SYNTHESIS OF LINKERS AND MEDIATORS FOR ELECTROCHEMICAL REACTOR DESIGN AND ENANTIOPURE SYNTHON PREPARATION

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SYNTHESIS OF LINKERS AND MEDIATORS FOR ELECTROCHEMICAL REACTOR DESIGN AND ENANTIOPURE SYNTHON PREPARATION

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ABSTRACT

SYNTHESIS OF LINKERS AND MEDIATORS FOR ELECTROCHEMICAL REACTOR DESIGN AND ENANTIOPURE SYNTHON PREPARATION

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The production of enantiopure compounds can be achieved by using dehydrogenases as biocatalysts catalyzing reduction reactions of prochiral compounds such as ketones, aldehydes and nitriles. These dehydrogenases are cofactor dependent enzyme where cofactor is Nicotinamide dinucleotite having some restrictions that limits usage of dehydrogenases in organic synthesis including instability of cofactor in water and high cost. Therefore suitable regeneration method is needed and developed which are enzymatic and electrochemical.

We will use an electrochemical approach for the regeneration of reduced co-factors which has been shown in principal with mediators like pentamethylcyclopentadienyl rhodium bipyridine complexes or ferrocenes. This project is European Union project, whose name is Development of Electrochemical Reactors Using Dehydrogenases for Enantiopure Synthon Preparations.

All active compounds; mediator, cofactor and enzyme, will be immobilized on the electrode surface of the constructed reactor surface. Therefore only educts and

products will exist in the reactor medium. A gas diffusion electrode will be employed as a counter electrode; which delivers clear protons to the system. Mediator will carry electrons to the cofactor for cofactor regeneration. Then enzyme will use the cofactor and convert substrates to the product in high stereoselectivity.

Our part in this project is the synthesis of mediator and suitable linkers for enzyme, cofactor and mediator immobilization. In the first part of the study, Linkers which contain thiol group and disulfide linkage were synthesized because working electrode made of by gold nano particles and immobilization carried out by the help of these groups on gold nano surface. In the second part of the study, mediators were synthesized which are pentamethylcyclopentadienyl rhodium bipyridine complexes and ferrocene derivatives. Synthesized mediators were reacted with linkers by using Click Chemistry and by imine formation in order to convert mediator to the thiol functionalized form.

Keywords: dehydrogenases, Mediator, Linker for enzyme immobilization, electrochemical reactor.

ÖZ

ENANSİYOMERİK SAF SİNTONLARIN SENTEZİNDE KULLANILACAK ELEKTROKİMYASAL REAKTÖR İÇİN ARABULUCU VE BAĞLAYICI BİLEŞİKLERİN TASARIMI VE SENTEZLENMESİ

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Eylül 2009, 78 sayfa

Aldehit, keton ve nitril gibi prokiral maddelerin indirgenme reaksiyonlarını gerçekleştiren dehidrogenazlar optikçe saf maddelerin sentezi için kullanılabilirler. Bu enzimler indirgenme reaksiyonlarını katalizlemek için nikotinamid dinükleotitte kofaktör olarak ihtiyaç duyarlar ve bu kofaktörün dayanıksız ve pahalı olması nedeniyle enzimin organik sentezlerde kullanılması sınırlıdır. Bu nedenle enzimatik ve elektrokimyasal olmak üzere kofaktör dönüşüm sistemleri geliştirilmiştir.

Bizim projemizde kofaktörün indirgenmiş halinin döngüsünü sağlamak için elektrokimyasal yöntem kullanılacaktır. Avrupa birliği projesi olan bu projenin amacı optikçe aktif maddelerin sentezi için elektrokimyasal reaktör geliştirilmesidir.

Bu projede, reaktör yüzeyleri geliştirilecek ve arabulucu bileşikler, kofaktör ve enzim gibi biyolojik önem taşıyan aktif bileşikler reaktör yüzeyine bağlanacaktır. Reaktör substratları ürünlere yüksek seçicilikte dönüştürecek ve bütün aktif bileşikler yüzeye tutturulduğu için ürünlerin ayrılması bir problem oluşturmayacaktır. Gaz membran elektrotu ise ortama proton sağlayacak ve sistemin potansiyelini 1 volt'a

kadar düşürecek ve istenmeyen reaksiyonların gerçekleşmesi engelleyecektir. Ortamdaki arabulucu bileşikler elektronları kofaktöre taşıyacak ve kofaktörün dönüşümünü sağlayacaktır, enzim ise kofaktörü kullanarak ketonları yüksek seçicilikle alkollere dönüştürecektir.

Biz ise bu projede arabulucu ve bağlayıcı bileşiklerin planlanması ve sentezlenmesidir. Bağlayıcı bileşikler enzimin, arabulucu bileşiklerin ve kofaktörün elektrot yüzeyine tutturulmasında kullanılacaktır. İkinci kısımda ise bipiridin rodyum kompleksleri ve ferrosen türevleri aracı moleküller olarak sentezlenmiştir. Çalışmanın birinci kısmında yapısında tiyol ve disülfit bağı içeren bağlayıcı moleküller sentezlenmiştir çünkü bu bileşiklerin altın yüzeyinde tutturulması bu gruplarla altının etkileşmesi sayesinde olmaktadır.

Çalışmanın ikinci kısmında ise pentametilsiklopentadienil rodyum bipiridil kompleksleri ve ferrosen türevleri sentezlenmiştir. En son bölümde ise sentezlenen bağlayıcı bileşikler ve arabulucu bileşikler reaksiyona sokulmuştur.

Anahtar kelimeler: Dehidrogenazlar, arabulucu moleküller, enzim immobilizasyonu için bağlayıcı moleküller, elektrokimyasal reaktör.

To My Family,

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I am deeply indebted to my supervisor Prof. Dr. Ayhan S. Demir whose guidance, help, suggestions and encouragement helped me througout my study. It is a great honor for me to be his student and his intellectual approach will continue to inspire me.

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INTRODUCTION

1. 1 Enzymes in Organic Chemistry

Synthetic organic chemistry is used for stereocontrolled synthesis of a very large number of complex molecules. As the field has developed, it's constrains and aims has also changed. Scientists have tried to find new techniques for preparing complex, water soluble biochemicals and development of environmentally friendly acceptable synthetic procedure. Enzymes are able to catalyze organic transformations in water so they are called as green catalyst [1, 2].

Enzymes are huge molecules catalyzing mostly biological reactions and have an important role in natural life [1, 2, 3]. In addition they catalyze reactions involving both unnatural as well as natural substrates. Enzymes have following properties:

1. They carry out the reactions under very mild conditions; at room temperature, in the water, without the substrate- functional group protection [2, 3].
2. They accelerate the rate of the reaction by a factor of 10^8-10^{10}compared to non enzymatic reactions [1,3]
3. They catalyze stereoselective reactions and show high selectivity to substrates. They perform their reactions in a stereo- and regiospecific manner [1, 3, 4].

4. They are used as a powerful approach for the synthesis of fine chemicals, commodity and even bulk chemicals. They are also catalyze organic transformations which is difficult to synthesize by using traditional method [1, 5]
5. They are affected from reaction conditions; their catalytic activity may strongly influenced by the concentration of salt, substrates and products, pH of buffer. Inhibition can occur at high salt, product and substrates concentration [1, 3]
6. They have very broad range of substrates; they can accept unnatural and natural substances as substrates [1, 2, 3].
7. They are chiral due to this enantiomerically pure product and intermediates are usually obtained instead of racemates which is used in food and feed industry or as a building block in the preparation of therapeutic compounds such as herbicides and insecticides [1, 2, 4].
8. Enzymes catalyze the reactions with high selectivity and yields which decrease the need for purification [1,6,7]
9. They catalyze a broad spectrum of reactions including oxidations, reductions, carbon carbon (C-C) bond formations, C-C bond cleavages, hydrolysis, additions, eliminations and isomerizations. Enzymes which are commonly used in organic synthesis and their reactions are given in Table 1 [2].

Table 1: Classification of enzymes

Enzyme class	Reaction type
Oxidoreductases	Oxidation, reduction: oxygenation of C-H, C-C, C=C bonds or overall removel or addition of hydrogen atom equivalents
Transferases	Transfer of groups: aldehydic, ketonic, acyl, sugar, phosphoryl or methyl
Hydrolases	Hydrolysis- formation of esters, amides, lactones, lactams, epoxides, nitriles, anhydrides, glycosides
Lyases	Addition and elimination of small molecules on C=C, C=N, C=O bonds
Isomerases	Isomerizations such as racemization, epimerization
Ligases	Formation-cleavage of C-O, C-S, C-N, C-C bonds with concomitant triphosphate cleavage.

Instability, higher cost and narrow substrate specifity are the most serious drawbacks of the enzymes [1,2,3] but there has seen a revolution in this area in the past fifteen years due to new developments in chemistry and biology to overcome these problems [1].

1. Large numbers of enzymatic reactions have been demonstrated for biotransformation. New techniques have been developed to increase the

stability of enzyme and the catalytic activity of enzyme. Genetic engineering has been used to develop new techniques [1].

2. Immobilization methods have been used to facilitate the recovery of enzyme to reuse [8].
3. New enzymes and reactions have been found [1]
4. New technologies have been made possible to produce proteins and enzymes in low cost [1]

1.2 Dehydrogenases in Organic Chemistry

Synthesis of chiral products has very important applications in several areas such as food, feed industries, preparation of therapeutic compounds and asymmetric synthesis. Enzymes are used in synthetic organic chemistry for the stereoselective synthesis of chiral precursors. Redox enzymes, which are oxygenases, oxidases, dehydrogenases, are one of the classes of enzymes catalyzing oxidation and reduction reactions that are widespread in biological system in metabolic pathway for the energy conversion such as citric acid cycle and photosynthesis. Especially reduction reactions catalyzed by dehdyrogenases are used for the preparation of chiral synthons since dehydrogenases uses prochiral compounds which have sp^2 center as a substrates and converts to chiral precursor by transferring hydride to the prochiral center. Besides, synthesis of biologically important compounds is possible by using dehydrogenases with suitable substrates [1, 3, 9].

Dehydrogenases can be used for the synthesis of aminoacids which have important role in human nutrition and industrial scale applications [4]. For example Glutamate dehydrogenase (GluDH) was utilized for the synthesis of L-Glutamate by using α-ketoglutamarate as a starting material [10].

Dehydrogenases are also important for the synthesis of α-hydroxy acids which are crucial due to their involvement in the preparation of many

pharmaceuticals such as cephalosporin, anti- obesity drugs, penicillin and cosmetic products. Most of the dehdyrogenases can synthesize α-hydroxy acids form the reduction of α-ketoacids [4].

Furthermore enzymatic reduction of carbonyl compounds are catalyzed by dehdyrogenases and used for the preparation of several enantiomeric primary and secondary alcohols that are used as a building block for the synthesis of many pharmaceuticals, cosmetics and agricultural products [4]. Besides Enantiomeric alcohols can also be converted to other functional groups like halogens amine and azides [11, 12]. Generally inexpensive prochiral ketones are used as a starting material for the enzymatic synthesis of alcohols in high yields in organic reactions [4].

Dehdyrogenases is also used for the asymmetric applications if substrate is steroid. Steroid reactions involve the introduction of hydroxyl group at different positions. Synthesized molecules have biological application, for example cholic acid was converted to the 12-ketoursodeoxycholic acid that is used for the synthesis of ursodeoxycholic acid that has important applications in therapy and cholesterol gall stones [4, 13, 14].

In brief using of dehydrogenases in organic synthesis is very important and popular because of the synthesis of several chiral products which are valuable for the synthesis of several natural products and applicable in many areas.

1.2.1 General Reactions of Dehydrogenases

Redox enzymes divided into three cathegories; dehydrogenases, oxygenases and oxidases [3]. They need cofactor to catalyze their reactions which are nicotinamide nucleotide NAD(P)H, heme, flavin, pyrolidine quiloline quiline (PQQ). The differences between cofactors are their redox potentials, binding constants and

the mode of regenerations [4]. 80% of redox enzymes require NADH and 10% of them need NAD(P)H to catalyze their reactions, only fewer enzymes require other cofactors [4]. Enzymes, their reactions and cofactors are given in Table 2

Table 2: Redox enzymes, their reactions and cofactors

Enzyme class	**Reaction**	**Redox cofactor**
Dehydrogenases	Reductions of aliphatic, aromatics aldehydes and ketones, alkenes, steroids and C=N bond.	NAD(P)H, flavin,PQQ
Oxygenases	Oxidations of primary and secondary alcohols, epoxidations, hydroxylation of alkenes, aromatic compounds, Bayer-Villiger oxidations, peroxidases formations.	Flavin,NAD(P)H, non-heme structure.
Oxidases	Biotransformation in non-natural organic compounds	NAD(P)H, heme,PQQ

Dehydrogenases are one of the redox enzymes catalyzing reduction reactions. Most of them are NAD(P)H dependent. Nicotinamide dinucleotite and the analogous 2'-phosphate are involved in many reduction reactions [Figure 1].

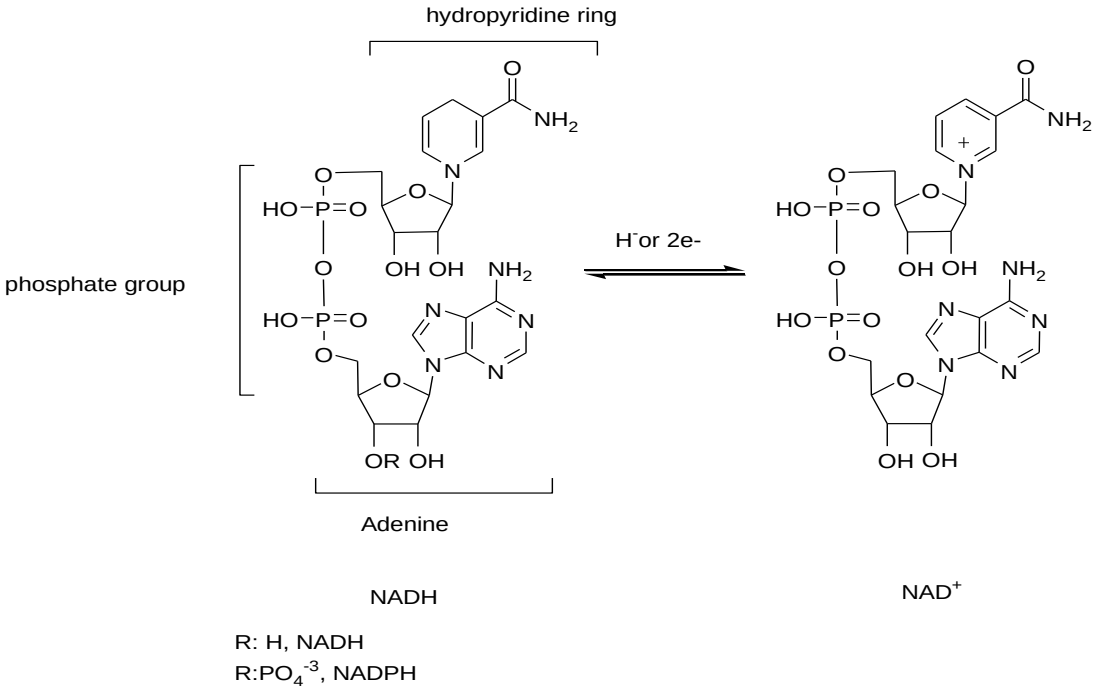
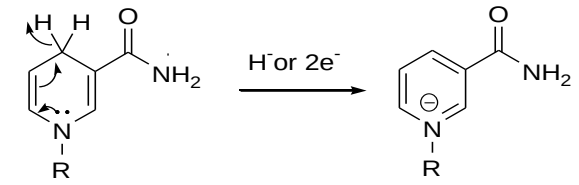


Figure 1: Structure of Nicotinamide Adenine Dinucleotite

Nicotinamide dinucleotite and phosphate analog not only change in their structure but also change in their process. NAD^+/NADH couple is utilized in catabolic pathway whereas $NADP^+$/NADPH couple is used for biosynthetic process. NADH is the mostly used form of cofactor for reduction reactions [9].

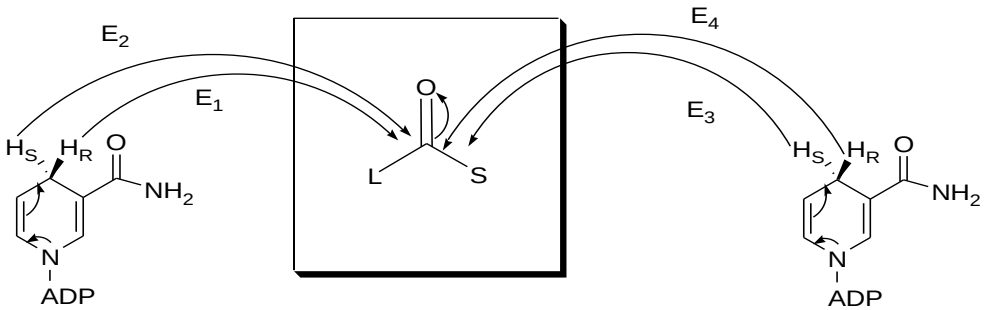
The nicotinamide cofactor is redox active and exists in oxidized and reduced forms which are in equilibrium and converted to each other during the enzymatic reactions. It accepts a hydride or two electrons to form reduced form of the cofactor (NAD(P)H). Hydride is transferred from reduced substrate to oxidized form of the cofactor or from NAD(P)H to oxidized substrates [Figure 2]. These reactions are stereoselective and characteristics of individual enzymes. Enzymes transfer one of the diastereotopic methylene hydrogens to a substrate with high enantiofacial or diastereofacial selectivity [1].



R: Adenine Diphosphate

Figure 2: Hydride generation in water

Dehydrogenases use prochiral compounds and convert to the chiral precursors at the end of the reaction in their general reactions. Nicotinamide dinucleotite plays an important role for this stereoselective conversion. NAD(P)H and $NAD(P)^+$ have two diastereotopic hydrogens (*pro-R* and *pro-S* hydrogens). These hydrogens can be transferred as a hydride to the oxidized substrates. Substrates have carbonyl, nitrile and alkene groups in their structure, and also have two diastereotopic or enantiotopic faces (*re* or *si* face). There are four possibilities for this conversion however generally *pro-R* hydrogens of cofactor is transferred to the *re* face of a carbonyl substrates [1, 3] [Figure 3].



E_1: pro-R/si face
E_2: pro-S/ si face
E_3: Pro- S/re face
E_4: Pro-R/re face

Figure 3: Stereoselectivity of dehydrogenases

Dehydrogenases can accept aldehydes, ketones and diketones as a substrate [2, 3, 15]. Ketones can be aromatic, aliphatic, cyclic and bicyclic [Figure 4]. Primary alcohol, secondary alcohol and α-hyroxy ketones from reduction of alkenes, ketones and diketones are synthesized stereoselectively [3].

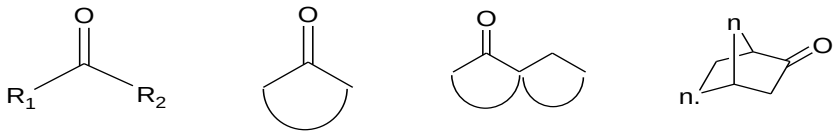


Figure 4: Substrates for reduction of ketones

Beside dehydrogenases reduce nitriles which are important group for the preparation of aminoacid analogs [3, 15]. Generaly α- ketoacids are used for this type of conversions and imino group is the intermediate in the reaction pathway [3, 15] [Figure 5].

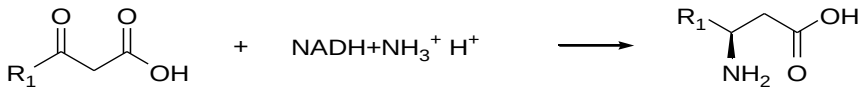


Figure 5: Reduction of nitrile by dehydrogenases

Reduction of alkenes and steroids are also carried out by dehydrogenases. To achieve the reduction of alkenes, there must be electron withdrawing group which can be nitro (NO_2) or halogens [Figure 6] [3, 15].

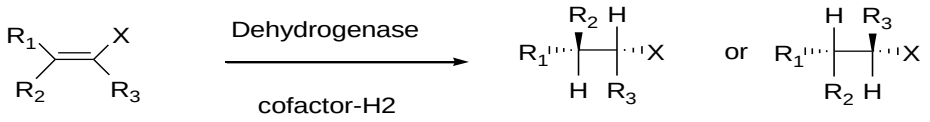


Figure 6: Reduction of alkenes by dehydrogenases

Finally, biological transformations of steroids can be done by dehydrogenases. These transformations are of great interest with respect to the production of pharmaceuticals and also can be used for the asymmetric synthesis. Reduction of the double bond in the structure of steroids carried out by dehydrogenases with high enantioselectively [3, 10, 15].

1.2.2 NAD(P)H-NAD(P)$^+$ Regeneration System

Nicotinamide dinucleotitide that are important for the biotransformation catalyzed by dehydrogenases, has important properties. They are usually not covalently bound to the enzymes and readily dissociate. Besides they are instable and this is important drawback of nicotinamide cofactor [4]. Both oxidized and reduced forms of the cofactor are instable in aqueous medium since, hydropyridine is converted to 6- hydroxyl- tetrahydropyridine in the presence of water [16] [Figure 7].

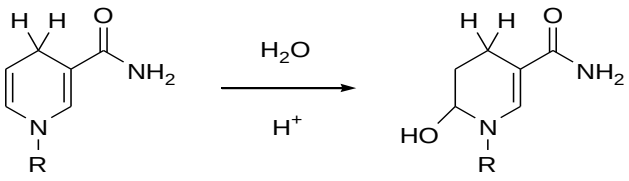


Figure 7: Reaction of hydropyridine in water

The oxidized form of cofactor [$NAD(P)^+$] is generally stable in acidic medium whereas reduced [NAD(P)H] form is stable in basic medium [4]. In acidic medium, decomposition of NAD(P)H occurs by protanation, epimerization and cyclization [Figure 8] [16].

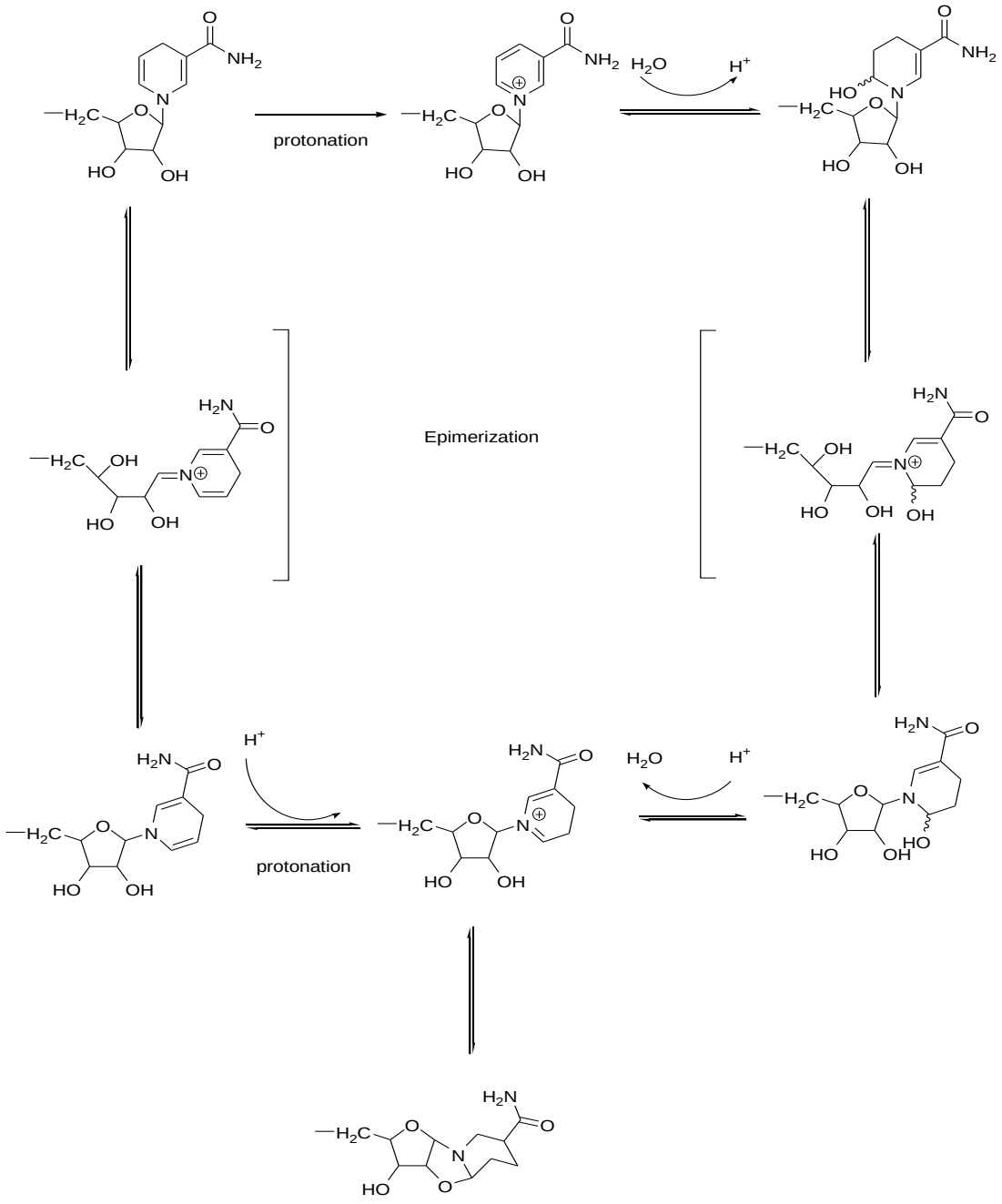


Figure 8: Decomposition reaction of NAD(P)H in acidic medium

In basic medium, NAD(P)H decomposes by hydrolytic attack to the nicotinamide ribose bond or nucleophilic addition to the nicotinamide ring [Figure 9] [17].

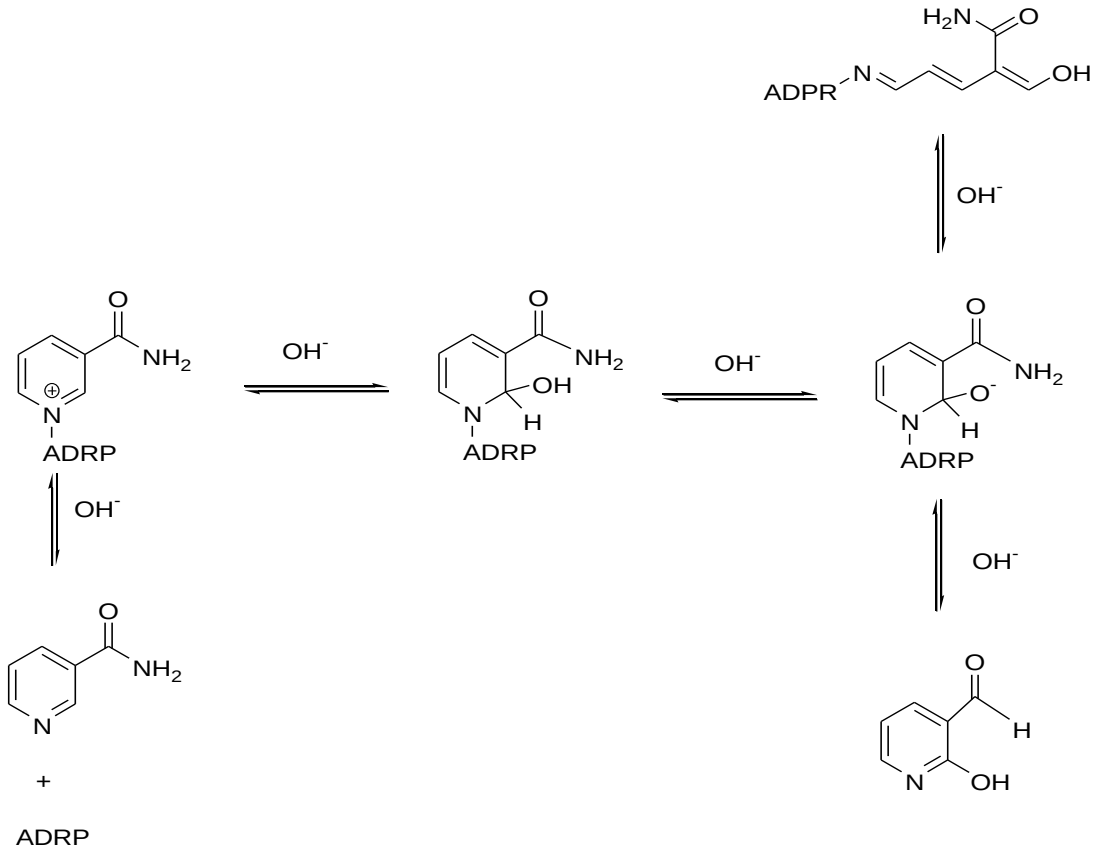


Figure 9: Decomposition reaction of NAD(P)H in basic medium

Both the oxidized and reduced forms are present in reaction medium during the enzymatic reaction because there is equilibrium between reduced and oxidized form of the cofactor. Therefore, pH range has a profound effect on stability of the cofactor. pH range should be 7-7.5 for reduction where NAD-NADH couple is used as cofactor while 8-8.5 for NADP-NADPH couple [1,16]

Nicotinamide cofactors are too expensive to be used as stoichiometric reagents. It's price change from 175 Euro for 10 mg of NADH to 333 Euro for 10 mg of NADPH. Besides, there is no alternative way for cofactor that is; they cannot be replaced by more economical way such as synthetic materials [3, 4]. Therefore regeneration of cofactor is needed to use dehydrogenases in organic synthesis [1, 2, 3, 4, 18, 6].

Important thing is the lower the cost of the cofactor [1, 4]. Therefore regeneration method must recycle the cofactor 10^2-10^6 times in order to be economical. Side reactions of the cofactor lead to accumulation and inhibition of the forward reaction so method has to increase the formation of enzymatically active form of the cofactor because enzyme accepts 1,4 –dihydro-NAD or NADP instead of 1,6 or 1,2 dihydro pyridine formed as a side reactions during the enzymatic reaction. Thermodynamically favored regeneration method can also be used to decrease the cost of the cofactor by catalyzing the unfavorable reactions. Besides regeneration method has to be compatible with the synthetic reactions, practical, inexpensive and convenient. This purpose can be achieved by using available, inexpensive biological molecules and reagents. Another property of the regeneration method is related to the reaction that should be operated under conditions in which enzyme and cofactors are stable enough to achieve high turnover number and selectivity. Besides reaction has to simplify the work up, purification and leads to negligible by product formation because by products formation sometimes cause the inhibition of enzyme used for biotransformation. Furthermore purification is problematic due to the formation of byproducts [1, 4, 19]

Two methods have been developed for regeneration of the cofactor to satisfy these properties: enzymatic and electrochemical methods [1, 2, 4].

Enzymatic regeneration method is divided into two parts: coupled - substrate regeneration, coupled -enzyme regeneration system [3, 4]. In the coupled-substrate

system; regeneration is achieved by using one enzyme with two substrates. Enzyme work both oxidized and reduced forms of the cofactor to convert substrates into the products [Figure 10] [3, 4, 19].

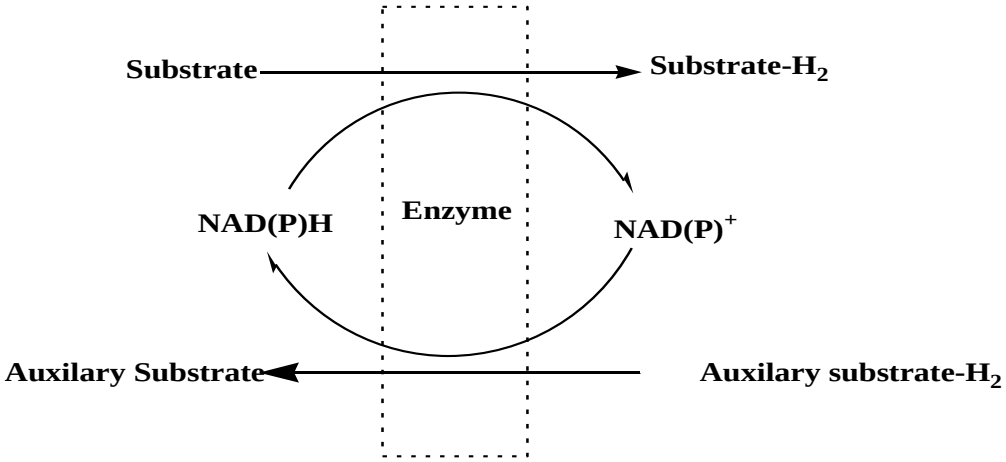


Figure 10: Coupled-substrate system

Some limitations of the system restrict the usage of this regeneration method such as deactivation or inhibition of enzyme by large amount of auxiliary products, less efficiency of the system due to division of the activity of enzyme between two substrates and difficulty of purification of products from large amount of auxiliary substrates [3, 4, 19].

Regeneration of cofactor is achieved by using two enzyme and substrates in the coupled- enzyme system. One enzyme utilizes the reduced form of the cofactor whereas other enzyme uses oxidized from of the cofactor [Figure 11] [3, 4, 19] .This method is more applicable than substrate-coupled system.

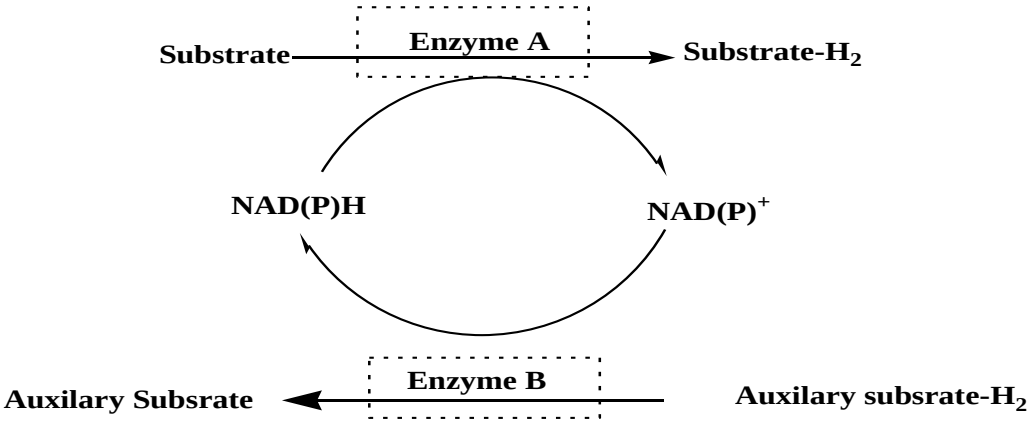


Figure 11: Coupled-enzyme system

Formate dehydrogenases, glucose dehydrogenases, alcohol dehydrogenases and aldehyde dehdyrogenases are enzymes which use oxidized form of the cofactor while glutamate dehydrogenases, lactate dehydrogenases are enzymes which use the reduced form of the cofactor [3, 4, 19].

Enzyme-coupled regeneration method has some requirements. Substrates and enzymes have to be chosen carefully. Enzyme must have different specifities for their substrates in order to catalyze their reactions independently from each others. Otherwise substrates compete for the active site of enzymes which diminish the activity of the enzymes. This system also has some drawbacks such as inhibition of enzymes from the products of the reactions, being not applicable to all dehydrogenases and difficult separation of products at the end of the reaction [3].

1.2.3 Electrochemical regeneration method

Electrochemistry is used for regeneration of different cofactors and coenzymes and is used for both recycling of oxidized and reduced forms of the cofactor [4, 20, 21].

These systems have several advantages over enzymatic regeneration systems. The supply of redox equivalents is mass free, only electrons are transferred which are the cheapest redox equivalents. Beside; second enzyme and cosubstrate are not required so it does not lead to the production of by-products and this also decreases the possibility of enzyme inhibition by educts. Furthermore there is no need to use second enzyme so it decreases the cost of the regeneration system [4, 22, 23]. It divides into two; indirect and direct electrochemical method.

1.2.4 Direct Electrochemical method

Regeneration of the $NAD(P)^+$ occurs on the anode surface in direct electrochemical methods [Figure 12]. High overpotential is needed since conversion occurs at 900mV on the standard calomel electrode [18].

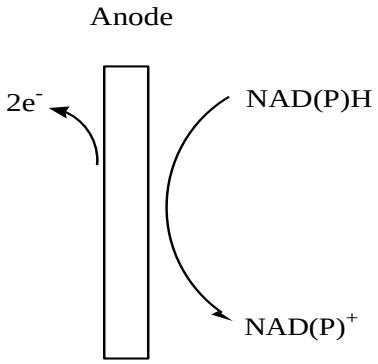


Figure 12: Direct electrochemical method

Direct reduction is taken place in two steps. In the first step one electron is transferred to the $NADP^+$ by forming radicalic species. In the second step protanation

and second electron transfer take place. However dimerization of radicalic species occurs after the first step, before protanation, which is the inactive form of the cofactor. Another undesirable reaction takes place at the protanation step, because the hydrogenation is not selective and enzymatically active 1,4-NAD(P)H and inactive 1,6-NAD(P)H forms [Figure 13]. Therefore this system is suitable for the oxidation-stable substrates [2, 4, 5, 24].

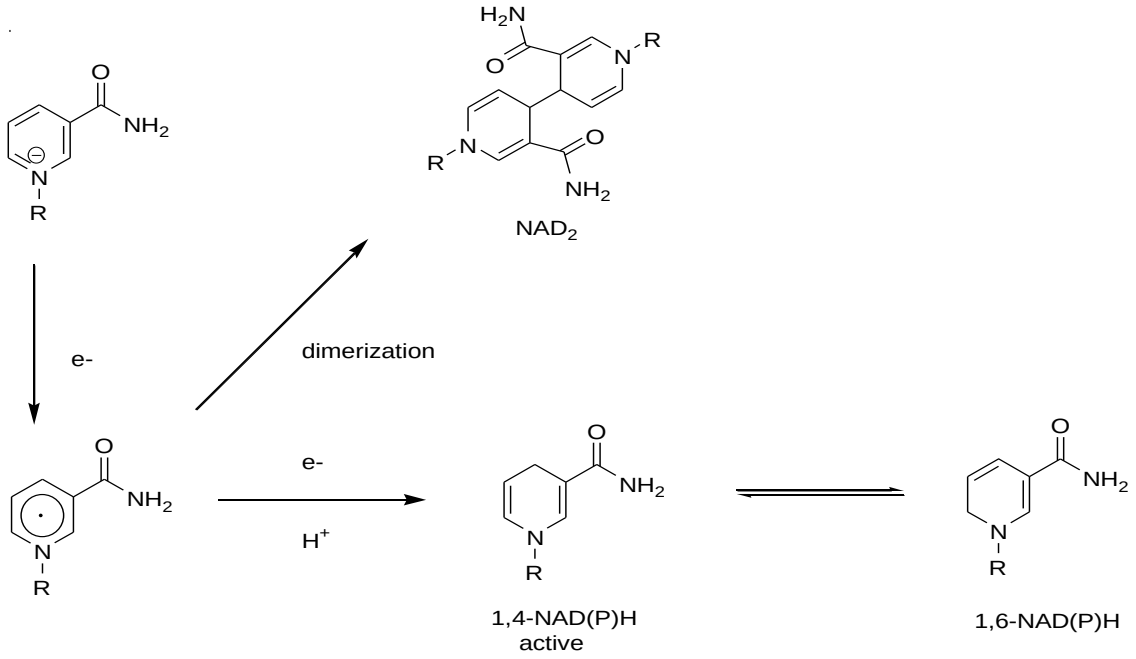


Figure 13: Formation of 1, 4-NAD(P)H and side products in direct electrochemical method

Due to the problems faced with in direct electrochemical method, few examples have been reported by using nicotinamide cofactor. In these examples, to overcome the dimerization step low concentration of cofactor is used because it is thought that side reactions are negligible at low concentrations [18].

1.2.5 Indirect Electrochemical Method

High overpotential needed in direct electrochemical method limits the application of this method. Indirect electrochemical method has been developed to overcome limitations of direct electrochemical methods. In this system, mediators are used as electron carriers which are chemical redox agents instead of direct conversion of nicotinamide cofactor on the anode surface [18] [Figure14].

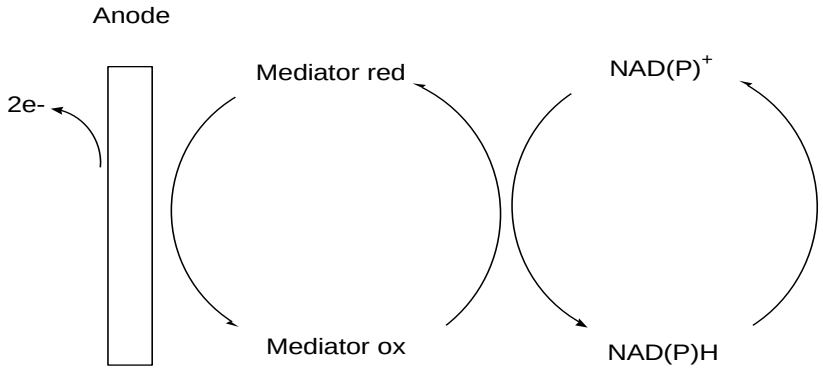


Figure 14: Indirect electrochemical method

Mediators must have some characteristics which make them to be used in this regeneration system. Firstly; two electrons or one hydride is to be transferred at one stage to the substrate. Otherwise if one electron transfer takes place the same problems occur which is seen direct regeneration system. It has to enhance formation of 1,4-NAD(P)H which is active form of the cofactor. Beside; electrochemical activation of mediator must be possible at less negative value then -900mV to prevent the direct regeneration of cofactor [22].

Different metal complexes are used as a mediator. The reason why metal complexes are used is that they increase the electron transfer, lower electron density

within the ligand and increase the solubility of the mediators in medium where reaction has been carried out [25].

The first mediator that met all the requirements which was developed by Steckhan and coworkers, was 2-2'-bipridyl rhodium complex [26, 27, 28].This complex was used for the conversion of cyclohexanone to cyclohexanol. Regeneration cycle was small for cofactor and mediator [29].

Pentamethylcyclopentadienyl (Cp*) was inserted to the rhodium complex to increase the performance of mediator [Figure 15] [30]. Selectivity of enzymatically active form of the cofactor elevated. Then penthamethylcyclopentadienyl 2-2'-bipyridiyl rhodium complex $[Cp^*Rh(bpy)H_2O]^{2+}$ was utilized with different substrates, in electrochemical reactors and aqueous-organic- two phase system. Immobilization of enzymes was also carried out to increase the turnover number of both cofactor and mediator in reactors [31, 32].

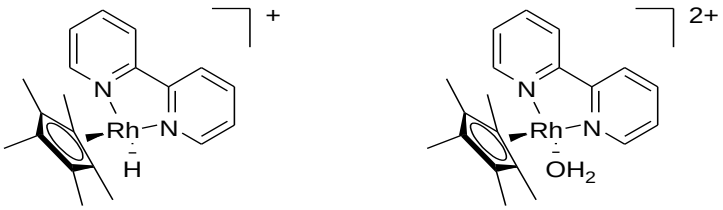


Figure 15: Pentamethylcyclopentadienyl bipyridiyl rhodium complex

The stability of mediator is studied at different temperatures and pHs and found that mediator did not loss its activity when temperature was lower than 80^oC and pH between 5 and 10. The mechanism of mediator was also studied at indirect electrochemical method to understand the function of rhodium contained mediator [Figure 16] [26, 27, 28].

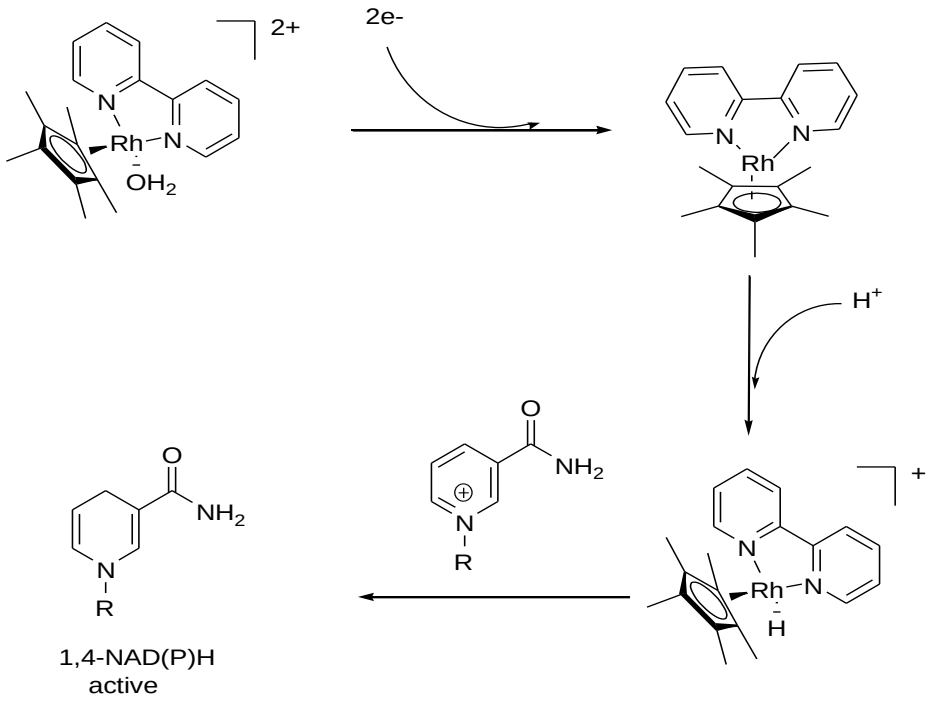


Figure 16: Reduction of $[Cp^*Rh(bpy)H_2O]^{2+}$ with $NAD(P)^+$

In the first step, water in the structure of $[Cp^*Rh(bpy)H_2O]^{2+}$ was converted to the $[Cp^*Rh(bpy)H]^+$ which acts as hydride reagent. Then $[Cp^*Rh(bpy)H]^+$ reacts with oxidized form of the cofactor and1,4-NAD(P)H forms [Figure 16] [26, 27, 28].

In addition to the rhodium bipyridyl complexes; ferrocenes and violegens have also been studied [Figure 17] [9, 15, 33, 34, 35, 36].

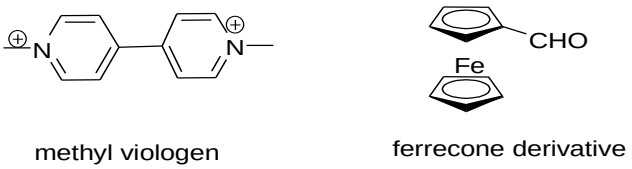


Figure 17: Mediators used indirect electrochemical methods for cofactor regeneration

1.2.6 Immobilization of Enzymes

Enzymes are called “immobilized” if its mobility is restricted by chemical or physical means. The reasons why immobilization is needed are that stability of many enzymes under operational conditions is low, recovery of enzyme from substrates and products are difficult in water and enzymes are not applied to industrial processes because enzymes show limited tolerance to high concentrations of substrates and products [37, 38].

Several immobilization techniques have been developed to overcome these limitations. Immobilization process provides high stability to enzyme, allows repeated and continuous use, easy separation, prevent protein contamination in the products and substrates so it increases the applications of enzymes [3, 37, 39]

Immobilized enzymes were classified for the first time in 1971 at the first Enzyme Engineering Conference in Henniker (USA). Immobilization methods were divided into four [38]:

1. Adsorption
2. Covalent binding
3. Crosslinking
4. Entrapment into gel, matrix or membranes

Adsorption is the simplest and the oldest method of immobilizing an enzyme onto a water-insoluble carrier**.** Enzymes are held the surface of the carrier by physical forces (Wander walls) [Figure18]. The main advantages of adsorption are that it is simple to carry out and has little influence on the conformation of the biocatalyst. This method is not harmful for the enzymes because coupling conditions which is employed in other methods of immobilization are not necessary for this method. However this method is relative weak therefore adsorbed biocatalyst are

easily desorbed by temperature fluctuations, changes in substrates and ionic concentrations [37, 38].

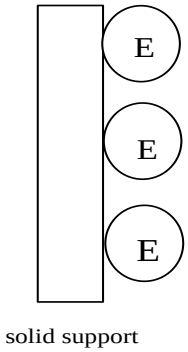


Figure 18: Immobilization by adsorption

Covalent binding involves the formation of a covalent bond between enzyme and a support material [Figure 19]. There are some functional groups on the surface of the support making bond with the functional groups belonging the aminoacid residues on the surface of enzyme. Hydroxy group (OH), amino group (NH_2), carboxylic group (COOH) and the sulfydryl group (SH) are the suitable functional groups for participation in covalent bond formation [37, 38].

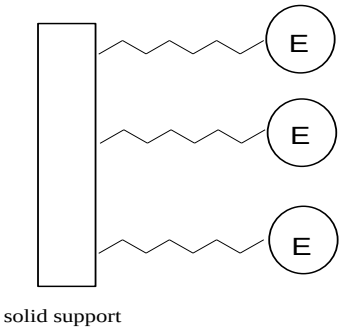


Figure 19: Immobilization by covalent binding

Covalent attachment can be achieved by either direct linkage between the components or via spacer (linker). Covalent attachment is carried out in the two steps. First functional groups on support material are activated by a specific reagent and second the enzyme is added in a coupling reaction to form a covalent bond with a support material. Support material can be inorganic carriers, natural and synthetic polymers [15, 38].

A frequently encountered drawback of this method is that it harms to the enzyme and leads to conformational changes and loss of catalytic activity of the enzyme. However activation of functional group on the surface of carrier instead of enzyme decreases the risk of decreasing catalytic activity of enzyme. There are some methods for the activation of groups such as using cyanogen bromide for activation of OH group or chloride for activation of COOH group [37, 38].

Crossslinking is support free and involves joining the cells to each other to form a large three dimensional complex structure and can be done by chemical and physical methods [Figure 20]. Chemical methods of crosslinking normally involve chemical bond formation. Bi- or multifunctional reagents such as glutaraldehyde and toluene diisocyanate are used for covalent bond formation. Physical crosslinking is carried out by flocculating agents such as polyamines, polyethyleneimine, polystyrene sulfanates and various phosphates [37, 38].

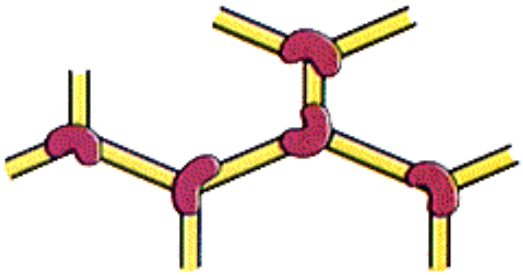


Figure 20: Immobilization with Crosslinking

Encapsulation of enzymes and cells can be achieved by enveloping the biological materials within the various forms of semi permeable membranes [Figure 21]. Coimmobilization has advantage of encapsulation that is enzymes may be immobilized in any desired combination to suit particular applications [37, 38].

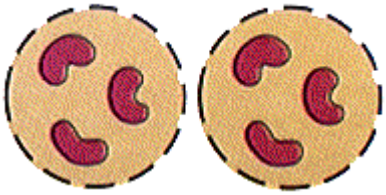


Figure 21: Encapsulation of enzyme

Immobilization in sol- gel matrix has great attention in recent years since it is possible to immobilize antibodies, proteins and organic molecules as well as enzymes.

This method has advantages over classical immobilization techniques including covalent binding, adsorption and crosslinking to a suitable carrier matrix because of the simplicity of preparation, low temperature encapsulation, easy immobilization, chemical inertness, mechanical stability, and negligible swelling behavior [40, 41, 42]. Besides it can construct with organic or inorganic material and it can carry hydrophilic or hydrophobic properties. Small molecules and ions can also go inside the sol- gel. Furthermore this method is applicable for construction of biosensor and electrochemical systems [43].

Metal oxides are used in sol-gel method where metal is titania, silica, alumina and other metal. Silica is commonly used since it is possible to immobilize transition metal complexes for electrochemical application and the catalytic activity of enzyme remains the same in the silica based sol-gel method [41, 43].

Immobilization is carried out in two steps. In the first steps the hydrolysis of metal alkoxides is carried out in the presence of an acid and all metal alkoxides are converted to metal hydroxides [$M(OH)_4$]. Enzyme is added to sol- gel in the second step. As The degree of cross-linking from polycondensation increase the gel becomes viscous and solidifies. Condensation is quite fast and metal network grows around the protein. A porous network is formed around the protein and trapping it inside the sol- gel [41, 43].

1.3 Electrochemical Reactor

To use dehydrogenases in organic synthesis for the optically pure enantiomers, suitable regeneration system is needed. Much effort has focused on improving this regeneration process with electrochemical methods receiving increased attention. Electrochemical microreactor can be a solution for regeneration of the cofactor.

Microreactor have some advantages, decreasing reagent and processing costs, enhancing temperature control, improving process conditions, conversion and selectivity and used for synthesis of organic molecules [44].

Electrochemical reactors use the converse charge flow from the electrode via a mediator, via the co-factor and via the redox-active enzyme to the substrate in order to perform reductive or oxidative chemical transformations. First attempts for the construction of reactors for the enzymatic degration of hazardous materials have been made, e.g., for the degradation of azo dyes [45]. There are few examples about electrochemical reactor are available in the literature.

Batch reactor was designed by coating of methyl violegens and lipamide dehydrogenase on porous RVC electrodes with a Nafion film, long term regeneration was accomplished [46].

Kee et. al [47] report about a laminar flow- based microreactors for electrochemical regeneration of NADH. Immobilization of the biological specials was not done; purification was problematic.

Yoon et. al [48] report laminal flow- based electrochemical microreactor. FAD was used as mediator for the regeneration of NADH without immobilization of the component.

Cheikhov and coworkers developed an electrochemical microreactor. $FAD/FADH_2$ was used as redox mediators. As a model reaction Chiral L- Lactate was synthesized from pyruvate by using LDH as an enzyme [49].

In conclusion all of the regeneration system is not suitable for all dehydrogenases and electrochemical method has a future for cofactor regeneration which has few examples about the usage of electrochemical reactor for cofactor

regeneration so much effort has to be made for development of electrochemical reactor.

1.4 Aim of the Work

General problem of usage of dehydrogenases in organic synthesis is the instability and high cost of cofactor that is needed for carrying out the reaction. Electrochemical reactors can be a solution to overcome these problems.

In EU project, we have tried to develop an electrochemical reactor. Our part in this study is to synthesize mediators and linkers for immobilization of cofactor, mediator and enzyme. In the first part, alkene thiol and disulfides planned to synthesize as linkers because immobilization will be done on gold electrodes and these groups are necessary for the successful immobilization.

In the second part, ferrocenes derivatives and pentamethylcyclopentadienyl rhodium bipyridine complexes will be synthesized as a mediator whose function is to shuttle electrons between electrode surfaces to the cofactor for NAD(P)H recycling.

CHAPTER 2

RESULTS AND DISCUSSION

2.1 Synthesis of linker

Redox reactions have important application in many areas. Much effort has to be done to increase the usage of dehydrogenases in organic synthesis by providing the cofactor regeneration. Electrochemical reactor has future for cofactor regeneration.

In our project we will try to develop an electrochemical reactor by using dehydrogenases for enantiopure synthon preparation [Figure 22].

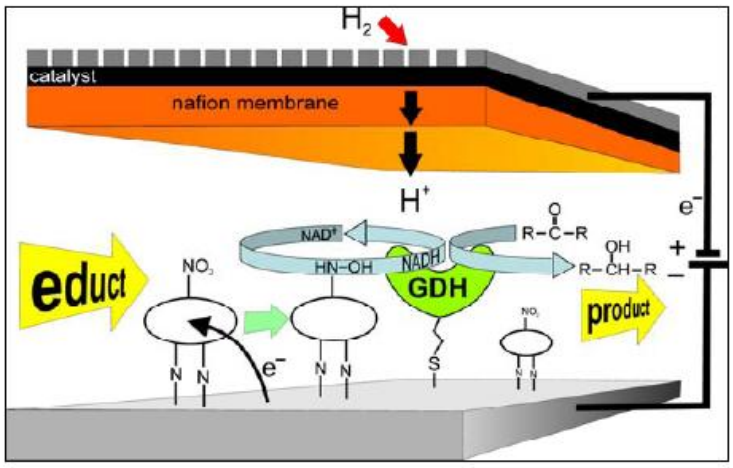


Figure 22: Model for electrochemical reactor

In this system linkers for immobilization of mediators, enzyme and cofactor will be synthesized and all biologically active compounds will be immobilized on the working electrode. In this system only educt and product will exist in the reaction medium and purification will not be problematic. Besides there is a gas diffusion electrode will be employed for the production of clear protons to the catalyst and this leads to decrease in cell voltage, hence undesired side reaction will be minimized.

In this system, working electrode will be prepared from one partner and immobilization of mediator, enzyme and cofactor will be carried out by silica sol- gel method. Gold nanoparticles will present in silica sol-gel film in order to increase the electroactive surface of the electrode. Moreover thiol functionalized mediator or cofactors will also be immobilize on the gold nanostructure to generate efficient three dimensional chains for electron transfer across the bioactive surface layer. In order to synthesize thiol functionalized mediator; suitable linker synthesis is very important.

Our part in this project is to synthesize suitable linker for enzyme, cofactor and mediator immobilization in addition to mediator synthesis. Orgonosulfur compounds which include dialkyl sulfides, dialkyl disulfides and thiols have been investigated on gold. These groups interact with the gold surface strongly. General approach for the immobilization of biomolecules is to synthesize linker which contains thiol and disulfides with functional group such as amine, carboxylic acid or hydroxyl group [50, 51]. Furthermore linker containing disulfide group in its structure breaks into two during immobilization of disulfides on gold surface [Figure 23] [50, 52, 53, 54].

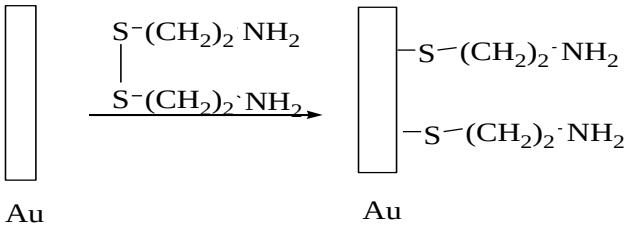
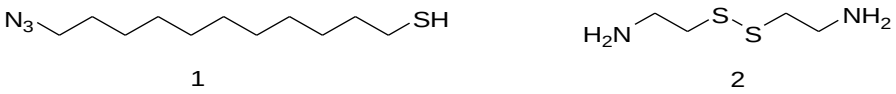


Figure 23: Immobilization of disulfides on gold nano surface

Therefore linkers containing thiol and disulfide units in their structures with terminal azide and amine groups were synthesized. The length of the linker is very important for the flexibility of the mediator after the reaction between mediator and linker. 11- azido-undecan-11-thiol **1** and cystamine dimers **2** were synthesized in our study [Figure 24]



1 2

Figure 24: Linker which is synthesized in this study

11-Bromoundecan-1-ol was used as a starting material for the synthesis of azido-undecan-11-thiol **1**. In the first step, 11-bromoundecan-1-ol was reacted with sodium azide (NaN_3) to convert bromo group to azido group with 87% yield .Then hydroxyl group in the structure of synthesized molecule was activated and converted to good leaving group with NEt_3 and methanesulfonyl chloride. Compound **5** was synthesized with 87% yield. Then mesylated form of the hydroxyl group was reacted with potassium thioacetate by doing reflux in methanol and substitution reaction of mesylate group took place with potassium thioacetate with 80% yield. This

conversion was supported by ^{1}HNMR. There is a singlet at 3.0 ppm which is belonging to -CH_3 group attached to the mesylate group was disappeared and –CH_3 group attached to the carbonyl group was formed at 2.2 ppm [55]. Eventually thioacetate unit was deprotected with K_2CO_3 in methanol 80% yield because immobilization is not done with the protected form of thiol [Figure 25] [56].

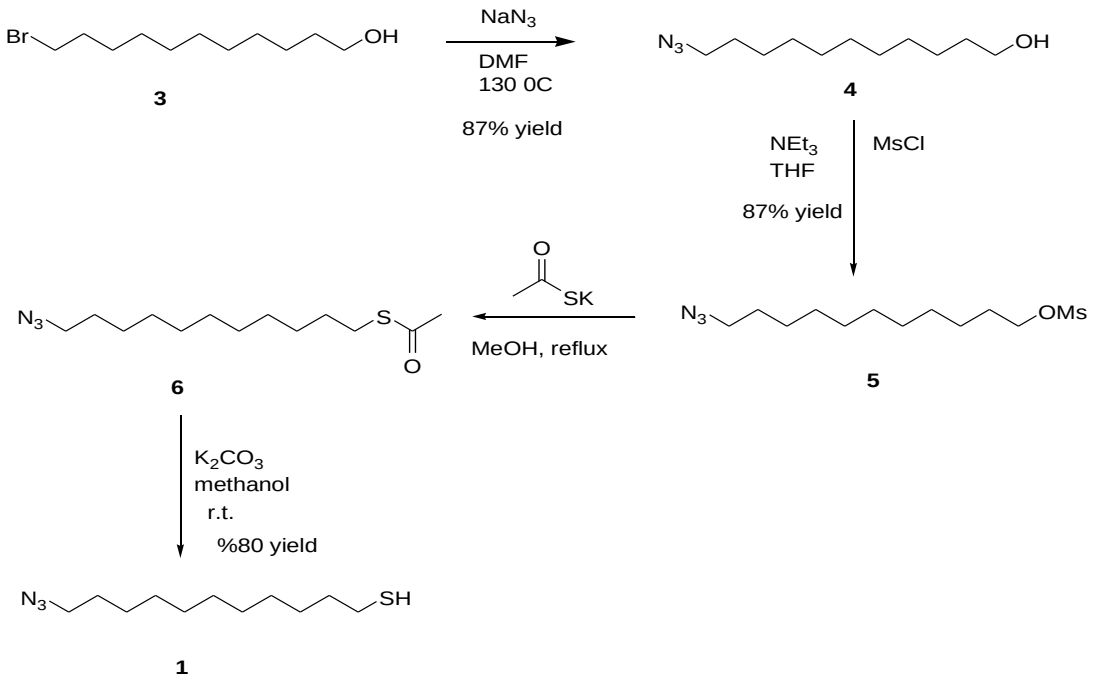


Figure 25: Synthesis of 1-azidoundecan-11 thiol

^{1}HNMR and ^{13}CNMR of the linker were taken to support the product [Figure 26]. Disappearance of methyl peak at 2.2 ppm means that deprotection step was carried out successfully.

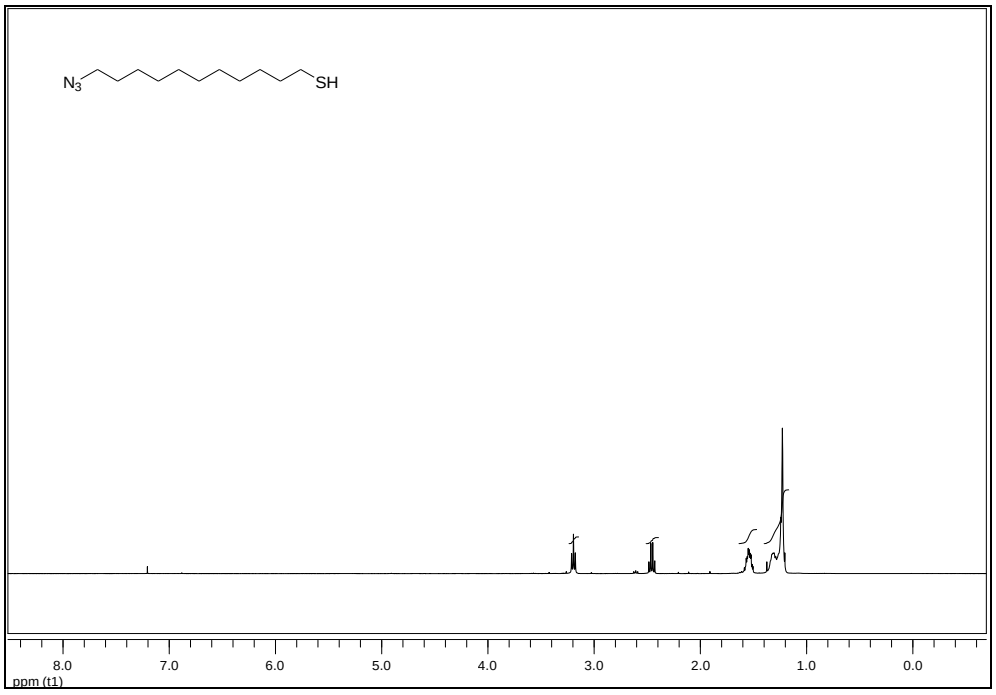


Figure 26:[1]HNMR of 1-azidoundecan-11-thiol

Synthesis of cystamine dimer was carried out in one step by starting form cystamine **7.** In the presence of $NaBH_4$ and cupper salt, cystamine was converted to cystamine dimers **2** [Figure 27] [57].

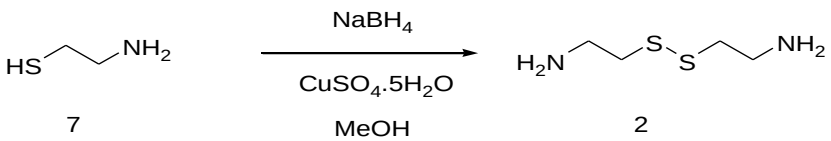


Figure 27: Formation of cystamine dimer

Characterization of this compound was done by ^{1}HNMR by taking integrals of $–CH_2$ groups. Integrals were calculated as four. This indicates that dimerization was carried out [Figure 28].

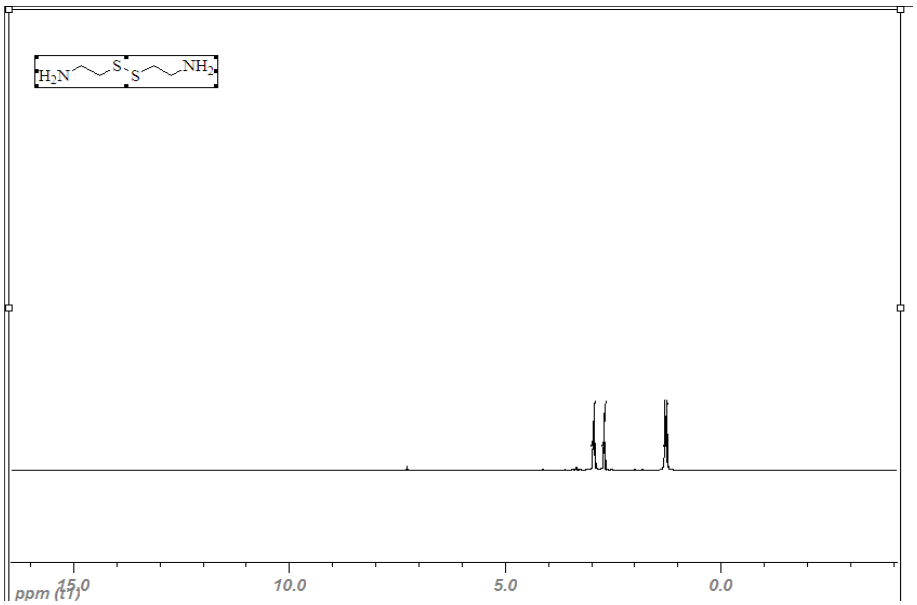


Figure 28: ^{1}HNMR of cystamine dimer

Synthesized linkers were used to react with mediators that is planned to synthesize in this study. Linker having azide functional group was utilized for 1,3-dipolar cyclo addition reaction whereas linker having amine functional group was reacted with the mediator having aldehyde functionality [Figure 29].

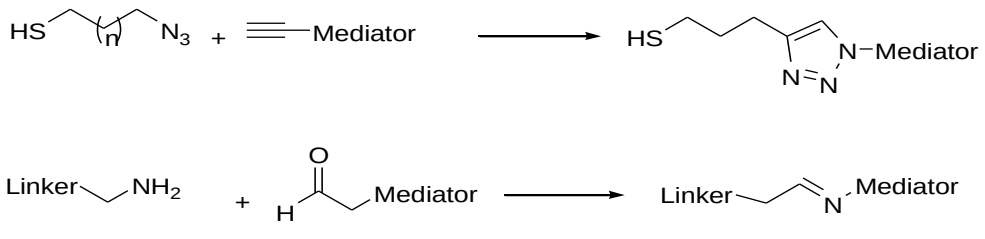


Figure 29: Coupling reactions of linkers with mediator

2.2 Synthesis of mediators

It is very difficult to find a suitable mediator that fulfils all of the requirements for NAD(P)H regeneration effectively. Possible mediators are mercaptohydrochinones, dimethylferrocenes, viologen mediators or pentamethyl-cyclo-pentadienyl rhodium bipyridine complexes. Among them penthamethylcyclopentadienyl rhodium bipyridine was an efficient mediator [28] and it has been applied into several model reaction catalyzed by dehydrogenases, batch reactors and aqueous-organic-two phase systems.

The first application was carried out by Steckhan et. al [30], mediator was used in the conversion of pyruvate to D- lactate by D-lactate dehydrogenases. Turnover number for mediator was 14 and for cofactor was 7. This type of mediator was also used in batch reactor as well as electrochemical enzyme membrane reactor.

Begel and coworkers by using dialysis membrane electrochemical reactor and ultrafiltration dialysis membrane electrochemical reactor to keep the catalyst close to the working electrode. By the help of two reactor %100 conversion of cayclohexanone to cyclohexanol was observed [47].

Hildebrand et al. [31, 32] reported a batch reactor with a carbon felt as a working electrode, platinum grid in a dialysis sack as counter electrode and a

reference electrode of Ag/AgCI, and a high selectivity was observed for the synthesis of (R)-phenylethanol. The immobilized enzyme was also used with the same type of reactor and causes the higher turnover number and enantiomeric excess.

Cofactor regeneration was achieved in an organic two phase system. High turnover number was observed [32].

Therefore we synthesized pentamethylcycopentadienyl rhodium bipyridine and ferrocene derivatives as mediators [Figure 30].

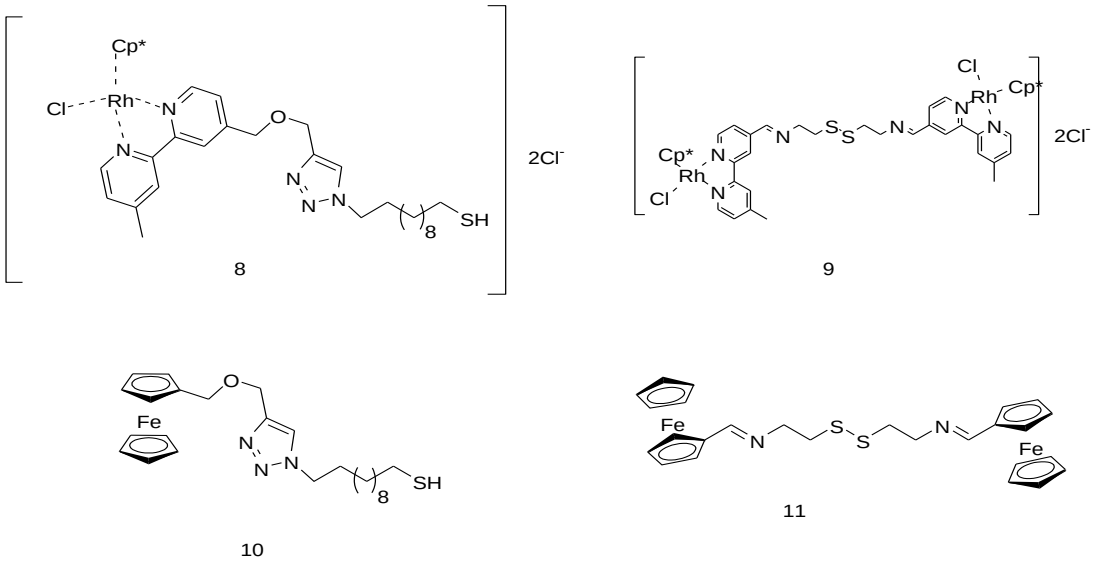


Figure 30: Mediators synthesized in this study

For the synthesis of mediator **8**, 4-4' dimethyl 2-2'-bipyridiyl was used as starting compound and converted into 2-(4-methylpyridin-2-yl)-4-((prop-2-ynyloxy) methyl) pyridine in three steps. In the first step; allylic oxidation of 4-4' dimethyl 2-2', bipyridine was carried out by using SeO_2 [58]. Characterization was done by ^{1}HNMR and formation of the compound was understood from the singlet signal at

4.93 ppm since this signal belongs the $–CH_2$ group attach to the oxygen group. Then crude product was converted directly to the alcohol with $NaBH_4$ and mono alcohol and dialcohol were isolated at the end of the reaction. The desired product yield was 83%. In the second step; mono alcohol was reacted with propargyl bromide in the presence of NaH and desired product **14** containing terminal alkyne functionality was synthesized. The formation of the product was supported by taking ^{1}HNMR, there were two peaks support this conversion. One of them was doublet signal at 4.20 ppm that was the peak of two hydrogen atoms between oxygen and alkyne groups. Other one was a triplet signal at 2.44 ppm that is the peak of hydrogen belongs to the alkyne group [Figure 31].

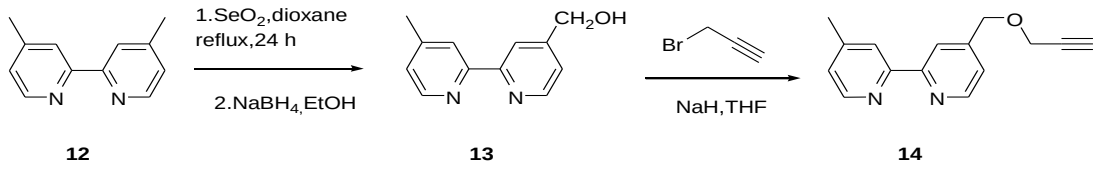


Figure 31: Synthesis of 2-(4-methylpyridin-2-yl)-4-((prop-2-ynyloxy) methyl) pyridine

Alkyne functionality in the structure of 2-(4-methylpyridin-2-yl)-4-((prop-2-ynyloxy) methyl) pyridine, was very important because 1,3-dipolar cycloaddition reaction was carried out with linker contain azido group. Coupling of terminal alkyne group with azido group in linker **1** was done by click chemistry that is used for the synthesis of triazoles with $CuSO_4.5H_2O$ in t-butanol-water mixture and sodium ascorbate and 11-4-(4-(((2-(4-methylpyridin-2-yl) pyridin-4-yl) methoxy) methyl)-1*H*-1,2,3-triazol-1-yl)undecane-1-thiol **15** was synthesized [59]. Yield of the reaction was 7% for this step that was low. The reason for that was the coordination of the cupper with nitrogen atoms of bipyridine or solubility of the reagents in t-butanol and water mixture

First attempt was made by using the cupper salt in stoichiometric amount instead of catalytic amount but yield increased slightly. Then it is looked to the literature for new procedures and it is found that Bathophenanthrolinedisulfonic acid disodium salt hydrate was used to prevent the coordination of salt but it didn't work with our reagent [Figure 32] [60].

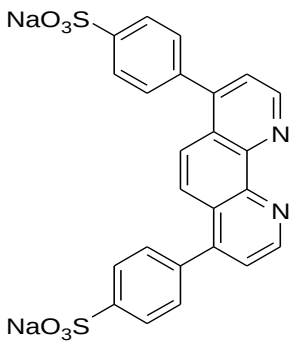
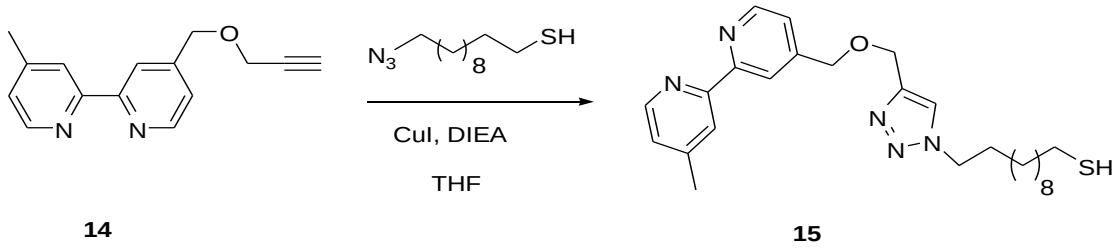


Figure 32: Structure of Bathophenanthrolinedisulfonic acid, disodium salt hydrate

Procedure was changed and instead of water- t-butanol mixture, THF was utilized as a solvent and reaction was carried out with CuI under inert atmosphere in the presence of diisopropyl ethyl amine (DIEA). %100 conversion was observed with our reagent in TLC [Figure 33] [61].



14 **15**

Figure 33: Synthesis of 11-(4-(((2-(4-methylpyridin-2-yl)pyridin-4-yl)methoxy)methyl)-1*H*-1,2,3-triazol-1-yl)undecane-1-thiol

This conversion was supported by ^{1}HNMR. The peak in the structure of 2-(4-methylpyridin-2-yl)-4-((prop-2-ynyloxy)methyl)pyridine, belonging to hydrogen atom attached to triple bond at 2.44 ppm was disappeared and peak was appeared which is in the structure of triazole, at 7.58 ppm as a singlet [Figure 34].

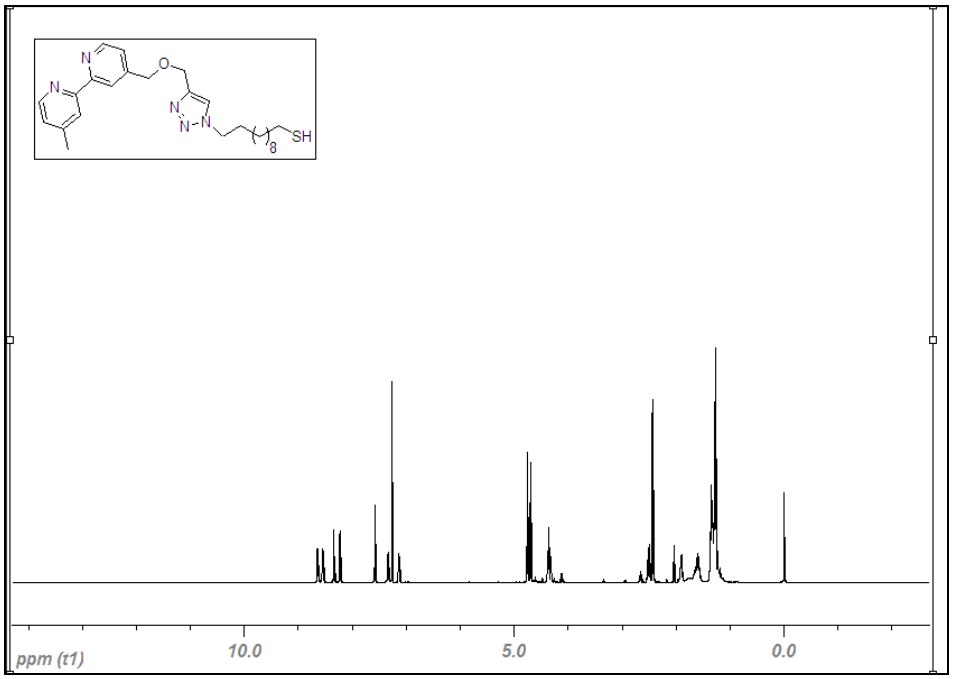


Figure 34: ^{1}HNMR spectrum of 11-4-(4-(((2-(4-methylpyridin-2-yl)pyridin-4-yl)methoxy)methyl)-1*H*-1,2,3-triazol-1-yl)undecane-1-thiol

After finding a suitable procedure, 11-4-(4-(((2-(4-methylpyridin-2-yl)pyridin-4-yl)methoxy)methyl)-1*H*-1,2,3-triazol-1-yl)undecane-1-thiol **15** was converted to the rhodium salt [Figure 35] [28].

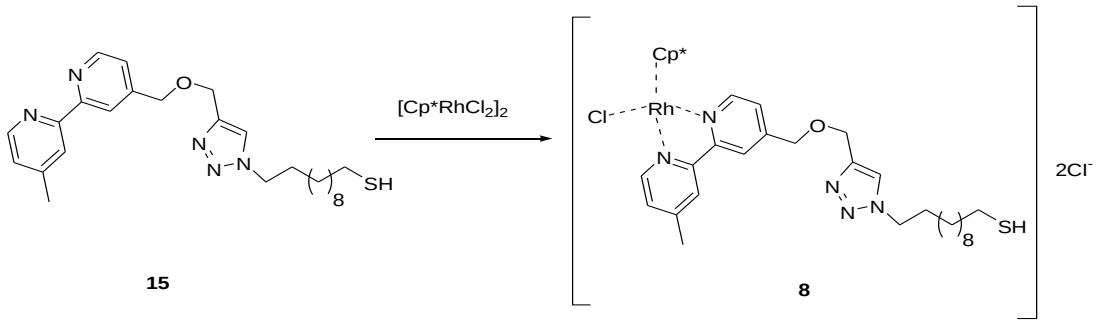


Figure 35: Synthesis of rhodium complex

To understand whether this conversion was carried out or not, ^{1}HNMR and ^{13}CNMR was taken in acetone-d_6, DMSO, water-d_6. NMR data were not clear because molecular weight of the mediator was very high and ^{13}CNMR could not be recorded. ^{1}HNMR taken in DMSO was clearer than the others. The peaks in aromatic region shift to the low field; this is the evidence for this conversion. Peaks seen in aromatic region shifted to the 7.71 ppm to 8.91 ppm compared to the peaks of 11-4-(4-(((2-(4-methylpyridin-2-yl)pyridin-4-yl)methoxy)methyl)-1*H*-1,2,3-triazol-1-yl)undecane-1-thiol [Figure 36].

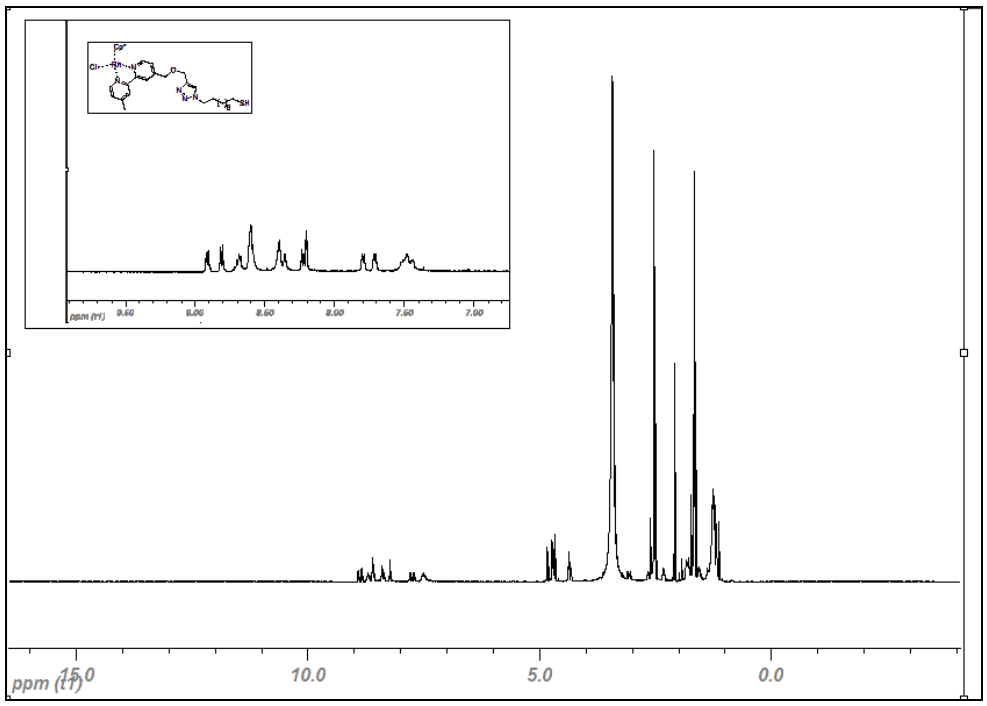


Figure 36: ^{1}HNMR of rhodium complex

In our mediator, there is a chlorine group instead of hydroxyl group in the mediator which is important for the production of hydride [Figure 15, 16]. This does not create any problem because in the buffer, chloride is converted to H_2O directly.

For the synthesis of mediator **9,** 4-4' dimethyl 2-2' bipyridiyl was used again as a starting compound. Firstly; Allylic oxidation of 4-4' dimethyl 2-2' bipyridiyl was carried out by using SeO_2 and reduction was done by $NaBH_4$ and mono alcohol was separated by using flash column chromatography [58]. Synthesized molecule **13** was converted to aldehyde by doing oxidation reaction with MnO_2. Yield of the final product was 58% [Figure 37] [62].

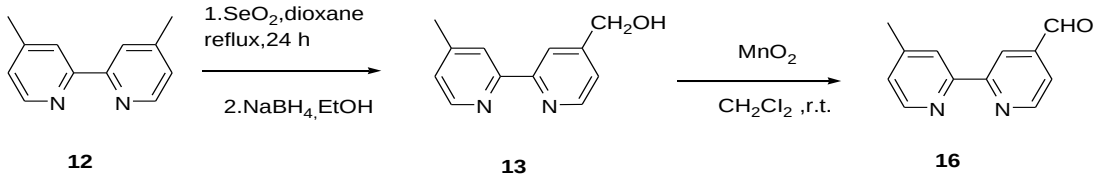


Figure 37: Synthesis of 2-(4-methylpyridin-2-yl)pyridine-4-carbaldehyde

In ^{1}HNMR there is a peak at 10.18 ppm belonging to aldehyde group is the evidence for this conversion [Figure 38].

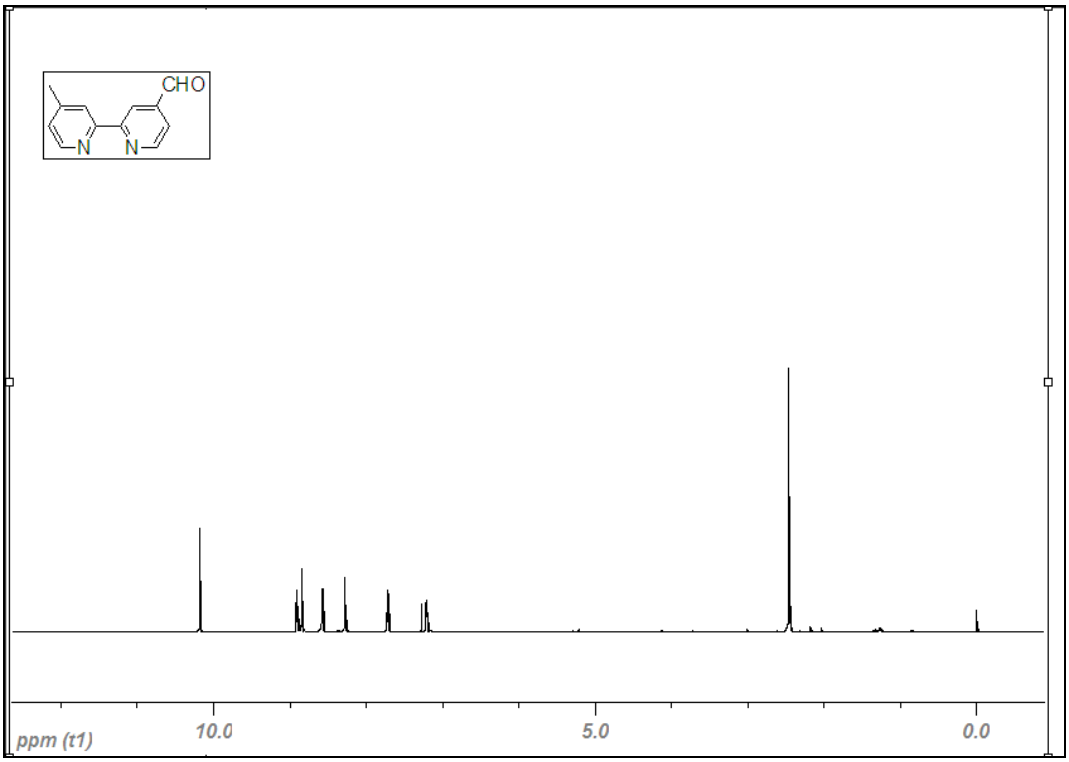


Figure 38: ^{1}HNMR of 2-(4-methylpyridin-2-yl)pyridine-4-carbaldehyde

Bipyridine aldehyde **16** and cystamine dimer **2** was reacted in the presence of molecular sieve in CH_2CI_2, compound **17** was formed with 57% yield. Eventually compound **17** was converted to the rhodium salt [Figure 39].

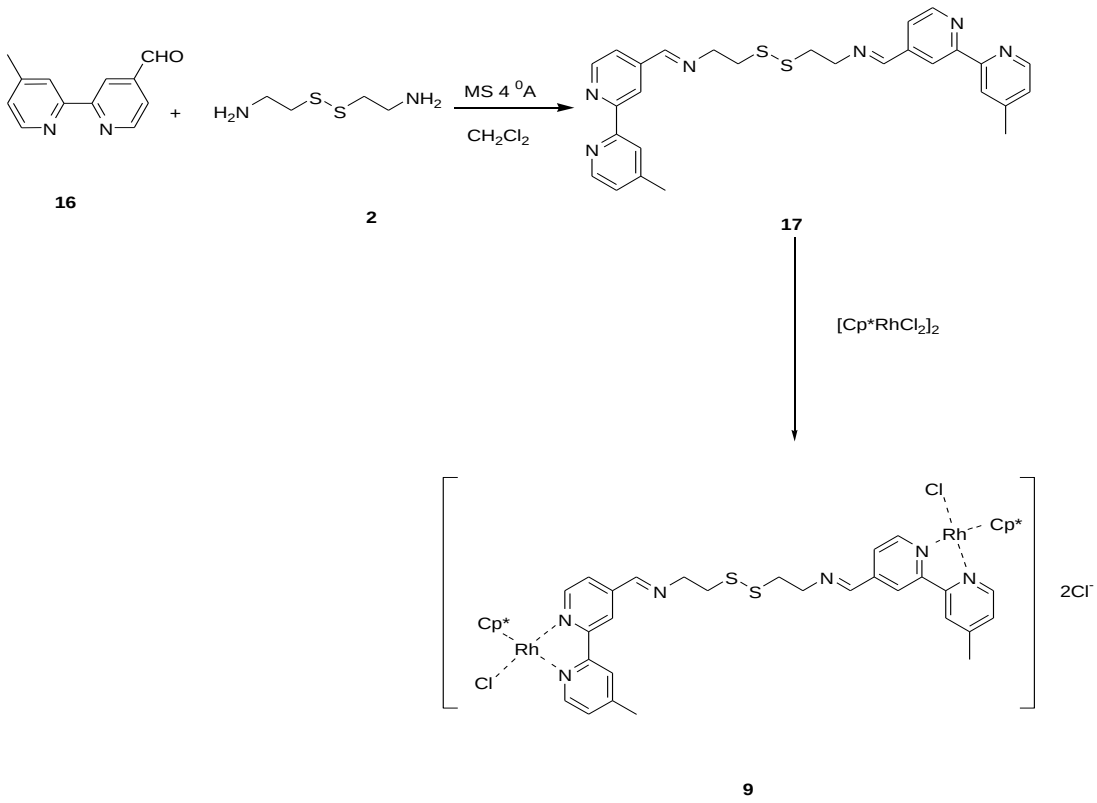


Figure 39: Formation of (E)-2-((4-methyl-2-2'-pyridin-4-yl)methyleneamino)ethane-1-thiol dimers rhodium complex

The reason for the conversion of cystamine into cystamine dimers in the linker synthesis was that there is an iminium formation in the synthesis of mediator **9;** intramolecular attack of thiol group to the imine occurs after the first step [Figure 40].

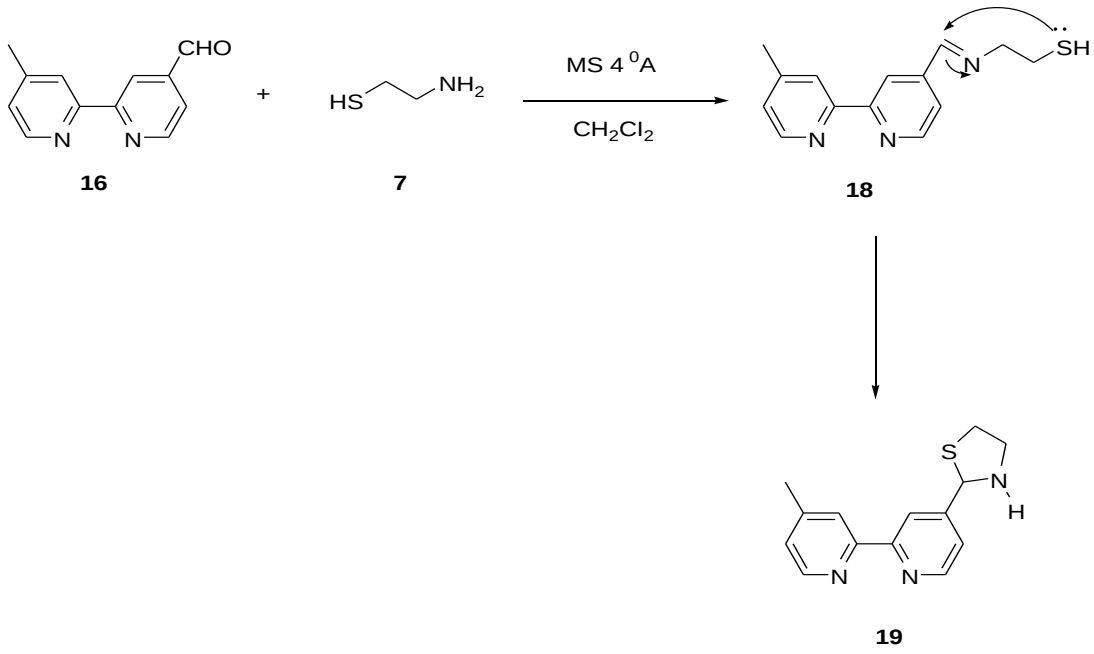


Figure 40: Intramolecular attack of cystamine to imino group

For the synthesis of ferrocene derivatives, the same procedure used for the synthesis of bipyridine rhodium complexes, was employed. Ferrocene carboxyaldehyde **20** was used as a starting material and transformed to the corresponding alcohol **21** by reacting with $NaBH_4$ in methanol. In ^{1}HNMR peaks seven hydrogens of ferrocene come together and two hydrogens come separately. However in ^{13}CNMR, it is expected to see five different peaks but only three of them were observed since they are not differentiated in NMR Then ferrocene alcohol was reacted with propargyl bromide in the presence of NaH and **22** was synthesized quantitatively [Figure 41].

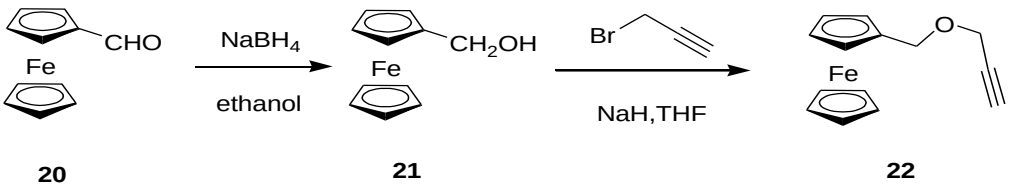


Figure 41: Synthesis of ferrocenyl methoxyprop-1-yne

According to the ^{1}HNMR, formation of compound **22** was understood. Acetylene hydrogen gave triplet signal at 2.41 ppm by coupling with the $–CH_2$ group and due to high electron density of ferrocene, ferrocene hydrogens came at 4 ppm. After synthesis of compound **22**, click chemistry was used for the synthesis of triazole by coupling **22** with 1-azidoundecan-11-thiol **1** with $CuSO_4$ and sodium ascorbate in the presence of 1:1 t-butanol- water mixture. Finally ferrocene derivatived mediator **10** was synthesized [Figure 42].

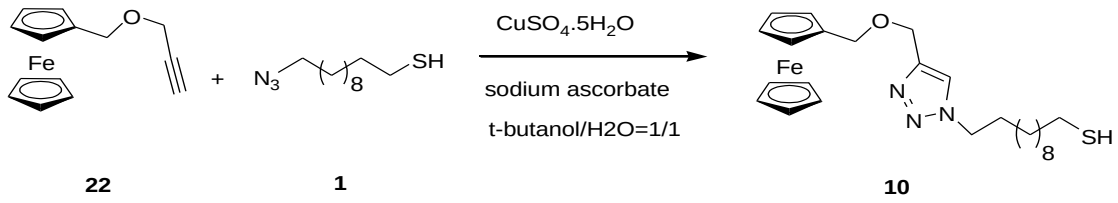


Figure 42: Synthesis of ferrocenyl 11-(4-(ethoxymethyl)-1*H*-1,2,3-triazol-1-yl)undecane-1-thiol

Disappearance of the peak at 2.41 (acetylene hydrogen) and formation of peak at 7.45 (hydrogen in structure of triazole) was the evidence for the synthesis of mediator [Figure 43].

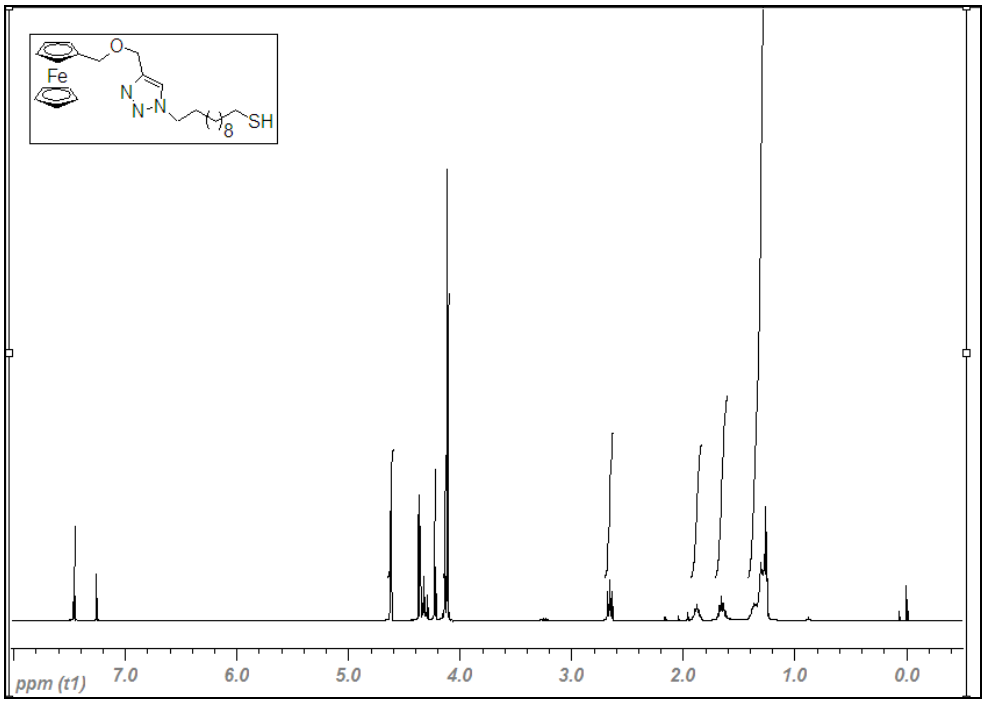


Figure 43: ^{1}HNMR spectrum of ferrocenyl 11-(4-(ethoxymethyl)-1*H*-1,2,3-triazol-1-yl)undecane-1-thiol

Synthesis of ferrocene derived mediator **11** was done in a single step by the reaction of ferrocene carboxyaldehyde and cystamine dimers in CH_2Cl_2. Final product was synthesized quantitatively [Figure 44].

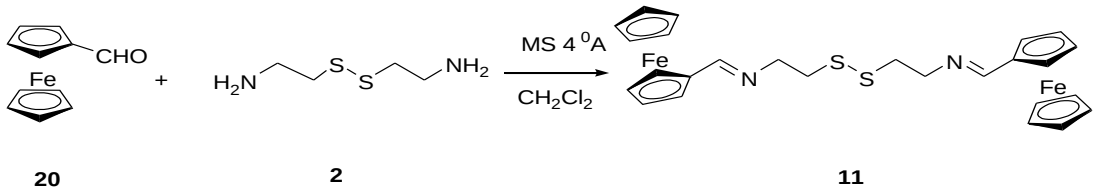


Figure 44: Synthesis of ferrocenyl (E)-2-(ethylideneamino)ethanethiol dimers

[1]HNMR was taken to support the formation of this mediator. Peaks around 4 ppm belong to the ferrocene ring hydrogens and imine hydrogens were appeared at 8.08 ppm [Figure 45].

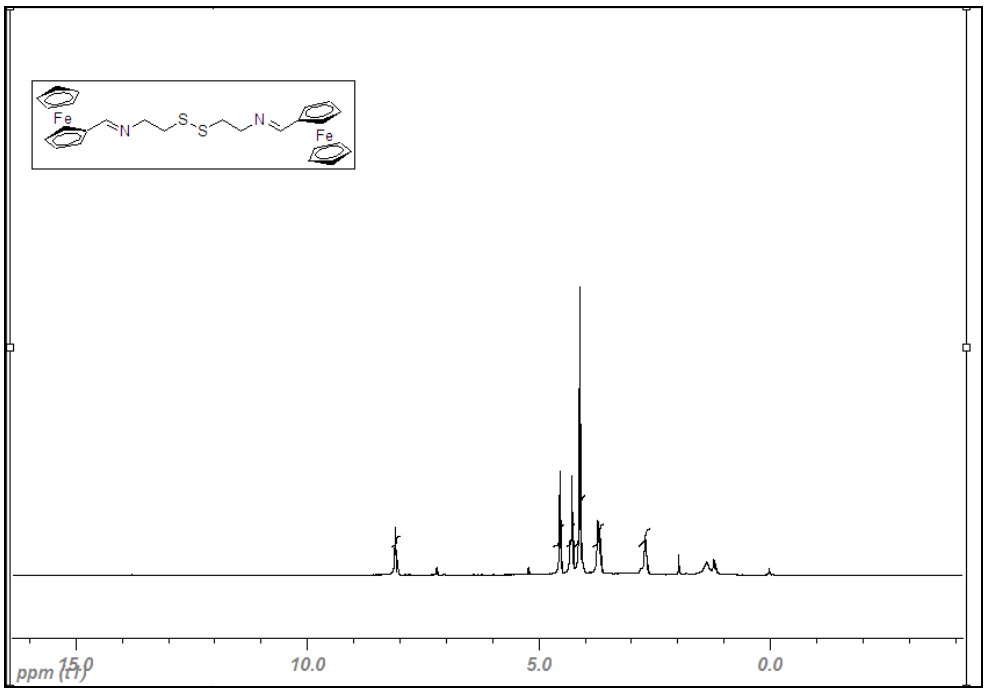


Figure 45: [1]HNMR spectrum of ferrocenyl (E)-2-(ethylideneamino)ethanethiol dimers

Finally four mediators which was the ferrocene and bipyridine derivatives were synthesized. These mediators will be used in the development of electrochemical reactor.

CHAPTER 3

EXPERIMENTAL

NMR spectra were recorded on a Bruker DPX 400. Chemical shifts δ are reported in ppm relative to $CHCl_3$ (^1H: δ=7.26) and $CDCl_3$ (^{13}C: δ=77.0) as an internal standard; coupling constants are reported in Hz. Column chromatography was conducted on silica gel 60 (mesh size 40-63 um). TLC was carried out on aliminium sheets precoated with silica gel $60F_{254}$ (Merck) and the spots were visiualized with UV light (λ=254 nm).

3.1 Synthesis of the Linker

11-bromo undecan-1-ol and NaN_3 were purchased (Aldrich) and used as obtained. The others were prepared from the reaction was these two substances. Published procedures were used for the synthesis of linkers [55, 56, 57].

Synthesis of 11-azido- undecan-1-ol 4:

10.0 g (40mmol) 11-bromoundecan-1-ol and 2.86 g (44mmol) NaN_3 was mixed in 150 ml DMF and reflux was done under inert atmosphere for 24 hours. After cooling to room temperature, water was added and extraction was done by ethyl acetate three times. Organic phase was collected and washed with water three times. Then resulting solution was dried over $MgSO_4$ and solution was removed under reduced pressure. Product which was pale yellow oil was taken. This reaction was quantitative and purification was not done.

^{1}H NMR ($CDCl_3$, 400 MHz) δ:1.25-1.41 (14H, m), 1.46 (1H,bs), 1.52-1.64 (4H,m), 3.26 (2H,t, *J=7.0 Hz*), 3.64 (2H, t, *J=6.6 Hz*). ^{13}C NMR ($CDCI_3$, 100 MHz) 25.5, 26.4, 28.6, 28.9, 29.1, 29.2, 29.3, 32.5, 51.2, 62.8.

Synthesis of 1-azidoundecan-11-methylsulfonate 5:

1.0 g of 11-azidoundecan-1-ol was put in 100 ml balloon and 35 ml dry THF was added. To the stirred solution 1ml methanesulfonyl chloride was added under inert atmosphere. 1.76 ml triethyl amine was mixed with 5 ml dry THF in a separate vial and added to the balloon slowly. The resulting mixture was mixed at room temperature for two hours. After completion of reaction 35 ml ice-cold water was added to this mixture and extraction was carried out with diethyl ether. The combined solution was washed with 0.1 M HCI, water, $NaBH_4$ and water. Then organic phase was dried over MgSO4 and solvent was removed under reduced pressure. Resulting pale yellow oil was product with 83% yields.

^{1}H NMR ($CDCl_3$, 400 MHz) δ:1.24-1.46 (14H, m), 1.55-1.65 (2H, m), 1.69-1.79 (2H,m), 3.00 (3H,s), 3.26 (2H, t, *J=7.0 Hz*), 4.22 (2H,t, *J=6.6 Hz*).

^{13}C NMR ($CDCl_3$, 400 MHz) 25.4, 26.7, 28.8, 29.0, 29.1, 29.3, 29.4, 37.3, 51.4, 70.2

Synthesis of 1-azidoundecan-11-thioacetate 6:

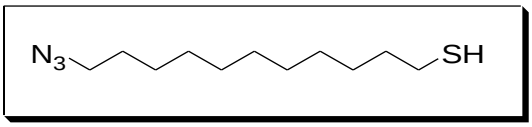
1.0 g 1 azidoundeca-11-methylsulfonate and 0.77 g photasium thioacetate was mixed in methanol. Reflux was done for three hours under inert atmosphere. After completion of reaction methanol was removed under reduced pressure and cold water was added to the mixture. Extraction was carried out diethyl ether and combined organic phases were washed with water. Then solution was dried over $MgSO_4$ and solvent was under removed under reduced pressure. Yield was yellow oil.

^{1}H NMR ($CDCl_3$ 400 MHz) δ:1.16-1.38 (14H,m), 1.44-1.60 (4H,m), 2.25 (3H,s), 2.78 (2H,t,*J=7.3Hz*), 3.18 (2H,t,*J=6.9 Hz*). ^{13}CNMR ($CDCI_3$, 100MHz) 26.1, 26.7, 28.8, 28.8, 29.1, 29.1, 29.4, 29.4, 30.6, 51.5, 58.5, 72.9, 95, 9.

Synthesis of 1- azidoundecan-11 thiol 1:

Potassium carbonate (593 mg, 4,3 mmol) was added to 1-azidoundecan-11-thioacetate (600 mg, 2,2 mmol) in methanol (5 mL). The mixture was stirred for 4 h under nitrogen at room temperature and then poured into $NaHCO_3$. The mixture was

extracted with $CHCl_3$ and washed with brine. The organic phase was dried with anhydrous $MgSO_4$, and the solvent was evaporated.



^{1}H NMR ($CDCl_3$, 400 mHz) δ: 1.16-1.38 (14H, m), 1.46-1.60 (4H,m), 2.40-2.49 (2H,m), 3.14-3.22 (2H,m).

Synthesis of cystamine dimer 2:

A solution of $CuS0_4$ (0.025 g, 0.1 mmol) in methanol (50 mL) is added to $NaBH_4$ (190.0 mg, 5 mmol) and the mixture was stirred under reflux for 3 h. The mixture was cooled to room temperature under nitrogen. After adding a solution of cystamine (771.0 mg, 10 mmol) in methanol (10 mL), the reaction mixture was stirred at room temperature for 3 h. After completion of the reaction, the solids are removed by filtration. Methanol was evaporated under reduced pressure to give pure disulfide.

^{1}H NMR ($CDCl_3$, 400 mHz) δ: 1.27 (4H, s), 2.65-2.75 (4H, m), 2.90-3.00 (4H,m). ^{13}CNMR ($CDCl_3$, 100MHz) 40.6, 42.6.

3.2 Synthesis of Mediators

4-4'- dimethyl -2-2' bipyridine , SeO_2, propargyl bromide, $NaBH_4$, Ferrocene carbaldehyde and cystamine were purchased from Aldrich and used as a chemical for the synthesis of mediators. Published procedures were used for the synthesis of mediators [58, 59, 60, 61, 62].

Synthesis of (2-(4-methylpyridin-2-yl) pyridin-4-yl) methanol 13:

2.0 g (1 4-equivalent) 4-4 dimethyl -2-2' bipryridine stirred in 1-4 dioxane and 2.0 g (1.6 equivalent) SeO_2 was added to this solution. Reflux was done under inert atmosphere by 24 hours. Filtration was done and solvent was removed under reduced pressure and resulting solid was dissolved in chloroform and filtered three times to get rid of SeO_2 by products. Methanol was added and $NaBH_4$ was added in O^oC to this solution for reduction reaction. Then solution was stirred at room temperature for one hour. Methanol was removed by using rotavapour and Na_2CO_3 was added to resulting solution and extraction was done by using chloroform. Organic phases was collected and dried over $MgSO_4$. After filtration, solution was removed under reduced pressure. Column chromatography was done with 1:1 ethyl acetate, hexane and 5% triethyl amine. (2-(4-methylpyridin-2-yl) pyridin-4-yl) methanol was taken with 83% yield.

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.45 (3H, s), 4.93 (2H, s), 8.65 (H, d,*J=5.0 Hz*), 8.54 (H,d,*J=4.9 Hz*), 8.35-8.36 (1H, m), 8.23-8.25 (1H,m), 7.33-7.37 (1H,m), 7.12-7.17 (1H,m) ^{13}C NMR ($CDCl_3$,100 MHz) 21.3, 63.4, 118.8, 121.2, 122.4, 124.9, 148.5, 148.9, 149.3, 151.6, 155.9, 156.2.

Synthesis of 2-(4-methylpyridin-2-yl)-4-((prop-2-ynyloxy) methyl) pyridine 14:

720.0 mg (10 equivalent) NaH was suspended in dry THF and 600.0 mg (1 equivalent) (2-(4-methylpyridin-2-yl) pyridin-4-yl)methanol was added to this solution. After 10 minutes 1.5 equivalents propargyl bromide was added. After completion of reaction, $NaHCO_3$ was added to the mixture and extraction was done by chloroform. Organic phase was combined and dried over $MgSO_4$. After filtration, solution was removed under reduced pressure and purification was done by column chromatography.

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.38 (3H, s), 2.41 (2H, t, *J=2.4 Hz*), 4.20 (2H, d,*J=2.4 Hz*), 4.64 (2H,s), 7.05-7.06 (1H, m), 7.06-7.08(1H,m), 8.16-8.18 (1H,m), 8.27-8.29 (1H,m), 8.46 (1H,d,*J=5.0 Hz*), 8.57 (1H,d,*J=5.0 Hz*).

Synthesis of 11-4-(4-(((2-(4-methylpyridin-2-yl)pyridin-4-yl)methoxy)methyl)-1*H*-1,2,3-triazol-1-yl)undecane-1-thiol 15:

0.24 mg (0.25 mmol) 11-azidoundecan-11-thiol and 0.5 mg (2.10 mmol) (2-(4-methylpyridin-2-yl) pyridin-4-yl) methanol was mixed with THF and CuI (12 mmol, 23 mg), and DIEA (0.986 g, 7.6 mmol). Solution was stirred 24 hours at room temperature. After completion of reaction extraction was carried out with ethyl acetate three times. Then combined organic phase was dried over $MgSO_4$ and solvent was evaporated under reduced pressure and purification was done with column chromatography.

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.10-1.40 (14H,m), 1.52-1.63 (2H,m), 1.77-1.88 (2H,m), 2.43 (3H,s), 2.47-2.55 (2H,m), 4.34 (2H, t, *J=7.5 Hz*), 4.69 (2H,s), 4.75 (2H,s), 7.1(1H,s), 7.34 (1H,s), 7.58 (1H,s), 8.23 (1H,s), 8.34 (1H,s), 8.53 (1H,d,*J=4.2 Hz*), 8.64 (1H,d, *J=4.8Hz*). ^{13}CNMR ($CDCl_3$, 100MHz) 21.2, 24.6, 26.5, 28.3, 28.9, 29.0, 29.2, 29.3, 29.4, 30.3, 34.0, 50.4, 64.6, 70.9, 119.4, 121.9, 122.0, 122.4, 124.8, 143.5, 144.7, 148.2, 148.9, 149.3, 155.8, 156.4.

Synthesis of 11-4-(4-(((2-(4-methylpyridin-2-yl)pyridin-4-yl)methoxy)methyl)-1*H*-1,2,3-triazol-1-yl)undecane-1-thiol rhodium complex 8:

To 0, 25 mmole Rh dimer in 5 ml MeOH is 5 mmol of 4-(4-(((2-(4-methylpyridin-2-yl)pyridin-4-yl)methoxy)methyl)-1H-1.2.3-triazol-1-yl)butane-1-thiol were added. After 30 minutes, mixture is filtrated and MeOH is removed under reduced pressure. Then, ether is added to give a suspension and the mixture is stirred overnight. After completion of reaction, the mixture is filtrated to give Rh complex.

Synthesis of 2-(4-methylpyridin-2-yl)pyridine-4-carbaldehyde 16:

The bipyridine alcohol (5.00 g, 35.0 mmol) was dissolved in CH_2CI_2 (100 mL), and activated MnO_2 (20.0 g) was added at room temperature over a 4-h period. The suspension was stirred overnight and filtration was done and the pale brown filtrate was evaporated under reduced pressure to yield crude product. Purification by column chromatography (silica; ethyl acetate-toluene, 1:1) was carried out and product was taken with 58% yield as white crystals.

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.45 (3H,s), 7.19 (1H,d,*J=5.0 Hz*), 7.71-7.73 (1H,m), 8.28 (1H,s), 8.58 (1H,d,*J= 4.9 Hz*), 8.83 (1H,s), 8.89 (1H, d, *J=4.9 Hz*), 10.18 (1H,s). ^{13}C NMR ($CDCl_3$,100 MHz) 21.2, 120.6, 121.3, 122.1, 125.4, 142.7, 148.4, 149.2, 150.3, 154.8, 158.3, 191.8

Synthesis of (E)-2-((2-(4-methylpyridin-2-yl) pyridin-4-yl) methyleneamino) ethanethiol dimers 17:

1 mmol cystamine dimer and 0.5 mmol aldehyde are dissolved in 2 ml CH_2Cl_2. After 2 h, the reaction mixture is filtrated. The solvent is removed under reduced pressure.

^{1}HNMR ($CDCl_3$, 400 MHz) δ:2.35 (6H, s), 3.00 (4H,t, *J=6.6 Hz*), 3.90 (4H, t, *J=6.4 Hz*), 6.98-7.07 (4H,m), 7.55-7.63 (2H,m), 8.10-8.20 (4H,m), 8.29 (2H,s), 8.40-8.46 (4H,m), 8.53 (2H,s), 8.62 (2H, d, *J=8.6 Hz*).^{13}CNMR ($CDCl_3$, 100MHz) 21.3, 63.4, 118.8, 121.5, 122.4, 124.9, 148.5, 148.9, 149.3, 151.6, 155.9, 156.2.

Synthesis of (E)-2-((2-(4-methylpyridin-2-yl)pyridin-4-yl) methyleneamino) ethanethioldimers rhodium complex 9:

To 0,25 mmole Rh dimer in 5 ml MeOH is 5 mmol of E)-3-(2-(4-methylpyridin-2-yl)pyridin-4-ylimino)propane-1-thiol dimers were added. After 30 minutes, mixture is filtrated and MeOH is removed under reduced pressure. Then, ether is added to give a suspension and the mixture is stirred overnight. After completion of reaction, the mixture is filtrated to give Rh complex.

Synthesis of Ferrocenyl methanol 21:

2 mmol ferrocene carboxyaldehyde was dissolved in ethanol and 2 equivalent $NaBH_4$ was added to stirring solution slowly. After 2 hours, saturated $NaHCO_3$ was added to the solution and extraction was carried out with ethyl acetate. Combined organic phase was dried over $MgSO_4$ and solvent was removed under reduced pressure.

^{1}HNMR($CDCl_3$,400 MHz) δ: 4.16- 4.19 (7H,m) 4.24 (2H, t, *J= 1.8 Hz*), 4.33 (2H,d,*J=5.6 Hz*). ^{13}CNMR ($CDCl_3$, 100MHz) 60.8, 67.8, 68.3.

Synthesis of ferrocenyl methoxyprop-1-yne 22:

0.55 g (23 mmol) NaH was suspended in dry THF and 0.5 g (2.3 mmol) ferrocene methyl alcohol was added to this solution. After 10 minutes 0.41 g (3.4 mmol) propargyl bromide was added. After completion of reaction, $NaHCO_3$ was added to the mixture and extraction was done by chloroform. Organic phase was combined and dried over $MgSO_4$. After filtration, Solution was removed under reduced pressure and purification was done by column chromatography.

^{1}HNMR ($CDCl_3$, 400MHz) 2.42 (1H, t, *J= 2.4 Hz*), 4.11 (2H, d, *J= 2.4 Hz*), 4.12-4.15 (7H, m), 4.24 (2H, t, *J=1.8 Hz*), 4.38 (2H, s). ^{13}CNMR ($CDCl_3$, 100MHz) 56.4, 67.5, 68.5, 68.6, 69.4, 74.3, 79.9, 82, 4.

Synthesis of ferrocenyl 11-(4-(ethoxymethyl)-1*H*-1,2,3-triazol-1-yl)undecane-1-thiol10:

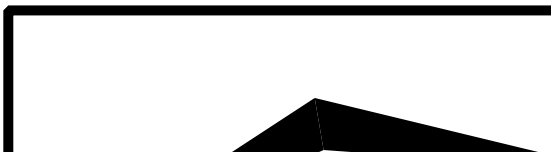
0.55 g (2.4 mmol) 11-azidoundecan-1-thiol and 0.6 g (2.4mmol) ferrocenyl methoxyprop-1-yne was mixed with 1:1 ratio t-butanol and water. %5 mole $CuSO_4$ and 10% mole sodium ascorbate was added to stirring solution. Solution was stirred 24 hours at room temperature. Mixture was poured to water and filtered and product was taken.

^{1}H NMR ($CDCl_3$,400 MHz) δ: 1.14-1.32 (14H,m), 1.42-1.55 (2H,m), 1.76-1.90 (2H,m), 2.66 (2H, t,*J=7.4 Hz*), 3.41 (2H,s), 4.10 (5H,s), 4.13 (2H,t, *J=1.8 Hz*),

4.23 (2H,t, *J=1.8 Hz*), 4.36 (2H,s), 4.62 (2H,s), 7.45 (1H,s) ^{13}CNMR ($CDCl_3$, 100MHz) 26.5, 28.5, 28.9, 29.2, 29.4, 29.4, 30.3, 39.1, 50.2, 63.4, 68.4, 68.5, 68.7, 69.5, 82.5, 83.0, 84.8, 85.4, 96.1.

Synthesis of ferrocenyl (E)-2-(ethylideneamino)ethanethioldimers 11:

1 mmole cystamine dimer and 0, 5 mmole ferrocene carbaldehyde are dissolved in 2 ml CH_2Cl_2. After 2 h, the reaction mixture is filtrated. The solvent is removed under reduced pressure.



^{1}HNMR ($CDCl_3$,400 MHz) δ: 2.60-2.80 (4H,m), 3.60-3.78 (4H,m), 4.12 (10H, s), 4.28 (4H,s), 4.55 (4H,s), 8.08 (2H,s).

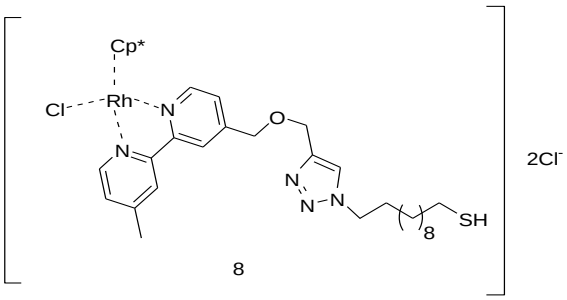
CHAPTER 4

CONCLUSION

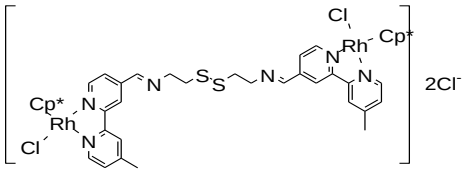
The study can be divided into two parts. In the first part synthesis of linker was carried out, linkers containing thiol and disulfide group were synthesized in order to use this compounds for immobilization of the enzyme, cofactor and mediator in electrochemical reactor working electrode surface**.** 1-azido-undeca-11-thiol **1** and cystamine dimers **2** were synthesized in good yield.

In the second part of the study, mediator synthesis was done; pentamethylcyclopentadienyl rhodium complexes and ferrocene derivatives were synthesized in good yield. For the synthesis of mediators first, 2-(4-methylpyridin-2-yl)-4-((prop-2-ynyloxy) methyl) pyridine **14**, 2-(4-methylpyridin-2-yl) pyridine-4-carbaldehyde **16** and ferrocenyl methoxyprop-1-yne **22** were synthesized. Then 2-(4-methylpyridin-2-yl)-4-((prop-2-ynyloxy) methyl) pyridine **14** ferrocenyl methoxyprop-1-yne **22** were reacted with 1-azido-undecan-11- thiol. Furthermore synthesized 2-(4-methylpyridin-2-yl) pyridine-4-carbaldehyde and commercially available ferrocene carboxyaldehyde were reacted with cystamine dimers. Mediator **11** was synthesized quantitatively. Eventually bipyridine derivatives; 11-4-(4-(((2-(4-

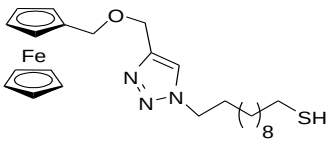
methylpyridin-2-yl)pyridin-4-yl)methoxy)methyl)-1*H*-1,2,3-triazol-1-yl)undecane-1-thiol **8** and (E)-3-(2-(4-methylpyridin-2-yl)pyridin-4-ylimino)propane-1-thiol dimers **9** were converted to the rhodium complex and four mediators were synthesized.



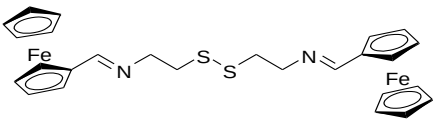
8



9



10



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APPENDIX A:

NMR DATA

NMR spectra were recorded on a Bruker DPX 400. Chemical shifts δ are reported in ppm relative to $CHCl_3$ (^1H: δ=7.27), $CDCl_3$ (^{13}C: δ=77.0) and CCl_4 (^{13}C: δ=96.4) as internal standards. ^1H and ^{13}C NMR spectra of products are given below.

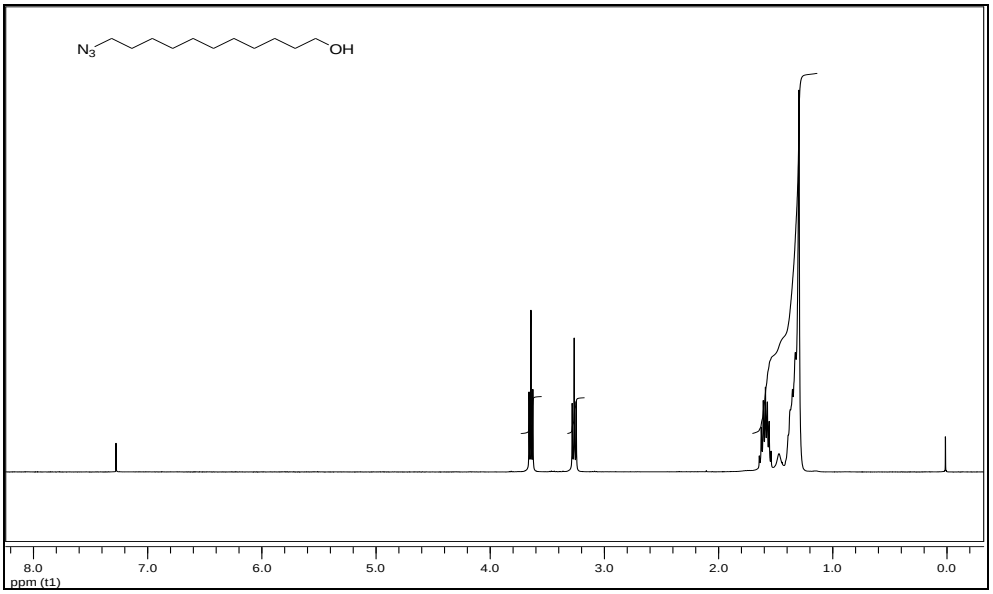


Figure 46. ^1H NMR spectrum of (4)

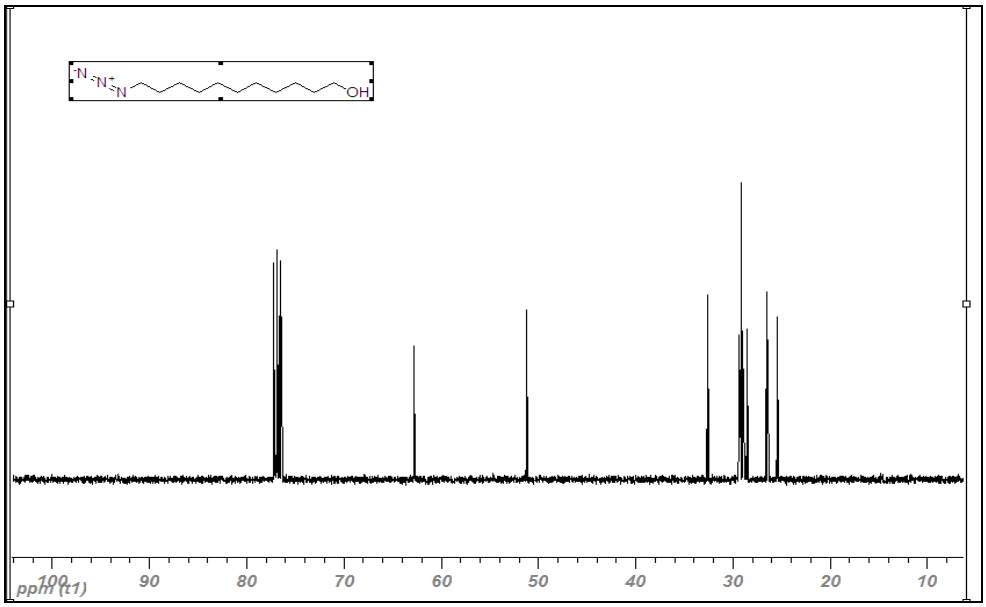


Figure 47. ^{13}C NMR spectrum of (4)

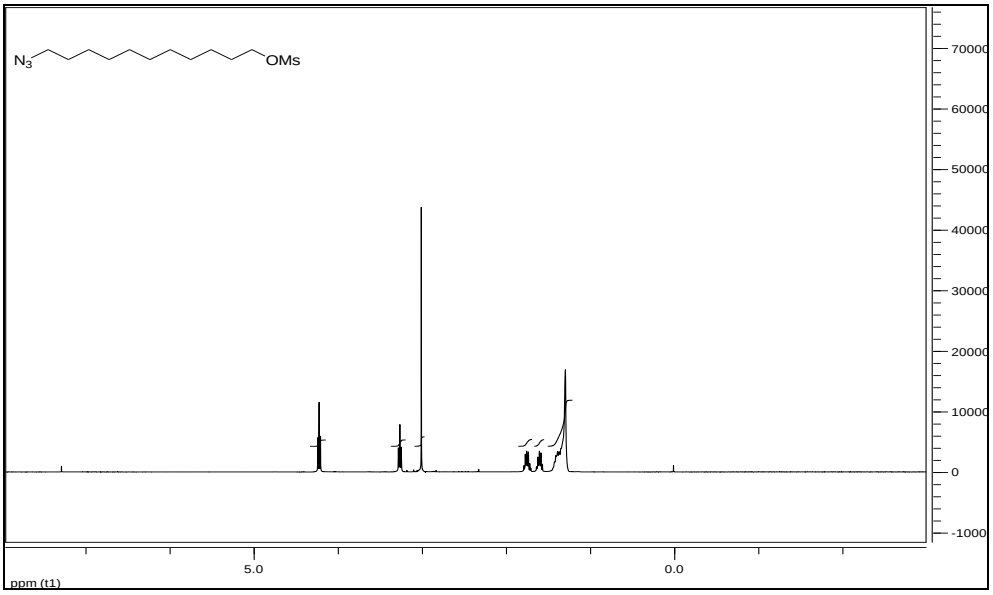


Figure 48. ^{1}H NMR spectrum of (5)

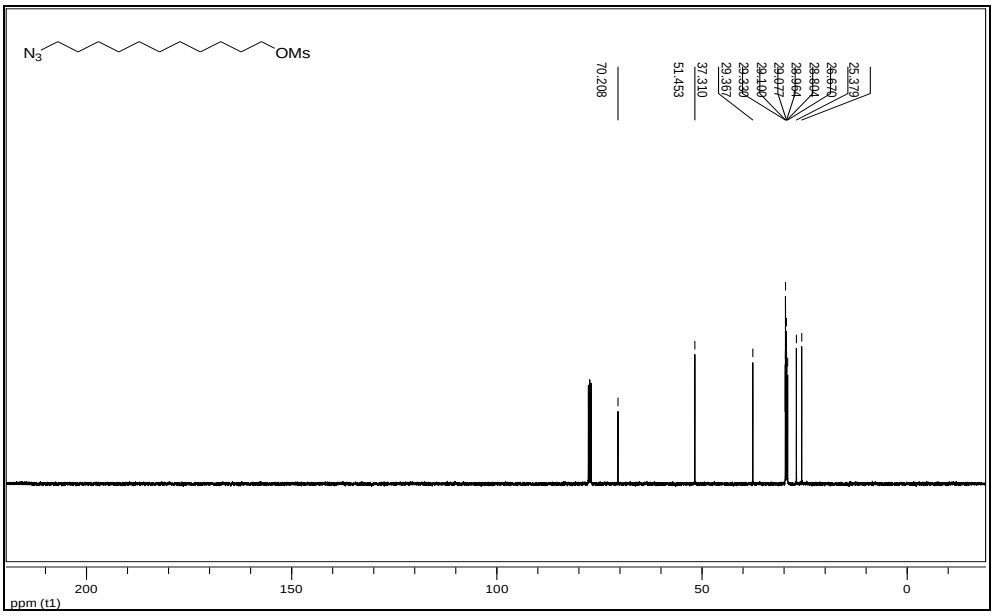


Figure 49. ^{13}C NMR of spectrum of (5)

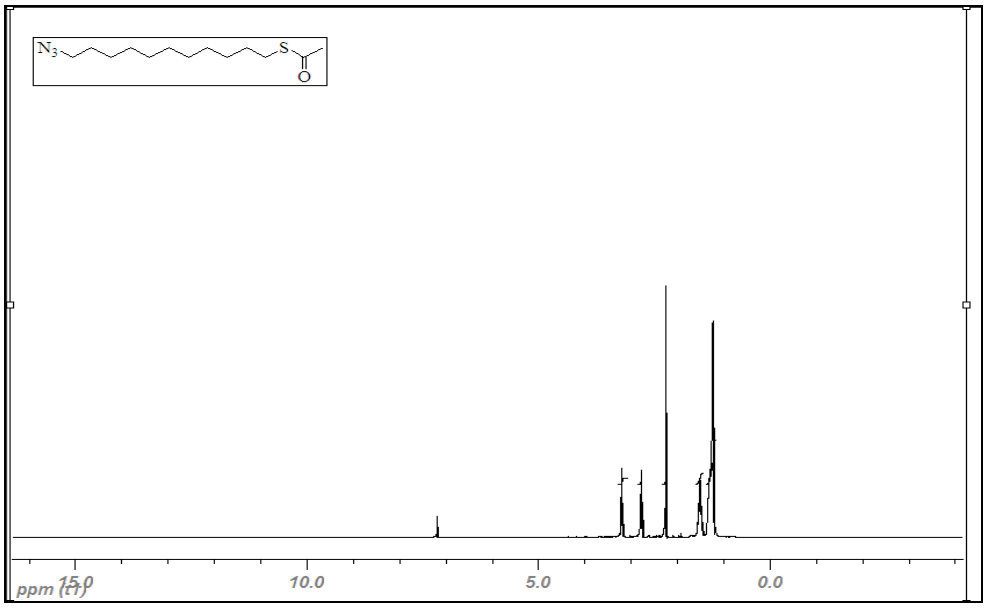


Figure 50. ^{1}H NMR spectrum of (6)

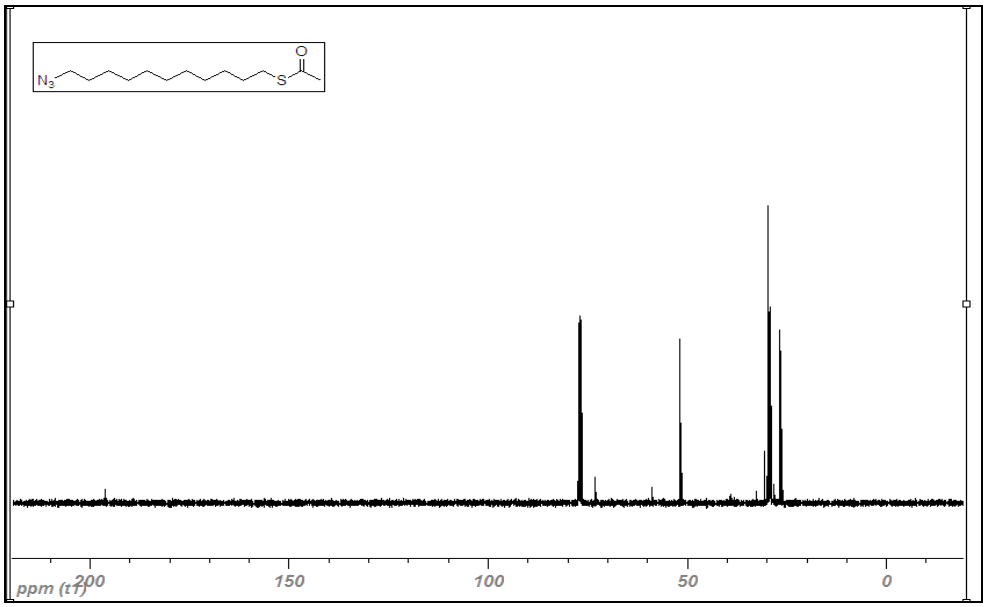


Figure 51. ^{13} CNMR spectrum of (6)

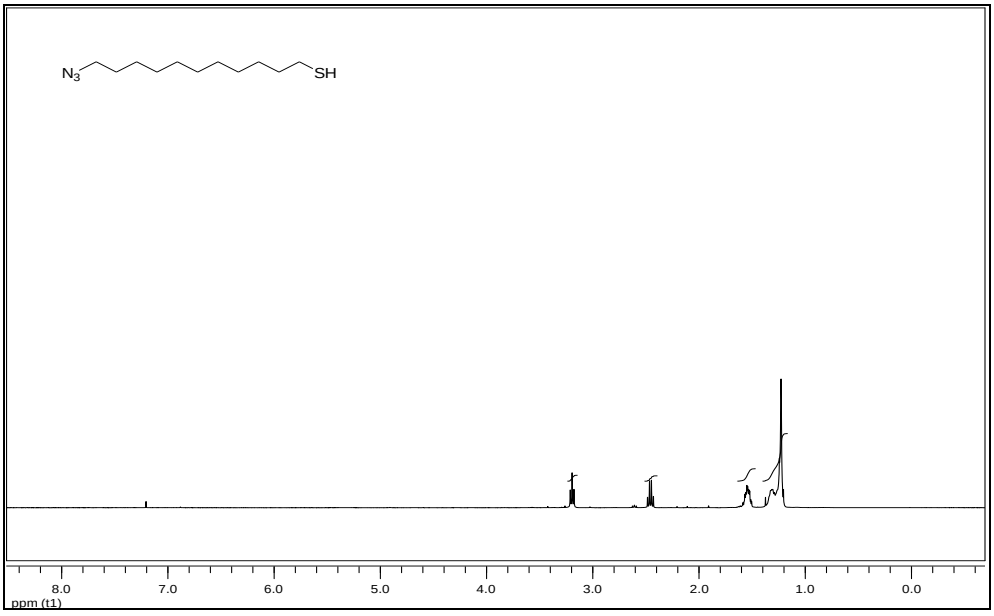


Figure 52. ^{1}H NMR spectrum of (1)

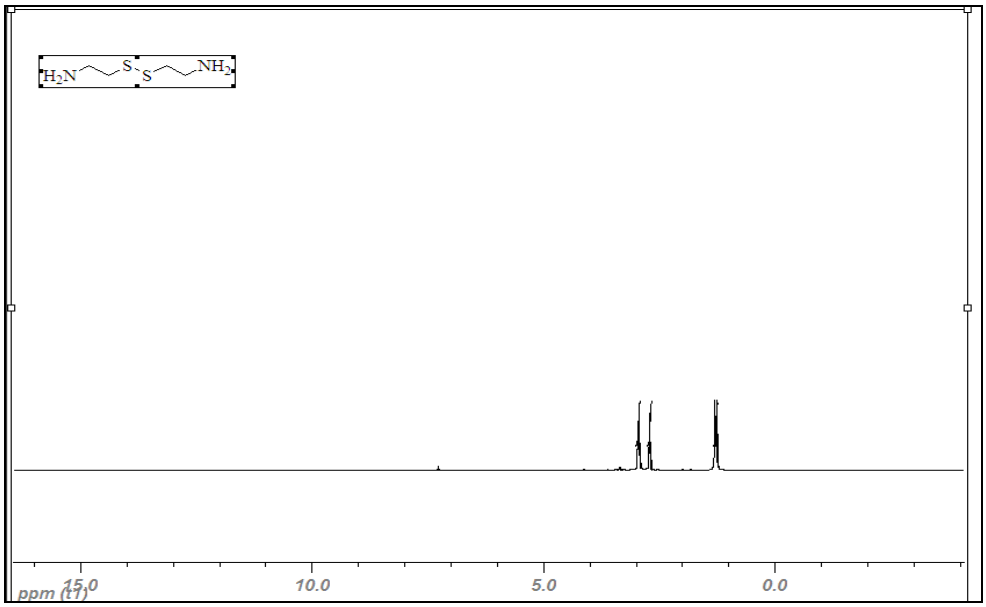


Figure 53. ^{1}H NMR spectrum of (2)

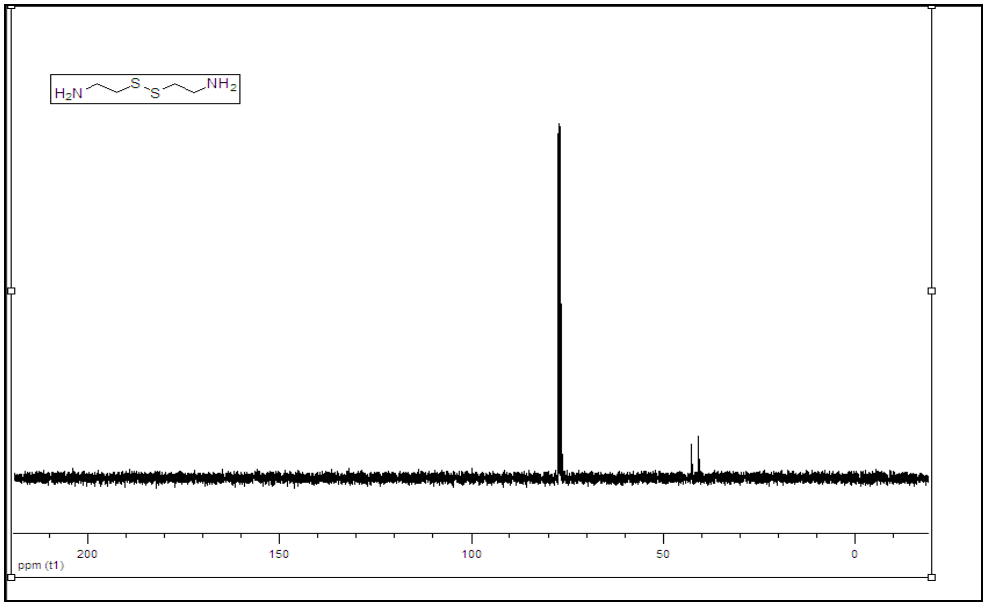


Figure 54. ^{13}CNMR spectrum of (2)

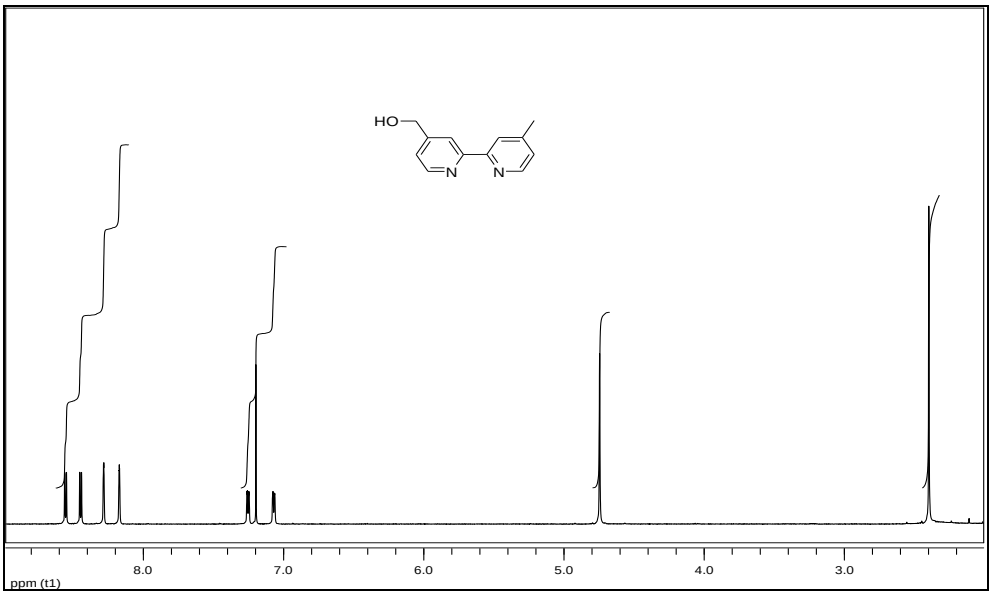


Figure 55. ^{1}H NMR spectrum of (13)

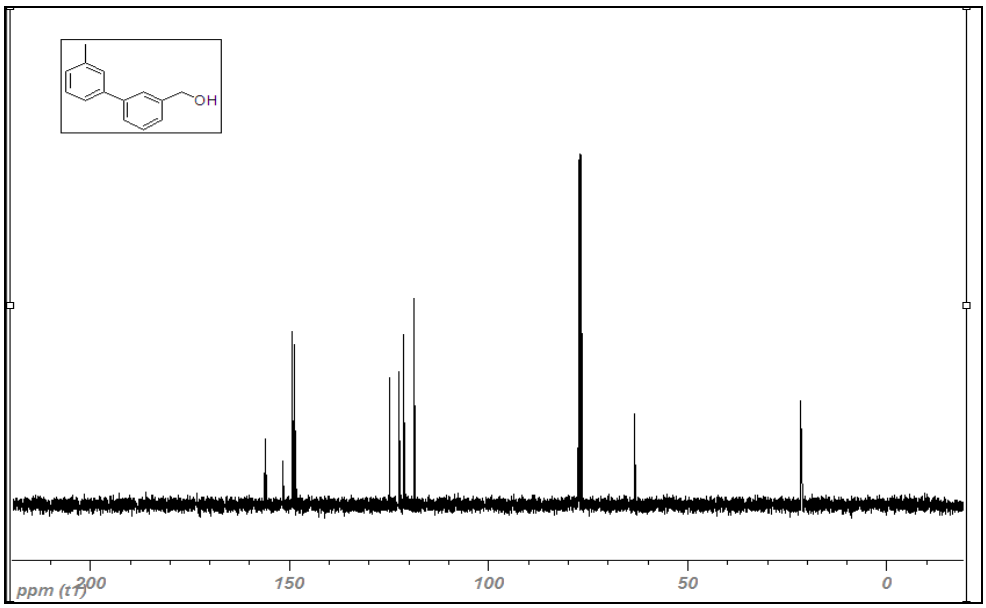


Figure 56. ^{13}CNMR spectrum of (13)

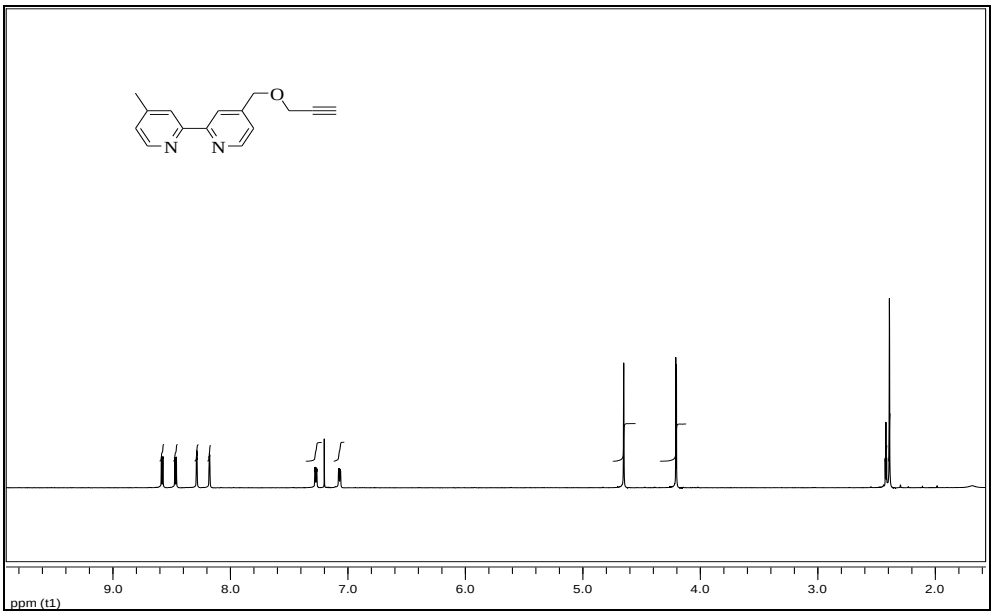


Figure 57. ^{1}H NMR spectrum of (14)

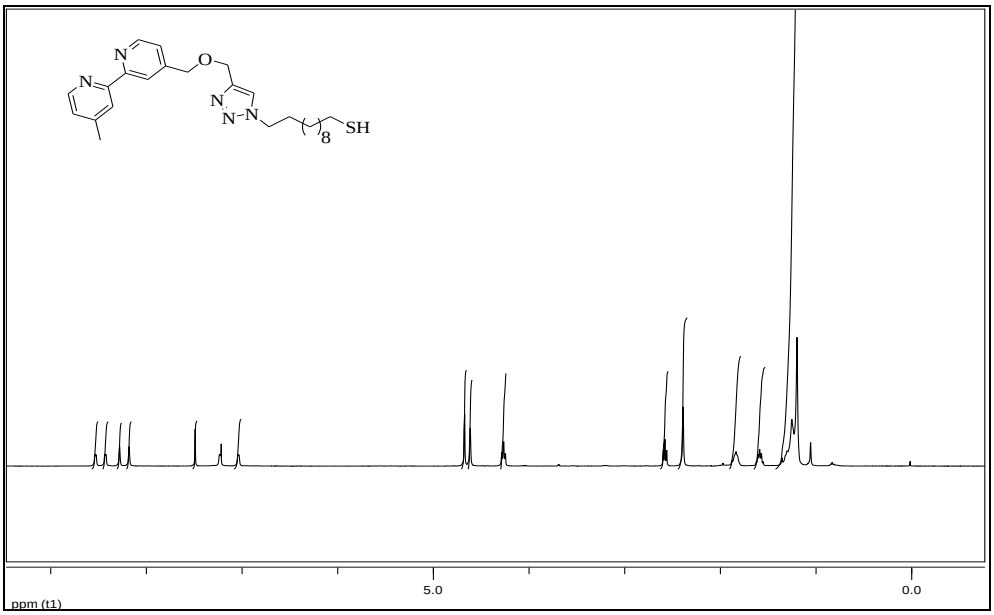


Figure 58. ^{1}H NMR spectrum of (15)

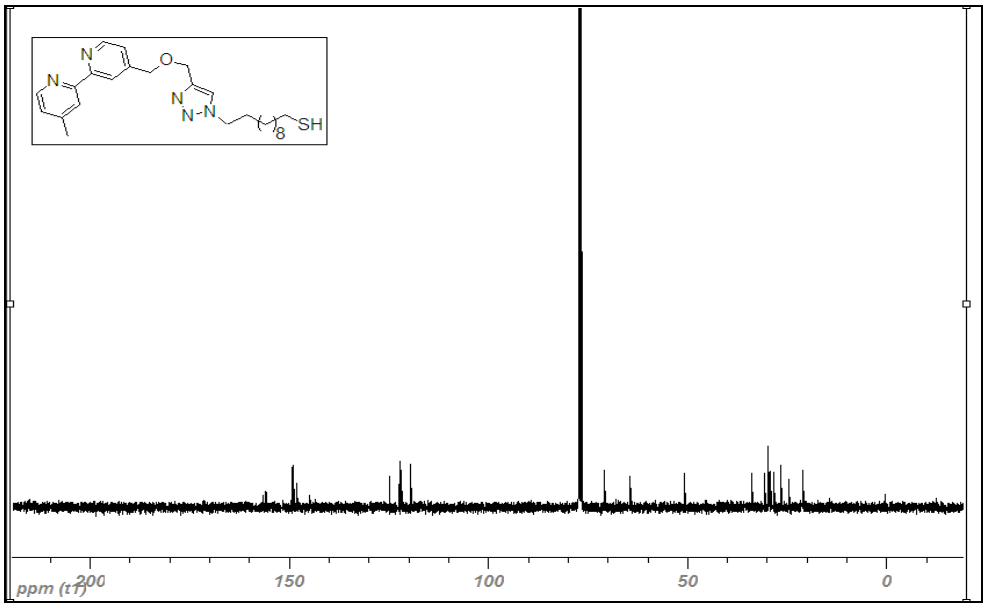


Figure 59. ^{13}CNMR spectrum of (15)

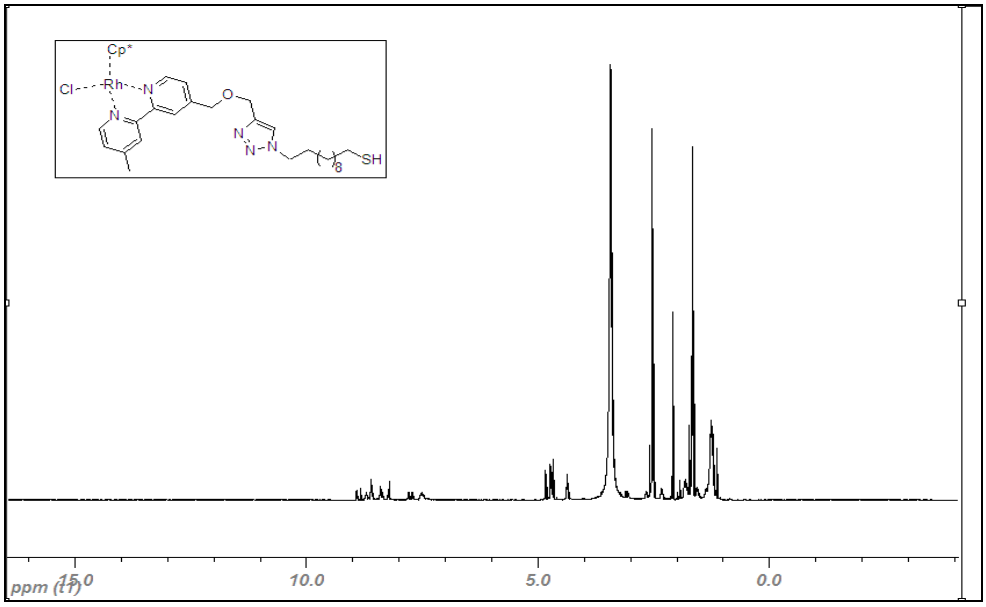


Figure 60. ^{1}HNMR spectrum of (8)

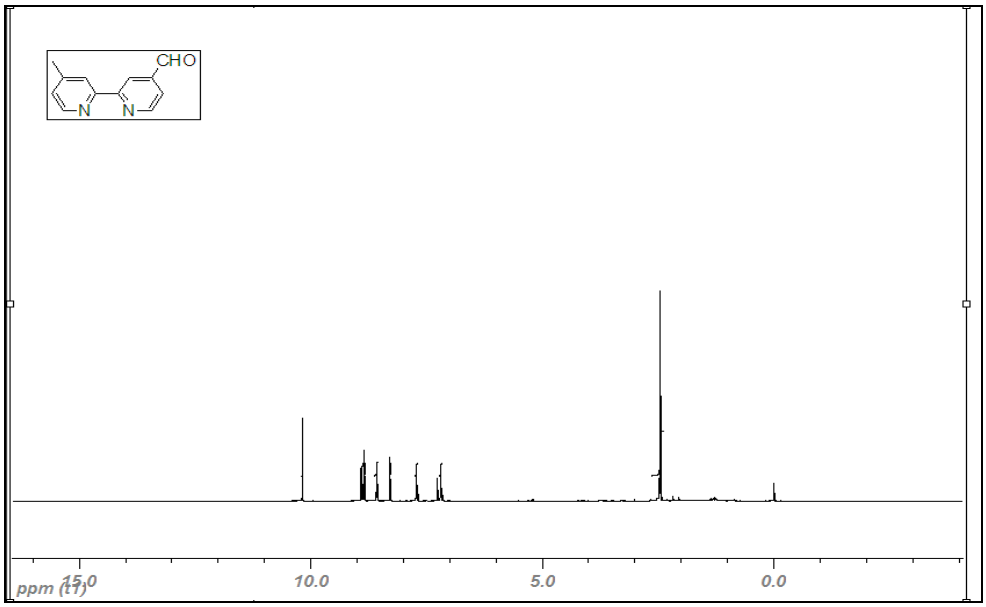


Figure 61. ^{1}HNMR spectrum of (16)

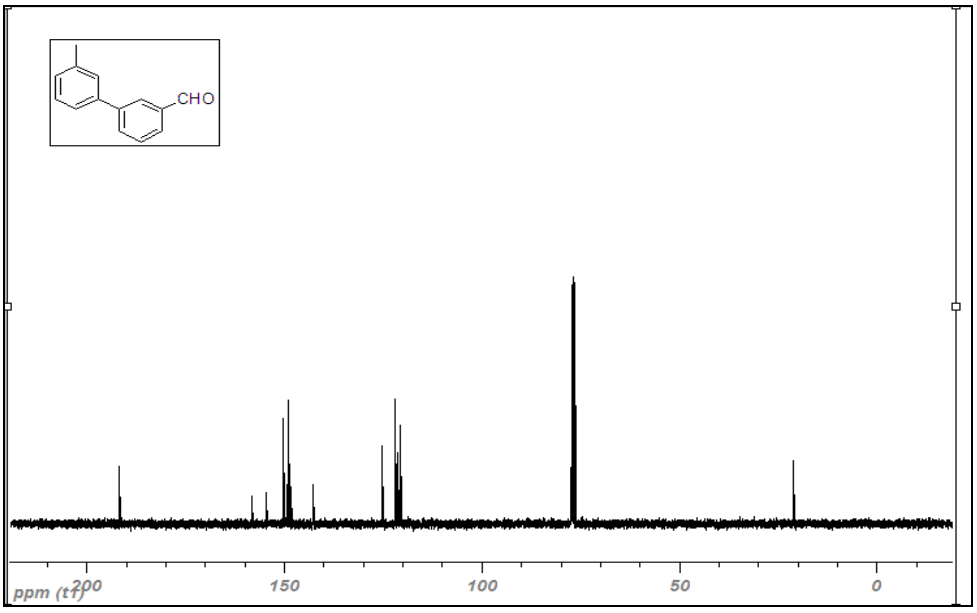


Figure 62. ^{13}CNMR spectrum of (16)

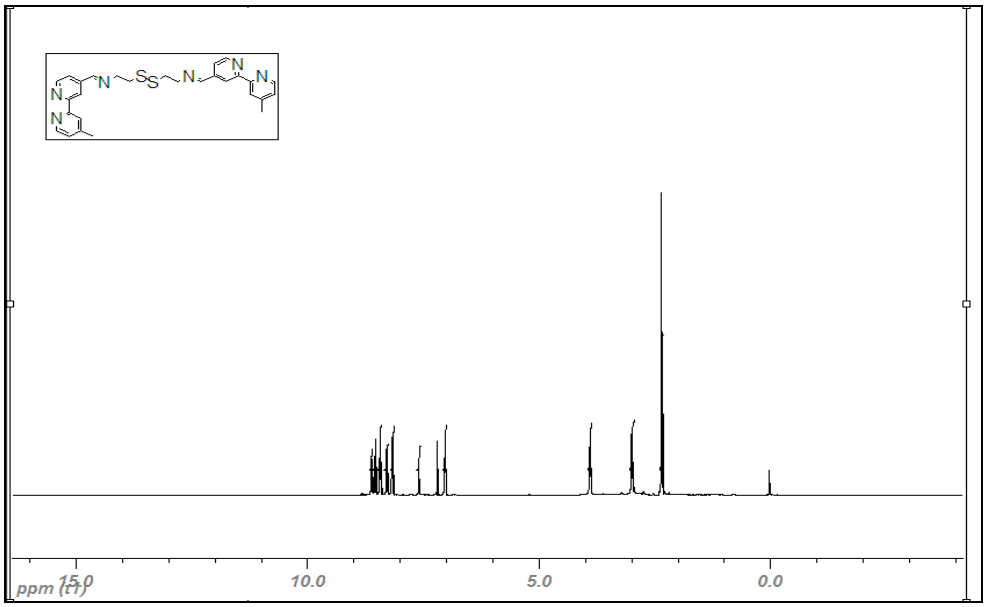
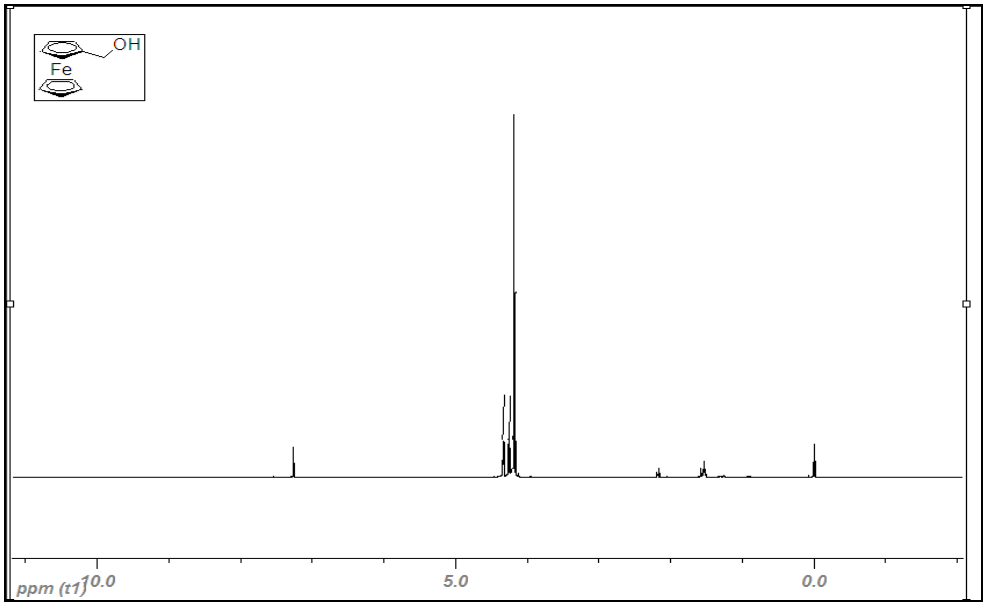


Figure 63. ^{1}HNMR spectrum of (17)



,Figure 64. ^{1}HNMR spectrum of (21)

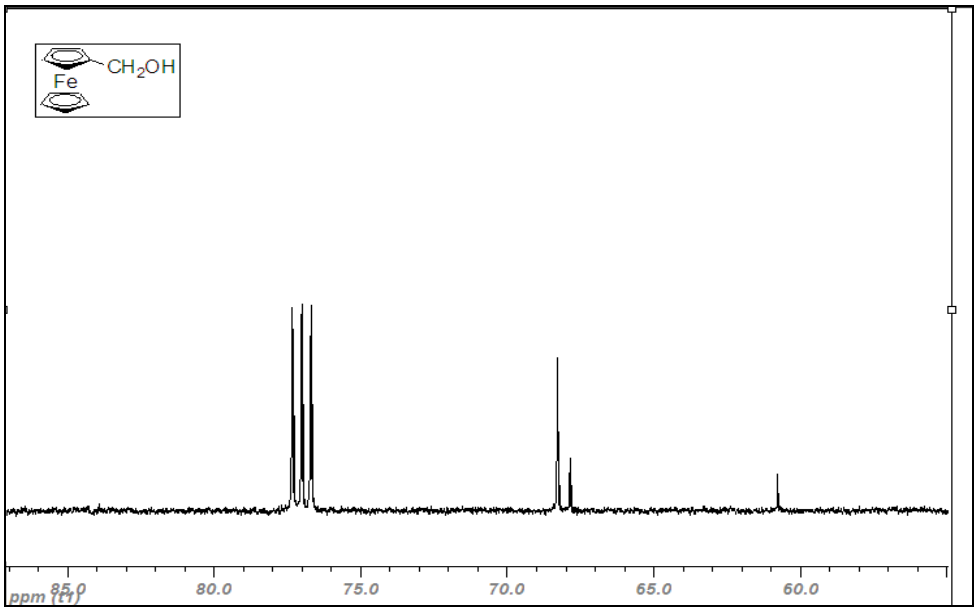


Figure 65. ^{13}CNMR spectrum of (21)

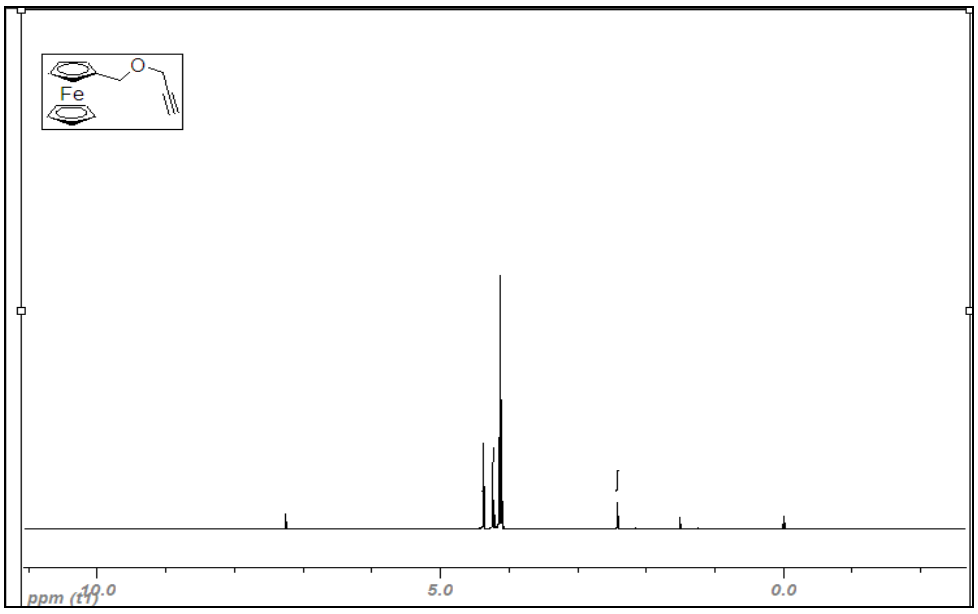


Figure 66. ^{1}HNMR spectrum of (22)

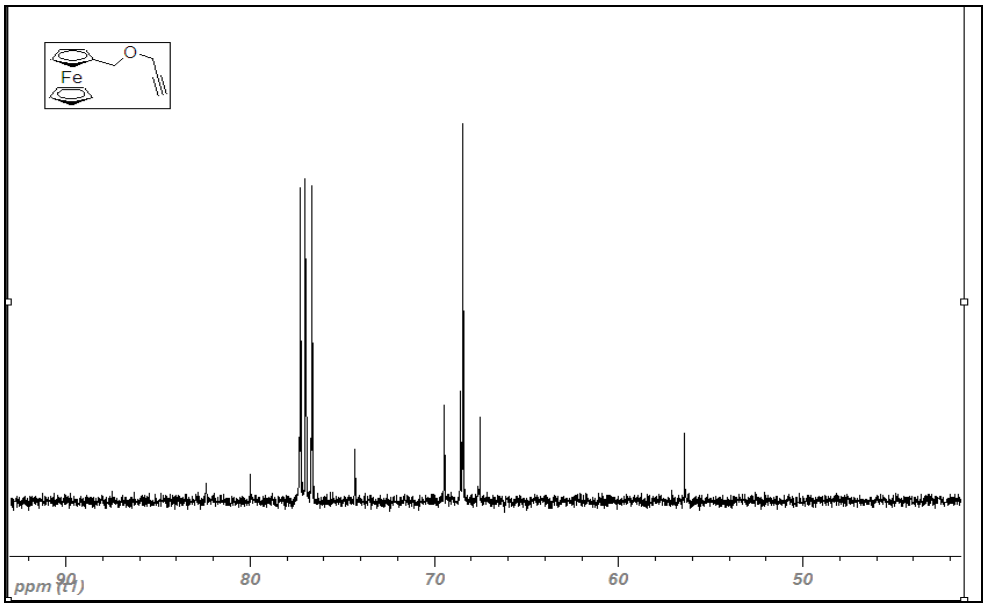


Figure 67. ^{13}CNMR spectrum of (22)

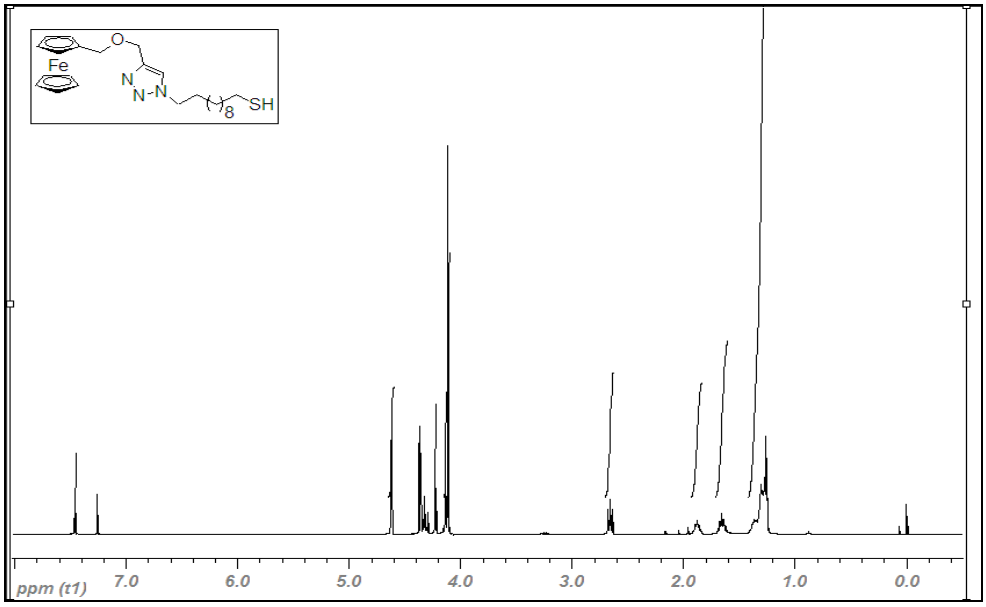


Figure 68. ^{1}HNMR spectrum of (10)

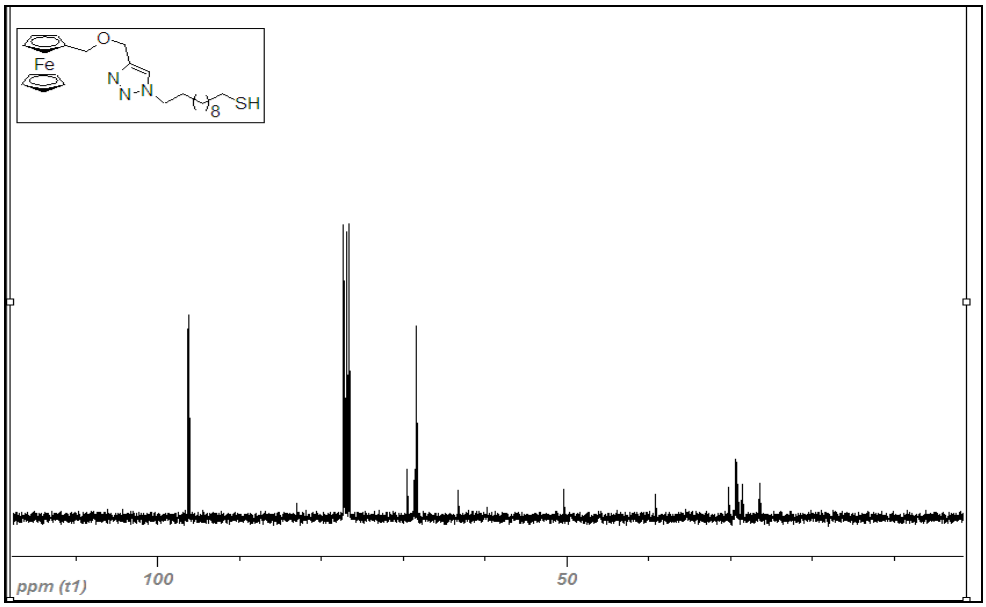


Figure 69. ^{13}CNMR spectrum of (10)