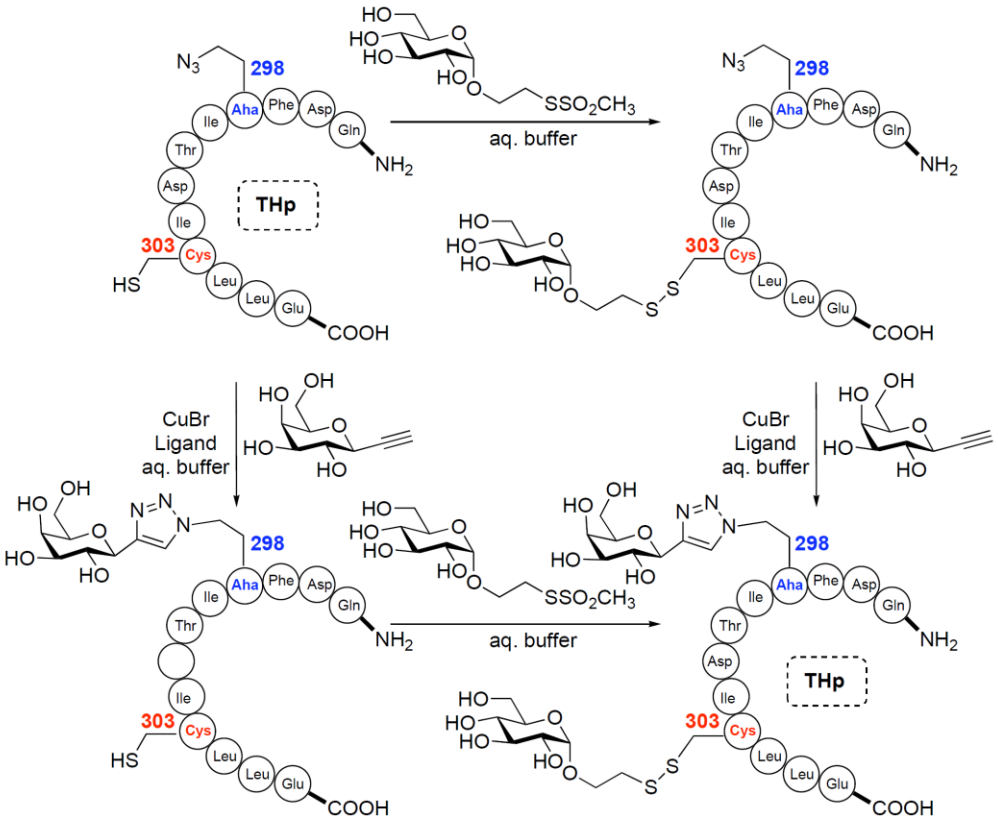


Figure 11. Selective protein conversion is seen through the pattern of the glyco-MTS glycosylation reaction with the glyco-CCHC reaction[20]

1.7.1 Synthesis of Azido Amino Acids

Due to the broad chemical variety spanned by the proteinogenic amino acids, there are several ways of introducing azide functionality.[21] The most commonly used method based on the usage of di-azo transfer reagents. The di-azo transfer reactions are generally used for introducing di-azo functionality on methylene groups which is activated and for changing primary amines to azide groups. The most di-azo transfer reagents are organic sulfonyl-azide derivatives, because the sulfonyl moiety has governing capacity of electron-withdrawing for di-azo transfer reaction. However, in the last decade, many alternatives have been developed. Some of them are shown below figure. [21]

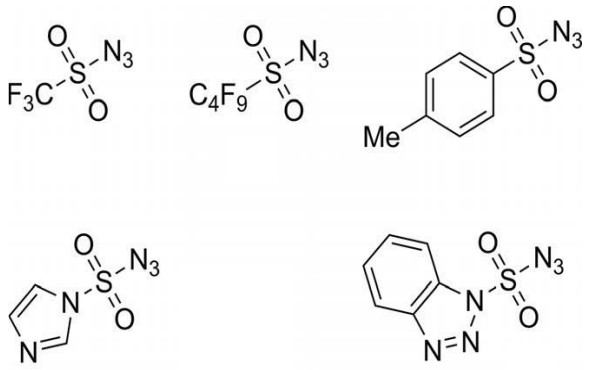


Figure 12. Diazotransfer reagents [21]

Most of the time, triflyl azide has been the choice of reagent for synthesizing azide derivatives from free amino groups. But triflyl azide is not commercially available and it must be prepared recently prior to use. And also it is very toxic and explosive chemical. Another disadvantage of triflyl azide is the formation of triflyl amide in the reaction that can be difficult to eliminate.

A safer alternative, imidazole-1-sulfonyl azide, is used as an alternative to triflyl azide, its preparation is a simple one pot procedure by using low-priced starting materials. [22] Additionally, its hydrochloride salt is commercially available. However a major disadvantage for this reagent is the need for copper or zinc ions as a catalyzer.

The other di-azo transfer reagent is benzotriazol-1-sulfonyl azide whose preparation is similar to imidazole-1-sulfonyl azide and its results are very promising for the synthesis of optically active azide derivatised alfa amino acids.[23]

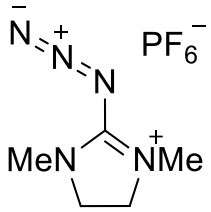


Figure 13. The molecular structure of 2-azido-1,3-dimethyl imidazolinium hexafluorophosphate (ADMP)

The one of important reagent for synthesizing azido compounds is 2-azido-1,3-dimethyl imidazoliniumhexafluorophosphate (ADMP). ADMP was prepared from 2-chloro-1,3-dimethyl imidazolinium chloride.[24] Firstly, with anion exchange reaction, chloride salt is converted to its hexafluorophosphate salt, then this salt and sodium azide in acetonitrile gives the final product is ADMP via a substitution reaction. The method that utilizes ADMP is very safe and ADMP is both crystalline and stable. It is an effective di-azo transfer reagent for primary amines, and also it doesn't need any metal salt such as copper. Various alkyl and aryl azide compounds can be obtained directly from the corresponding primary amines or hydroxyl groups with high yields using this diazo-transfer and azide substitution.[24-25]

CHAPTER 2

RESULT & DISCUSSION

2.1. Synthesis of Azido Amino Acids by using Di-azo Transfer Reagent ADMP

The synthesis of azido amino is achieved mostly by using di-azo transfer reagents but in certain cases azide substitution reactants were also used. The advantage of ADMP is that it makes both di-azo transfer and azide substitution possible. In di-azo transfer reaction, ADMP is used as di-azo transfer reagent, where the nuclophile between carbonyl groups attacks to terminal nitrogen atom and after rearrangement, di-azo compound is formed. ADMP can also act azide transfer reagent like Mitsunobu, the nucleophile attacks the carbon atom bound to azide functional group, after the rearrangement, the azido compound is obtained.

One of the advantage of ADMP is being very safe. It is not explosive, it be stored at -20°C and it is commercially available. For biochemistry and peptide chemistry, ADMP can be a useful reagent because it does not need any metal ions as a catalyst. The presence of metal ions is a problem during the peptide synthesis since these ions can be captured by peptides or other biomolecules and couldn't be removed later. In addition, even the trace amount of the metal residues after the synthesıs can cause toxic effects.

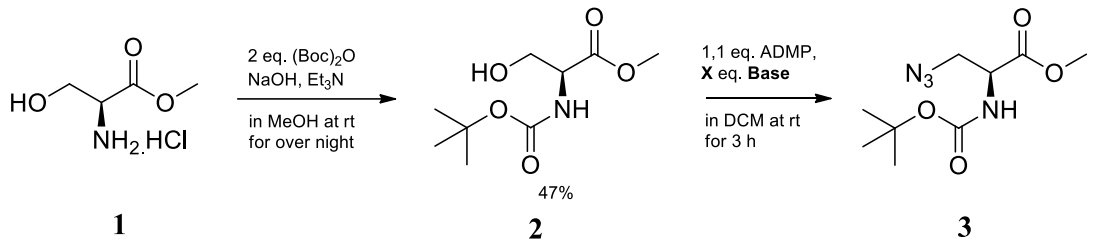


Figure 14. The reaction scheme of azide substitution with serine derivative

By using L-Serine methyl ester hydrochloride (**1**) salt as the starting material, we obtained Boc-L-Serine methyl ester (**2**) in the presence of boc anhydride, then, we tried the azidation of primary hydroxyl position was achieved and the desired product (**3**) was isolated in 15% yield. A side reaction, which is elimation to give alpha beta unsaturated ester, was dominant and accounted approximately for 40% of the product mixture. Attempts to eliminate this side reaction by changing the equivalence of base and type of base were not successful.

Table 3. The yield of azide substitution reaction at different temperatures

X (eq)	Base	Yield
2.5	DBU	15%
1.5	DBU	15%
2	DMAP	15%
2	KOt-Bu	13%

This experiment is also tried at low temperatures with DBU base but the yield of reaction was much lower under these conditions.

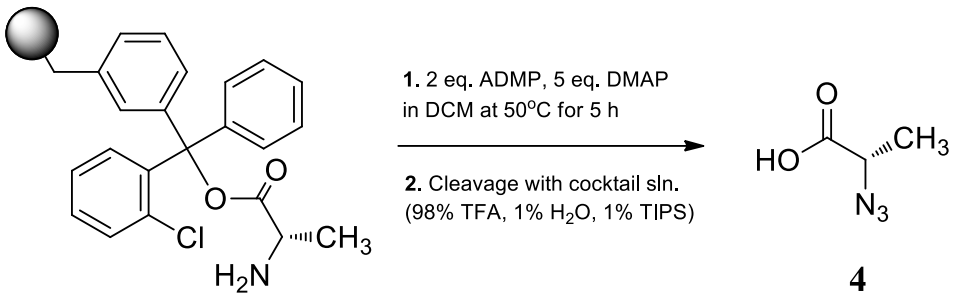


Figure 15. The reaction scheme of di-azo tranfer with alanine derivative

We also tried ADMP's di-azo transfer property in the solid phase synthesis. L-Alanine-2-chlorotrityl resin was used in this experiment and the reaction was performed at 50 °C, with 2 equivalent ADMP and 5 equivalent DMAP as a base. Based on NMR, the conversion of the reaction is quantitative.

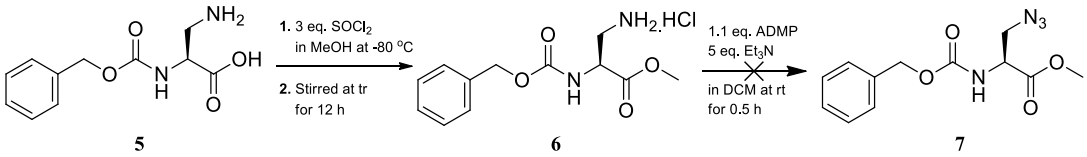


Figure 16. The reaction scheme of di-azo transfer with DAP derivative

To test the di-azo transfer ability of ADMP, some test reactions are also performed. 2-3 diamino propanoic acid (DAP) derivative (**5**), which has carboxybenzyl protection group at alpha amino group, was used as a starting material. After the esterification reaction, we tried to synthesize azido compound (**7**) but the reaction didn't proceed in the expected way. Interestingly the products didn't contain any UV active group according to TLC analysis so we thought maybe there is another reactivity pattern for ADMP such as reduction since deprotection of Cbz group generally requires reduction conditions.

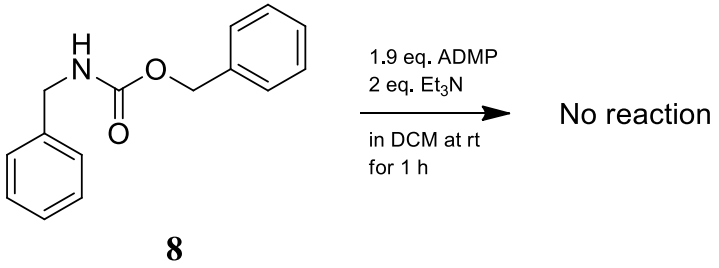


Figure 17. The reaction scheme of di-azo transfer with benzyl amine derivative

We tried this reaction with Cbz protected benzyl amine but benzyl amine (**8**) didn't react with ADMP, no change was observed after 1 hour showing that reduction of Cbz group with ADMP is not a viable reaction pathway.

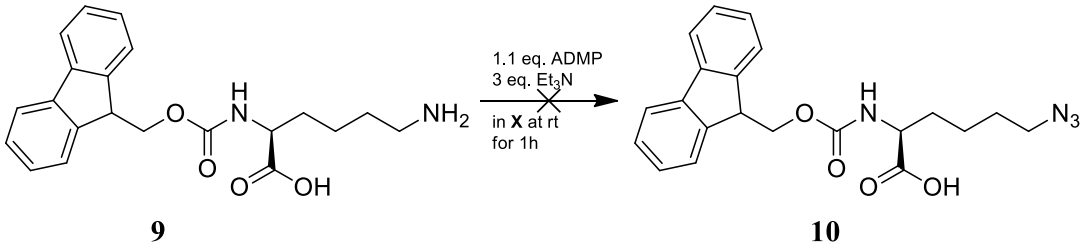


Figure 18. The reaction scheme of di-azo transfer with Fmoc-lysine

Table 4. The result of reactions between ADMP and Fmoc-lysine at different solvents

Solvent	Result
DCM	No reaction
MeOH	No reaction
H_2O	No reaction

We tried to tranfer the di-azo group to Fmoc protected Lysine (**9**) with ADMP, but we faced solubility problem with this amino acid. The reaction was performed in different solvents, but none of them gave the expected product.

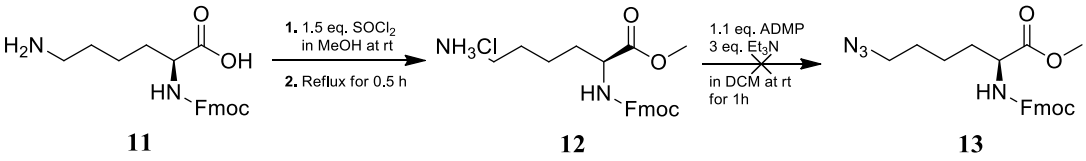


Figure 19. The reaction scheme of di-azo transfer with Fmoc-lysine derivative

To overcome this problem, we tried azidation of Fmoc protected Lysine methyl ester (**12**), but it did not also give the expected product **13**. Since a variety of bases can deprotect the Fmoc group, there are a limited number of conditions that can be applied for the di-azo transfer reaction. Therefore we started to use the Boc protection group.

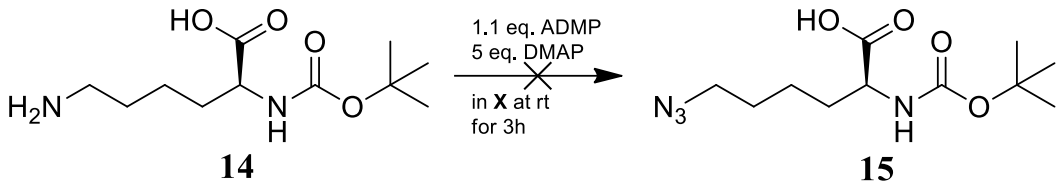


Figure 20. The reaction scheme of di-azo transfer with Boc-lysine

Table 5. The result of reactions between ADMP and Boc-lysine at different solvents

Solvent	Result
DCM	X
ACN	X

We continued our experiments by using Boc protected lysine (**14**), but towards the end of our trial, it became clear that when the carboxylic acid group exists, the ADMP acts azide transfer reagent, not di-azo transfer reagent. It preferentially reacts with carboxylic acid group and resulted a complex product mixture.

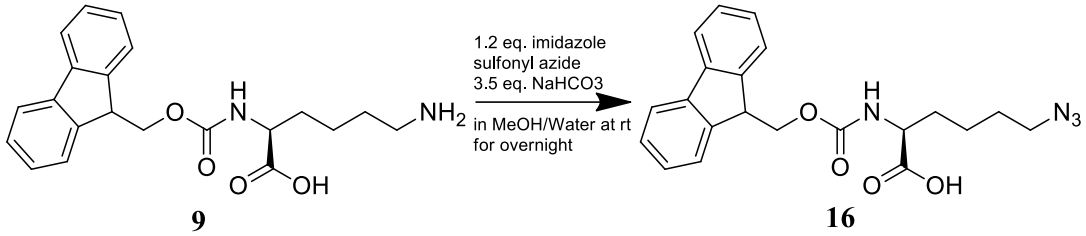


Figure 21. The reaction scheme of di-azo transfer with Fmoc-lysine and imidazole sulfonyl azide

As a result of all these experiments, we concluded ADMP is not a good reagent to synthesize azido amino acids and we synthesized the azido-Fmoc-Lysine (**9**) with imidazole sulfonyl azide based on literature procedure.

2.2. Chlorotoxin and its derivative synthesis

The chlorotoxin can be synthesized easily by using Boc-SPPS strategy, but in the Fmoc based strategy, the length of peptide is very limited, after certain length of

peptide sequence, due to aggregation of peptide backbone under basic conditions, the yields of the each coupling steps decrease and produce lots of side products which are sometime very difficul to separate from desired product.. There are some reports on the synthesis of CLTX using Fmoc based SPPS but their yields are lower than 1% after purification and folding.

Nevertheless, we also performed the synthesis of the chlorotoxin by using Fmoc-SPPS method, all in once to see this problem. In the synthesis of chlorotoxin, after addition of first 18 amino acids, the small amount of resin is taken from peptide reactor and the peptide (**pep 1**) bound to the resin was cleaved and deprotected by using cleavage cocktail. The HPLC and LC-MS results show the yield of peptide synthesis was going well.

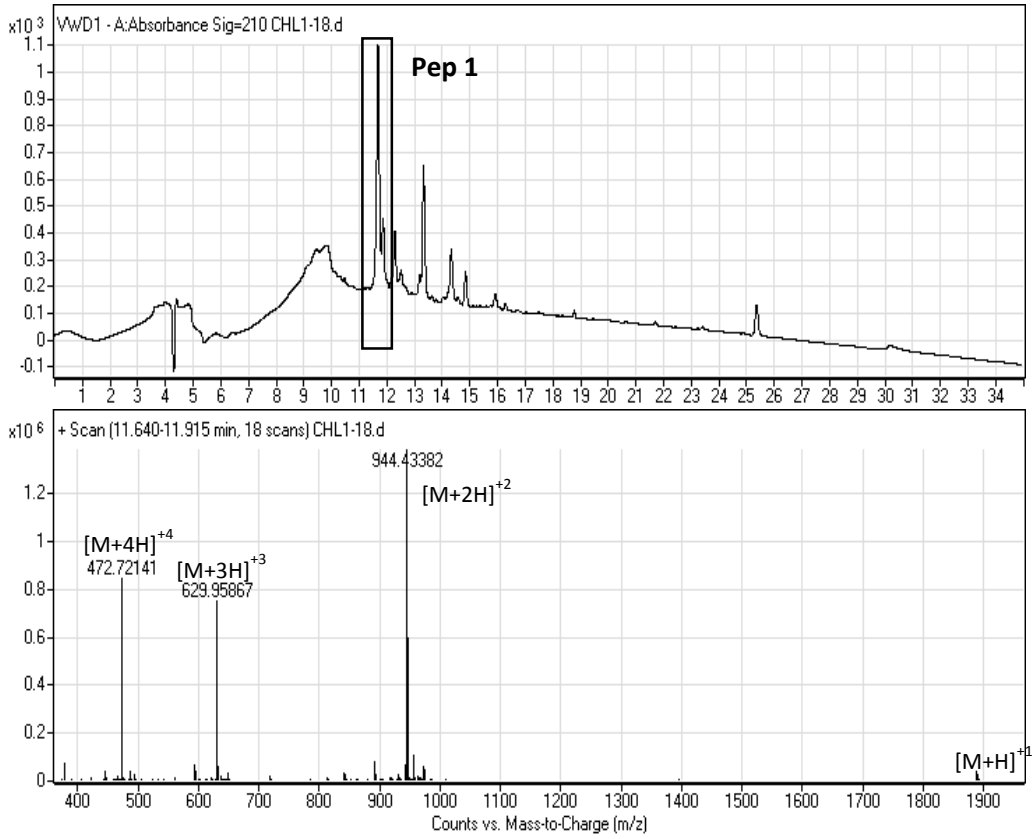


Figure 22. The UV absorbance and mass spectrum of chlorotoxin part containing 18 aminoacids (pep 1)

After the synthesis was completed without the deprotection of the last Fmoc protection on the N-terminal of the peptide the small amount of resin was taken and these resin treated with cleavage cocktail. The expected product was chlorotoxin which with Fmoc residue (**pep 2**) at the N-terminal as confirmed by in the HPLC and LC-MS

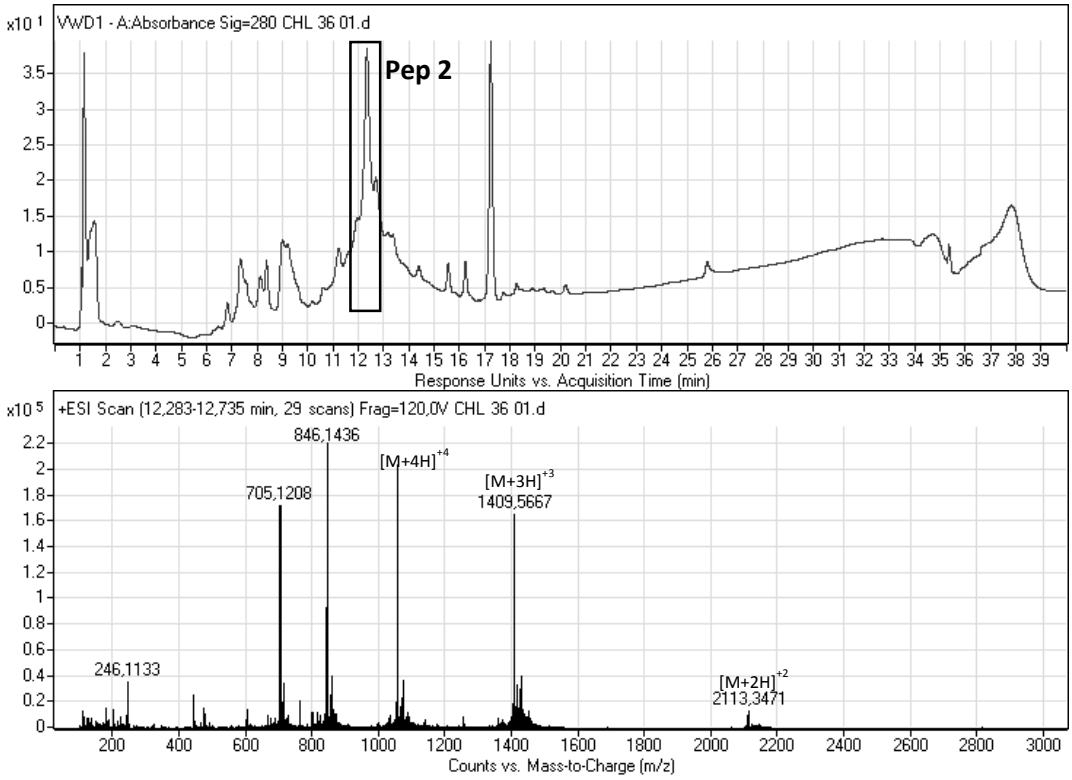


Figure 23. The UV absorbance and mass spectrum of Fmoc-chlorotoxin (pep 2)

Then, the peptidyl resin in the main reaction vessel was treated with piperidine solution in order to deprotect final Fmoc residue by using cleavage cocktail, the unprotected peptide was obtained but the HPLC and LC-MS results showed the product (**pep 3**) contained many products, and chlorotoxin was one of them.

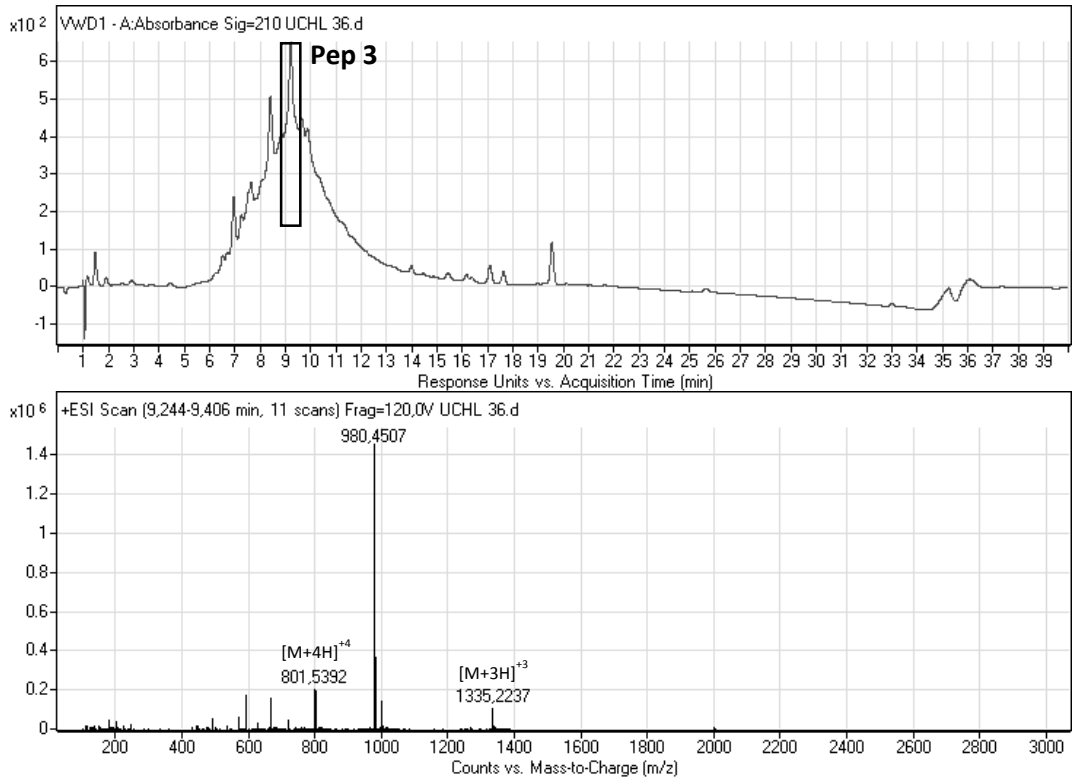


Figure 24. The UV absorbance and mass spectrum of chlorotoxin (pep 3)

By using reverse phase HPLC semi-preparative C-18 columns, the purification of chlorotoxin was performed but we did not get the desired product in pure form.

During the synthesis of chlorotoxin, we faced many issues. One of the problems was resulted because of the presences of disulfide bridges. Chlorotoxin peptide contains 8 cysteine residues which form 4 disulfide bounds, the cause of problem is the formation of secondary structure of peptide after cleavage from resin. Chlorotoxin peptide can be made with different disulfide bridges, however these bridges affect the secondary structure and cause many signals at different retention times at the HPLC or LC-MS that belong to chlorotoxin. For preventing this problem, DTT is used for breaking the disulfide bridges during measurements.

After we tried the synthesis of chlorotoxin by using SPPS method based on Fmoc chemistry, we decided to use native chemical ligation for the synthesis for a better yield. First of all, the ligation point should be determined and optimized for chlorotoxin peptide. For this purpose, the test peptides were synthesized to see the

ligation reaction working effectively. Firstly, we decided to do the ligation between 18th and 19th amino acids and we synthesized a model peptide which mimick N-terminal of CLTXwhose sequence is CGYKG, the cysteine residue at the N-termini would be used at the ligation process. The design of the peptide is based on the first 5 amino acid of peptide, which would be used at chlorotoxin synthesis, whose sequence is CGGKG, we changed one of the glysine with tyrosine for easily detection of the peptide in the HPLC since tyrosine residue has a strong absorbance at 280 nm. This peptide (**pep 4**) was synthesized by using Fmoc-SPPS method and the purification of this peptide was performed.

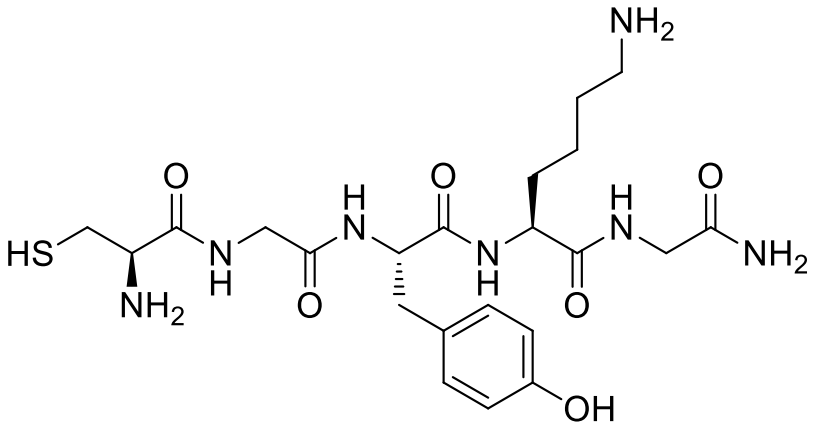


Figure 25. The molecular structure of CGGKG peptide (pep 4)

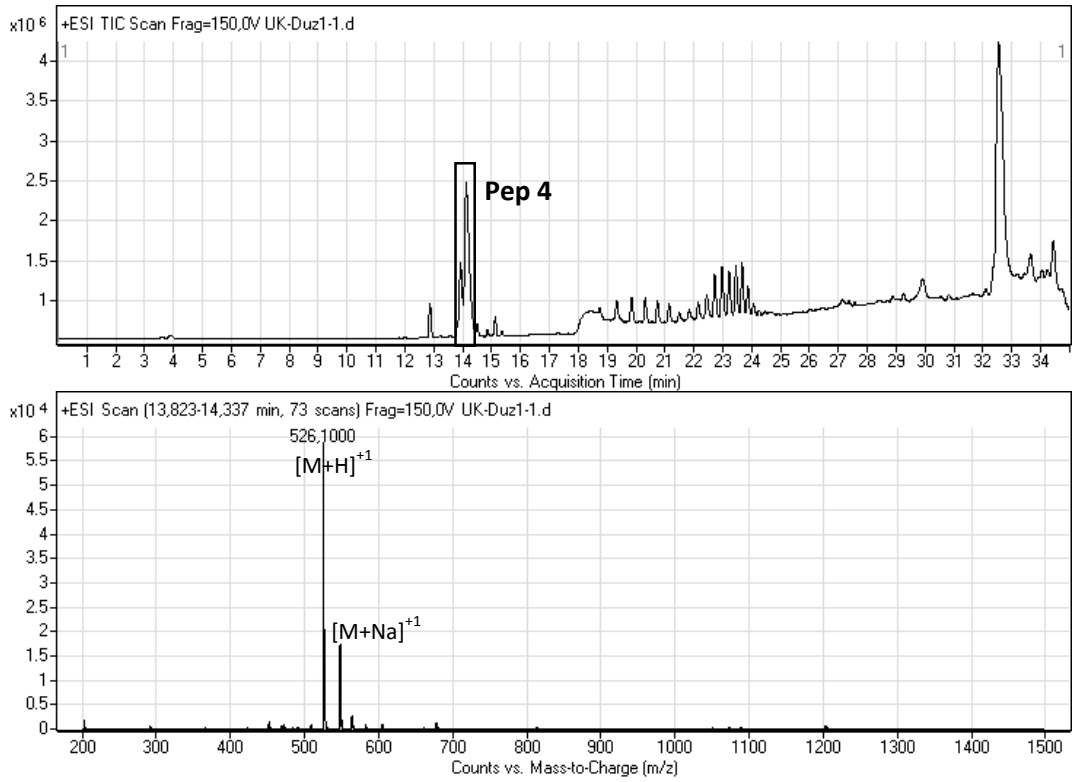


Figure 26. The mass chromatogram and spectrum of CGGKG peptide (pep 4)

Additionally, the peptide whose sequence is MYRDC-Dbz, (**pep 5**) which contains N-acyl-benzimidazolinone at the C-termini was synthesized as a model peptide which mimicks the C-terminal of CLTX, but the yield of peptide synthesis was very low and the peptide was not stable. The reason of these problems was probably cysteine residue which is next to Dbz. The cysteine may attack the carbonyl group which is bound to Dbz group and decompose the structure due to close proximity of thiols to Dbz group.

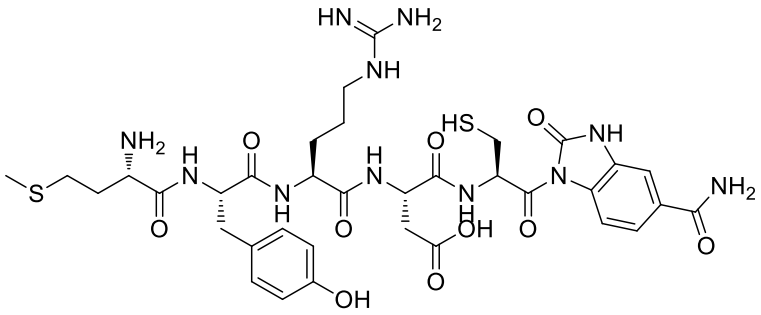


Figure 27. The molecular structure of MYRDC-Dbz peptide (pep 5)

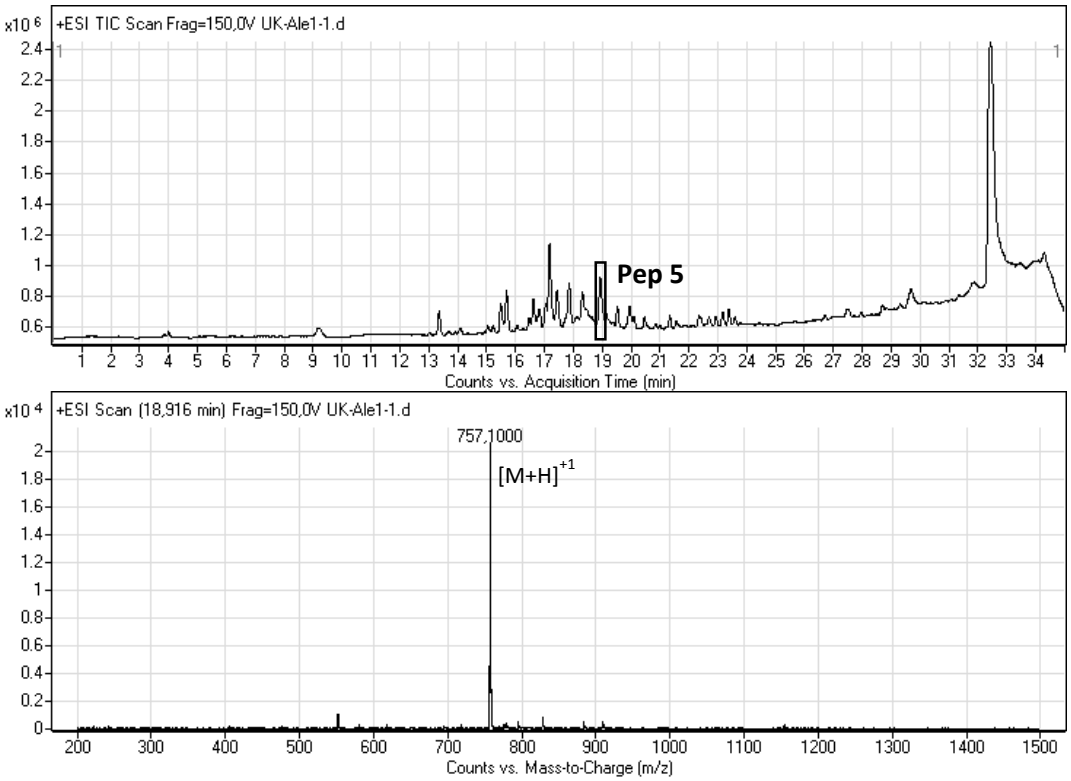


Figure 28. The mass chromatogram and spectrum of MYRDC-Dbz peptide (pep 5)

Then, for solving this problem, we decided to change the ligation position that would be between 15th and 16th amino acids. In this method, the peptide which contains Dbz part would not have any cysteine residue at near to C-termini for stabilizing the peptide. The peptide whose sequence is Fmoc-QMYRK-Dbz (**pep 6**) was synthesized for testing our new method, and it was stable enough to work with.

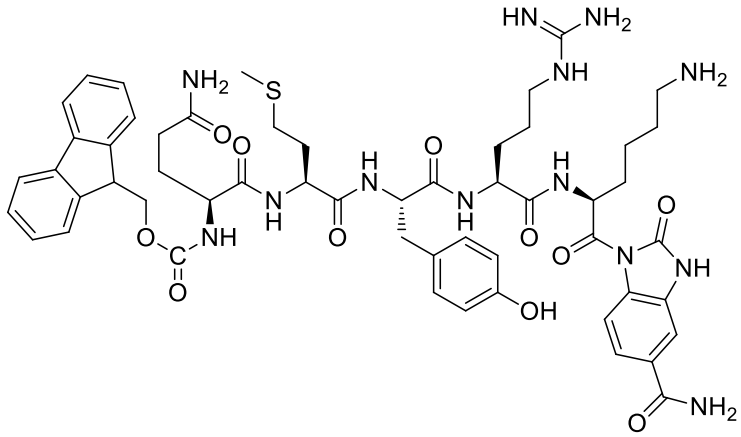


Figure 29. The molecular structure of Fmoc-QMYRK-Dbz peptide (pep 6)

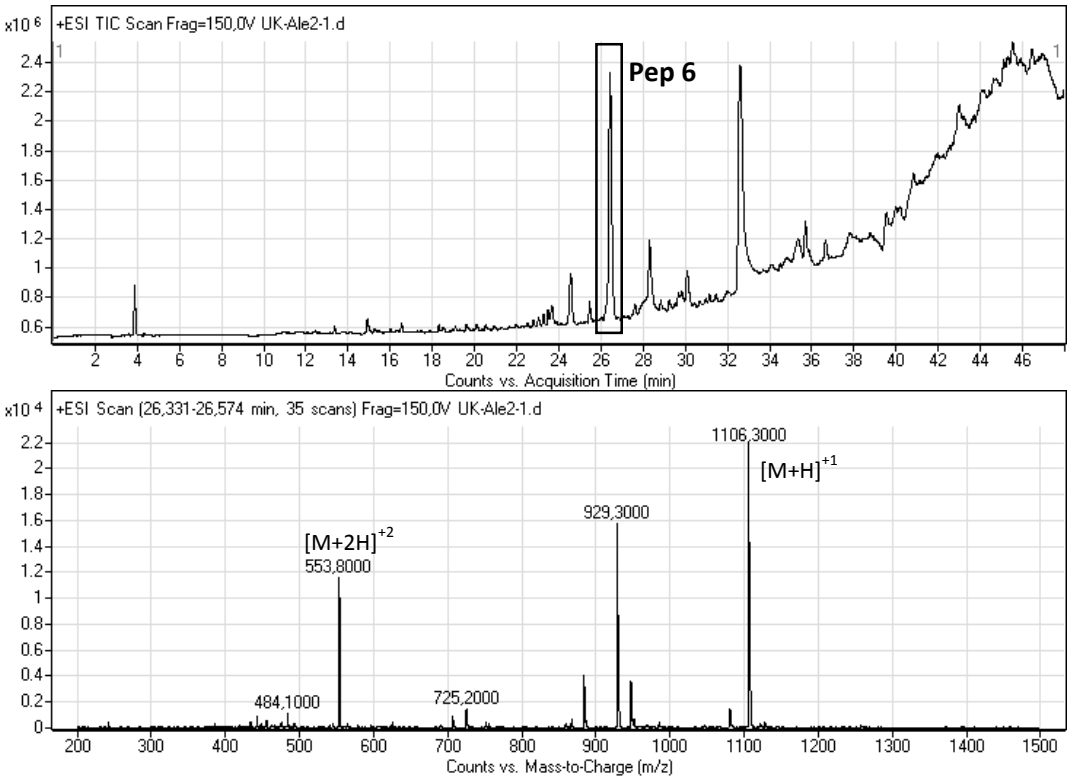


Figure 30. The mass chromatogram and spectrum of Fmoc-QMYRK-Dbz peptide (pep 6)

There are two different signal at the spectrum belonging to our peptide having Dbz group, due to structural isomers of the peptide. The peptide bond could form at the meta or para nitrogen atoms at Dbz structure, the structural isomers have different retention times. .

Table 6. The peptides designs for ligation reactions.

	N-Termini	C-termini
First design	MCMPCFTTDHQMARKCDDC-(Nbz)	HS-CGGKGRGKCYGPQ-CLCR
	19 amino acids + Nbz group	17 amino acids
Alternative design	MCMPCFTTDHQMARK-(Nbz)	HS-CDDCCGGKGRGKC-YGPQCLCR
	15 amino acids + Nbz group	21 amino acids

After the synthesis of test peptides, we started to synthesize real targets whose sequences are CDDCCGGKGRGKCYGPQCLCR (**pep 7**) and Fmoc-MCMPCFTTDHQMAR-K-Dbz (**pep 8**). The same synthetic method was used for obtaining these peptides.

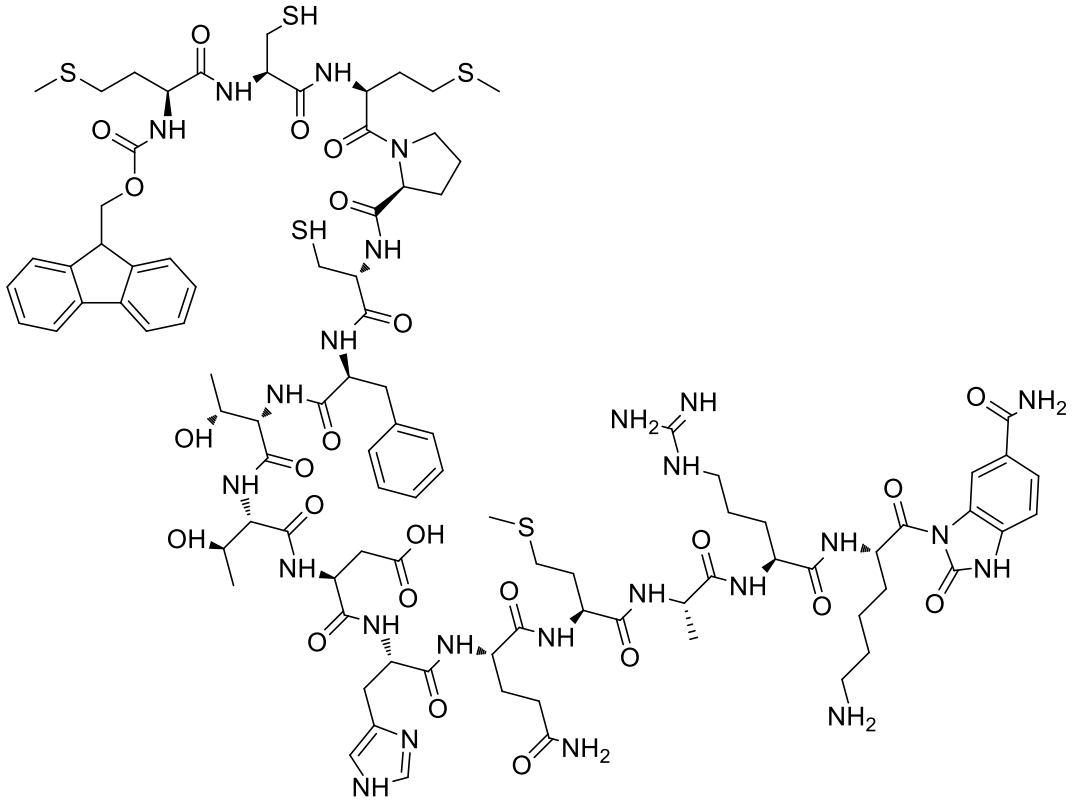


Figure 31. The molecular structure of Fmoc-MCMPCFTTDHQMARK-Dbz peptide (pep 7).

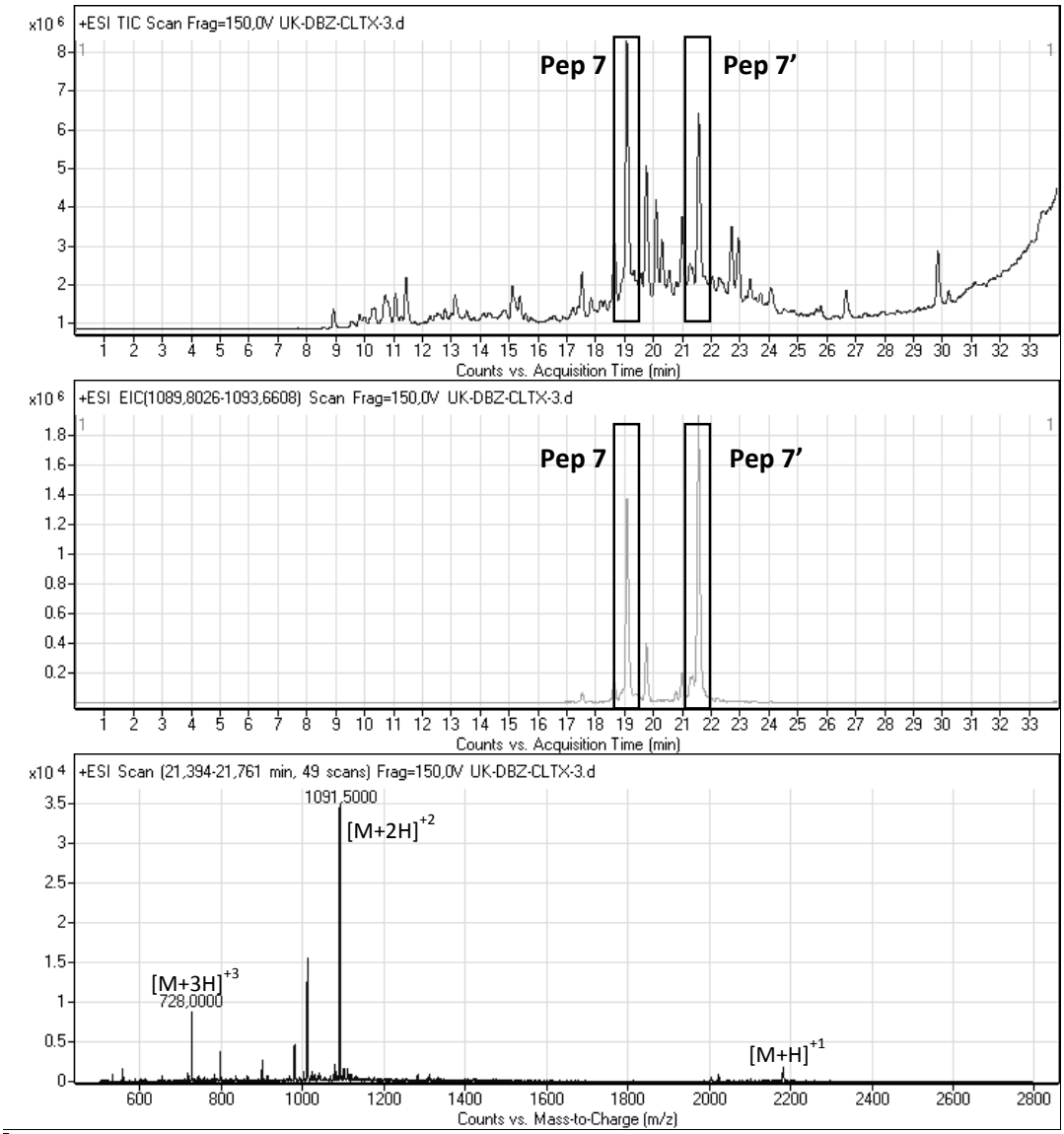


Figure 32. The mass chromatogram and spectrum of Fmoc-MCMPCFTTD-HQMARK-Dbz peptide (pep 7).

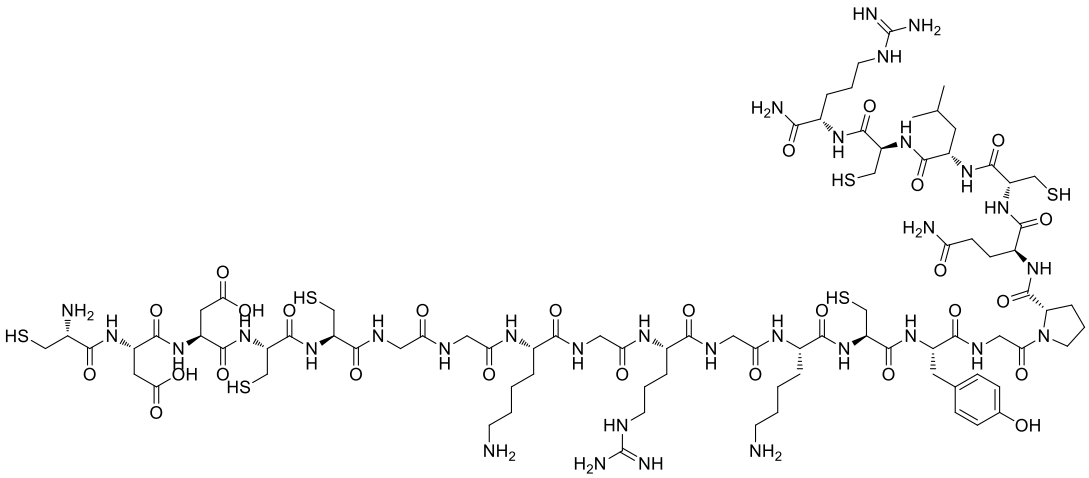


Figure 33. The molecular structure of CDDCCGGKGRGKCYGPQCLCR peptide (pep 8).

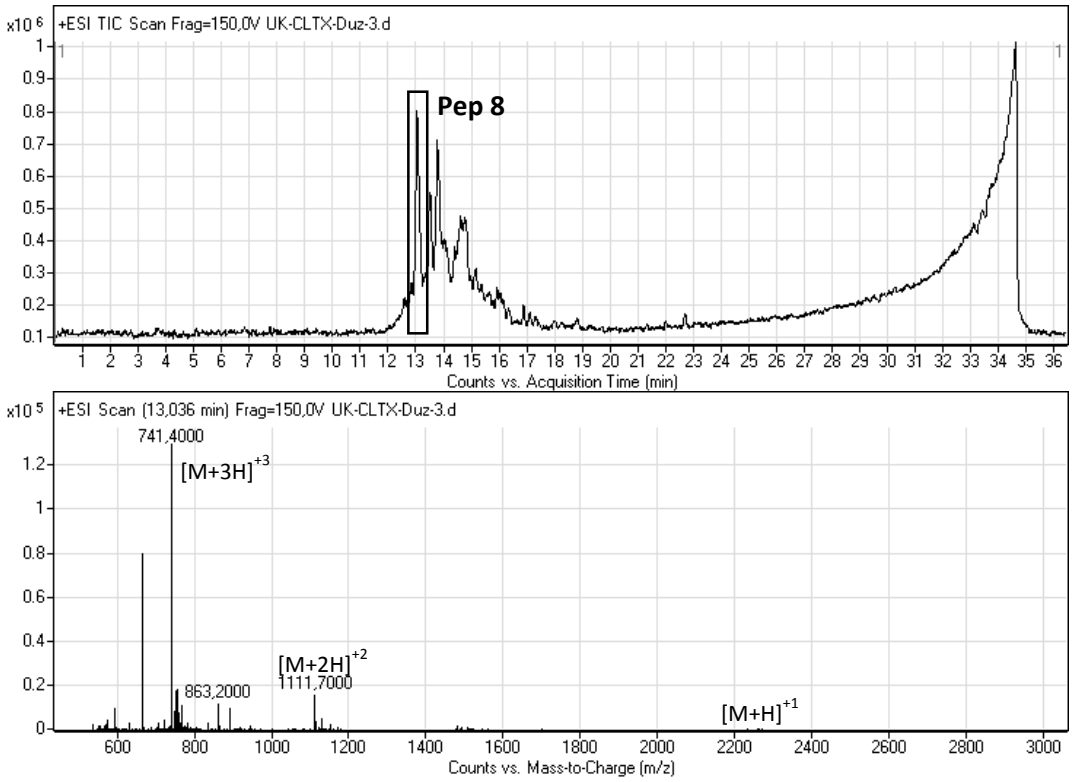


Figure 34. The mass chromatogram and spectrum of CDDCCGGKGRGKCYGP-QCLCR peptide (pep 8).

After the synthesis of the peptides, the ligation reaction of these peptides was performed in the ligation buffer. At 0 hour and 1 hour, the small amount samples were taken from reaction mixture and their HPLC measurement was performed.

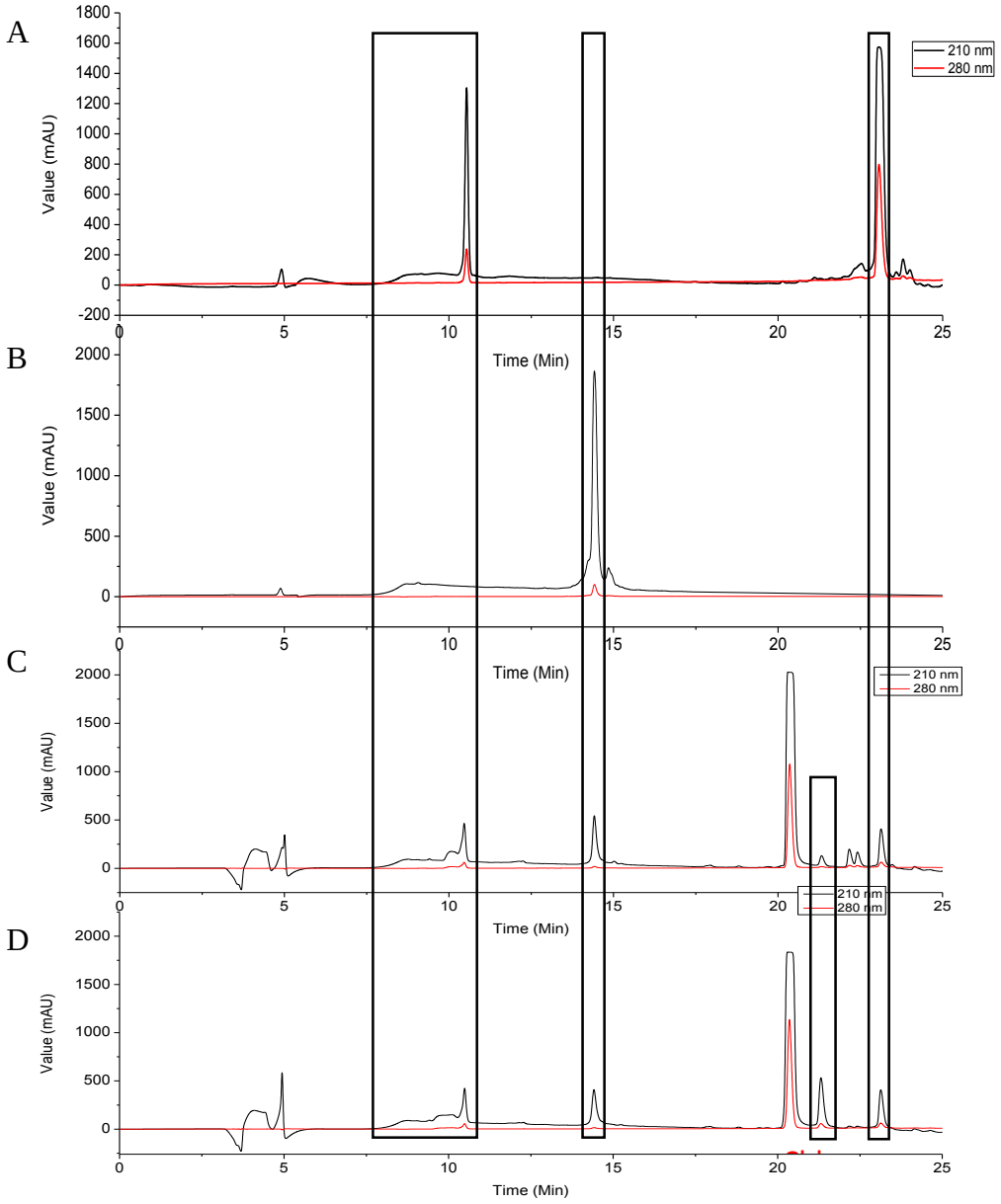


Figure 35. The HPLC chromatograms of A) Pep 7 which is containing Dbz group B) Pep 8 which is linear unprotected peptide C) The ligation reaction at 0 hour D) the ligation reaction at 1 hour.

The product of ligation is was observed as a new appearing peak with a retention time of 21 minutes as seen at the last HPLC chromatogram. In the result of isolation of this product and measurement of its mass, the product is Fmoc-Chlorotoxin which was confirmed by LC-MS analysis.

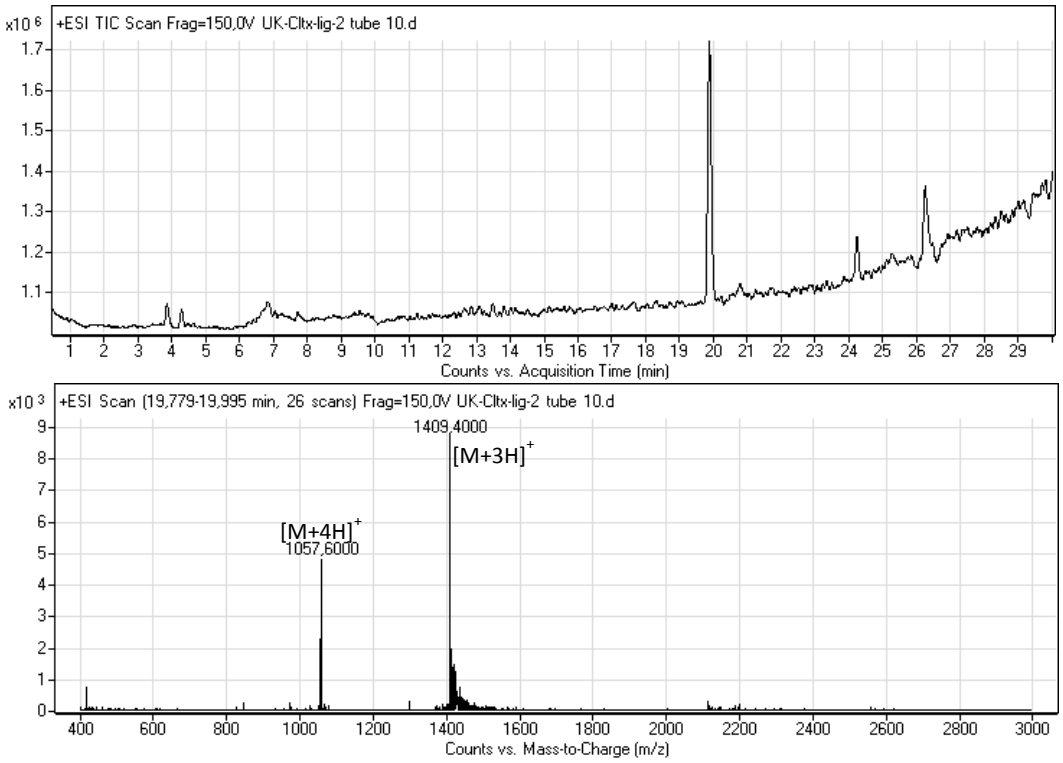


Figure 36. The mass chromatogram and spectrum of ligation product (Fmoc-Chlorotoxin).

CHAPTER 3

CONCLUSION

To conclude, in this study, we tried to find the new and efficient method for synthesizing azido amino acids by the reaction between hydroxyl and free amine groups of amino acids, and ADMP but only hydroxyl group of serine amino acid gave a positive outcome and the other experiments failed despite changing some parameters. The reason behind these problems is that ADMP is unstable and also very reactive with other functional group such as carboxylic acid. The azido amino acid was synthesized by using known method including imidazole-1-sulfonyl azide.

In addition, in this study, the chlorotoxin was synthesized first time by using native chemical ligation. The reason of using method is that the directly synthesis of chlorotoxin by using Fmoc-SPPS strategy has a very low yield and the native chemical ligation method is used to increase this yield. The synthesis of shorter peptides and their ligation is more efficient method to get longer peptides. In the result of native chemical ligation, Fmoc protected chlorotoxin was obtained.

CHAPTER 4

EXPERIMENTAL

4.1 Materials and Methods

For the synthesis of artificial amino acids, all reaction 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) data were recorded in d_6-DMSO and $CDCl_3$ 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

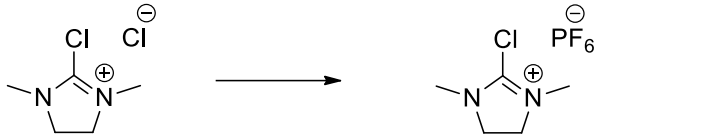
HPLC purification of the cleaved peptides were performed with Dionex Ultimate 3000 Series equipped with a variable wavelengths absorbance detectors using a reverse phase C18 column (Hypersil Gold, 12 µm, 250 x 10 mm). A binary gradient of water (0.1% TFA) and acetonitrile (0.08% TFA) was used with a 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, 4.6x150 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 4.6x150 mm 3-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.*

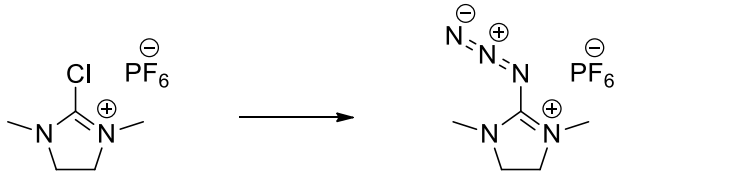
4.2 The reaction procedures for the azido amino acids synthesis



2-Chloro-1,3-dimethylimidazolinium Hexafluorophosphate

Synthesis of 2-Chloro-1,3-dimethylimidazolinium Hexafluorophosphate

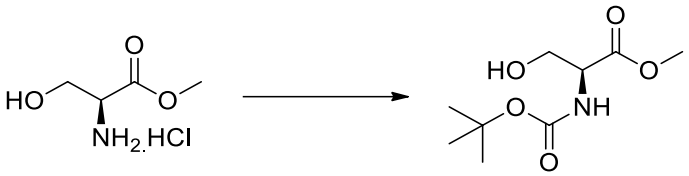
Based on literature procedure[24], to 2-chloro-1,3-dimethylimidazolinium chloride (2.35 g, 13.9 mmol) in CH_3CN (3.8 mL) solution was added potassium hexafluorophosphate (2.5 g, 13.6 mmol) in CH_3CN (9.5 mL), and the solution was stirred for 10 min at room temperature. The solution was filtered by using a celite pad and the solvent is evaporated in vacuo. The residue was dissolved with a small amount of CH_3CN and the solution was poured into Et_2O to form solid precipitate, which was gathered by suction filtration to afford chloroimidazolinium hexafluorophosphate (3.33 g) in 84% yield.



2-Azido-1,3-dimethylimidazolinium Hexafluorophosphate (ADMP)

Synthesis of 2-Azido-1,3-dimethyl imidazolinium Hexafluorophosphate (ADMP)

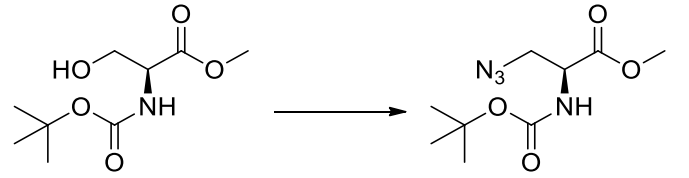
Based on literature procedure[24], to 2-Chloro-1,3-dimethylimidazolinium Hexafluorophosphate (3.18 g, 11.4 mmol) in CH_3CN (12 mL) solution was added sodium azide (1.1 g, 16.9 mmol) at 0 °C, and the solution was stirred for 45 min. The solution was filtered through a celite pad, and the solvent is evaporated in vacuo. The crude product was dissolved in a small volume of CH_3CN, and the solution was poured into Et_2O to form solid precipitate, which was gathered by suction filtration Yield: 2.74 g, 84%. ^{1}H-NMR (400 MHz, DMSO): δ = 3.74 (s, 4 H) 3.01 (s, 6 H) ppm.



(*S*)-methyl 2-((*tert*-butoxycarbonyl)amino)
-3-hydroxypropanoate **2**

Synthesis of (S)-methyl 2-((tert-butoxycarbonyl)amino)-3-hydroxypropanoate (2)

Based on literature procedure[26], to a solution of L-serinemethylester hydrochloride (740 mg, 4.76 mmol) in methanol (13.5 mL) was added a solution of 1 M NaOH (1.5 mL), Et_3N (1.5 mL) and $(Boc)_2O$ (2.08 g, 9.51 mmol, 2.0 equiv). The solution was mixed for overnight at room temperature. The solvents was evaporated in vacuo, and 10 ml ethyl acetate added. The pH of mixture adjusted to 2 with 3 M HCl solution. The solutions was extracted with ethyl acetate. Theorganic layers was washed with water, dried with $MgSO_4$, and solvents was evaporated in vacuo. The product was a colorless liquid (865 mg, 83%) after purified by silica columns chromatography (1:1 hexane: ethyl acetate). ^1H NMR (400 MHz, $CDCl_3$): δ 1.40 (s, 9H), 2.80 (br s, 1H), 3.72 (s, 3H), 3.80-3.86 (m, 1H), 3.90-3.95 (m,1H), 4.32 (br s,1H), 5.48 (br d, 1H) ppm

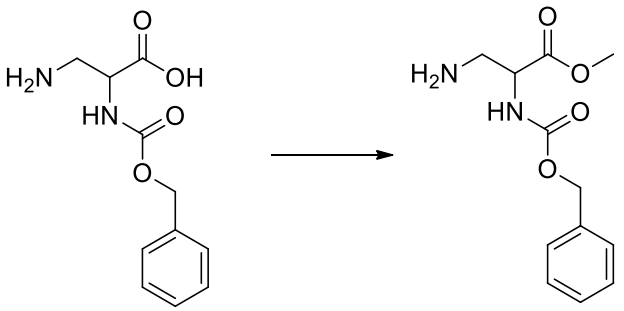


(*S*)-methyl 3-azido-2-((*tert*-butoxycarbonyl)
amino)propanoate (**3**)

Synthesis of (S)-methyl 3-azido-2-((tert-butoxycarbonyl)amino)propanoate (3)

Boc-L-serine methyl ester (135 mg, 0.62 mmol) in THF (3.1 mL) and ADMP (214 mg, 0.75 mmol), and DBU (0.1 mL, 0.87 mmol) mixed at room temperature, and the solution was mixed for 15 min. The reaction was stopped by adding saturated NH_4Cl solution, and organic products were extracted with CH_2Cl_2. The organic layers was washed with brine, and dried overs $MgSO_4$. The $CH_2Cl_2$was evaporated in vacuo to obtain the crude compounds that were purified by flash silicas column

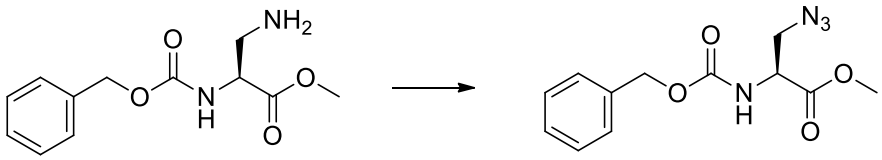
chromatography (25:6 hexane–EtOAc) to give pure product. Yield: 23 mg, 15% ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.44 (s, 9 H), 3.61-3.67 (m, 2 H), 4.28 (m, 1 H), 5.31 (br s, 1 H)



methyl 3-amino-2-(((benzyloxy)carbonyl) amino)propanoate (**6**)

Synthesis of methyl 3-amino-2-(((benzyloxy)carbonyl)amino)propanoate (6)

Based on literature procedure[27], thionyl chloride (0.1 mL, 1.4 mmol) is slowly added to methanol (2 mL) with stirring at 0 ^{0}C in an ices bath.After stirring for 30 minutes, N-alpha-Cbz-L-2,3- diaminopropiomc acid (Z-Dap-OH) (119 mg, 0.5 mmol) is added and the reactions is stirred for overnight at r.t. The solvents is evaporated in vacuo, and the resulting white solid is triturated in ether (3 mL) and filtered to give the methyl ester as the hydrochloride salt ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.02 (t, 1H), 3.16 (t, 1H), 3.67 (s,3H), 4.42 (m, 1H), 5.07 (s, 2H), 7.34 (m, 5H), 7.91 (d, 1H), 8.31 (br s, 3H).

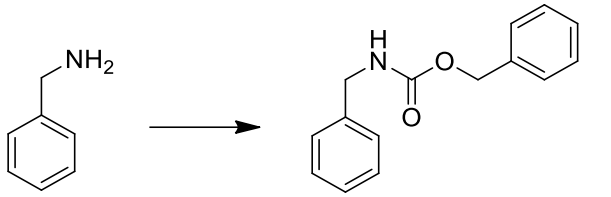


(*S*)-methyl 3-azido-2-(((benzyloxy) carbonyl)amino)propanoate (**7**)

Synthesis of (S)-methyl 3-azido-2-(((benzyloxy)carbonyl)amino)propanoate (7)

ADMP (342 mg, 1.2 mmol) in dichloromethane (2 mL) was addedto the solution of Cbz-Dap methyl ester (252 mg, 1 mmol) and Et_3N (0.35 ml, 2.5 mmol) in dichloromethane (2 mL) at the room temperature. The solution is stirred for 2 h.The reaction was quenched with 20 ml $NaHCO_3$ solution, and the organic layers were

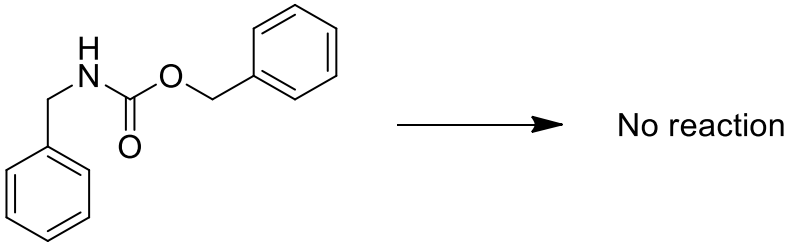
extracted with dichloromethane. The organic layers was washed withbrine and then dried with $MgSO_4$. The solvents was evaporated in vacuum to obtain the crude compound, which was purified by flash silica column chromatography. The products of purification didn't contain the expected product.



benzyl benzylcarbamate (**8**)

Synthesis of benzyl benzylcarbamate (8)

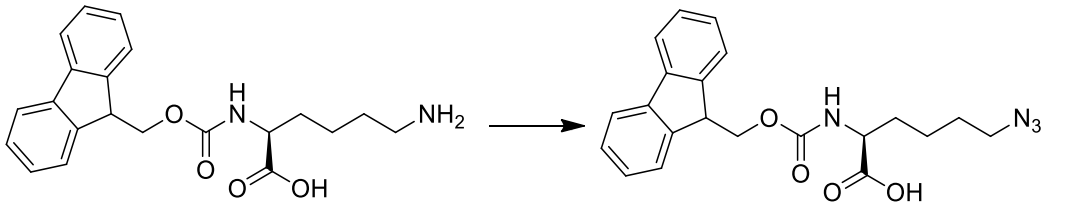
Based on literature procedure[28], the benzyl amine (428 mg, 4.0 mmol) and DIPEA (0.7 ml, 4.0 mmol) was added to 20 ml of dichloromethane and the resulting solution was cooled to 0 °C. A solution of the benzyl chloroformate (0.629 ml, 4.4 mmol in 1.0 ml dichloromethane) was added drop-wise to the amine solution while stirring. The reaction solution was stirred for 40 min at 0 °C and then allowed to warm to r.t. for 2h. The solution was washed with 0.5 M HCl followed by brine. After dryingover $MgSO_4$, the solvents was evaporated under vacuo and yield: 83%, 800 mg. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35-7.25 (m, 10H), 5.12 (s, 2H), 5.05 (s,1H), 4.41 (d, J= 4 Hz, 2H)



Synthesis of compound by using benzyl benzylcarbamate (8)

ADMP (250 mg, 0.9 mmol) was added to a mixtureof benzyl benzylcarbamate (121 mg, 0.5 mmol) and Et_3N (0.14 ml,1 mmol) in CH_2Cl_2 (2 mL) at the room temperature. The solution is mixed for 30 min.The reaction was finished with aq.$NaHCO_3$ (10 mL), and the organic materials were extracted with CH_2Cl_2 (3x15 mL). The combined

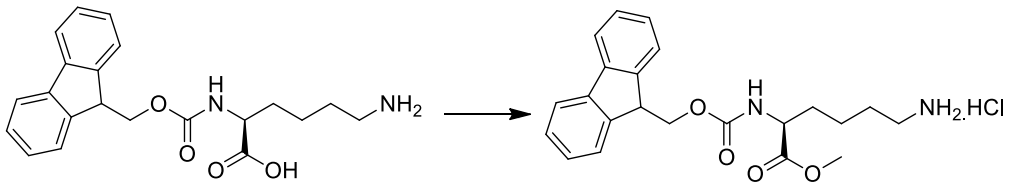
extracts was washed with water and brine and then dried over$MgSO_4$. The solvents were evaporated in vacuo to afford the crude compound, but the starting material did not give any reaction, there were no product.



(*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl) amino)-6-azidohexanoic acid (**10**)

Synthesis of (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-azidohexanoic acid (10)

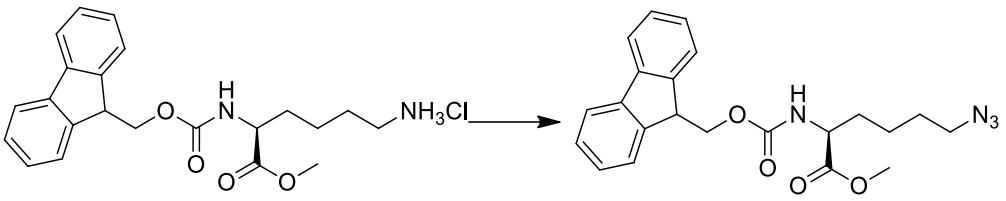
ADMP (171 mg, 0.6 mmol) in methanol (1 mL) were added to the solution of Fmoc-Lys (185 mg, 0.5 mmol) and Et_3N (0.14 ml, 1 mmol) in methanol (1 mL) at the rooms temperature. The mixture is stirred for 30 min. The solvent was evaporated in vacuo, and the residues was redissolved in CH_2Cl_2 (50 mL). The solution was washed with 50 ml of 1 M HCl solution, and the water phase was extracted with CH_2Cl_2 (3 x 50mL). The organic extracts were washed with water and brine and then dried over anhydrous $MgSO_4$. The solvents were evaporated under vacuo. The solid didn't contain the expected product.



(*S*)-methyl 2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-6-aminohexanoate hydrochloride (**12**)

Synthesis of S)-methyl 2-((((9H-fluoren-9-yl) methoxy)carbonyl)amino)-6-aminohexanoate hydrochloride (12)

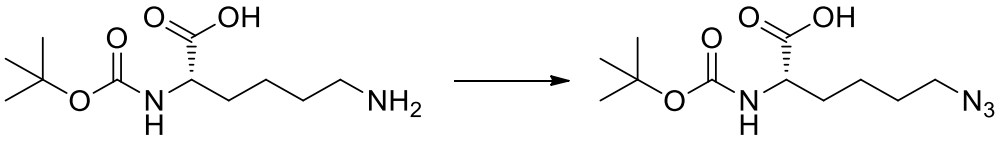
Based on literature procedure[29], Fmoc-Lys (921 mg, 2.5 mmol) was esterified using a $SOCl_2$ (0.27 ml, 3.75 mmol,) in 15 ml MeOH reagent under reflux for 45 min. The solvents were evaporated under vacuo and the residue wasrecrystallized from MeOH/Et_2O to give the ester 0.88 g Fmoc-Lys-OMe•HCl (yield 84%).



(*S*)-methyl 2-((((9*H*-fluoren-9-yl)methoxy)carbonyl) amino)-6-azidohexanoate (**13**)

Synthesis of (S)-methyl 2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-azidohexanoate (13)

ADMP (171 mg, 0.6 mmol) in CH_2Cl_2 (1 mL) was added to a mixture of S)-methyl 2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-aminohexanoate hydrochloride (210 mg, 0.5 mmol) and Et_3N (0.31 ml, 1.5 mmol) in dichloromethane (1 mL) at the room temperature. The mixture is mixed for 30 min. The reaction was stopped with aq.$NaHCO_3$ (10 mL), and the organic materials were extracted with CH_2Cl_2 (3x15 mL). The organic extracts was washed with water and brine and then drieds over anhydrous $MgSO_4$.. The solvent was evaporated under vacuo. The solid didn't contain the expected product.

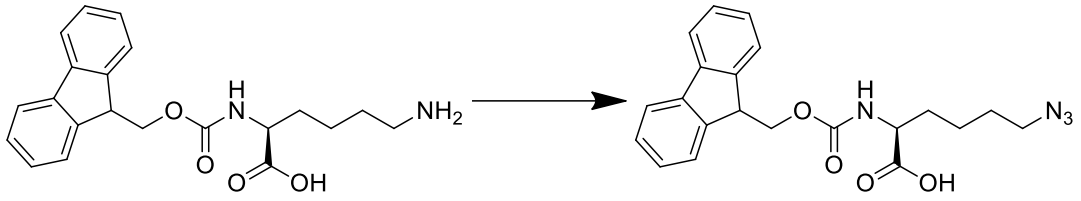


(*S*)-6-azido-2-((*tert*-butoxycarbonyl) amino)hexanoic acid (**15**)

Synthesis of (S)-6-azido-2-((tert-butoxycarbonyl) amino)hexanoic acid (15)

To a solution of Boc-L-lysine (135 mg, 0.5 mmol) in THF (2.5 mL), ADMP (173 mg, 0.6 mmol), and DBU (0.08 mL, 0.7 mmol) were added at r.t., and the mixture was stirred for 15 min. The reaction was stopped with sat. aq NH_4Cl, and organic products were extracted with CH_2Cl_2. The combined extracts were washed with brine, and then dried with anhydrou $MgSO_4$.. The solvent was evaporated in vacuo to obtain the crude compounds that were purified by flash silica column chromatography (silica gel, 25:6

hexane–EtOAc) to gives pure product. The products of purification didn't contain the expected product.

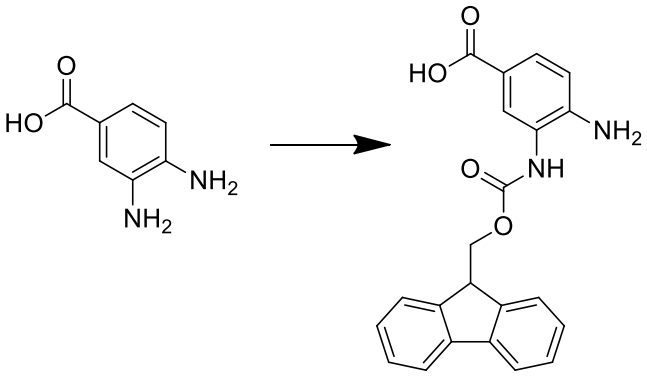


(*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-6-azidohexanoic acid (**16**)

Synthesis of (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-azido hexanoic acid (16)

Based on literature procedure[30], to a stirred mixture of Fmoc-lysine (1.84 g, 5 mmol) and $CuSO_4$ (0.012g, 0.08 mmol) in MeOH/Water 8:2, 40 mL) at rooms temperature was added $NaHCO_3$ (1.5 g, 17.5 mmol) followed by imidazole-1-sulfonyl azide (1.25g, 6 mmol). The pH of the reaction mixture was repeatedly regulated to pH 8 by adding of $NaHCO_3$. The reaction mixture was stirred for overnight and the pH was acidified at 2 using 1 M HCl. The mixture was extract with ethyl acetate (4x75 mL), then the combined organic phase was washed with brine (50mL),dried over anh.s Mg_2SO4, filtered and concentrated in order to obtain the product as a yellowish oil which was used without further purification.^1H NMR (400 MHz, $CDCl_3$):7.75 (2H,d, J=7.4Hz),7.78 (2H, d, J=7.4 Hz), 7.36 (2H, t, J=7.4 Hz), 7.29 (2H, t, J=7.4 Hz), 5.3 (1H, d), 4.2(2H,d), 4.12 (1H, t, J=6.9 Hz), 3.8 (1H, m), 3.2 (2H, t), 1.9-1.1 (6H,m)

4.3 The reaction procedure for synthesizing chlorotoxin and its derivatives



3-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)
-4-aminobenzoic acid

Synthesis of 3-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)-4-aminobenzoic acid

Based on literature procedure[18], to a solution of 3,4-diaminobenzoic acid (1 g, 6.6 mmol) in $NaHCO_3$(aq) (84 mg,) water/acetonitrile (1:1, 20mL) was added N-(9-fluorenylmethyloxy carbonyloxy) succinimide (2.2 g, 6.6 mmol) in small amounts.. After the night, acidification with 1 M HCl(aq) formed a precipitate at was filtered, washed with methanol, cold ether and hexane. The solid was dried under vacuum affording the expected compound as a pale white solid (1.7 g, 70%). ^{1}H NMR (400 MHz, d_6-DMSO,):d=4.29-4.40 (m, 3H), 6.72 (d,1H), 7.33-7.36 (m, 2H), 7.44 (t,2H), 7.51 (d,1H), 7.72-7.80 (m, 3H), 7.91 (d, J=7.4Hz), 8.78 (s, 1H)

4.4 General procedure for solid phase peptide synthesis

Loading amino acid and coupling with an Fmoc-protected amino acid:

169 mg (0.1 mmol) Rink Amide resin is weighed in reaction vessel in which the desired peptide will be synthesized. It's washed with DMF (2x) and swelled in 5-6mL DMF for 40-45min. After Fmoc deprotection, Fmoc-protected amino acid (0.45 mmol, 4.5 eq.) (Fmoc-protected 3,4-diaminobenzoic acid acts like Fmoc-protected

amino acid) dissolved in HBTU (0.8 mL, 0.5 M in DMF) then DIEA (140µL) is added. After addition of DIEA, solution is mixed and added to the resin in 30 seconds at max. Mixture is allowed to stand for 15 minutes and agitated in every 5 min. Then the solution is filtered and the resin washed with DMF. This process repeated one more time. The reaction can be controlled with the Kaiser test.

Deprotection:

The Fmoc protection group is unprotected by treating the pre-swollen resin with %20 piperidine in dimthylformamide for 2x10 min (2x10 mL). Then the solution is emptied and the resin celaned with dimthylformamide.

Final Deprotection:

After deprotection of Fmoc of the very last amino acid residue, resin is washed with DMF (4 × 2 mL), DCM (4 × 2 mL) then dried under vacuum.
As an alternative way, final Fmoc deprotection can also be done in solution phase, after cleavage. In order to do that, completely dry powder is dissolved in 20% Piperidine in DMF solution. After the mixture is mixed for 10 min. with sonication, it is precipitated with methyl-tertbutyl ether. Precipitate is centrifuged and supernatant is poured off. The process is repeated 3-4 times until the supernatant becomes neutral by checking with pH paper.

Cleavage:

95% TFA, 2.5% decontaminated water and 2.5% TIPS solution – so called cleavage cocktail – is used to cleave the peptide sequence from resin. This cocktail (each 50 mg peptidyl resin is needed 1 mL cocktail solution) added to the resin and waited for 2 or 3 hour. Then the solution is collected. The peptide is triturated by addition of ice-

cold diethyl ether. The solid is filtered off. The product is dissolved in distilled water and lyophilized.

Different procedure for peptide containing Dbz group:

After peptide elongation, the resin was washed with dichloromethane and 100 mg p-nitrochloroformate in 10 ml dichlorometane was added peptide reactor. The resin was bubbled with N_2 for 40 min. This process repeated one more time. Then, it was washed with dichlorometane and treated with 10 ml 0.5 M DIEA in DMF for 20 min and, finally, it washed with DMF and dichlorometane. The peptidyl-resin was dried under vacuum and the peptide was cleaved by using cleavage cocktail. The final deprotection of Fmoc is not performed.

Native Chemical Ligation:

Ligations were performed at room temperature in the following ligation buffer,: 6 M Gn.HCl, 200 mM potassium phosphate, 200 mM 4-mercaptophenylaceticacid (MPAA), 20 mM tris(2-carboxyethyl) phosphine hydrochloride (TCEP.HCl).
Take 2.5 ml of stock solution of 6M Gn.HCl (guanidine hydrochloride) and 0.2 M K_2HPO4 and filter using a 0.2 µm syringe-driven filter into a 20 mL scintillation vial containing 14.3 mg TCEP.HCL (0.05 mmol) and 84.1 mg MPAA (0.5 mmol). Buffer was degassed under nitrogen for 15 min before the reaction. And pH was fitted to 7.0 with 2 M NaOH.
In a 20mL scintillation vial, the peptide containing Dbz group ($1.5x10^{-3}$ mmol) and linear peptide ($1.8x10^{-3}$ mmol) were dissolved in ligation buffer (0.75 mL) to achieve a final concentration of 2 mM with regards to the peptide containing Dbz group. The reaction solution was mixed at room temperature and monitored by HPLC.

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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

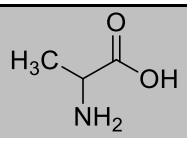
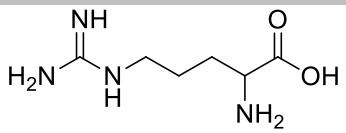
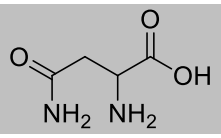
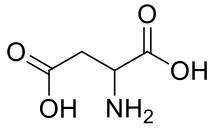
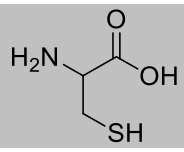
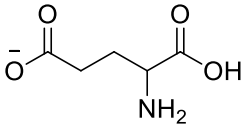
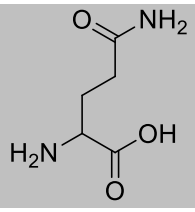
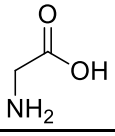
Name	3-Letter code	1-Letter code	Structure
Alanine	Ala	A	
Arginine	Arg	R	
Asparagine	Asn	N	
Aspartic acid	Asp	D	
Cysteine	Cys	C	
Glutamic acid	Glu	E	
Glutamine	Gln	Q	
Glycine	Gly	G	

Table A.1. List Of 21 Amino Acids Found In Nature (Continued)

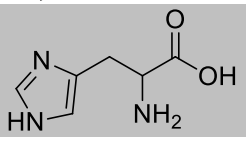
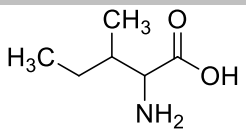
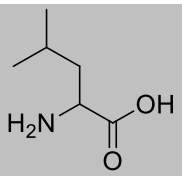
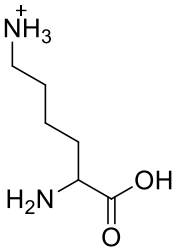
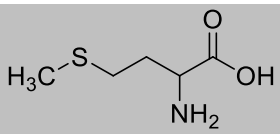
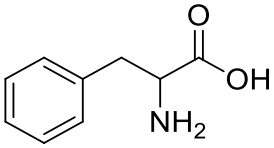
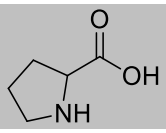
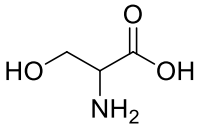
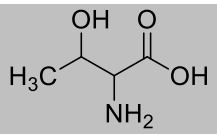
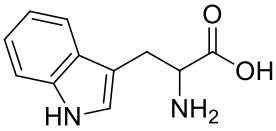
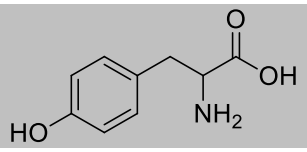
Histidine	His	H	
Isoleucine	Ile	I	
Leucine	Leu	L	
Lysine	Lys	K	
Methionine	Met	M	
Phenylalanine	Phe	F	
Proline	Pro	P	
Serine	Ser	S	
Threonine	Thr	T	
Tryptophan	Trp	W	

Table A.1. List Of 21 Amino Acids Found In Nature (Continued)

Tyrosine	Tyr	Y	
Valine	Val	V