SYNTHESIS OF METAL BINDING ARTIFICIAL AMINO ACIDS

A THESIS SUBMITED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

GÖZDE EŞAN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
CHEMISTRY

NOVEMBER 2014

Approval of the thesis:

SYNTHESIS OF METAL BINDING ARTIFICIAL AMINO ACIDS

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ABSTRACT

SYNTHESIS OF METAL BINDING ARTIFICIAL AMINO ACIDS

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November 2014, 55 pages

A large variety of peptides with different physical and chemical properties can be acquired by using different amino acids. Through chemical peptide synthesis, utilizing unnatural synthetic amino acids beside 21 natural amino acids, divergent peptides can also be obtained easily. Specifically amino acid and peptide conjugates of metals have received considerable attention due to their important role in many biological processes with unique structural properties. Moreover, it is well known that some metal ions are essential for living organisms and important for biochemical reactions as well as metal-amino acids and peptide complexes.

The target of this project is to obtain metal binding artificial amino acids and to use these artificial amino acids in pre-designed peptide sequence. For this purpose, initially a bipyridine and pyridine bistriazole amino acid derivatives that can coordinate to a wide variety of metal ions were tried to be synthesized using Stille coupling reactions and Huisgen cycloaddition reactions, respectively. Consequently, only diamine derivative of pyridine bistriazole could be synthesized. After that synthesis, tetrapeptide including lysine (K), phenylalanine (F) and glutamic acid (E) was coupled to the two side of diamine derivative. Having the parallel β-hairpin structure and the metal binding units in the middle of this sequence owing to triazoles could give a chance metal transport and release in biological systems.

Keywords: Bipyridine, bistriazole, click, metal binding amino acids

ÖZ

METAL İYONLARINA BAĞLANABİLEN YAPAY AMİNO ASİTLERİN SENTEZİ

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Kasım 2014, 55 sayfa

Peptitleri oluşturan amino asitlerin çeşitliliği, çok farklı fiziksel ve kimyasal özelliğe sahip peptit yapılarının elde edilmesine mükemmel bir olanak sağlamaktadır. Kimyasal peptit sentezi sayesinde 21 tane doğal amino asitin yanı sıra, doğal olmayan, sentetik amino asitlerin kullanımı ile de farklı özelliklere sahip peptitler elde etmek mümkündür. Özellikle amino asitlerin ve peptitlerin metal konjugatları birçok biyolojik sistemde rol almaktadır ve bunun yanında özgün yapısal özellikleri sayesinde bu konjugatlara büyük ilgi duyulmaktadır. Ayrıca, metallerin amino asit ve peptitlerle oluşturduğu komplekslerin yanı sıra metal iyonları da canlı organizmalarda ve biyokimyasal tepkimelerde oldukça büyük önem taşımaktadır. Bu çalışmanın amacı, metal iyonlarına bağlanabilen yapay amino asitler sentezlemek ve bu yapay amino asiti içeren peptitler sentezlemektir. Bu amaç doğrultusunda öncelikle bipiridin ve piridin bistriazol amino asit türevleri sırasıyla Stille kenetlenmesi ve Huisgen halkasal katılma tepkimeleri kullanılarak sentezlenmeye çalışılmıştır. Sonuç olarak yalnızca piridin bistriazol diamin türevi sentezlenebilmiştir. Bu sentezin ardından lizin(K), fenilalanin(F), ve glutamik asit (E) içeren dörtlü peptit sentezlenip diamin türevinin iki ucuna kenetlenme tepkimesi gerçekleştirilmiştir. Bu sayede saç tokası peptitlerinin geometrisine gelebilen ve metal iyonuna bağlanabilen yapısıyla biyolojik sistemlerde metal iyonlarının taşınmasına olanak sağlayabilecektir.

Anahtar Kelimeler: Bipiridin,bis-triazol, metale bağlanabilen amino asitler

to my beloved family…

ACKNOWLEDGEMENTS

I am greatly appreciative to my thesis supervisor *Assist. Prof. Dr. Salih ÖZÇUBUKÇU* for his guidance and support throughout my study. Without his greatest patience and encouragement it was impossible to accomplish my experiments. Being one of his first students was a great chance and I am sure that his scientific and personal support will bridge over throughout my life.

I would like to express my appreciation to my lovely labmates; *Güzide AYKENT, Meniz TEZCAN, Melek PARLAK, Gökçil BİLİR, Ulvi Oral KARACA,* and for their kind friendship and motivations. They were all great at sharing their knowledge and experiences during my study.

I also would like to thank my friends *Esmeray ÜSTÜNYAĞIZ* and *Ezgi ŞAHİN*.

Merve ERGUN, Başak ÖZTÜRK, Seda OKUMUŞ, Ayşegül HİSAR, Selin ŞENCANLI, Feriye ŞENOL and *Tolga KAPTI* are gratefully acknowledged who helped me during my master degree. They were always with me with their profound knowledge and valuable friendship and I know it will never end.

I would like to thank specially my dear friend *Emre TÜRKSELÇİ*. He was always with me and ready to support me all the time and his existence in my life always makes me happy.

Last but definitely not the least I would like to express my deepest thanks to my family especially my mother *Nuray EŞAN*, my father *Akif EŞAN* and my sister *Özge EŞAN MOSER* for their supports, encouragements, patience and the most important their endless love throughout my whole life. It would have been impossible to complete this work without their love and support.

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LIST OF ABBREVIATIONS

ADMP : 2-azido-1,3-dimethylimidazolinium hexafluorophosphate

AIBN : 2,2′-Azobis(2-methylpropionitrile)

Bipy/ Bpy : Bipyridine

Boc : *tert*-butyloxycarbonyl

BPO : Benzoyl peroxide

DBU : 1,8-Diazabicyclo[5.4.0]undec-7-ene

DCM : Dichloromethane

DFT : Density Functional Theory

DIEA : Diisopropylethylamine

DMF : *N*,*N*-Dimethylformamide

DMSO : Dimethyl sulfoxide

EDC : 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

EPR : Electron Paramagnetic Resonance Spectroscopy

Et_2O : Diethyl ether

EtOAc : Ethyl acetate

EXAFS : Extended X-ray Absorption Fine Structure

Fmoc : Fluorenylmethyloxycarbonyl

Glu/ E : Glutamic acid

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

HEPES : *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid

Hex : Hexane

HOBT : Hydroxybenzotriazole

HPLC : High Performance Liquid Chromatography

LC-MS : Liquid Chromatography–Mass Spectrometry

LiHMDS : Lithium bis(trimethylsilyl)amide

Lys/ K : Lysine

***m*CPBA** : *meta*-chloroperbenzoic acid

NBS : *N*-Bromosuccinimide

NMR : Nuclear Magnetic Resonance Spectroscopy

Phe/ F : Phenylalanine

PTC : Phase- Transfer Catalyst

RP-HPLC : Reversed- Phase High Performance Liquid Chromatography

SPPS : Solid Phase Peptide Synthesis

TFA : Trifluoroacetic acid

TFE : 2,2,2-Trifluoroethanol

THF : Tetrahydrofuran

TIPS : Triisopropylsilane

TLC : Thin Layer Chromatography

TMS : Trimethylsilyl

TMSCl : Trimethylsilyl chloride

TsCl : *p*-Toluenesulfonyl chloride

TTr. CuCl : tris(1-benzyl-1*H*-1,2,3-triazol-4-yl)methanol-Cu(I)

UV-VIS : Ultraviolet–Visible Spectroscopy

XANES : X-ray Absorption Near Edge Structure

CHAPTER 1

INTRODUCTION

There is a huge interest in artificial amino acids and the synthesis of those especially that can bind metal ions due to their effect on the structure and the stability of the peptides or proteins which they incorporated into.[1,2]

1.1. Metal Binding Amino Acids

Metal ions are usually coordinated by nitrogen, oxygen or sulfur centers belonging to amino acids. These centers may be provided by main-chain amino and carbonyl groups. However generally metal binding is achieved by the amino acid side chains. Especially, carboxylate groups of aspartic and glutamic acid; hydroxyl groups of serine, threonine, tyrosine; the ring nitrogen atom of histidine and tryptophan enable to bind metals as donor groups. Other side chains that bind metals ions include carbonyl groups (less often amino groups) of asparagine and glutamine; thiol group of cysteine and thioether of methionine.[3] (Table 1)

Table 1. Metal binding sites of some natural amino acids

	Amino acid	**Metal binding site**
1.	Asparagine	Carbonyl group (less often amino group)
2.	Aspartic acid	Carboxylate group
3.	Cysteine	Thiol group
4.	Glutamic acid	Carboxylate group
5.	Glutamine	Carbonyl group (less often amino group)
6.	Histidine	Ring nitrogen atom
7.	Methionine	Thioether
8.	Serine	Hydroxyl group
9.	Threonine	Hydroxyl group
10.	Tryptophan	Ring nitrogen atom
11.	Tyrosine	Hydroxyl group

In detail, for example, L-histidine is an important amino acid which is responsible in the strong metal coordination in proteins.[4] It has three binding atoms which coordinate to metal ions; nitrogen of amine group, nitrogen at the imidazole ring and oxygen of carboxylate group.[5] (Figure 1)

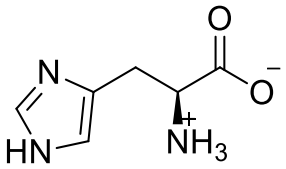


Figure 1. L-Histidine; tridentate ligand.

The configuration distributions of Zn(II)/His solution complexes at different pH values were investigated by Zhou and his co-workers[6] using solid-state NMR. Based on the solid state NMR experiments, they proposed Zn(II)/His binding models under different pH values and verified by DFT theoretical calculations (Figure 2).

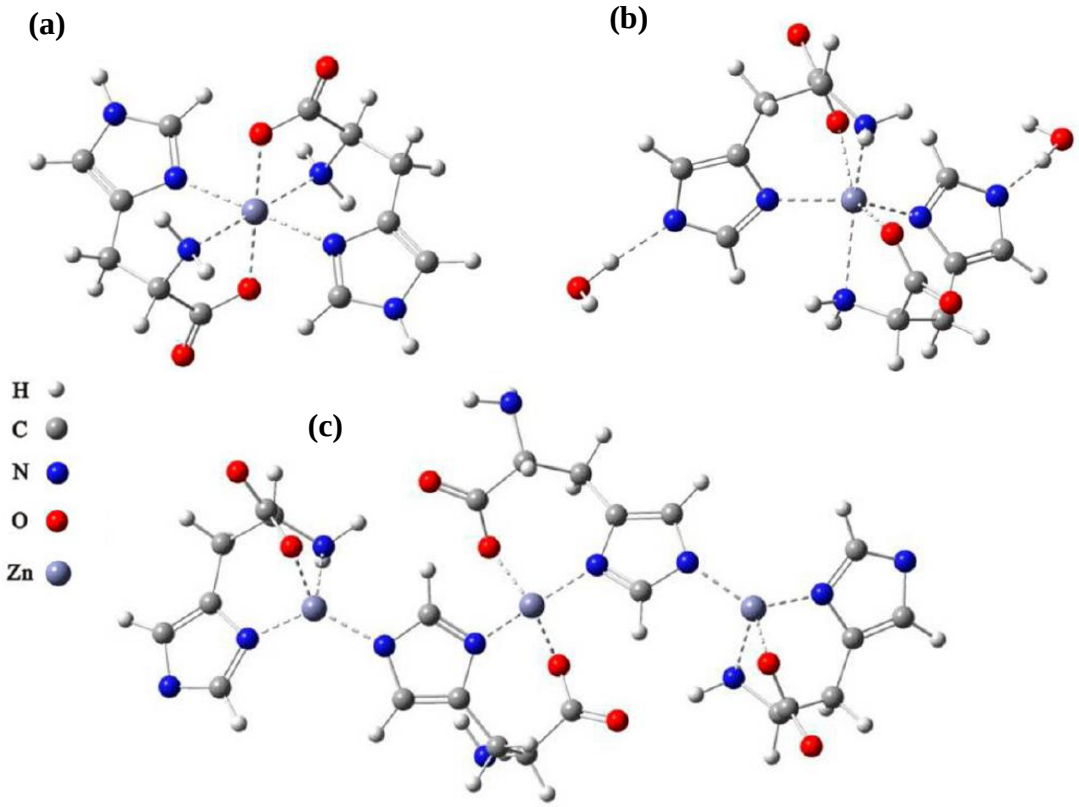


Figure 2. Optimized geometries of histidine−Zn(II) complexes at different pH values: (a) 7.5, (b) 11 and (c) 14.

The solution complexes configuration of different divalent transition metal ions with L-Histidine were studied by Meijuan Yu and his co-workers.[5] In that study they showed that although all metal-histidine complexes have distorted octahedral geometries at pH 6.0 with a molar ratio 1:2, the complexes of histidine with Cu(II) and Zn(II) have more disordered configurations than complexes with Mn(II), Co(II) and Ni(II) based on the extended X-ray absorption fine structure (EXAFS) and X-ray absorption near edge structure (XANES) results (Figure 3). Moreover it is indicated that the bond distances between histidine and the metals ions demonstrate the relative stabilities of complexes and the order follows the Irving–Williams series which states that the stability of the high-spin complexes formed by the divalent ions of first row transition metals follows the order $Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$.

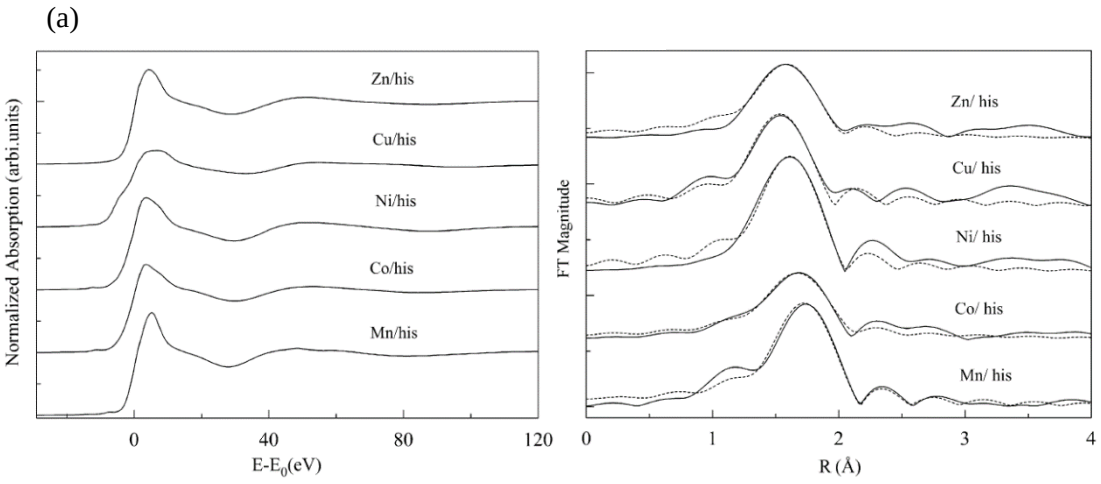


Figure 3. (a) Comparison of XANES spectra at the K edges of Zn, Cu, Ni, Co and Mn of the five Metal/His solutions. **(b)** EXAFS fit results of the Fourier Transform at the K edges of Zn, Cu, Ni, Co and Mn in the five Metal/His solutions.[5]

1.2. Metal Binding Peptides

Peptides can efficiently and specifically bind many metal ions. Furthermore having different possible metal binding sites in the sequence of peptides allow to obtain complexes with great variety of conformations by altering energetic and steric constraints.[7]

Copper complex of a glycine-histidine-lysine (GHK-Cu) is the most famous naturally occurring tripeptide-metal complex due to its anti-aging activity.[8]

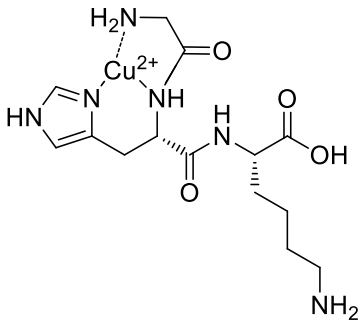


Figure 4. Copper complex of (S,Z)-6-amino-2-(2-(2-aminoacetamido)-3-(1H-imidazol-4-yl)acrylamido)hexanoic acid (GHK-Cu)

The metal binding tripeptide glycyl-L-histidyl-L-lysine (GHK) which has a high affinity for copper ion isolated from human plasma albumin in 1973 by Loren Pickart.[9] After its discovery, using different spectroscopic methods such as X-ray crystallography, EPR spectroscopy, X-ray absorption spectroscopy, and NMR spectroscopy, the molecular structure of the GHK-Cu complex was tried to figure out. In the Cu(II) complex of GHK, the copper ion is coordinated by three different nitrogen atom: form the imidazole of histidine, from the α-amino group of glycine, and the amide nitrogen of the glycine-histidine peptide bond (Figure 4).[8]

Afterwards the antioxidant, anti-inflammatory, regenerative, and wound healing effect of GHK-Cu complex has been demonstrated.[10]

Furthermore, due to its cell growth effect, it was suggested that the GHK plays a role in delivering copper into the cell which required for the cellular functions in the non-toxic form.[9]

1.3. Metal Binding Amino acids Derivative

Peptides which including metal binding sites in their side chain and peptide conjugates of metals have been extensively used in order to enhance binding affinities of metals and to control conformation of peptide sequences.[11]

Dirscherl et al.[11] have synthesized of Fmoc protected and modified amino acid chelates which can be used in solid-phase peptide synthesis and their metal complexes. For that purpose, as it is seen from Figure 5, they synthesized of Fmoc protected iminodiacetic acid amino acid using Fmoc-Lys and converted into its copper complex using $CuCl_2$ and base

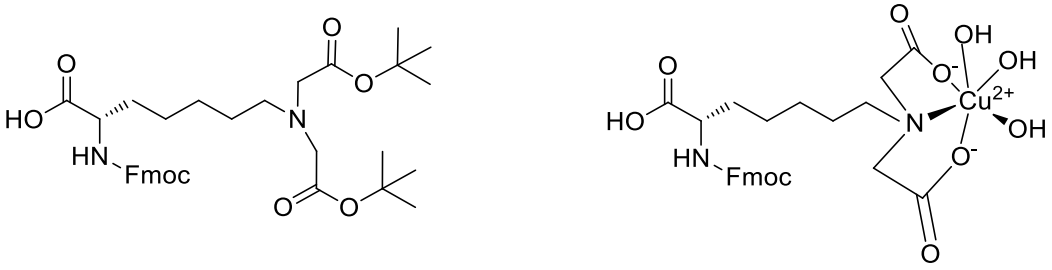


Figure 5. Fmoc protected iminodiacetic acid amino acid and its copper(II) complex.

Furthermore these group synthesized Fmoc protected dipyridylmethyl amine amino acid again using Fmoc-Lys (Figure 6). After that they obtained its metals complexes with Zn^{2+}, Cu^{2+}, and Ni^{2+} in a water/methanol solution.

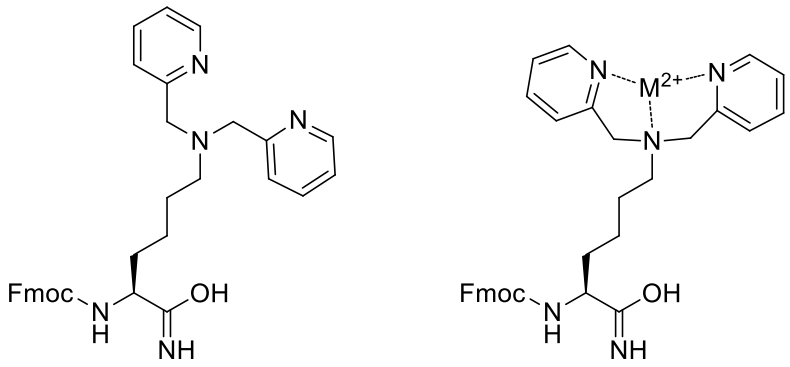
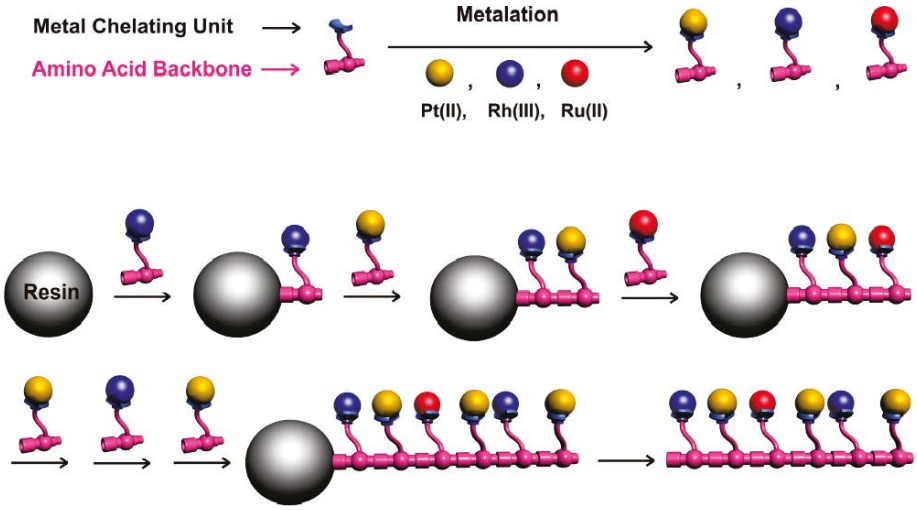


Figure 6. Fmoc protected dipyridylmethyl amine amino acid and its metal complex.

Another study with artificial amino acid derivative and its metal complexes was done by Vairaprakash and his co-workers.[12] They synthesized metalloamino acid derivatives and as shown in Scheme 1. They used these complexes to obtain peptides by SPPS.



Scheme 1. Synthesizing peptides using metalloamino acid derivatives by SPSS

In order to allow this amino acid derivative to bind a metal ion, they functionalized tyrosine by using terpyridine as a multidentate ligand and performed metalation step.

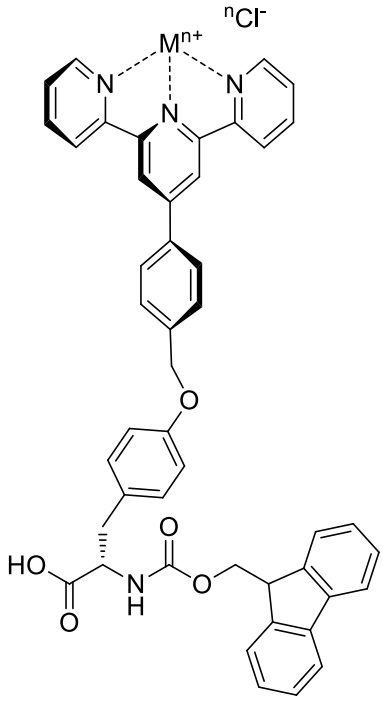


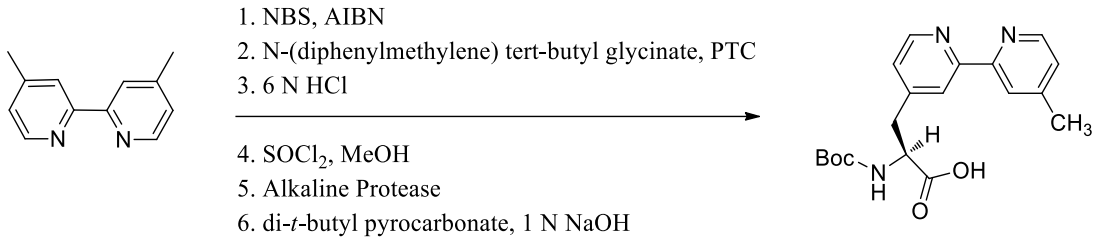
Figure 7. Fmoc protected tyrosine terpyridine metal complex

1.4. Bipyridine as Metal Ligand

Metal-binding amino acids include bipyridine as side chain because of their high metal binding affinity.[13]

2,2'-Bipyridine (bipy or bpy) is a bidentate chelating ligand, forming complexes with many transition metals.[14] Since its discovery at the end of nineteenth century[15], 2,2'-Bipyridine and its C-substituted derivatives have been used extensively as a metal binding ligand and functional systems due to its strong oxidation-reduction stability and ease of functionalization.[16]

The study of Bowler and Kise (1998) is an example of synthesis of an artificial bipyridyl amino acid.[17] They synthesized bipyridyl amino acid derivative starting with 4,4'-dimethyl-2,2'-bipyridine.(Scheme 2)



Scheme 2. The synthesis of a bipyridyl amino acid, 2-amino-3-(4'-methyl-2,2'-bipyridin-4-yl) propanoic acid

Futhermore, they incorporated this amino acid derivative into a 22 amino acid peptide sequence composed of alanine, histidine and lysine by Boc-SPPS method. Their peptide, with the sequence Acetyl–AKAAAAKAAAABpyAAAAHAAAHA–NH_2 The secondary structure of this peptide was characterized by circular dichroism (CD) spectropolarimetry and found an α-helix form in water.

These researchers report another study[13] that contains synthesis of metalloamino acid which is seen in Figure 8. In order to synthesizethis metalloamino acid, they used Boc-protected 2-amino-3-(4'-methyl-2,2'-bipyridin-4-yl) propanoic acid with *cis*-dichlorobis(2,2'-bipyridine)-ruthenium(II). They reported that the reason of using protected amino acid was being unsuccessful with the unprotected amino acid due to interference from the *R*-amino group.

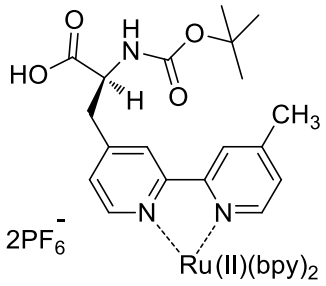
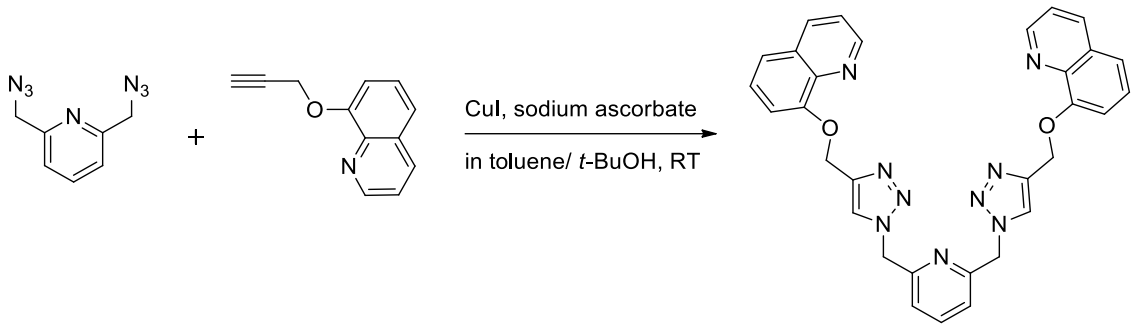


Figure 8. The metalloamino; produced by bipyridyl amino acid with cis-dichlorobis(2,2'-bipyridine)-ruthenium(II).

1.5. Triazole as Metal Ligand

Triazoles are very interesting ligands because they have the coordination geometries of both pyrazoles and imidazoles as to the arrangement of their three heteroatoms.[18] Pyridine-1,2,3-triazole ligands have been used as chelating agent recently since they ara easily available through copper catalyzed azide-alkyne cycloaddition (CuAAC) reaction, "*click*" chemistry strategy. This strategy facilitates the formation of 1,2,3-triazoles as 1,4-substituted isomers.[19] These multidentate ligands containing one pyridine and two triazole groups are strong coordination ligands to metal centers.[20] Therefore different derivatives such as pyridine-mono(triazoles), picolyl-mono(triazoles), pyridine-thiomethyl-mono(triazoles), pyridine-bis(triazoles), and lutidyl-bis(triazoles) have been synthesized and studied complexes with various transition metals; Cu(II), Ag(I), Re(I), Pd(II), Pt(II), Ru(II), Zn(II), Fe(II), Ir(III), Ni(II), or Co(II).[21]

The study of Narayanaswamy and his co-workers[22] is a great example for application of metal-binding pyridine-constrained traizole ligand as chemosensor for Zn^{2+}. Firstly, they have synthesized pyridine-constrained traizole-linked hydroxyquinoline ligand using click chemistry (Scheme 3).



Scheme 3. Synthesis of pyridine-constrained traizole-linked hydroxyquinoline ligand.

They studied the photophysical properties of this ligand in mixed aqueous medium (HEPES:CH_3CN, 1:9, pH 7.2) upon addition of various metal ions (Figure 9).

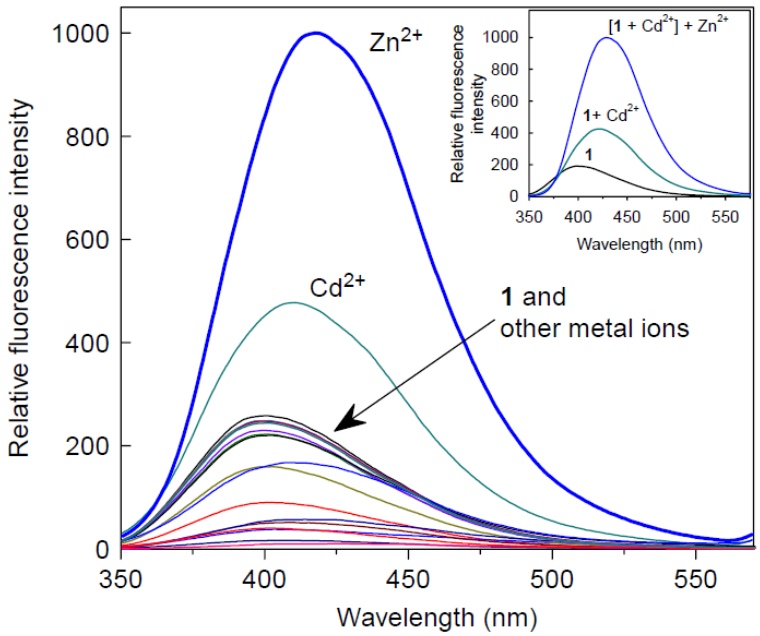
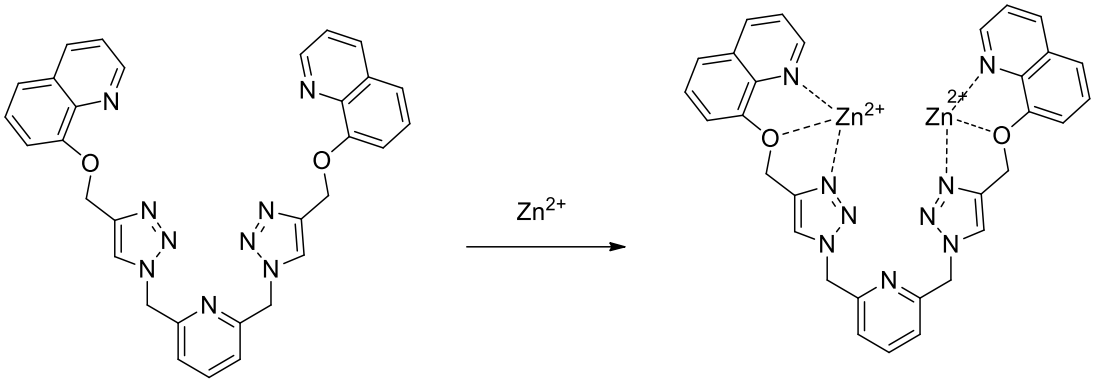


Figure 9. Fluorescence spectra of free ligand (20mM) and with added metal ions (25 equiv) Li^+, Na^+, Mg^{2+}, Sr^{2+}, Al^{3+}, In^{3+}, Hg^{2+}, Pb^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, Ag^+, Fe^{2+} and Fe^{3+}

According to their study this ligand exhibits a weak fluorescence emission band at 401 nm. In presence of Li^{+}, Na^{+}, Mg^{2+}, Sr^{2+}, Al^{3+}, In^{3+}, Hg^{2+}, Pb^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, Ag^{+}, Fe^{2+} and Fe^{3+} ions, the ligand showed nearly no change in the emission intensity although as it is seen in Figure 9 Zn^{2+} exhibited enhancement in the fluorescence emission.



Scheme 4. Binding mode of ligand-Zn^{2+} complex

1.6. Aim of the Study

The main objective of this project is to obtain metal binding artificial amino acid derivatives and to incorporate these artificial amino acids into pre-designed peptide sequence. For this purpose, initially a bipyridine and pyridine bistriazole amino acid derivatives that can coordinate to a wide variety of metal ions are tried to be synthesized using Stille coupling reactions and Huisgen 'Click Chemistry', respectively. Also with proper position of functional groups of bipyridine and pyridine bistriazole, peptides are going to be able to form β-hairpin structure. For the synthesis of peptides, certain natural amino acids such as lysine (K), phenylalanine (F) and glutamic acid (E) are preferred. Moreover, these peptides having the metal coordination part in the middle of the sequence can be used for transportation of metal ions and releasing in biological systems.

CHAPTER 2

RESULTS AND DISCUSSION

2.1. Bipyridine Metal Complexes

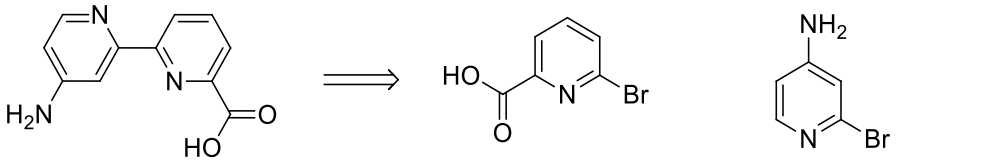
For several transition metal ions, nitrogen heterocycles act as very efficient and stable complexation agent.

Bipyridines are aromatic N-heterocycles which are formed by the coupling of two pyridine rings. They are bidentate ligands that coordinate to a wide variety of metal ions such as cobalt(II), copper(II), nickel(II), and ruthenium(II), by forming complexes.[23]

2.1.1. Synthesis of Bipyridine Amino Acid Derivatives

2.1.1.1. 4'-amino-[2,2'-bipyridine]-6-carboxylic acid (1)

In order to synthesize the bipyridine amino acid **1** that can coordinate to a metal ion, pyridine derivatives; 2-bromopyridine-4-amine **(2)** and 6-bromopicolinic acid **(3)** were synthesized for coupling reaction.



4'-amino-[2,2'-bipyridine]-6-carboxylic acid — 6-bromopicolinic acid — 2-bromopyridin-4-amine

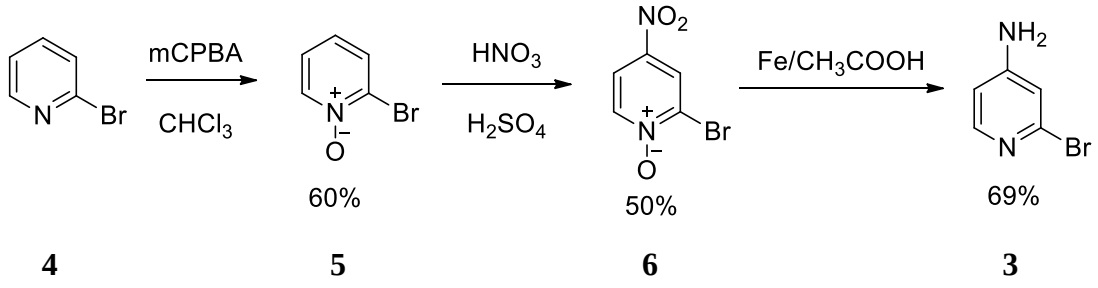
1 **2** **3**

Scheme 5. Retro synthesis of 4'-amino-[2,2'-bipyridine]-6-carboxylic acid**.**

Firstly, 2-bromopyridine **(4)** was converted to its *N*-oxide by oxidation with *m*-chloroperbenzoic acid, since pyridine *N*-oxides can be nitrated in moderate yield (60%) at the 4-position due to the electron donating effect of itself.

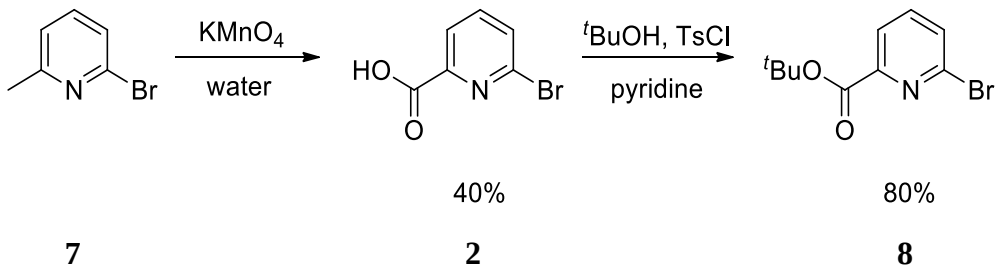
Nitration step of 2-bromopyridine *N*-oxide **(5)** was done using fuming HNO_3 and conc. H_2SO_4 as the nitrating agent at 100 °C with a yield of 50%. Then reduction of nitro group and *N*-oxide was performed with iron power under ultrasonication to give amino derivative **3** in 69% yield.

The resulting products were characterized by ^{1}H-NMR spectroscopy.

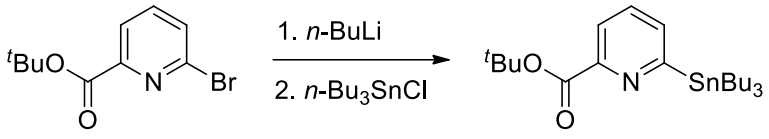


Scheme 6. Synthesis of 2-bromopyridine-4-amine starting with 2-bromopyridine.

On the other hand in order to synthesize the carboxylic acid part of the bipyridine amino acid **1**; 6-bromopicolinic acid **(2)** was prepared in one step by oxidation of the 2-bromo-6-methylpyridine **(7)** with $KMnO_4$ in water in 40% yield. After that 6-bromopicolinic acid was protected as *tert*-butyl ester **8** using tosyl chloride and *tert*-butanol in the presence of pyridine with a yield of 80%. Bromo group of **8** was first lithiated by *n*-BuLi in THF and then converted into tributyltin derivative **10** using *n*-Bu_3SnCl (55%).



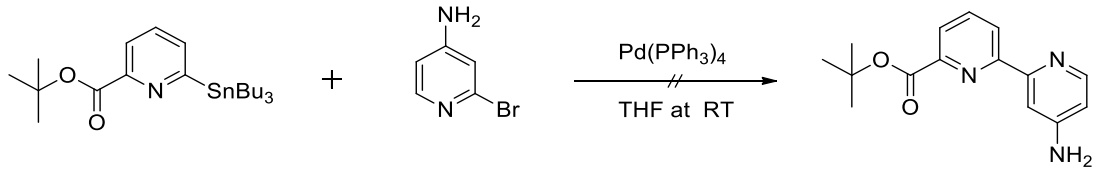
Scheme 7. Oxidation and esterification reactions of 2-bromo-6-methylpyridine, respectively**.**



8 **9**

Scheme 8. Lithiation of 8 and subsequent stannylation.

Tert-butyl ester **9** was coupled with bromopyridine **3** under Stille coupling conditions using tetrakis(triphenylphosphine)palladium(0) as catalyst in THF at room temperature under argon. Unfortunately, no product was obtained, only starting materials were recovered at the end of the reaction after 48 hours (Scheme 9).

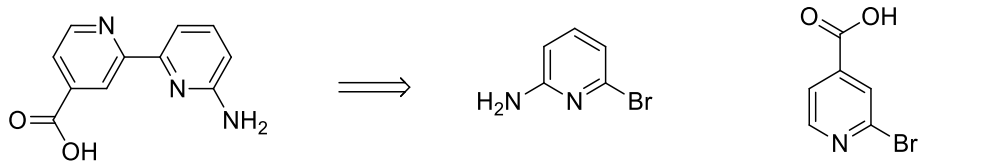


9 **3** **1**

Scheme 9. Stille coupling reaction to obtain amino acid derivative **1**.

2.1.1.2. 6'-amino-[2,2'-bipyridine]-4-carboxylic acid (10)

To synthesize another bipyridine amino acid **10** that can coordinate to a metal ion, pyridine derivatives; 6-bromopyridine-2-amine **(11)** and 2-bromoisonicotinic acid **(12)** was synthesized to use in coupling reaction.

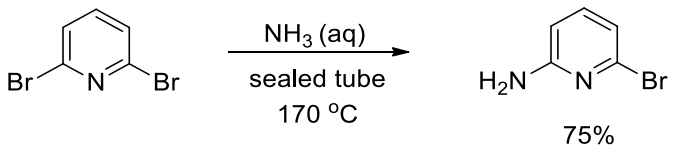


6'-amino-[2,2'-bipyridine]-4-carboxylic acid | 6-bromopyridin-2-amine | 2-bromoisonicotinic acid

10 **11** **12**

Scheme 10. Retro synthesis of 6'-amino-[2,2'-bipyridine]-4-carboxylic acid.

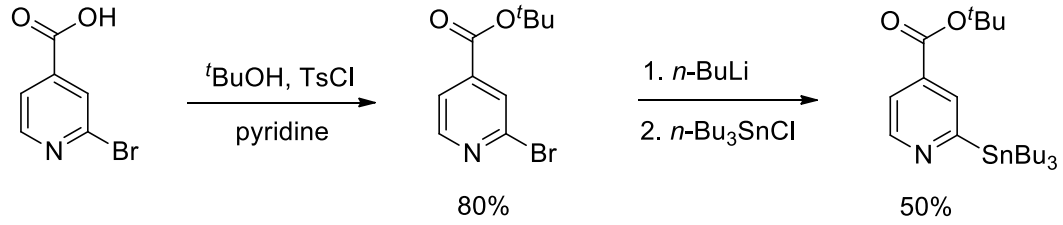
Monoamination reaction of 2,6-dibromopyridine with ammonia in sealed tube at 170 °C gave 6-bromo-2-aminopyridine in good yield (75%) (Scheme 11) which would undergo Stille coupling reaction with *tert*-butyl protected 2-bromoisonicotinic acid derivative **15**.



13 **11**

Scheme 11. Monoamination reaction of 2,6-dibromopyridine.

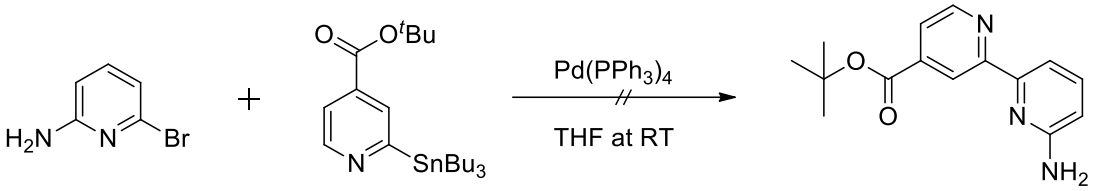
To perform Stille coupling reaction, *tert*-butyl protected 2-bromoisonicotinic acid **(14)** was reacted first with *n*-butyl lithium then tributyltin chloride in THF to get tin derivative **15** with a yield of 50%.



12 **14** **15**

Scheme 12. *Tert*-butyl esterification then lithiation of **14** and subsequent stannylation.

Unexpectedly, coupling reaction of **11** and **15** failed. The synthesis of the desired bipyrine derivative; 6'-amino-[2,2'-bipyridine]-4-carboxylic acid was not achieved using Stille coupling conditions (Scheme 13).

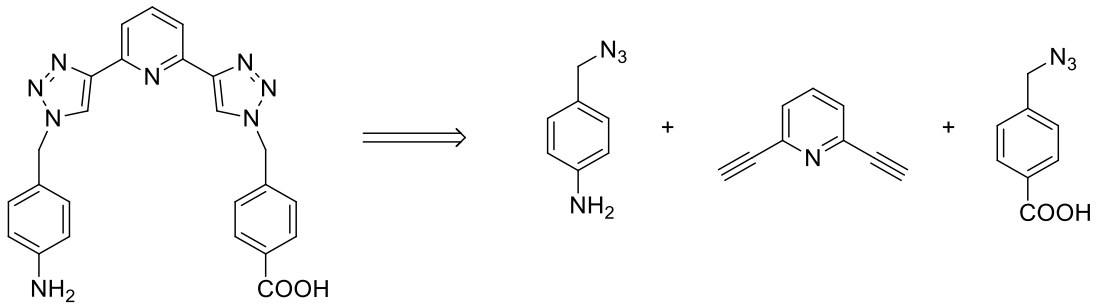


11 **15** **10**

Scheme 13. Stille coupling reaction of amino acid derivative **10**

2.2. Metal Binding Amino Acids Derivative Containing Pyridine Bistriazole Moiety

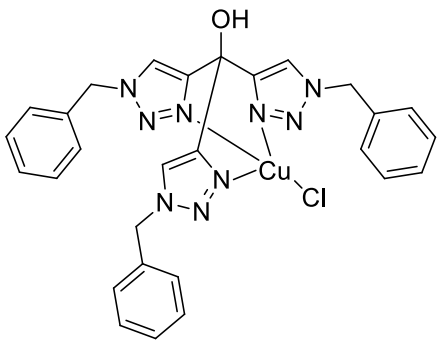
After having trouble with coupling reactions to get bipyridine derivatives, Huisgen 1,3-dipolar cycloaddition was thought to be a better way to construct metal binding units since triazoles are also good metal ligands. The triazoles resulting from the "click reaction" of azides and alkynes are potential ligand for the metal center.[21]



16 **17** **18** **19**

Scheme 14. Retro synthesis of 4-((4-(6-(1-(4-aminobenzyl)-1H-1,2,3-triazol-4-yl)pyridin-2-yl)-1H-1,2,3-triazol-1-yl)methyl)benzoic acid **(16)**.

For Huisgen 1,3-dipolar cycloaddition reaction, so-called '*click reaction*' a new type of catalyst was used: CuCl complex of tris(1-benzyl-1*H*-1,2,3-triazol-4-yl)methanol (TTr.CuCl) catalyzes the click reaction in water which provides lower catalyst loading and short reaction times at room temperature.[24]

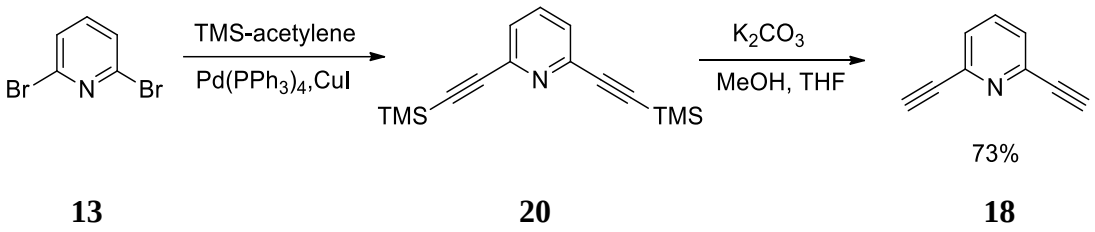


TTr.CuCl

Scheme 15. Structure of *click* catalyst:
CuCl complex of tris(1-benzyl-1*H*-1,2,3-triazol-4-yl)methanol.

2.2.1. Synthesis of Amino Acid Derivative

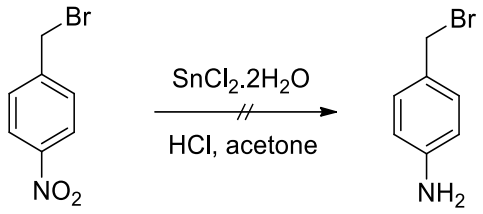
Firstly 2,6-dibromo pyridine was converted into 2,6-bis((trimethylsilyl)ethynyl)pyridine **(20)** using trimethylsilyacetylene with cross-coupling reaction in the presence of both palladium and copper catalysis. Then TMS group of alkyne was cleaved by K_2CO_3 in methanol to give **18** with a total yield of 73%.



13 **20** **18**

Scheme 16. Sonogashira cross coupling reaction of 2,6-dibromo pyridine with TMS-acetylene.

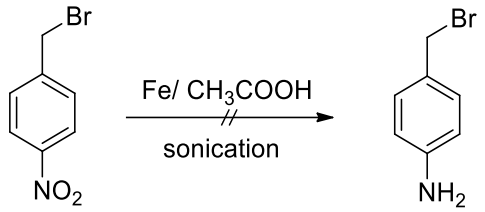
For the amino part of the artificial amino acid **16**, 1-(bromomethyl)-4-nitrobenzene was used as a starting material. Reduction of nitro group was tried with a mixture of stannous chloride and conc. hydrochloric acid at 50 °C. Unfortunately, no product was obtained.



21 **22**

Scheme 17. Reduction of nitro group to corresponding aniline derivative.

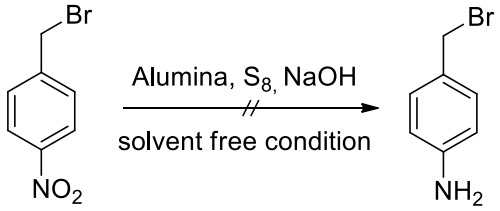
After that another method was tried for reduction reaction of **21**. Iron powder in acetic acid under sonication was treated to **21** as used in reduction of **6**. Based on the ^{1}H-NMR spectra, the synthesis of the desired product **22** was not achieved.



21 **22**

Scheme 18. Nitro reduction with iron powder in acidic media under sonication.

According to the literature[25] it was possible that aromatic nitro compounds could be reduced to the corresponding anilines through sulfur with alumina supported sodium hydroxide under solvent-free conditions. This reaction procedure was performed, but desired product was not obtained.

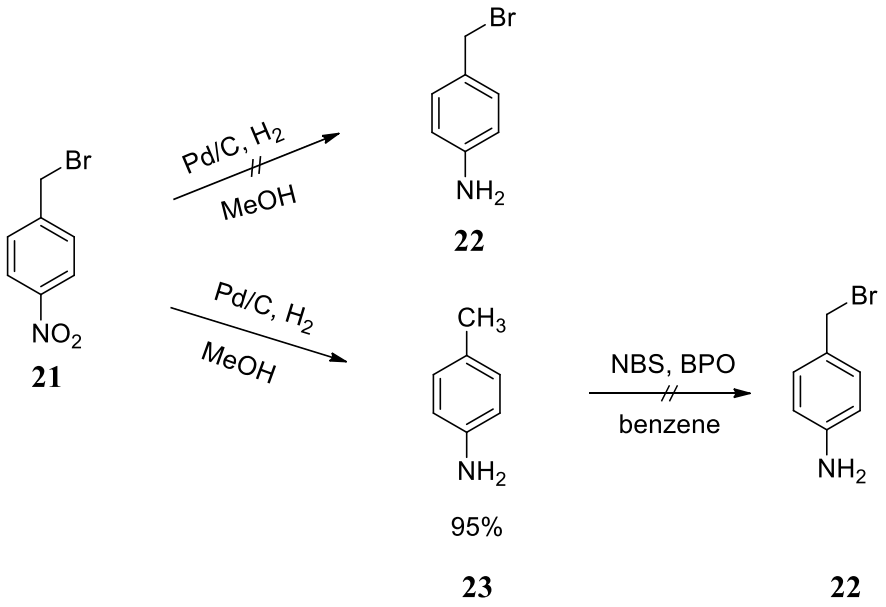


21 **22**

Scheme 19. Reduction of nitro group with sulfur in basic media under solvent-free conditions.

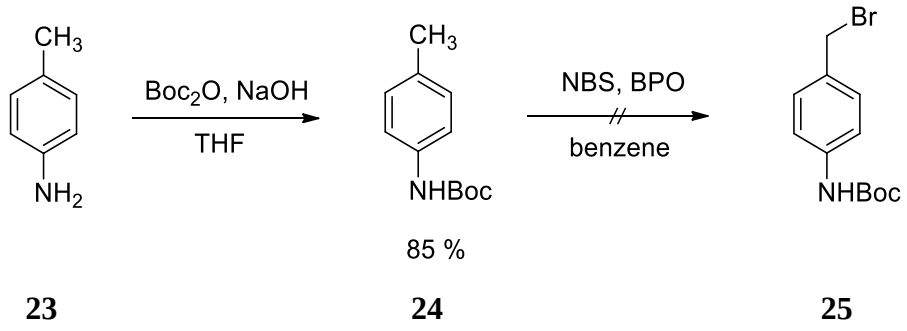
The other method was hydrogenation via palladium charcoal under an atmosphere of H_2 to reduce nitro group. Although the reduction of nitro was achieved, based on the ^{1}H-NMR results it was not the desired product but *p*-toluidine which was formed by the reduction of bromide group. Different H_2 pressures (3, 4 and 5 atm) and temperature (25, 30 and 40 °C) were applied but the product was always the same: *p*-toluidine with a yield of 95%.

Furthermore, *p*-toluidine was treated with NBS in the presence of benzoyl peroxide (BPO) in benzene under reflux temperature. Nevertheless benzyl bromide **22** could not be obtained.



Scheme 20. Scheme of hydrogenation and bromination**.**

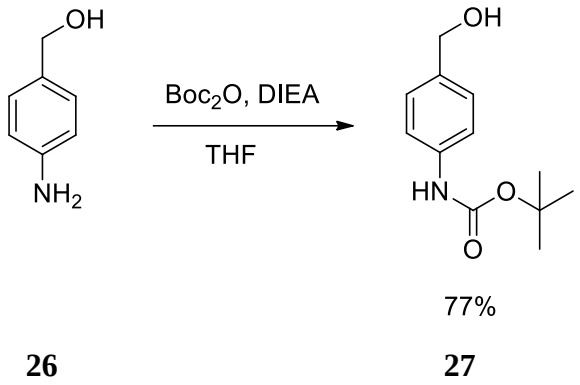
It was thought that protection of amino group of **23** was necessary to perform bromination step, so *p*-toluidine was treated with di-*tert*-butyl dicarbonate (Boc_2O) in basic medium to give **24** with 85% yield. When bromination step was performed with NBS after protection of aniline, the reaction was failed again.



Scheme 21. Amine protection with Boc_2O and bromination step.

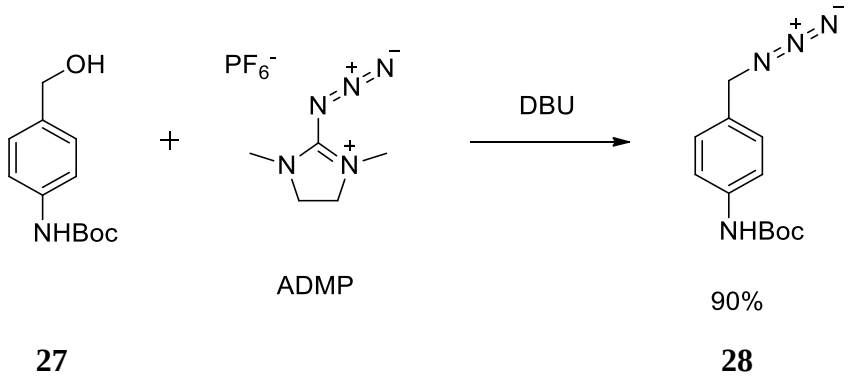
After performing many failing experiments for the amino part of the artificial amino acid **16**, (4-aminophenyl)methanol was used as a starting material. It was started with protection step of amino group. For that reason di-*tert*-butyl dicarbonate was added to the (4-aminophenyl)methanol in the presence of a base, DIEA in THF.to give **27** with a yield of 77 %

As a protecting group the *tert*-butyloxycarbonyl was chosen since it has stability in basic condition. This protection was important to prevent conversion of amine group to azide while preparing alkyl azide to use in Huisgen 1,3-dipolar cycloaddition reaction.



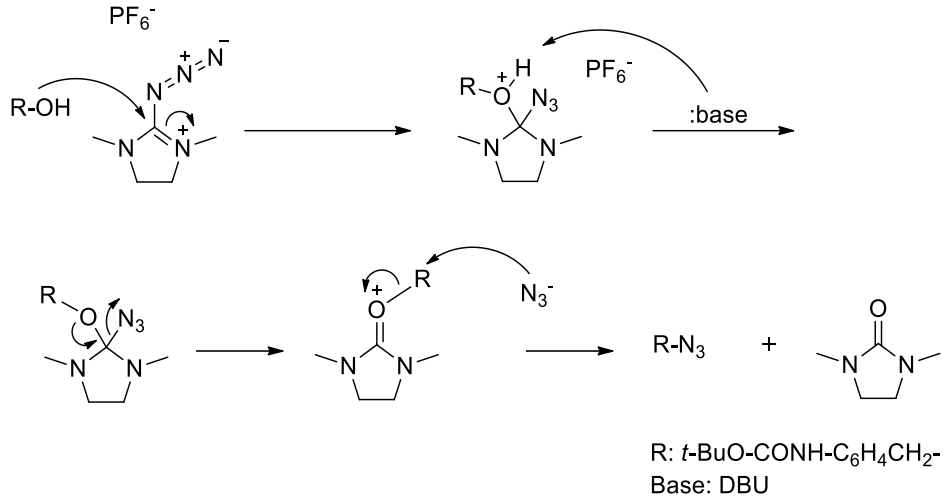
Scheme 22. Boc-protection of (4-aminophenyl)methanol.

After that 2-azido-1,3-dimethyl-imidazolinium hexafluorophosphate (ADMP) was used for the direct transformation of alcohol[26] **27** to corresponding azide in the presence of DBU to give **28** in 90% yield.



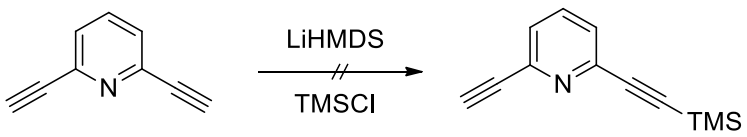
Scheme 23. The direct transformation of alcohol to azide with ADMP.

Proposed mechanism of the direct the direct transformation of alcohol to azide with ADMP is shown in Sheme 24.



Scheme 24. Proposed reaction mechanism of the direct transformation of alcohol to azide with ADMP.

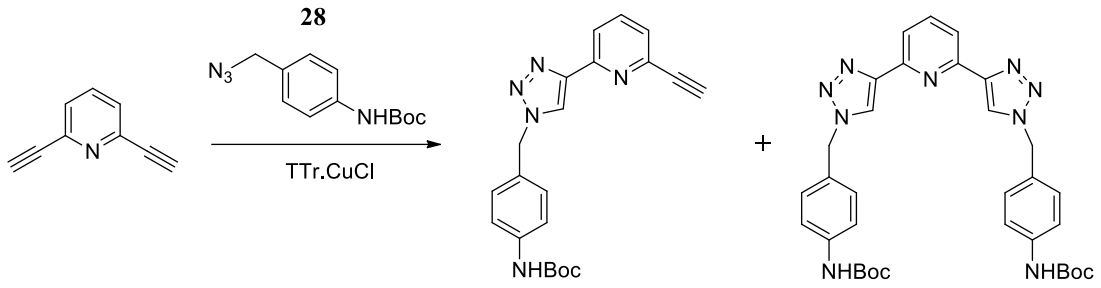
Synthesis of 2,6-diethynylpyridine **(18)** and *tert*-butyl (4-(azidomethyl)phenyl)carbamate **(28)** would be followed by 1,3-dipolar cycloaddition However to provide selectivity in cycloaddition reaction, firstly deprotonation of alkyne with one equivalent of lithium bis(trimethylsilyl)amide (LiHMDS) and then silylation with trimethylsilyl chloride (TMSCl) in THF was attempted but failed.



18 **29**

Scheme 25. Silylation of acetylene.

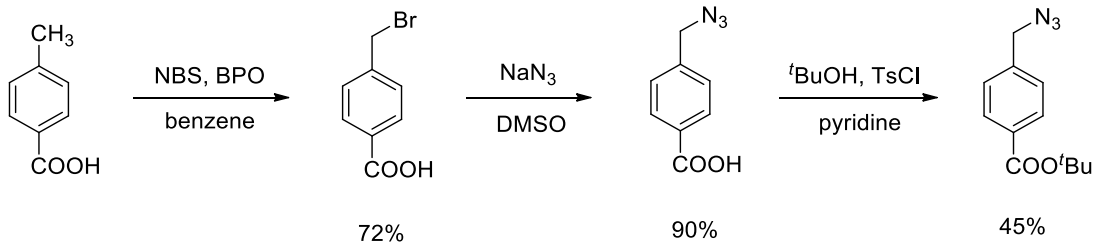
Alternatively, using 1 equivalent benzyl azide **28** and 2,6-diethynylpyridine **(18)**, Huisgen 1,3-dipolar cycloaddition was performed with TTr.CuCl catalyst in water at room temperature. Although 1 equivalent benzyl azide **28** was used, mono and di-product were obtained. These products were purified by column chromatography on silica gel.



18 **30** **31**

Scheme 26. Click reaction of 2,6-diethynylpyridine with (4-(azidomethyl)phenyl)carbamate.

For the carboxylic acid part of the artificial amino acid in order to use in 1,3-dipolar cycloaddition reaction, 4-methylbenzoic acid was treated with NBS and BPO to generate the benzyl bromide **33** with a yield of 72%. This radical substitution reaction was followed by nucleophilic substitution with 0.5 M NaN_3 in DMSO to obtain **34** in 90% yield.



72% 90% 45%

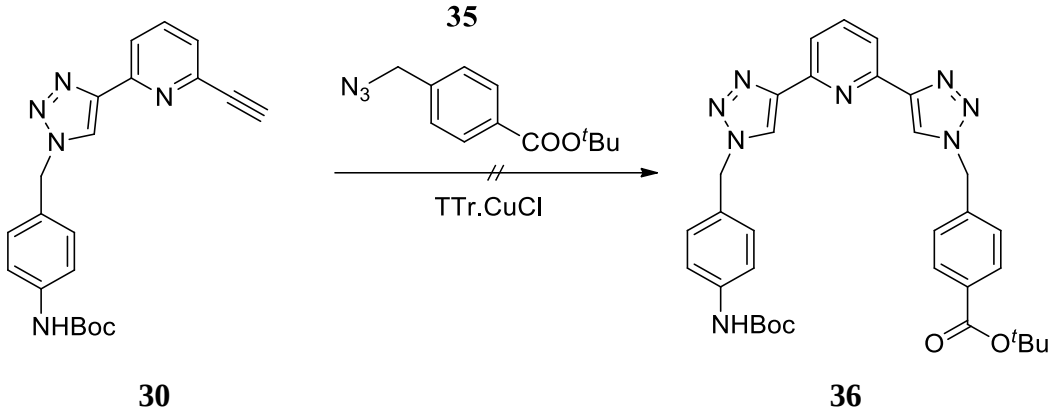
32 **33** **34** **35**

Scheme 27. Substitution reactions of 4-methylbenzoic acid and esterification step of carboxyl group.

1,3-dipolar cycloaddition reaction of 4-(azidomethyl)benzoic acid and mono-product **30** of first click reaction was performed using TTr.CuCl catalyst in water at room temperature. However on the basis of the NMR results, the synthesis of the desired product was not achieved.

In this case carboxylic acid was protected as *tert*-butyl ester using *tert*-butanol and tosyl chloride with 45% yield then click reaction was tried with **35** but the product was not obtained.

The alternative way to synthesize the target compound **16** was to perform Huisgen 1,3-dipolar cycloaddition reaction of 2,6-diethynylpyridine with firstly the carboxylic acid part then the amino part in order to obtain desired artificial amino acid: However 1,3-dipolar cycloaddition reaction of 2,6-diethynylpyridine **18** with *tert*-butyl 4-(azidomethyl)benzoate **35** was not achieved.

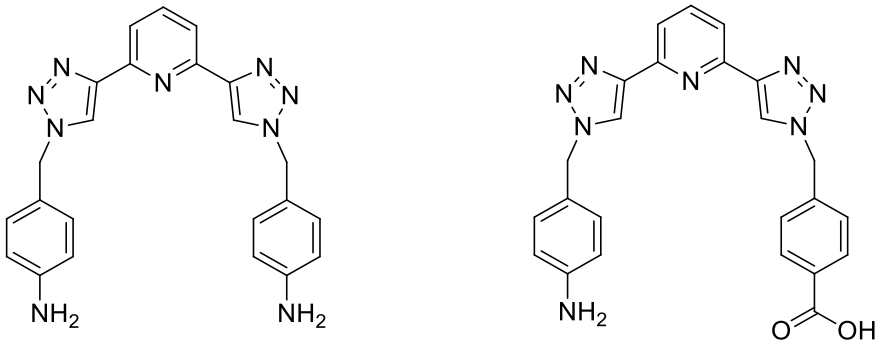


30 **36**

Scheme 28. The reaction scheme of Huisgen 1,3-dipolar cycloaddition.

2.2.2. Synthesis of Diamine Derivative

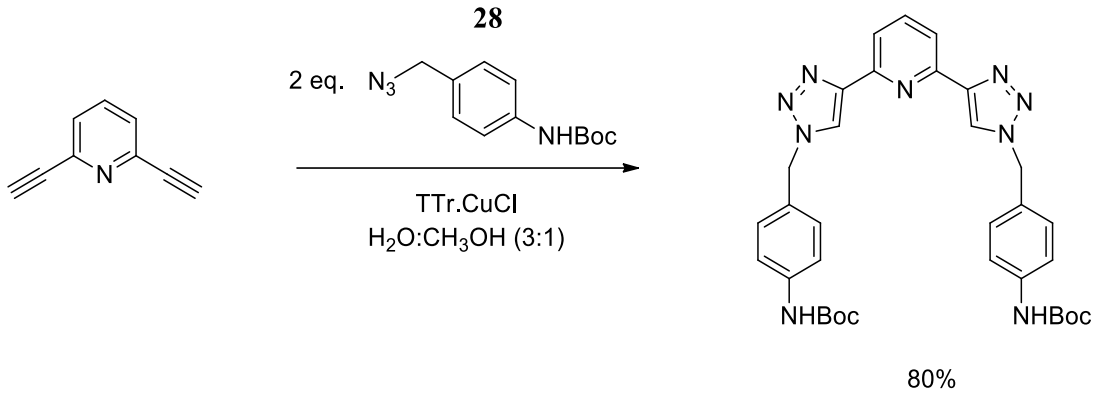
Since "click reaction" of *tert*-butyl 4-(azidomethyl)benzoate with pyridine derivative **30** which performed mono-cycloaddition with *tert*-butyl (4-(hydroxymethyl)phenyl)carbamate was not achieved in both two ways, the target compound was changed to diamine derivative which is also possible to incorporate into amino acid sequence.



37 **16**

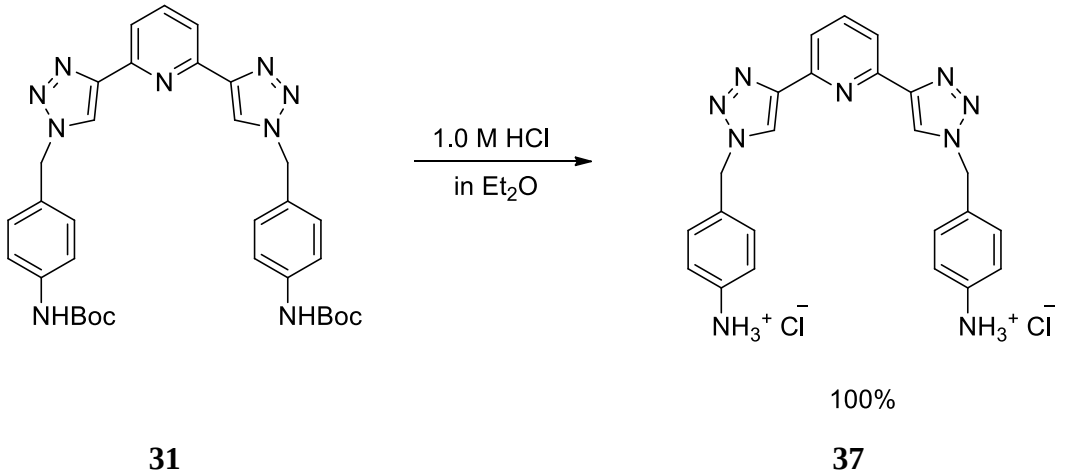
Figure 10. The molecular **s**tructures of bis-triazole containing diamine and amino acid derivative.

As shown in Scheme 25, diamine derivative was possible to get by click reaction, the same reaction was performed by 2 equivalents of azido **28** to synthesize only bis product with a yield of 80%. (Scheme 28)



Scheme 29. *Click* reaction of diamine 31.

In order to incorporate this diamine **31** into amino acid sequence, Boc protection group was cleaved. It was deprotected in acidic conditions by using 1.0 M HCl solution in diethyl ether at room temperature.



Scheme 30. Deprotection of Boc group of diamine derivative **31**.

Since our aim is to synthesize these derivatives to incorporate into peptide sequences to get beta-sheet structure, as it is shown in Figure 11 it is also possible to get beta sheet but in this way, it would be parallel instead of anti-parallel beta sheet structure.

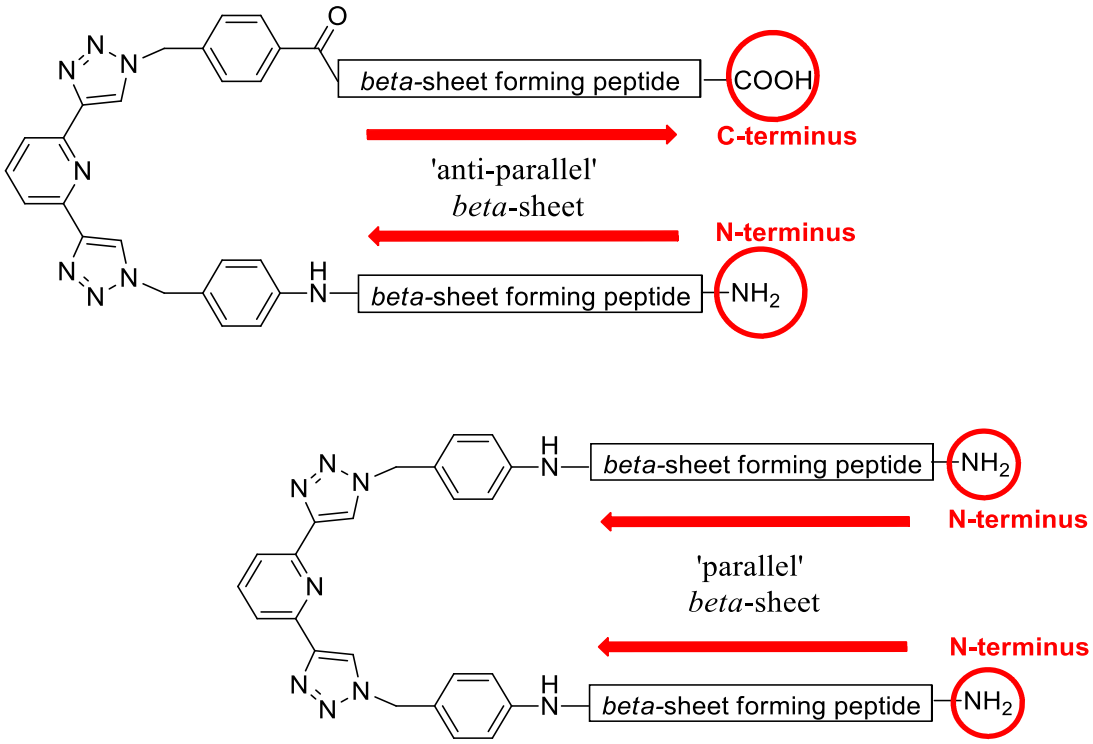


Figure 11. Schematic diagram of anti-parallel and parallel beta-sheet secondary structure of artificial amino acid containing peptides.

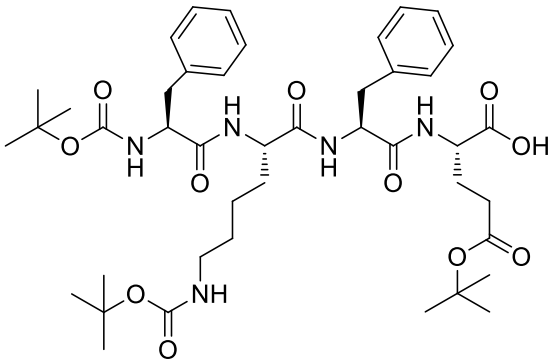
2.2.3 Synthesis of Peptides Having Diamine 37

To generate parallel beta-sheet forming peptide including triazoles as a metal binding units, diamine **37** was synthesized.

After that having hydrophilic glutamic acid (E), hydrophobic phenyl alanine (F) and lysine (K), tetra-peptide was designed with sequence of FKFE. This tetra-peptide was synthesized by Fmoc-SPPS method.

In order to couple this peptide with diamine **37** in solution phase, N-terminus of the peptide was protected with Boc protecting group. For this purpose Boc-Phe was used

while synthesizing the peptide by SPPS and the peptide was cleaved only from the resin without cleaving side chain protecting groups. For the cleavage of side chain protected peptide from the resin, TFE was used.



38

Figure 12. Side chain protected tetrapeptide (FKFE).

As it is seen in Figure 13, LC-MS analysis showed that side chain protected peptide was obtained successfully.

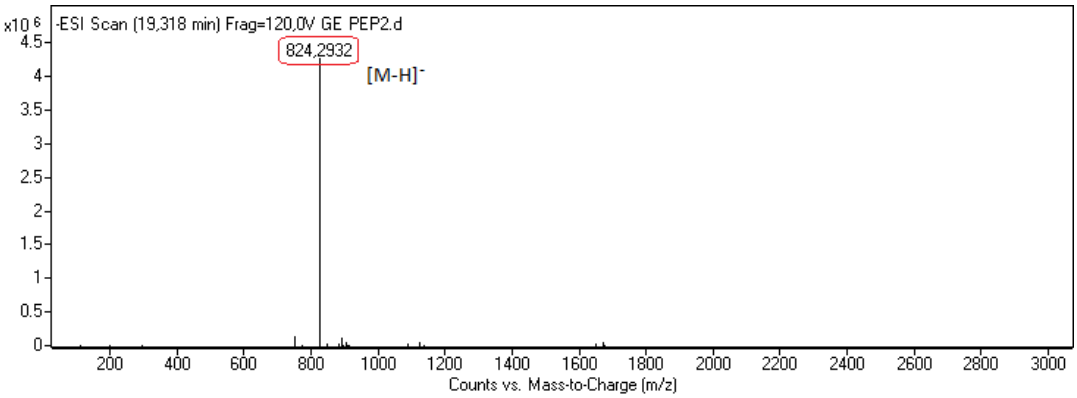
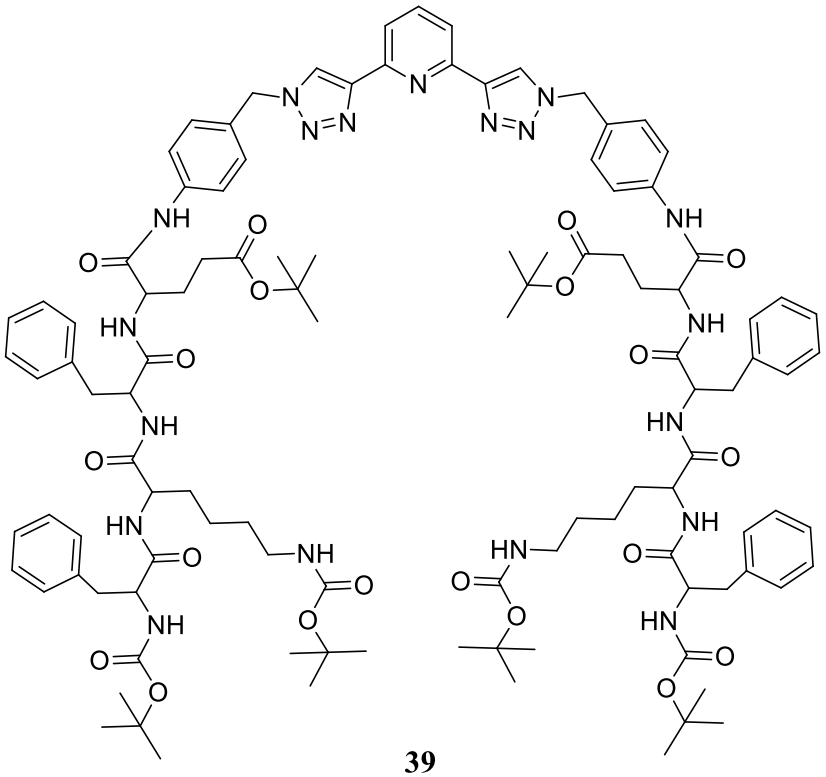


Figure 13. Mass spectrum of side chain protected tetrapeptide (FKFE) (38)

Coupling reaction of fully protected peptide and diamine **37** was performed in solution by using HOBt/ EDC methodology and DIEA at room temperature. The reaction was monitored by HPLC. The result of LC-MS analysis demonstrated that formation of new peptide bonds were achieved and mass of the desired peptide **39** was observed (Figure 15).



39

Figure 14. Structure of side chain protected artificial peptide **39**

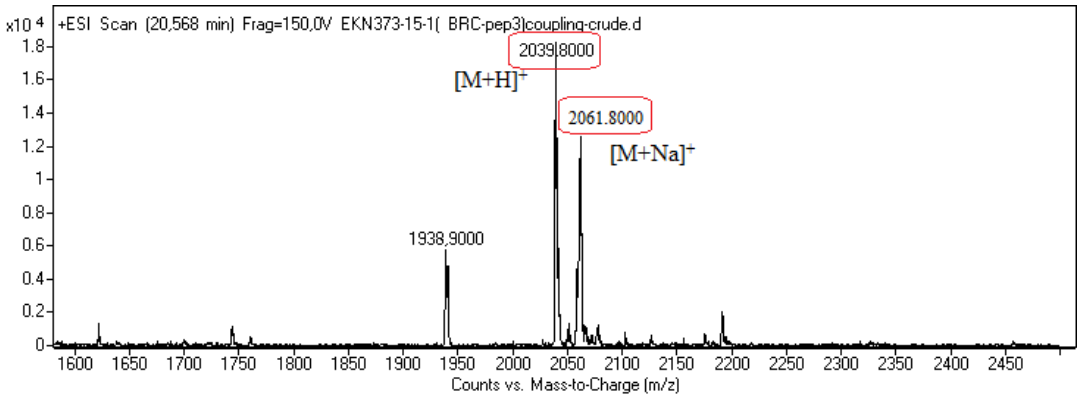
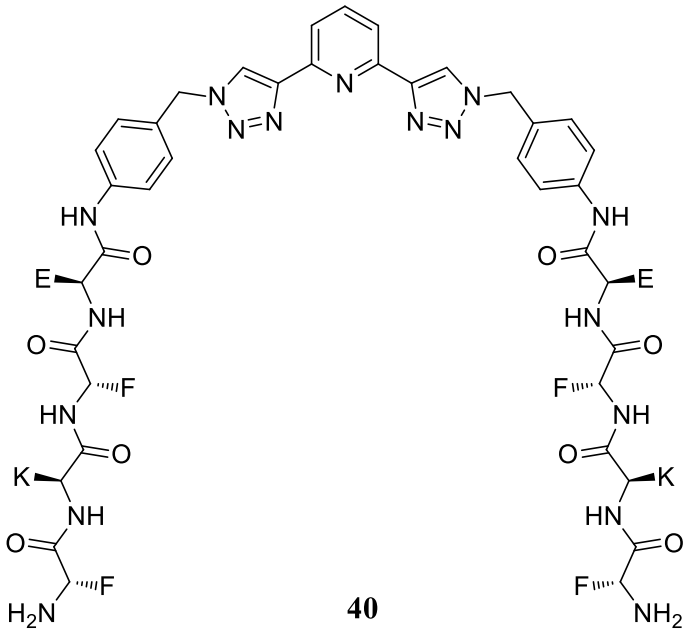


Figure 15. Mass spectrum of side chain protected artificial peptide (39)

Fully protected peptide **39** including pyridine bistriazole diamine in the middle of peptide sequence, was treated with TFA in DCM for deprotection of all the side chains. After deprotection peptide **40** was purified using RP-HPLC and as shown in Figure 17 the mass of desired artificial peptide **40** was observed according to LC-MS analysis.



40

Figure 16. Structure of artificial peptide **40** including pyridine bistriazole diamine derivative

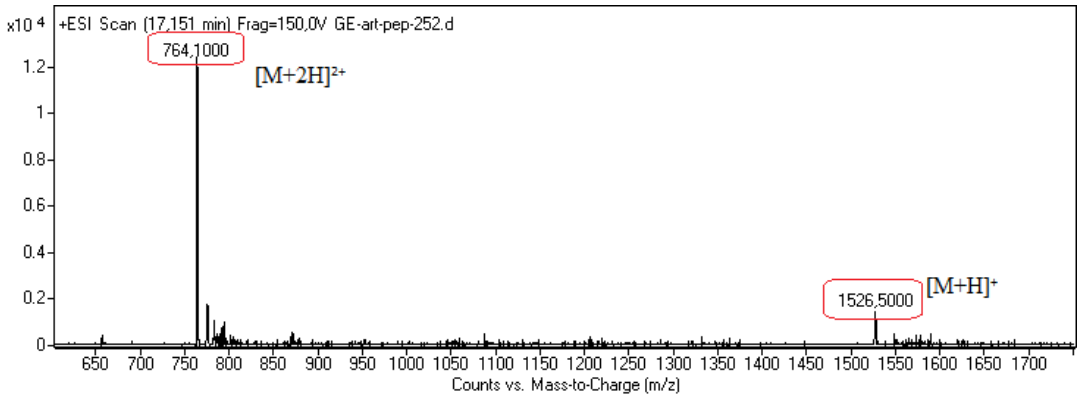
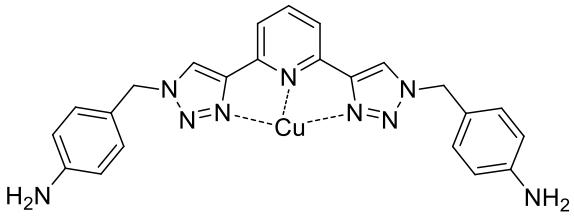


Figure 17. Mass spectrum of artificial peptide **(40)**

2.3. Metal Complex with Diamine 37

In order to show the binding affinity of diamine **37** to metal ions, copper (II) ion was chosen as a model metal ion.



41

Figure 18. Cu^{2+} complex of diamine **37**.

First of all, mass spectrum of diamine was measured in the presence and absence of copper ion. Figure 19 shows the mass spectrum of diamine **37**.

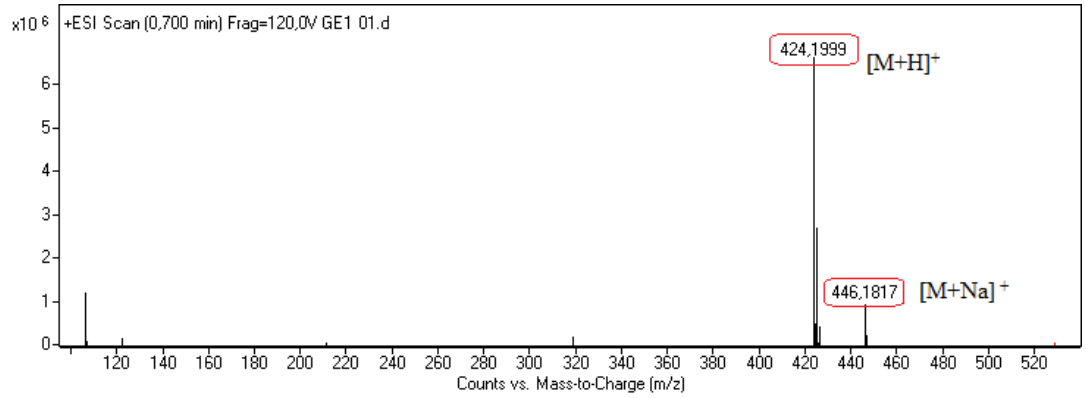


Figure 19. Mass spectrum of diamine derivative **37**.

In Figure 20, mass of diamine-Cu^{2+} complexes are seen as 1:1 and 1:2 stoichiometric ratio (Cu: diamine).

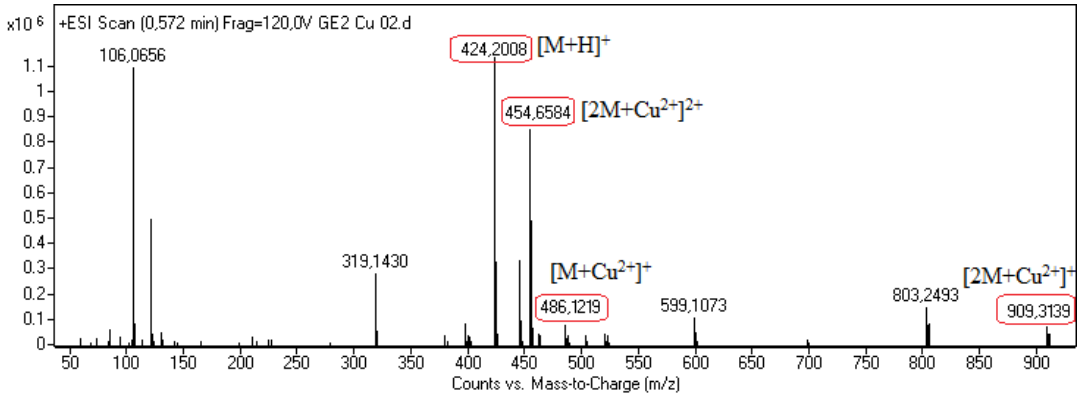


Figure 20. Mass spectrum of diamine- Cu^{2+}.complexes.

Moreover, binding affinity of copper to diamine was measured using UV-VIS spectrum titration. For this purpose, 0.1-0.5 µM diamine was titrated with 20-30 fold excess of copper (II) ions and the absorbance at 600 nm was plotted against the concentration of copper (II) ions. The graph was fitted to Hill Equation[27] to calculate the dissociation constant of copper diamine complex (Figure 21). K_d is found to be 1,22 x 10^{-6}.

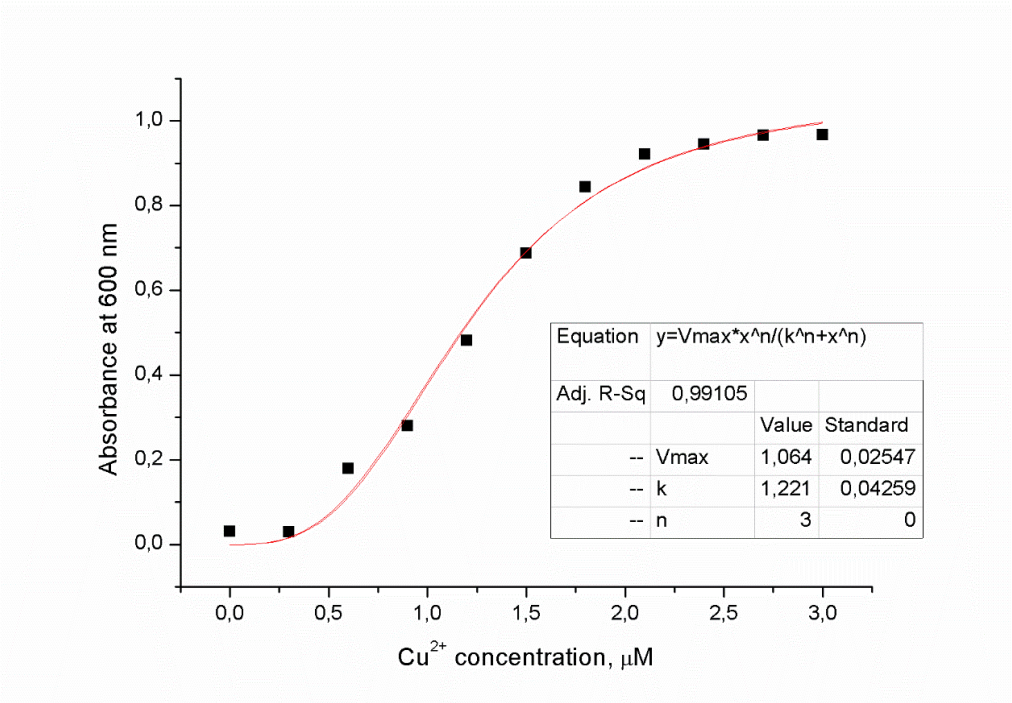


Figure 21. UV-Vis titration curve of diamine **37** with Cu^{2+}

CHAPTER 3

CONCLUSION

To conclude, in this study metal binding amino acids were tried to be synthesized. In order to do that firstly bipyridine derivative composed of amine and carboxylic acid functional groups were designed since bipyridine is known as a good bidentate ligand which can bind through nitrogen to wide variety of metal ions such as cobalt(II), copper(II), nickel(II), and ruthenium(II). Also with proper position of functional groups of bipyridine provides connection of two antiparallel β-strands as a hairpin loops.

So that two different bipyridine amino acid derivatives (**1** and **10**) were tried to be synthesized however last coupling reactions of pyridine derivatives of two designs were failed.

Other than that alternatively it was designed that constructing metal binding units with triazoles is possible since the triazoles and the pyridine in the middle of them resulting from the "click reaction" are good ligand for the metal ions.

In order to do that pyridine bistriazole amino acid derivative was designed. While synthesizing this with Huisgen 1,3-dipolar cycloaddition, a highly active catalyst TTr.CuCl was used. As a result, cycloaddition of amino part was achieved successfully while carboxylic acid part was failed. Then the target compound was changed to diamine derivative which is also possible to incorporate into amino acid sequence to form parallel *beta*-sheet structure. Including hydrophobic phenyl alanine, hydrophilic lysine and glutamic acid with a sequence of FKFE tetrapeptide was synthesized by SPPS method. This tetrapeptide was coupled to the two side of diamine.

Lastly binding of copper (II) ions to the pyridine bistriazole diamine derivative was showed by both mass spectroscopy and UV-VIS titration.

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.

Tetrahydrofuran (THF) was distilled from sodium/benzophenone. Other solvents such as toluene and diisopropylamine were distilled from CaH_2

Compounds were named by using ChemDraw Ultra 12.0. For the synthesis of pre-designed peptide, all natural amino acids were purchased from ChemImpex and for the synthesis of desired compounds, all the chemicals were purchased from Aldrich.

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}.

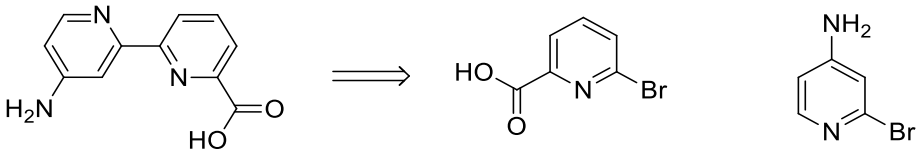
4.1.3. UV-Vis Spectroscopy

In order to measure binding affinity of the ligand to the metal ion double beam UV-VIS spectrum titration was scanned in the range 200-900 nm by using Shimadzu 2450 UV-Vis Spectrophotometer.

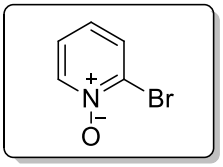
4.1.4. LC-MS-QTOF

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 4'-amino-[2,2'-bipyridine]-6-carboxylic acid



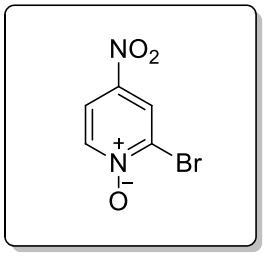
4.2.1. Synthesis of 2-bromopyridine 1-oxide Hydrochloride (5)



According to literature[27] to the solution of 2-bromopyridine (790 mg; 5 mmol) in 20 mL $CHCl_3$ 20 mol% excess of *m*CPBA (1.035 g; 6 mmol) was added. After 4 days the reaction mixture was extracted with 20% HCl solution. The acidic extracts were dried reduced pressure (630 mg, 60%).

^{1}H NMR (400 MHz, D_2O) δ ppm 8.27 (dd, *J*= 6.2, 1.6 Hz, 1H), 7.74 (dd, *J*= 7.8, 2.2 Hz, 1H), 7.4-7.3 (m, 2H)

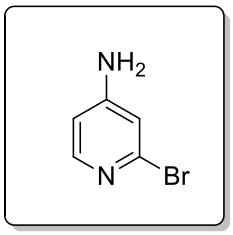
4.2.2. Synthesis of 2-bromo-4-nitropyridine 1-oxide (6)



To prepare the nitrating acid; 1.2 mL fuming HNO_3 were filled in an Erlenmeyer flask and slowly in portions 3.0 mL conc. H_2SO_4 were added and stirred in an ice bath. The nitrating acid was brought to 20 °C and was added dropwise within a few minutes to the **2** (630 mg, 3 mmol) which was heated to 60 °C. Then the reaction mixture was heated to 100 °C. After 24 hours the reaction mixture was cooled down to room temperature and was poured in a beaker containing 10 g of crunched ice. Satd. Na_2CO_3 solution was added until a pH value of 7-8 reached. The solvent was evaporated at a rotary evaporator and remaining solid was dissolved in acetone and the insoluble white salt was filtrated. The filtrate was dried under vacuum. The crude product was purified by column chromatography on silica gel (DCM: EtOAc; 5: 3); (109 mg, 50%).

^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm 8.53 (d, *J*= 3.1 Hz, 1H), 8.42 (d, *J*= 2.7 Hz, 1H), 8.09 (dd, *J*= 7.2, 3.1 Hz, 1H)

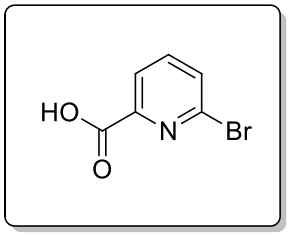
4.2.3. Synthesis of 2-bromopyridin-4-amine (3)



To the solution of **6** (380 mg, 1.7 mmol) in a mixture of 8 mL glacial acetic acid and 4 mL water was added activated iron powder (500 mg). The overall mixture was exposed to ultrasonication for 24 h at 30 °C. The reaction mixture was filtered to remove the iron powder residue and washed with ethyl acetate. To the filtrate, 2 M KOH was added and the basic layer was further extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (EtOAc: Hex; 1: 4); (206.5 mg, 69%).

^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm 7.95 (d, *J*= 5.6 Hz, 1H), 6.72 (d, *J*= 2.1 Hz, 1H), 6.47 (dd, *J*= 5.7, 2.1 Hz, 1H)

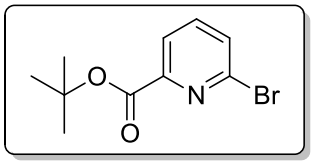
4.2.4. Synthesis of 6-bromopicolinic acid (2)



2-Bromo-6-methylpyridine (500 mg, 2.9 mmol) and $KMnO_4$ (462 mg, 2.92 mmol) were refluxed for 4 h in 80 mL H_2O. Another portion of $KMnO_4$ (462 mg, 2.92 mmol) was added, the resulting reaction mixture was refluxed for 20 h. The reaction mixture was filtered over celite and extracted with EtOAc. The aqueous layer was separated and acidified with 1 M HCl, adjusted to pH 3. The precipitate was filtered off, washed with H_2O, and DCM then dried to give **2**; (234 mg, 40%)

^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm 8.01 (dd, *J*= 7.3, 1.8 Hz, 1H), 7.78 (t, *J*= 7.2, 1H), 7.53 (dd, *J*= 8.6, 2.5 Hz, 1H)

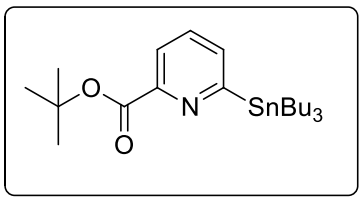
4.2.5. Synthesis of *tert*-butyl 6-bromopicolinate (8)



To the solution of **2** (100 mg, 0.5 mmol) in 3 mL ^{t}BuOH was added 0.5 mL pyridine and then TsCl (190 mg, 1 mmol) at 0 °C. The reaction mixture stirred at room temperature for 5h. The reaction was quenched with sat. $NaHCO_3$ and concentrated under reduced pressure. The desired product was collected by filtration, washed several times with water and dried in vacuo (103 mg, 80%).

^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm 7.98 (dd, *J*= 7.1, 1.4 Hz, 1H), 7.70-7.61 (m, 2H), 1.62 (s, 9H),

4.2.6. Synthesis of *tert*-butyl 3-(tributylstannyl) benzoate (9)

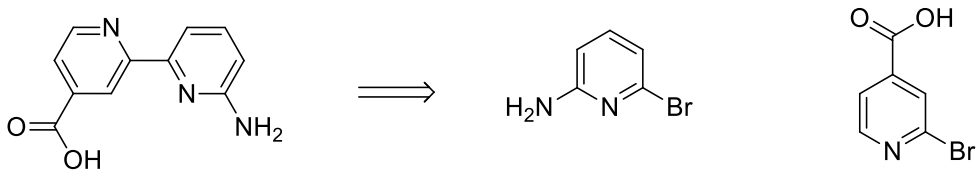


To the solution of **8** (103 mg, 0.4 mmol)in 5 mL anhydrous freshly distilled THF *n*-butyllithium (2.5 M in hexane, 0.4 mmol) was added and the solution was stirred for 1 h at -78 °C. After that Tributyltin chloride (107 µL, 0.4 mmol) was added and the solution was stirred for 1 h -78. °C and for 1 h at room temperature. The mixture was treated with satd. NH_4Cl solution and extracted

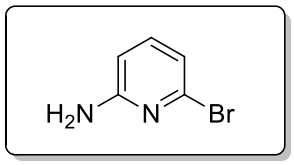
with diethyl ether. The organic phase was washed with brine, dried over MgSO4 and concentrated under reduced pressure to give **9**; (103 mg, 55%).

^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm 7.82 (dd, *J* = 7.2, 1.4 Hz, 1H), 7.75-7.59 (m, 2H), 1.70-1.62 (m, 6H), 1.40-1.28 (m, 12H), 0.94-0.90 (m, 9H)

4.3. Reaction Procedures for Synthesis of 6'-amino-[2,2'-bipyridine]-4-carboxylic acid



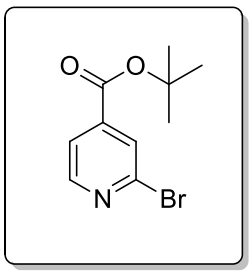
4.3.1. Synthesis of 6-bromopyridin-2-amine (11)



To the commercially available 2,6-bromopyridine (190 mg, 0.8 mmol) in sealed tube, 1 mL aqueous ammonia solution (28-32%) was added. Then it was heated to 170 °C for 24 h in sand bath. After the mixture was cooled to room temperature, the reaction mixture was partitioned between ethyl acetate and saturated Na_2CO_3 solution. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate. The combined organic phase was dried over anhydrous Mg_2SO_4. Solvent was removed by using evaporator (103.8 mg, 75%).

^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm 7.26 (t, *J*= 7.8 Hz, 1H), 6.81 (d, *J*= 7.5 Hz, 1H), 6.41 (d, *J*= 8.1 Hz, 1H), 4.59 (bs, 2H)

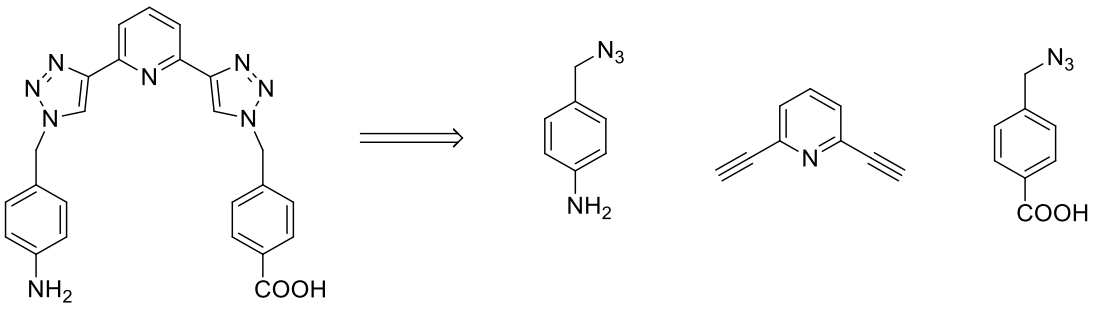
4.3.2. Synthesis of *tert*-butyl 2-bromoisonicotinate (14)



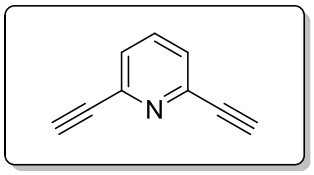
To the solution of commercially available 2-bromoisonicotinic acid (100 mg, 0.5 mmol) in 3 mL ^{t}BuOH was added 0.5 mL pyridine and then TsCl (190 mg, 1 mmol) at 0 °C. The reaction mixture stirred at room temperature for 5 h. The reaction was quenched with sat. $NaHCO_3$ and concentrated under reduced pressure and extracted with ethyl acetate. The combined organic phase was dried over anhydrous Mg_2SO_4. Solvent was removed under reduced pressure (103 mg, 80%).

^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm 7.55 (d, *J*= 8.2, 1H), 7.36 (s, 1H), 7.13 (dd, *J*= 8.6, 2.5 Hz, 1H), 1.26 (s, 9H)

4.4. Reaction Procedures for Synthesis of Amino acids Derivatives Containing Pyridine Bistriazole Moity



4.4.1. Synthesis of 2,6-diethynylpyridine (18)

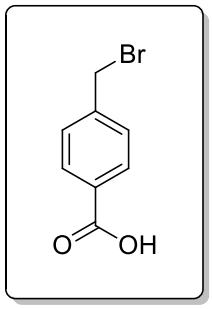


To an anhydrous toluene solution (50 mL) of $Pd(PPh_3)_4$ (577 mg, 0.5 mmol), CuI (95 mg, 0.5 mmol), and 2,6-dibromopyridine (1.18 g, 5.0 mmol), *i*Pr_2NH (10 mL) was added trimethylsilylacetylene (1.55 mL, 12.0 mmol) slowly at room temperature. The reaction mixture was stirred overnight and the resulting salts were filtered off. The filtrate was washed with 10 mL $NH_4Cl_{(aq)}$ and extracted with EtOAc. The combined organic layer was dried ($MgSO_4$) and evaporated. The crude product was subjected to filtration on silica gel (EtOAc: Hex; 1:4), and the filtrate was evaporated. To the crude product that obtained K_2CO_3 (6.9 g, 50 mmol), 10 mL THF

and 10 mL MeOH were added and the mixture was stirred at room temperature for 2 h. The reaction mixture was combined with H_2O and extracted with EtOAc for 3 times. The combined organic layer was washed with brine, dried ($MgSO_4$) and evaporated. The residue was subjected to column chromatography on silica gel (EtOAc: Hex; 1:4) to give **18**; (464 mg, 73%).

^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm 7.59 (dd, *J*= 9.8, 5.8 Hz, 1H), 7.38 (d, *J*= 7.8 Hz, 2H), 3.09 (s, 2H)

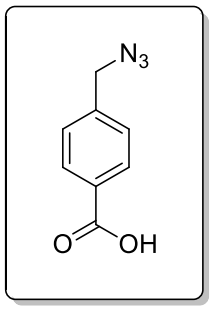
4.4.2. Synthesis of 4-(bromomethyl)benzoic acid (33)



A mixture of p-toluic acid (5.45 g, 40 mmol), benzoyl peroxide (4 g, 15 mmol) and NBS (11.4 g, 64 mmol) in 50 mL dry benzene was heated at reflux for 24 h. A solid precipitate was observed. After cooling to 25 °C, the precipitate was collected by filtration in orfer to remove polybromination products and extracted with water to remove succinimide. Then **33** was obtained by filtering, washing with hot water, drying in vacuo (6.2 g, 72%).

^{1}H NMR (400 MHz, *$(CD_3)_2CO$*) δ ppm 7.89 (d, *J* = 8.4 Hz, 2H), 7.46 (d, *J*=8.4 Hz, 2H), 4.58 (s, 2H),

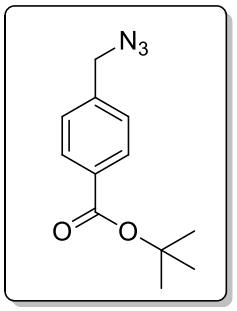
4.4.3. Synthesis of 4-(azidomethyl)benzoic acid (34)



A stock solution of 0.5 M was prepared by stirring in DMSO at room temperature. To 44 mL 0.5 M NaN_3, **4** (4.3 g, 20 mmol) was added at 25 °C and the mixture was stirred at room temperature until all the starting material had been consumed as monitored by TLC analysis. The reaction was quenched with water and the mixture was extracted with Et_2O. The organic layer was washed with brine, dried over $MgSO_4$, and the solvent removed in vacuum (3.19 g, 90%).

^{1}H NMR (400 MHz, *$(CD_3)_2CO$*) δ ppm 8.08 (d, *J*= 8.4 Hz, 2H), 7.54 (d, *J*= 8.1 Hz, 2H), 4.57 (s, 2H)

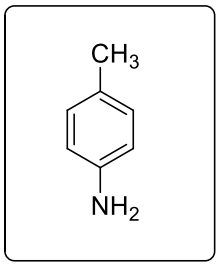
4.4.4. Synthesis of *tert*-butyl 4-(azidomethyl)benzoate (35)



To the solution of **34** (178 mg, 1 mmol) in 5 mL ^{t}BuOH was added 1 mL pyridine and then TsCl (380 mg, 2 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 5 h. The reaction was quenched with sat. $NaHCO_3$ and concentrated under reduced pressure. The desired product was collected by filtration, washed several times with water and dried in vacuo (100 mg, 45%).

^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm 7.92 (d, *J*= 8.2 Hz, 2H), 7.28 (d, *J*= 8.3 Hz, 2H), 4.30 (s, 2H), 1.51 (s, 9H)

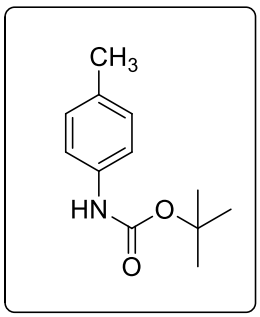
4.4.5. . Synthesis of *p*-toluidine(23)



To the solution of **21** (1.00 g, 4.63 mmol) in 30 mL methanol was added 70 mg Pd/C and the reaction was performed in hydrogenation reactor for 4 h at 30 °C and 3 atm H_2. After the reaction was completed, the solids was filtered by using celite. The solvent of filtrate was evaporated under vacuum to give pure **23**; (470 mg, 95%).

^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm 6.89 (d, *J*= 8.0 Hz, 2H), 6.53 (d, *J*= 8.3 Hz, 2H), 3.39 (bs, 2H), 2.16 (s, 3H),

4.4.6. Synthesis of *tert*-butyl *p*-tolylcarbamate (25)

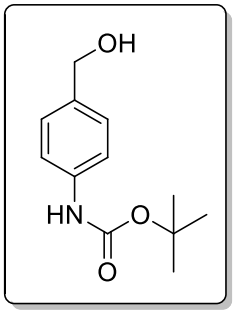


To the solution of **23** (470 mg, 4.4 mmol) in 40 mL anhydrous THF, DIEA (0.8 mL, 4.6 mmol) and Boc_2O (1.0 mL, 4.6 mmol) were added. The mixture was heated at reflux. After the completion of reaction, the mixture was cooled down and evaporated under vacuum. The residue was dissolved in EtOAc and extracted with 0.1 M HCl solution and dried over $MgSO_4$,

filtered and evaporated under vacuum. The crude product was purified by column chromatography on silica gel (EtOAc: Hex ; 1: 1); (700 mg, 75%).

^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm 7.16 (d, *J*= 8.2 Hz, 2H), 7.01 (d, *J*= 8.2 Hz, 2H), 2.21 (s, 3H), 1.44 (s, 9H)

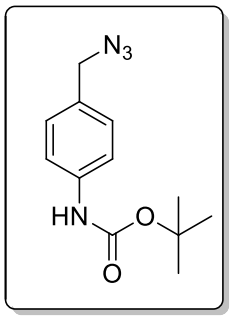
4.4.7. Synthesis of *tert*-butyl (4-(hydroxymethyl)phenyl)carbamate (27)



According to synthesis route in literature,[28] to the solution of p-amino-benzylalcohol (0.5 g., 4.06 mmol) in 40 mL anhydrous THF, DIEA (0.7 mL, 4.12 mmol) and Boc_2O (0.95 mL, 4.12 mmol) were added. The mixture was heated at reflux and monitored by TLC (EtOAc: Hex ; 1: 1). After the completion of reaction, the mixture was cooled down and evaporated under vacuum. The residue was dissolved in EtOAc and extracted with 0.1 M HCl solution and dried over $MgSO_4$, filtered and evaporated under vacuum. The crude product was purified by column chromatography on silica gel (EtOAc: Hex ; 1: 1); (700 mg, 77%).

^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm, 7.23 (d, *J*= 8.5 Hz, 2H), 7.29 (d, *J*= 8.4 Hz, 2H), 6.45 (bs, 1H), 4.56 (s, 2H), 1.44 (s, 9H)

4.4.8. Synthesis of *tert*-butyl (4-(azidomethyl)phenyl)carbamate (28)

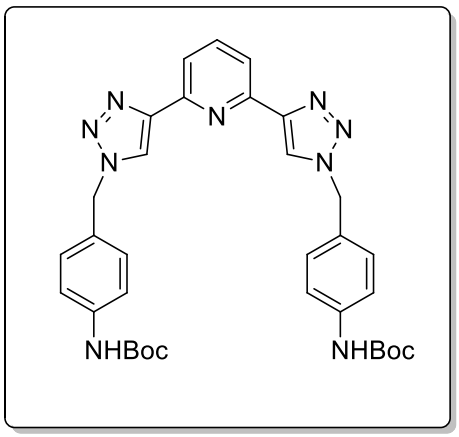


To the solution of **27** (223 mg, 1 mmol) in 5 mL anhydrous THF, ADMP (332 mg, 1.2 mmol), and DBU (0.15 mL, 1.3 mmol) were added at room temperature. The mixture was quenched with sat. aq NH_4Cl after stirring 3 h and organic materials extracted with CH_2Cl_2. Combined organic layers were washed with brine and dried over $MgSO_4$, then concentrated in vacuo. The crude product was purified by column chromatography on silica gel to give pure **28**; (224 mg, 90%).

^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm 7.31 (d, *J*= 8.3 Hz, 2H), 7.18 (d, *J*= 8.6 Hz, 2H), 4.20 (s, 2H), 1.45 (s, 9H)

4.5. Reaction Procedures for Synthesis of Diamine Derivative Containing Pyridine Bistriazole

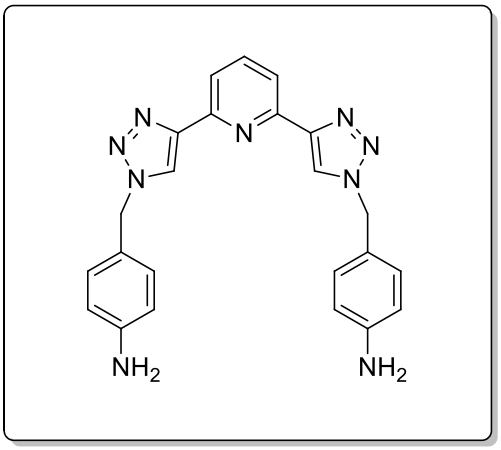
4.5.1. Synthesis of di-*tert*-butyl (((4,4'-(pyridine-2,6-diyl)bis(1H-1,2,3-triazole-4,1-diyl))bis(methylene))bis(4,1-phenylene))dicarbamate (31)



To the solution of **18** (63 mg, 0.5 mmol) in 2 mL MeOH, **28** (273 mg, 1.1 mmol) was added. To this mixture TTr.CuCl catalyst (3.0 mg, 0.5 mol %) and additional 2 mL water was added and stirred at 45 °C. The reaction was controlled by TLC (EtOAc: Hex ; 1: 1.5), and the mixture was cooled down after the completion of reaction and extracted with EtOAc washed with brine and dried over $MgSO_4$, then concentrated in vacuo. The crude product was purified by column chromatography on silica gel (EtOAc: Hex; 1: 1.5); (226 mg, 72%).

^{1}H NMR (400 MHz, *$CDCl_3$*) δ ppm, 8.00 (d, *J*= 7.8 Hz, 2H), 7.90 (s, 2H), 7.77 (t, *J*= 7.8 Hz, 1H), 7.33- 7.19 (m, 4H), 6.65 (s, 2H), 5.44 (s, 4H), 1.46 (s, 18H)

4.5.2. Synthesis of 4,4'-((4,4'-(pyridine-2,6-diyl)bis(1H-1,2,3-triazole-4,1-diyl))bis(methylene))dianiline Hydrochloride (37)



31 (226 mg, 0.36 mmol) was stirred in 5 mL of 1.0 M HCl solution in diethyl ether overnight at room temperature. Then the mixture dried under vacuum to give **37** (185 mg, 100%).

^{1}H NMR (400 MHz, *D_2O*) δ ppm 8.27 (s, 2H), 8.00 (t, *J*= 7.6 Hz, 1H), 7.82 (d, *J*= 7.3 Hz, 2H), 7.04 (d, *J*= 7.9 Hz, 4H), 6.88 (d, *J*= 7.6 Hz 4H), 5.27 (s, 4H)

^{13}C NMR (100 MHz, *D_2O*) δ ppm 53.1, 120.1, 122.9, 123.6, 129.2, 131.4, 134.5, 139.6, 147.4, 148.6

4.6. General Procedure for Solid Phase Peptide Synthesis

Loading first Fmoc-protected amino acid to 2-chlorotrityl chloride resin:

277 mg (0.25 mmol) resin is weighed in reaction vessel in which the desired peptide will be synthesized. It's washed with DMF (2x) and swelled in 2-3mL DMF for 10-15 min. Then, vessel is drained. In a separate scintillation vial, 0.375 mmol Fmoc-Glu(OtBu)-OH is dissolved in 1.5 mL DMF. To this solution, 326 μL DIEA (1.877 mmol) is added and the solution is transferred to same reaction vessel with resin. After overnight, substitution is checked via UV-spectroscopic methods. As a final step, 200 μL MeOH is added for capping without draining DMF and waited for 30 min. Lastly, vessel is drained, washed with DMF (5x) and DCM.

Deprotection:

The Fmoc protecting group is removed by treating the pre-swollen resin with 20% piperidine in DMF for 10 min (2x10 mL). Then the solution is drained and the resin washed with DMF.

Coupling with an Fmoc-protected amino acid:

Fmoc-protected amino acid (0.55 mmol, 5.5 eq.) dissolved in HBTU (1.00 mL, 0.5 M in DMF) then DIEA (200 μL) 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 1 hour and agitated in every 10 min. Then the solution is filtered off and the resin washed with DMF and DCM. The reaction progress is checked with the Kaiser test. Then deprotection step is repeated before coupling with another Fmoc-protected amino acid

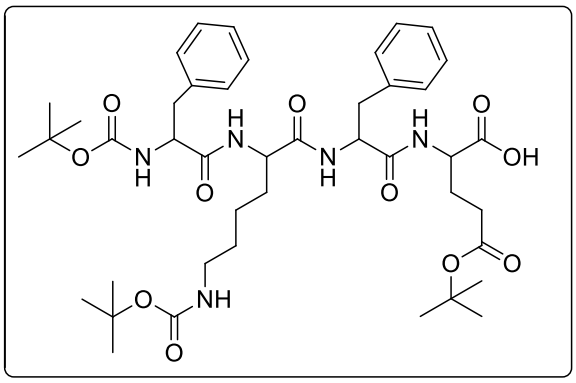
Final Deprotection:

After deprotection of Fmoc of the very last last amino acid residue, resin is washed with DMF (4 × 2 mL), DCM (4 × 2 mL) then dried under vacuum.

Cleavage:

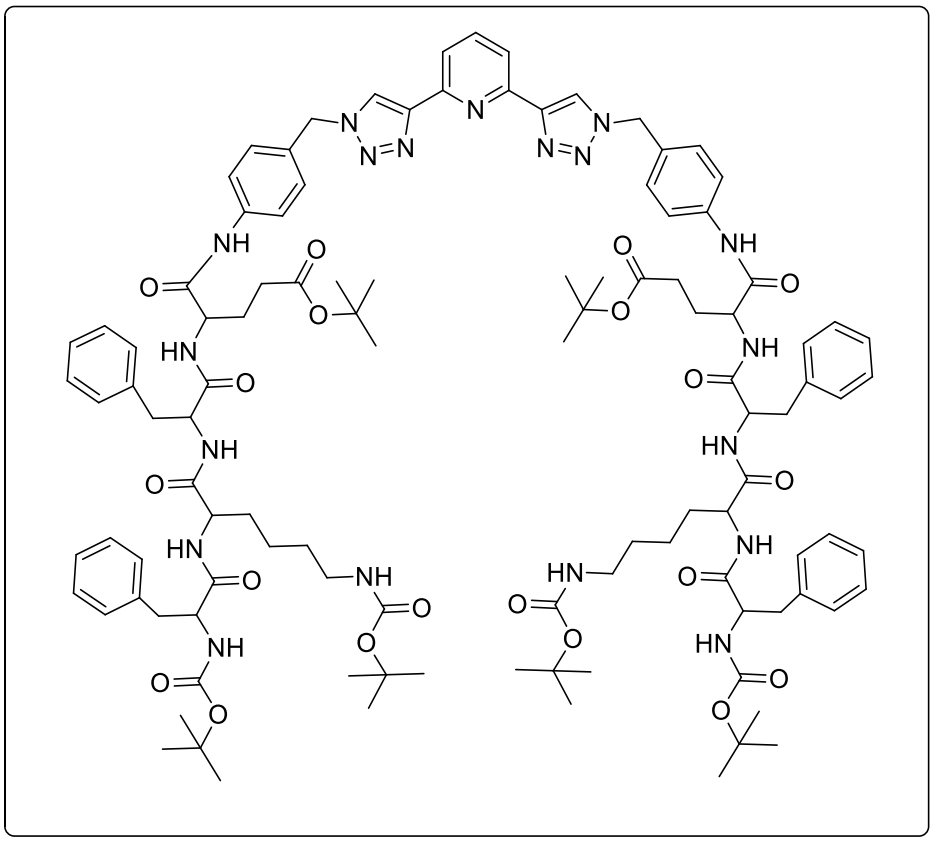
98% TFA, 1% DCM and 1% TIPS solution – so called cleavage cocktail – is used to cleave the peptide sequence from resin. This cocktail (2 x 1 mL) added to the resin and waited for 1 hour. Then the solution is collected. The peptide is triturated by addition of ice-cold diethyl ether and the resulting emulsion is centrifuged at 8500 rpm for 10 min. The solid is filtered off. The product is dissolved in distilled water and lyophilized.

4.6.1. Synthesis of Fully Protected FKFE (38)



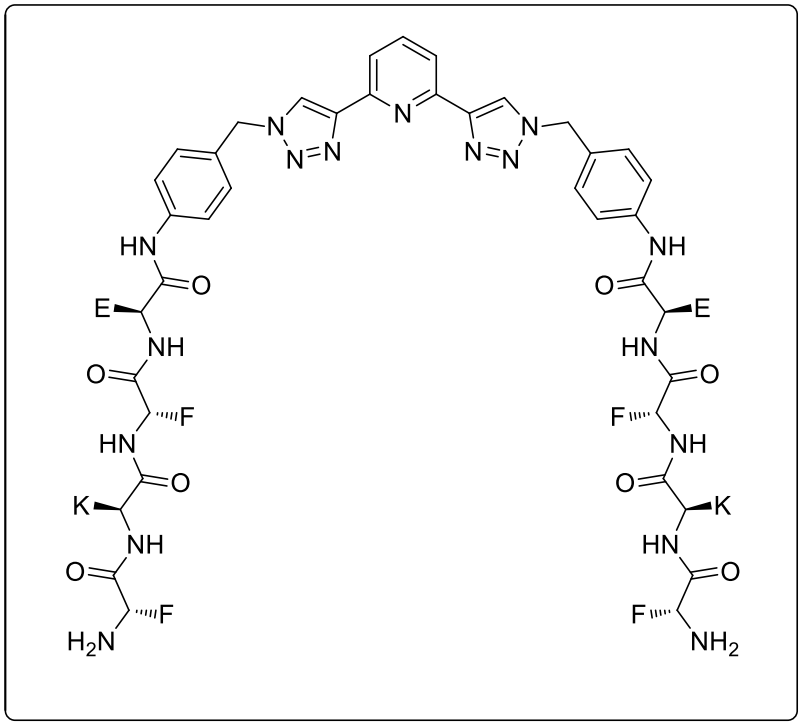
110 mg (0.1 mmol, subs: 0.91 mmol/g) Fmoc-Glu(O^t*Bu*)-2-chlorotrityl resin was weighed in reaction vessel. The resin was washed with DMF (2x) and swelled in 5-6 mL DMF for 40-45 min. The solution is drained and Fmoc group that attached to the first amino acid on the resin was deprotected by adding 10 mL of 20% piperidine in DMF (2x10 min). Then Fmoc-protected second amino acid (Fmoc-Phe-OH, 5.5 eq.; 213 mg, 0.55 mmol), was dissolved in 1.045 mL HBTU (0.5 M in DMF). DIEA (200 µL) was added to this amino acid solution, stirred for 30 seconds and added to the resin in the reactor. The second amino acid was coupled for 1 h. At the end of 1 h, resin was washed with DMF and Fmoc-protected third amino acid (Fmoc-Lys(Boc)-OH; 257.7 mg, 0.55 mmol) were coupled with the same procedure by starting with the Fmoc deprotection step. Last Boc-protected amino acid (Boc-Phe-OH; 145.9 mg, 0.55 mmol) were coupled after4 deprotection of Fmoc group and the resin was washed with DMF (4x5 mL), DCM (4x5 mL) and dried under vacuum. To cleave side chain protected peptide from the resin, 2mL; 20% TFE, 10% AcOH, 70% DCM solution was added to the resin and the mixture was stirred for 1 h (2x).and filtered and washed the resin with 4 mL; 20% TFE, 80% DCM solution two times. After the treatment of resin with cleavage cocktail, eluent was collected, evaporated in rotary and triturated with cold water.

4.6.2. Coupling Reaction of 4,4'-((4,4'-(pyridine-2,6-diyl)bis(1H-1,2,3-triazole-4,1-diyl))bis(methylene))dianiline Hydrochloride (37) and Side Chain Protected Boc-Phe-Lys-Phe-Glu-COOH (38)



To the solution of **37** (24 mg, 0.04 mmol) in 2 mL DCM, 2.25 equiv. fully protected peptide FKFE **38** (76.3 mg, 0.09 mmol), 8.5 equiv. DIEA (59.2 µL, 0.34 mmol), 4 equiv. HOBt (22 mg, 0.16 mmol) and 3.0 equiv. EDC (23 mg, 0.12 mmol) was added and stirred at room temperature. Once the reaction was completed after 3 weeks (monitored by HPLC) the reaction mixture was concentrated under reduced pressure (32 mg, 40%).

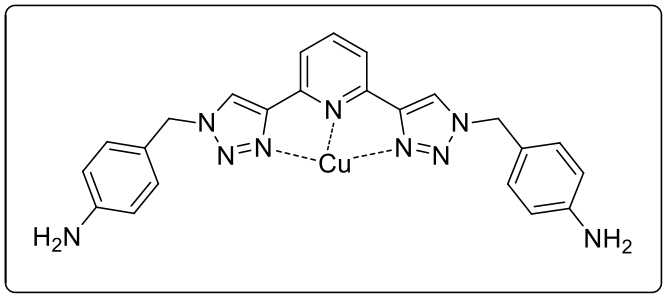
4.6.3. Deprotection of Artificial Peptide 39



95% TFA, 5% DCM solution was used to cleave the side chain protection groups. This solution (2 x 2 mL) added to **39** and water for 1 h. The peptide was triturated by addition of ice-cold diethyl ether and the resulting emulsion was centrifuged at 8500 rpm for 10 min. The solid was filtered off. The product was dissolved in distilled water and lyophilized. The artificial peptide **40** was purified using RP-HPLC

4.7. Cu(II) Complex of Diamine 37(41)

In order to prepare 0.01 M HEPES buffer containing 0.1 M NaCl at pH 7, 138 mg *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) and 292 mg NaCl was dissolved in 40 mL distilled water. The pH was adjusted to 7 with 4.0 M HCl and 4.0 M NaOH solutions. After that the total volume was brought up to 50 mL with distilled water. For the UV titrations 0.1 M $CuSO_4$ was prepared by dissolving 1.25 g $CuSO_4$. $5H_2O$ in 50 mL distilled water.



Diamine **37** was dissolved in HEPES buffer at pH 7.

In UV titrations for the determination of K_d of Cu-diamine complexes, 0.1-0.5 μM of diamine was titrated with 20-30 fold excess of Cu^{2+}using 0.1 M $CuSO_4$ solution.

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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

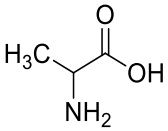
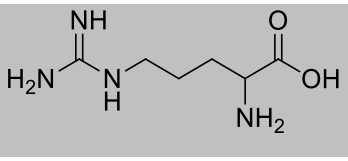
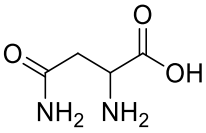
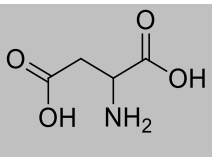
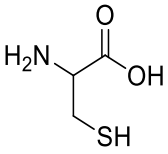
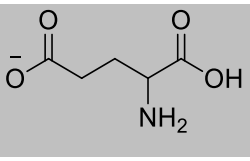
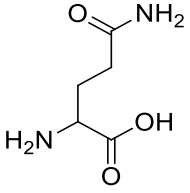
Name	3-Letter code	1-Letter code	Structure
Alanine	Ala	A	
Arginine	Arg	R	
Asparagine	Asn	N	
Aspartic acid	Asp	D	
Cysteine	Cys	C	
Glutamic acid	Glu	E	
Glutamine	Gln	Q	

Table A.1. List of 21 Amino acids Found in Nature (Continued)

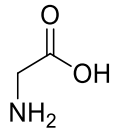
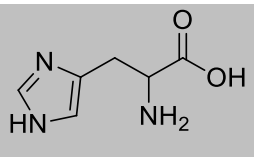
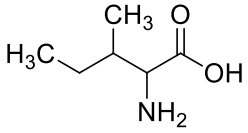
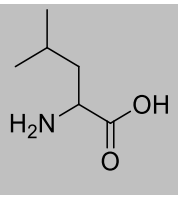
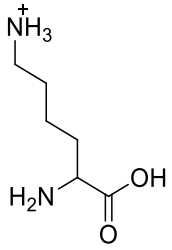
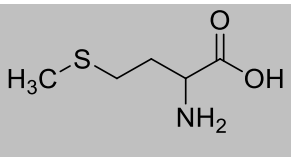
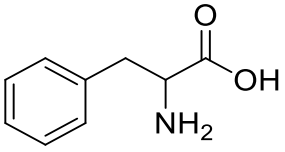
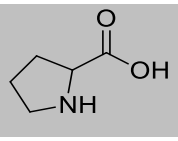
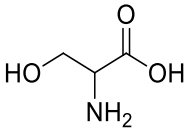
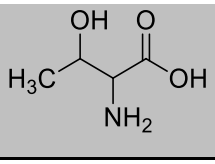
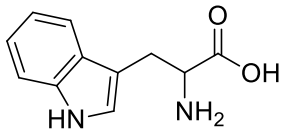
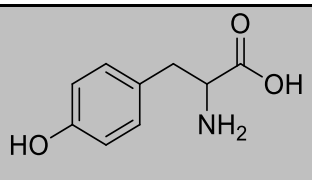
Glycine	**Gly**	**G**	
Histidine	His	H	
Isoleucine	Ile	I	
Leucine	Leu	L	
Lysine	Lys	K	
Methionine	Met	M	
Phenylalanine	Phe	F	
Proline	Pro	P	
Serine	Ser	S	
Threonine	Thr	T	

Table A.1. List of 21 Amino acids Found in Nature (Continued)

Tryptophan	**Trp**	**W**	
Tyrosine	Tyr	Y	
Valine	Val	V