SYNTHESIS OF NEW MEDIATORS FOR ELECTROCHEMICAL NAD/NADH RECYCLING

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

BY

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

FEBRUARY 2011

Approval of the thesis:

SYNTHESIS OF NEW MEDIATORS FOR ELECTROCHEMICAL NAD/NADH RECYCLING

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ABSTRACT

SYNTHESIS OF NEW MEDIATORS FOR ELECTROCHEMICAL NAD/NADH RECYCLING

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February 2011, 86 pages

The synthesis of enantiopure compounds can be achieved by using dehydrogenases as biocatalysts. For instance, reduction reactions of prochiral compounds (ketones, aldehydes and nitriles) into chiral compounds can be achieved by dehydrogenases. These dehydrogenases are cofactor dependent where cofactor is Nicotinamide Adenin Dinucleotite having some restrictions that confines usage of dehydrogenases in organic synthesis including instability of cofactor in water and high cost. Therefore, suitable recycling methods are required and developed which are enzymatic and electrochemical. We will use an electrochemical approach for the regeneration of reduced co-factors.

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 utilize the cofactor and

change the substrates to the products in high stereoselectivity. Our aim in this project is the synthesis of mediators and suitable linkers for enzyme, cofactor and mediator immobilization. In the first part of the study, mediators were synthesized which are pentamethylcyclopentadienyl rhodium bipyridine complexes. In the second part of the study, a conductive monomer (SNS) and linker were synthesized for immobilization of the enzyme. In the last part of the study, the reaction of galactitol dehydrogenase with monomer (SNS) was achieved.

Keywords: dehydrogenases, mediator, linker for enzyme immobilization, conductive monomer, galactitol deydrogenase, electrochemical reactor.

ÖZ

ELEKTROKİMYASAL NAD/NADH DÖNÜŞÜMÜ İÇİN YENİ MEDİYATÖRLERİN SENTEZİ

Khalily, Mohammad Aref
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Şubat 2011, 86 sayfa

Enansioseçici bileşikler, keton, aldehit ve nitril gibi prokiral bileşiklerin dehidrogenazlar ile biyokatalitik indirgenmeleri sonucu elde edilir. Dehidrogenazlar, Nikotinamit Adenin Dinükleotit kofaktörüne ihtiyaç duymaktadırlar ve bu kafaktörün suda kararsız olması ve maliyetinin yüksek olması nedeniyle, organic sentezde kullanımını sınırlıdır. Bu nedenle, bu kofaktörün tekrardan elde edilebilmesi için uygun bir rejenerasyon metodunun kullanılması ve geliştirilmesi gerekmektedir. En uygun rejenerasyon metodları enzimatik ve elektrokimyasal olup, biz bu çalışmada indirgenmiş olan kofaktörün tekrardan eldesi amacıyla elektrokimyasal yöntemi kullandık.

Bu projede, arabulucu bileşikler, kofaktör ve enzim, geliştirilmiş olan reaktör yüzeyine tutturulması ile yüksek enantiyoseçicilikle sentezlenmiş olan ürünün, başlangıç maddesinden ayrılması amaçlanmaktadır. İstenmeyen reaksiyonların gerçekleşmesini engellemek için gaz membrane elektrodu kullanılarak sistemin potansiyelinin 1 volta düşmesi sağlanacaktır. Gaz membran elektrodu ortama proton sağlarken, arabulucu bileşiklerin electron taşıması ile kofaktör rejenerasyonu gerçekleştirilecektir. Elektrot

yüzeyine tutturulmuş enzim, kofaktörü kullanarak, dönüşüm reaksiyonunu yüksek enantiyoseçicilikle katalizleyecektir.

Bu projede, enzimin, arabulucu bileşiklerin ve kofaktörün elektrot yüzeyine tutturulmasını sağlamak amacıyla yeni arabulucu ve bağlayıcı bileşikler tasarlanmış, bu bileşiklerin sentezini gerçekleştirilmiştir. Çalışmanın ilk kısmında pentametilsiklo pentadiyenil rodyum bipiridin kompleksleri içeren arabulucu bileşikler sentezlenmiştir. Projenin ikinci kısmında ise, enzimin elektrot yüzeyine tutturulmasını sağlamak amacıyla iletken monomer (SNS) ve bağlayıcılar sentezlenmiştir. Projenin son aşamasında ise galaktitol dehidrogenazın monomer (SNS) ile reaksiyonu gerçekleştirilmiştir.

Anahtar Kelimeler: Dehidrogenazlar, medyatör, enzim tutturulması için bağlayıcı moleküller, galaktitol dehidrogenaz, elektrokimyasal reaktör

To My Family,

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my supervisor Prof. Dr. Ayhan S. Demir not only for his guidance, support, encouragement, patience and for teaching us how to become a good scientist but also for listening and helping us in many ways.

I would like to express my sincere thanks to my family for everything. Althgouh I have to continue to my life away from them, I have never felt alone since they have always been supportive, and they have always shown their love and patience.

I wish to thank Demir's group members, present and past, for help, discussion, and the time we shared together.

I would like to thank to Seda Karayılan and Zehra Uzunoğlu for their kind help in our routine and special NMR analysis.

I would like to thank to EURUDESP for supplying financial support during my master work.

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INTRODUCTION

1.1 Biocatalysts in Organic Chemistry

The new developments in science and industry have created new environmental problems. The scientists have tried to find some solutions to these problems so that the new developments applicability maximized, the side effects and hazards are reduced to minimum. Synthetic organic chemistry is one of those flourishing area which has its own problems and constrains. Scientits have tried to perform organic reactions in aqueous medium, producing fewer waste products, no solvent utilization and less side reactions. In order to achieve this goal; organic chemists have developed the use of enzymes or biocatalysts in organic synthesis [1, 2]. Application of biocatalysts in organic chemistry has brought about the development of the new term "Green Chemistry" which is the design, enhancement, and use of chemical processes and products in such a way as to diminish or abolish the production of substances that are hazardous to human wellbeing and environment [3, 4].

Although there are some common prejudices against enzymes like they are receptive, costly, are only active on their natural substrates, and work only in their innate environment, but their vast number of advantages compensate for these exaggerated prejudices [4]

Enzymes are very efficient catalysts. They catalyze reactions very fast and the reaction rate is accelerated by 10^{8}-10^{10} times when compared to the conventional organic reactions. Moreover, they are used only in tinny amounts (10^{-3}-10^{-4} mole percentage of the substrate) which make them economical to be used [5].

Contrasting to heavy metals, for example, biocatalysts are environmental friendly chemicals since they are totally degradable. They work under mild reaction conditions in a pH range of 5-8 and in a temperature range of 20-40^{o}C. These mild reaction conditions give the adavantage of minimizing the side reactions like isomerization, racemization, rearrangement, and decomposition.

One of the most important characteristic of the enzymes is that they can accept enormous number of different substrates. In other words, they can catalyze many different types of organic reactions. Different organic reaction examples which are catalyzed by enzymes are listed below [4];

1. Hydrolysis- synthesis of esters, amides, lactone, lactams, ethers, acid anhydrides, epoxides and nitriles.
2. Oxidation- reduction of alkanes, alkenes, aromatics, alcohols, aldehydes and ketones, sulfides and sulfoides.
3. Addition-elimination of water, ammonia, hydrogen cyanide.
4. Halogenation and dehalogenation, alkylation and dealkylation, carboxylation and decarboxylation, isomerization, acylion, aldol reactions, and Michael additions have been reported.

In addition, enzymes generally perform under the same or similar conditions; it is expected to achieve several biocatalytic reactions in a reaction cascade in one flask. The use of multienzyme systems for simplifying reaction processes makes sequential reactions feasible. As any catalysts, enzymes accelerate a reaction just by reducing the energy barrier without being altered as a consequence of the reaction they promote (Figure 1).

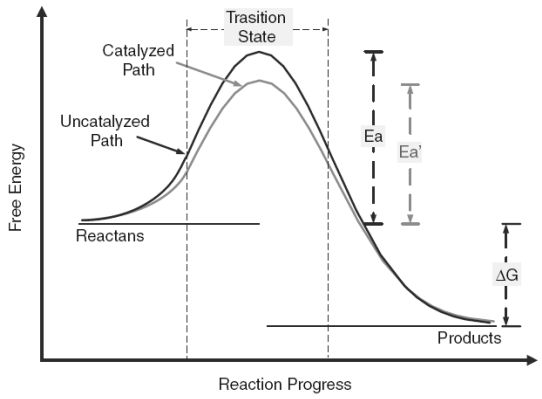


Figure 1: Mechanism of catalysis

(Ea and Ea' are the energies of activation of the uncatalyzed and catalyzed reaction. ΔG is the free energy change of the reaction)

Enzymes show three different types of selectivities which are very important and have wide application in asymmetric synthesis, pharmaceuticals and agrochemicals. Enzymes display high chemoselectivity; there is no need for functional groups to be protected. This gives the advantage of reducing the number of by-products in the reaction medium and as result the product is obtained without any purification. For example, hydrolysis of ester using enzymes does not demonstrate any tendency for acetal-cleavage. However; in the case of chemical catalysis, functional groups would react to a certain extent. Likewise, because of their three dimensional structure, enzymes could be able to differentiate functional groups of which regions are different in the same substrate that would lead to high regioselectivity in enzyme catalyzed reactions. Enzyme catalyzed reactions could be performed not only with their high chemoselectivity, regioselectivity but also with high enantioselectivity which could be the result of ezymes' chiral structure. Since they are chiral, any chirality present on the substrate will be recognized by the formation of enzyme-substrate complex that leads to the transformation of prochiral substrate into an optically active product

1.2. Redox Enzymes

The enzymes employed in redox reactions are called Oxidoreductases and are divided into three categories: dehydrogenases, oxygenases, and oxidases [6, 7]. Oxidoreductases play a significant role in the metabolism and energy conversion of living cells. As early as 1897 Buchner demonstrated in his famous experiment that such biocatalysts can also function in cell-free extracts. About 25% of the presently known enzymes are oxidoreductases [8].

Among those the most interesting catalysts for preparative and possible industrial applications are dehydrogenases or reductases classified in the groups EC 1.1.1 (Alcohol dehydrogenase, acting on the CH-OH group of donors), EC 1.2.1 (Dehydrogenases, acting on the aldehyde or oxo group of donors), EC 1.4.1 (Dehydrogenases, acting on the $CHNH_2$ group of donors), and using NAD^+ or $NADP^+$ as an acceptor (Figure 2). The most interesting characteristic of degydrogenases is that they can perform both oxidation nd reduction at the same time depending on the substrate and the form of the coenzyme as shown in Figure 2.

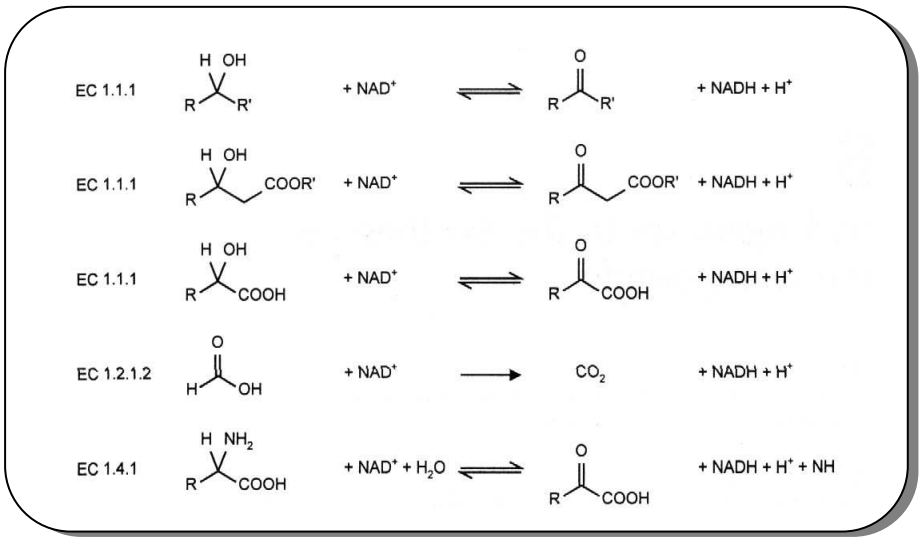


Figure 2: Enzyme-catalyzed reductions of prime interest in the production of fine chemicals

1.2.1 Dehydrogenases in chiral synthesis

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. 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. Stereochemistry has to be considered when studying xenobiotics like drugs, agrochemicals, food additives, flavours and fragnances [9] since enantiomers have different biological effects, smell, taste, etc.; due to the fact that all biochemical reactions taking place in organisms are governed by chiral catalysts, enzymes, which are highly selective with respect to the chirality of the molecule.

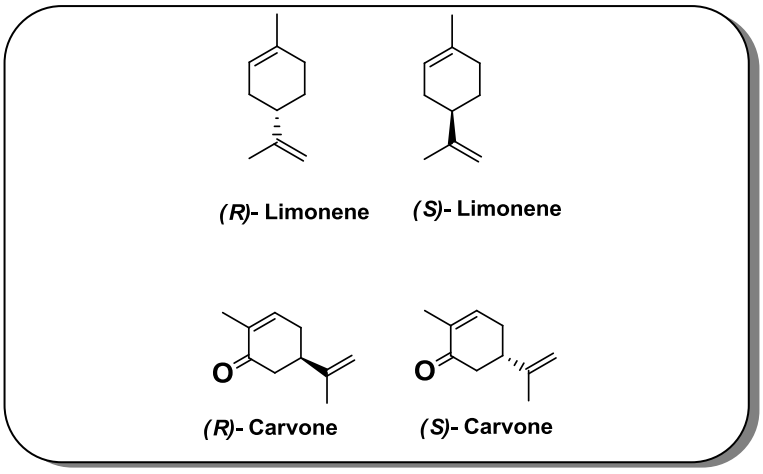


Figure 3: Enantiomers of limonene and carvone

Chiral molecules can demonstrate their different properties in many ways. For instance, one enantiomeric form of a compound called limonene (Figure 3) which is a terpene with isoprene units linked in rings is responsible for the odor of oranges and the other enantiomeric form is responsible for the odor of lemons. One enantiomer of the compound called carvone (Figure 3) is responsible for the fundamental nature of caraway and the other one is responsible for spearmint.

Differences in biological effects of enantiomers are not always responsible for the differences in taste, smell, etc.; they lead to sometimes very serious and tragic consequences as in thalidomide.

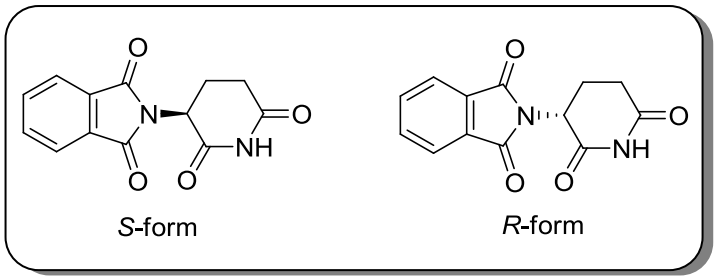


Figure 4: Enantiomers of thalidomide

The awareness of the stereoselectivity of drug action has intensified since the tragedies of thalidomide which was used to lessen the symptoms of morning sickness in pregnant women in 1963, however; it was recognized that thalidomide was the cause of horrible birth defects in many children born subsequent to the use of drug. Later, the intended curing effect of thalidomide for morning sickness was proved as *(R)*-form of it whereas the *(S)*-form of thalidomide is highly teratogenic [4, 5, 6]. The above example (Figure 4) shows the importance of enantioselectivity which is an exclusive property of biocatalysts.

Different types of chiral synthesis (amino acid synthesis, lactone synthesis, synthetic reactions involving alcohols, hydroxy acid and steroid synthesis, deracemization) are achieved by dehydrogenases, which may be the desired products or more often serve as key intermediate in the synthesis of pharmaceuticals, agrochemicals, or flavors. Examples of chiral amino acid and chiral alcohols synthesis are illustrated in Figure 5 and Figure 6 respectively.

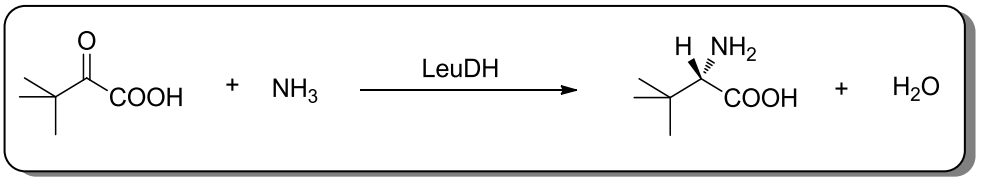


Figure 5: Reductive amination of 2-oxo-3,3-dimethyl butanoic acid with LeuDH, leucine dehydrogenase

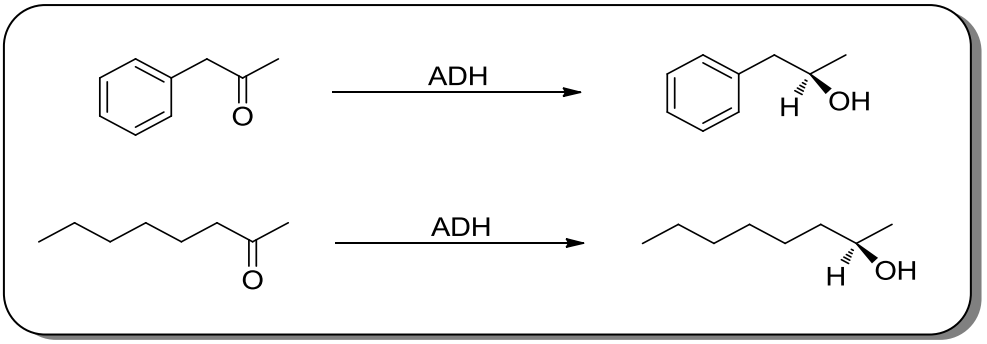


Figure 6: Reaction schemes for the synthesis of (*R*)-1-phenyl-2-propanol and (*R*)-2-octanol (ADH, alcohol dehydrogenase;

1.2.2 Importance of cofactors recycling

The oxidoreductases as catalysts require a coenzyme (cofactor) which functions as an electron carrier. If used in stoichiometric amounts the cost of coenzymes makes synthetic applications of redox enzymes prohibitively expensive for industrial applications.

The redox cofactor donates (or accepts) the chemical equivalents for reduction (or oxidation). From the total number of known redox enzymes about 80% require NAD(H), and about 10(%) require NAD(P)H. Fewer enzymes require flavines like Flavin mononucleotide (FMN), Flavin adenine dinucleotide (FAD) or Pyrroloquinoline quinone (PQQ). What all these coenzymes have in common is low stability, high costs (if they are used in stoichiometric amounts), and the fact that they cannot be replaced by synthetic compounds [10].

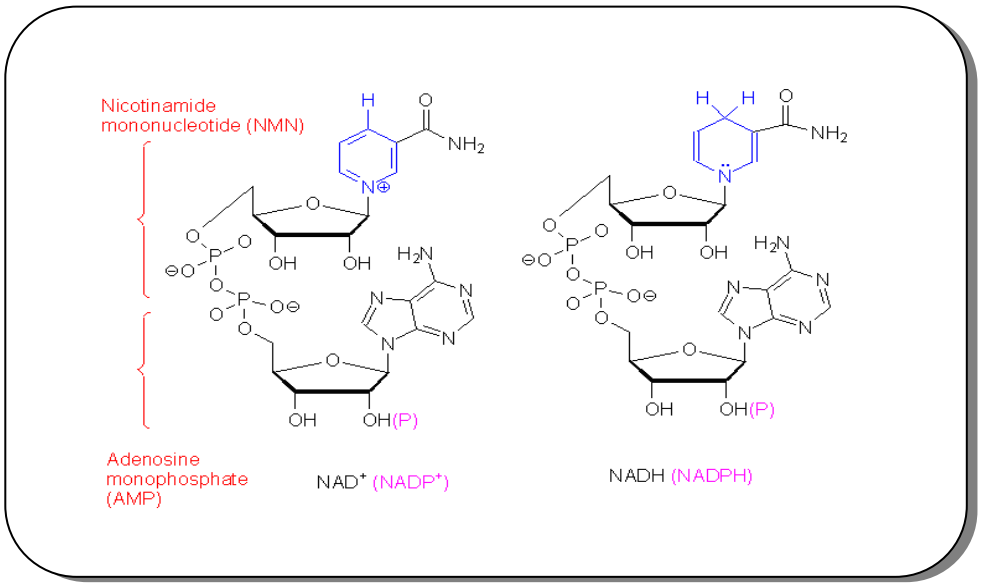


Figure 7: Structure of Nicotinamide Adenine Dinucleotite

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 the cofactor for reduction reactions [11]. 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 7]. 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].

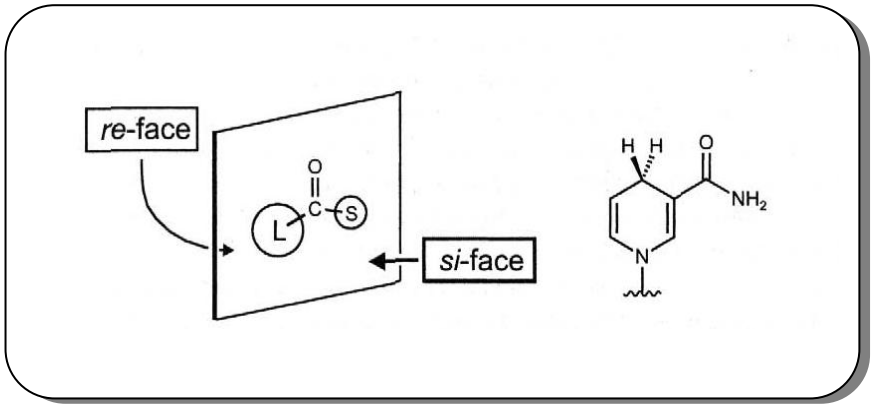


Figure 8: Delivery of the "hydride" from NADH to the *re* or *si* side of a prochiral ketone yielding either (*S*)- or (*R*)-alcohols. Depending on the dehydrogenase involved either the pro (*R*)- or the pro (*S*)-hydrogen at C-4 of the nicotinamide moiety of NAD(P)H is selectively transferred in the course of the reduction. *L* denotes the larger and *S* the smaller substituent at the central carbon.

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].

To utilize such reactions for the commercial synthesis of fine chemicals an efficient regeneration of the coenzyme(s) involved is needed. There are three main methods of cofactors regeneration: coupled-substrate, coupled-enzyme, and electrochemical method.

1.2.3 Substrate-coupled regeneration method

In substrate-coupled process the cofactor required for the transformation of the main substrate is constantly regenerated by addition of a second auxiliary substrate (donor) which is transformed by the same enzyme, but into the opposite direction (Figure 8). To shift the equilibrium of the reaction in the desired direction, the donor is usually applied in excess leading to turnover numbers of 10^3. Although this approach is applicable in principle to both directions of redox reactions, it has been mainly used for reductions because the equilibrium of dehydrogenase reactions lies heavily in favor of reductions [27]. Co-substrate and co-product are not chiral and in general can be easily separated from the compound of interest by distillation. Although the use of a single enzyme performing two reactions simultaneously appears elegant, some significant drawbacks are encountered in substrate-coupled recycling.

1. The overall efficiency of the process is limited since the enzymes's activity is distributed between both the substrate and the donor.
2. The product has to be purified from large amounts of auxiliary substrate used in excess.
3. Enzyme deactivation may occur when highly reactive carbonyl species such as acetaldehyde or cyckohexenone are used as redox-donors.
4. Enyme inhibition caused by the high concentrations of the auxiliary substrate-cosubstrate inhibition- is common.

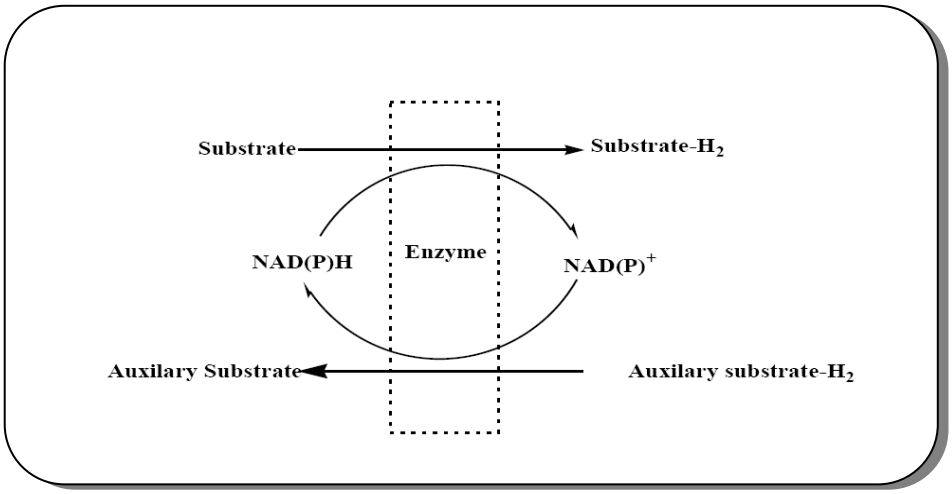


Figure 9: Coupled-substrate system

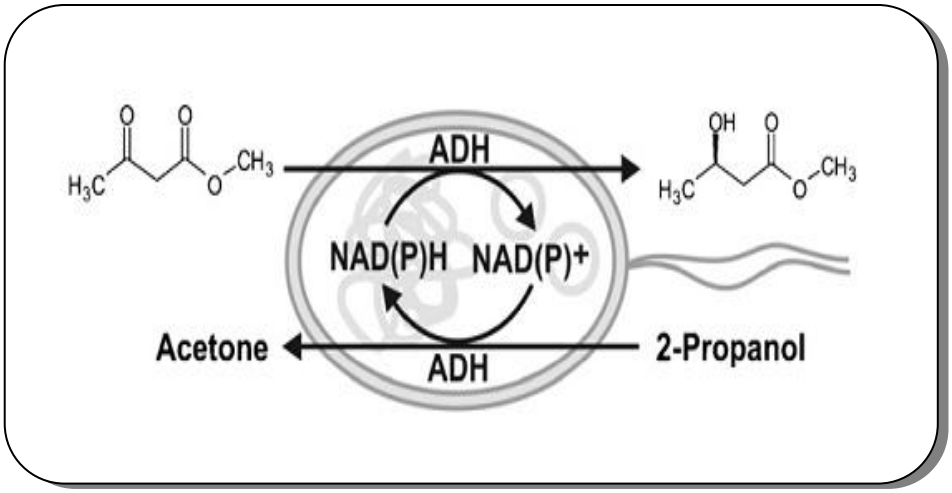


Figure 10: Reduction of methyl acetoacetate using alcohol dehydrogenase (ADH) in a substrate-coupled cofactor regeneration approach

1.2.4 Enzyme-coupled regeneration method

The enzyme-coupled approach is more advantageous (Figure 9). In this case the two parallel redox reactions –i.e. conversion of the main substrate and cofactor-recycling- are catalyzed by two different enzymes [27]. To achieve optimal results, both of the enzymes should have sufficiently different specifications for their receptive substrates whereupon the two enzymic reactions can proceed independently from each other and, as a consequence, both the substrate and the auxiliary substrate do not have to compete for the active site of a single enzyme, but are efficiently converted by the two biocatalysts independently.

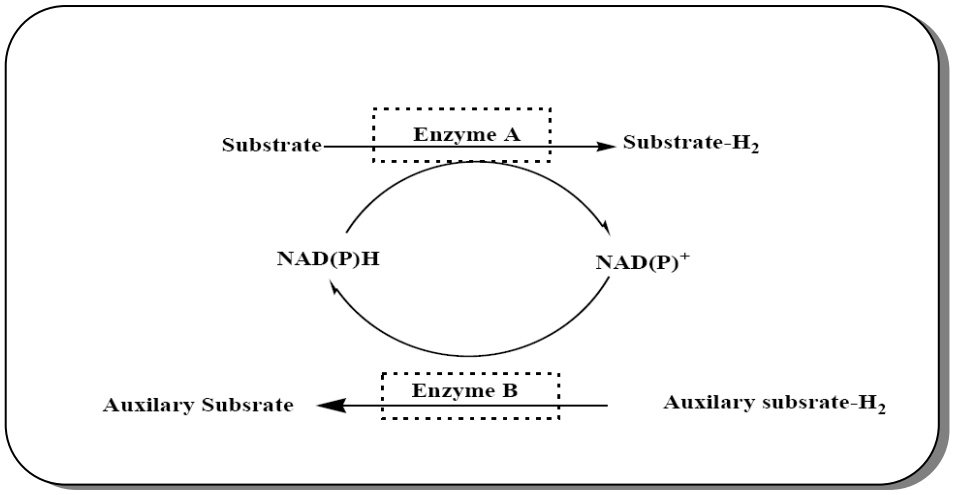


Figure 11: Coupled-enzyme system

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].

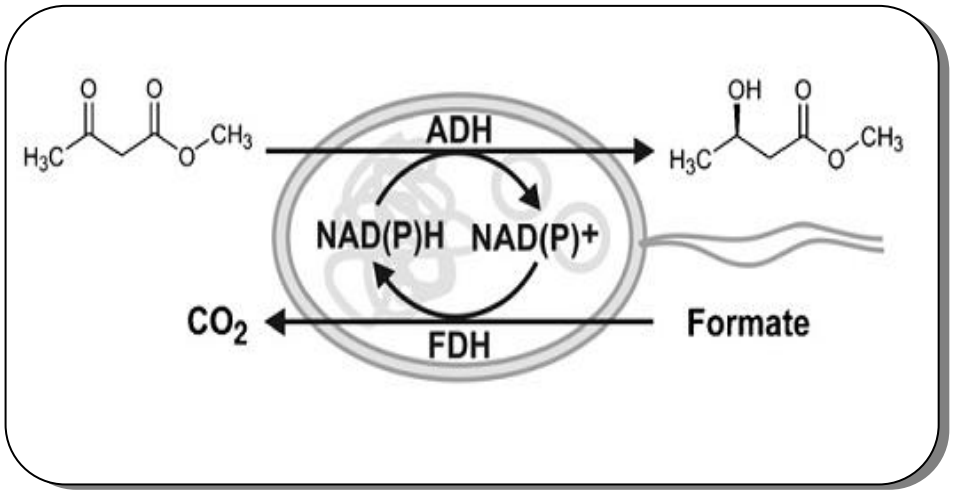


Figure 12: Reduction of methyl acetoacetate using alcohol dehydrogenase (ADH) with enzyme-coupled cofactor regeneration by formate dehydrogenase (FDH)

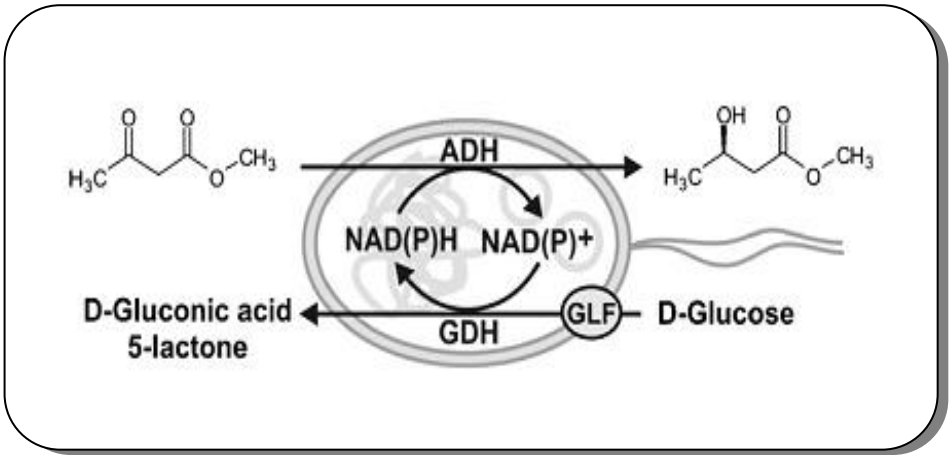


Figure 13: Reduction of methyl acetoacetate using alcohol dehydrogenase (ADH) with enzyme-coupled cofactor regeneration by glucose dehydrogenase (GDH).

1.2.5 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, 16, 17]. 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, 17, 18]. It divides into three; indirect electrochemical method, direct electrochemical method, and enzyme-coupled electrochemical regeneration (Figure 14).

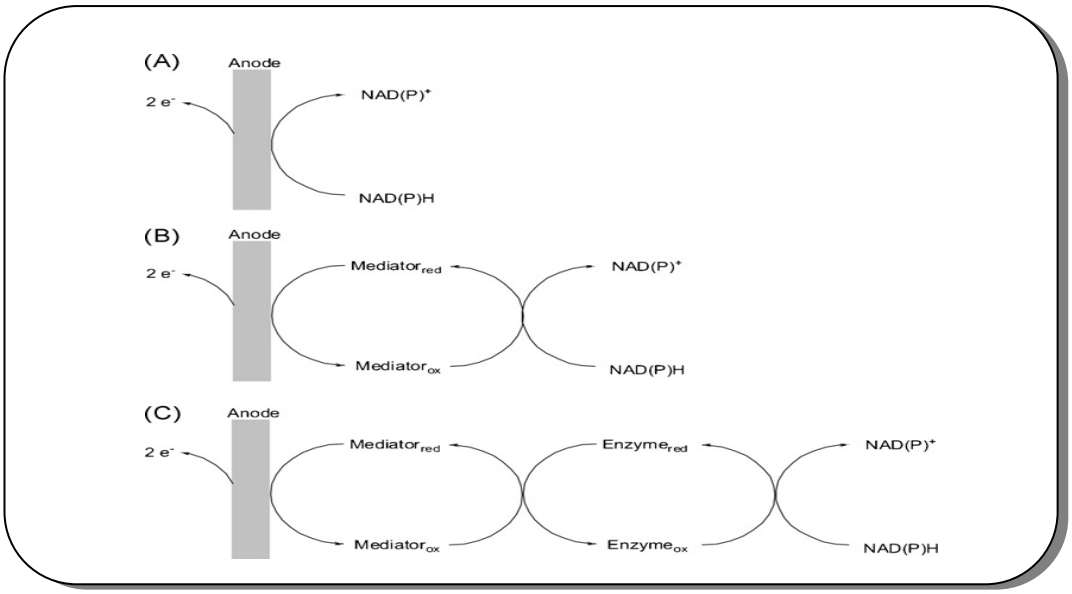


Figure 14: NAD (P) +-dependent oxidation reactions: (A) direct electrochemical regeneration, (B) indirect electrochemical regeneration, (C) enzyme-coupled electrochemical regeneration

1.2.6 Direct electrochemical method

The simplest method is the direct anodic oxidation of NAD(P)H. Regeneration of the $NAD(P)^+$ occurs on the anode surface in direct electrochemical methods (Figure 15). High overpotential is needed since conversion occurs at 900mV on the standard calomel electrode [19]. This reaction however, requires high overpotentials which can lead to undesired side-reactions.

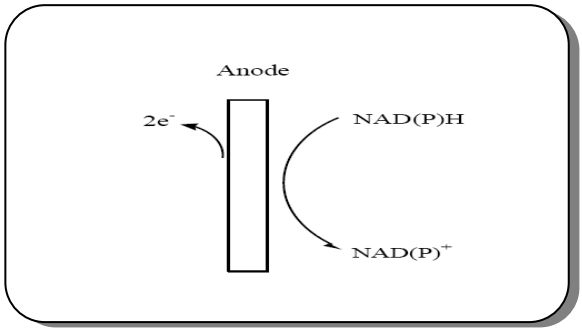


Figure 15: Direct electrochemical method

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 16). Therefore this system is suitable for the oxidation-stable substrates [2, 4, 5, 20].

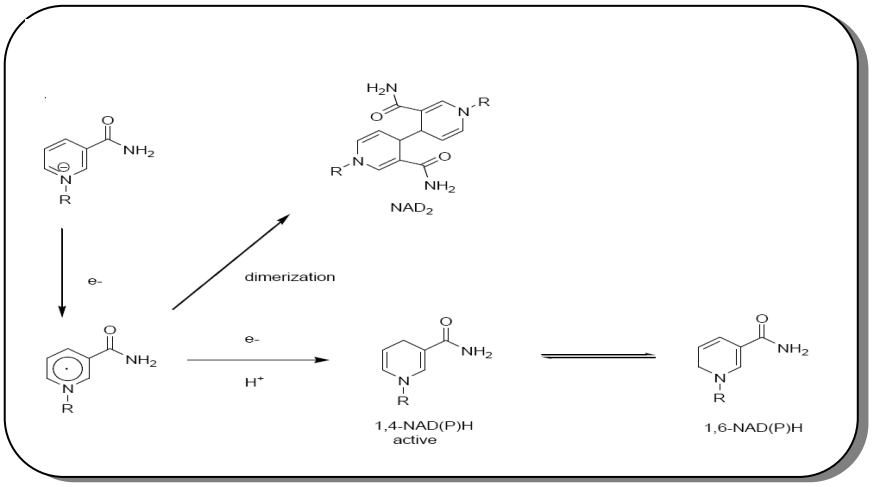


Figure 16: Formation of 1, 4-NAD (P) H and side products in direct electrochemical method

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 [19].

1.2.7 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 [19] [Figure17].

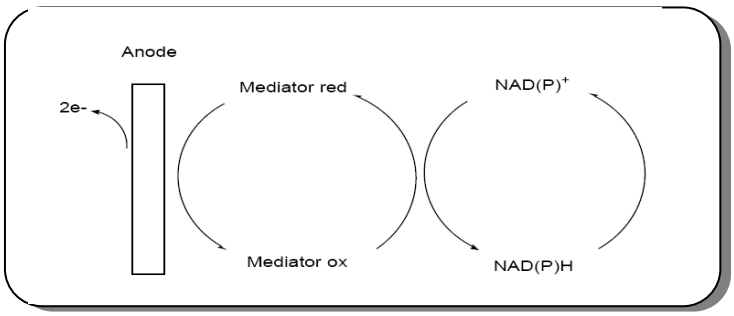


Figure 17: Indirect electrochemical method

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 in 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 [21].

The first mediator that met all the requirements which was developed by Steckhan and coworkers, was 2-2'-bipridyl rhodium complex [22, 23, 24]. This complex was used for the conversion of cyclohexanone to cyclohexanol. Regeneration cycle was small for cofactor and mediator [25]. Pentamethylcyclopentadienyl (Cp*) was inserted to the rhodium complex to increase the performance of mediator [26]. Selectivity of enzymatically active form of the cofactor elevated. Then penthamethylcyclopentadienyl 2-2'-bipyridiyl rhodium complex $[Cp^*Rh(bpy)H_2O]^{2+}$ [Figure 18(I)] was utilized with different substrates, in electrochemical reactors and aqueous-organic- two phase system.

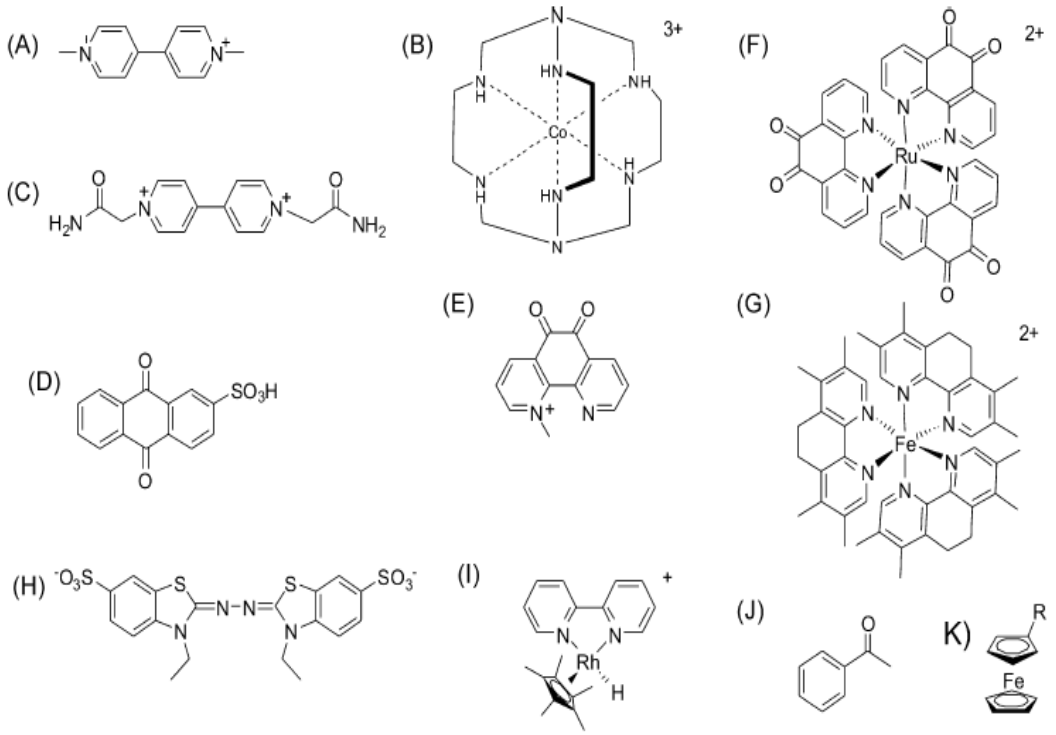


Figure 18: Substances used as electron shuttles (mediators) in electroenzymatic synthesis: (A) methyl viologen MV, (B) cobalt sepulchrate CoSep, (C) carboxamidomethyl viologen CAV, (D) anthraquinone 2-sulfonate AQ-2-S, (E) N-methyl-1,10-phenanthroline-5,6-dione, (F) tris(phenanthroline-5,6-dione)Ru(II), (G) tris(3,4,7,8- phenanthroline)Fe(II) Fe(tmphen)3, (H) 2,2_-azinobis(3-ethylbenzothiazoline)-6-sulfonate ABTS, (I) (2,2_-bipyridyl)(pentamethylcyclopentadienyl) rhodium complex, (J) acetophenone, (K) ferrocene derivatives

The stability of mediator (2,2-bipyridyl)(pentamethylcyclopentadienyl) rhodium complex) has been studied at different temperatures and pHs. It was found that mediator did not lose its activity when temperature was lower than 80°C 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 19] [22, 23, 24].

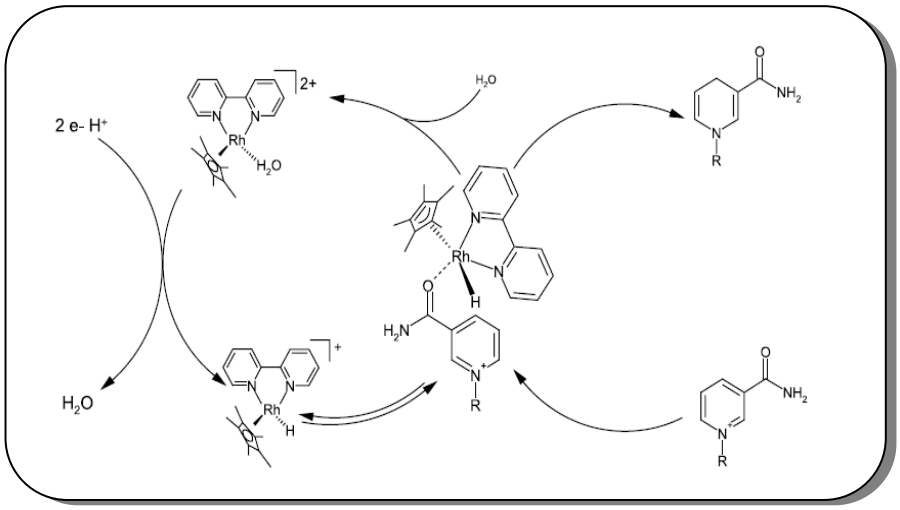


Figure 19: Mechanism of the regiospecific reduction of NAD(P)$^+$ by [Cp*Rh(bpy)(H2O)]$^{2+}$. The catalytically active Hydridorhodium complex [Cp*Rh(bpy)H]$^+$ is regenerated from its oxidized form either electrochemically (-750 mV vs Ag/AgCl) or via formate. The formation of an intermediate Rh-NAD complex was postulated to explain the high specificity of the hydride transfer .

1.3 Enzyme Immobilization

A biocatalyst is termed "immobilized" if its mobility has been restricted by chemical or physical means. This (artificial) limitation of mobility (immobilization) may be achieved by widely differing methods, such as binding the biocatalysts to one another or to carrier substances by entrapping in the network of a polymer matrix or by membrane confinement. Instead of homogeneous catalysis, in which substrate and catalyst are present in a homogeneous solution, immobilization makes possible heterogeneous catalysis, which can be of very considerable advantage [27].

1. Effeciently purification of biocatalyst from reaction mixture
2. Maintaining high bicatalytic activity in a small volume
3. Increasing the stability of the biocatalyst

This (artificial) limitation of mobility (immobilization) may be achieved by widely differing methods: Adsorption, Ionic Binding, Covalent Binding, Matrix Entrapment, and Membrane Confinement

1.3.1 Adsorption

In adsorption method, the enzyme is detained on the surface of a carrier by physical forces like van der Waal forces. It is the oldest and simplest method among other immobilization methods. The main advantages of this technique are that it is straightforward and has little influence on the conformation of the biocatalysts. The main drawback of the technique is that the enzymes are bound to the carrier softly. Changes in temperature, substrate concentration, and ionic concentration can easily remove the bound enzyme from the surface of the carrier [27].

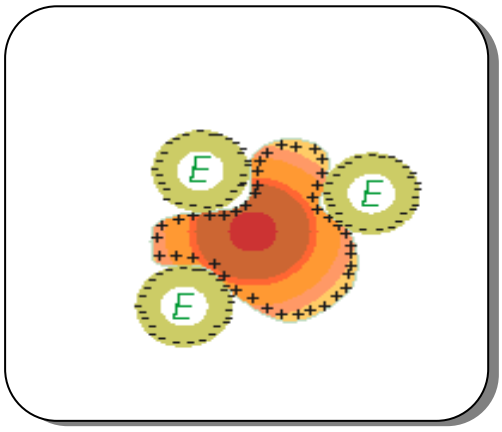


Figure 20: Enzyme (E) bound to a carrier by adsorption

Table 1: Biocatalysts immobilized by adsorption method [27]

Bioctalayst	Carrier
1. Alcohol dehydrogenase	Polyaminomethyl styrene
2. Alkaline phosphatase	Almunium
3. Enniatine synthetase	Propylagarose
4. Glucoamylase	Almunium oxide
5. Glucoamylase	Titania-activated glass
6. Glucose oxidase	Activated charcoal
7. Yeast cells	Charcoal pellets
8. Clostridia	Cellukose derivatives

1.3.2 Ionic Binding

In this technique, the charges or electrostatic attraction play role in binding the enzyme on a carrier. The main carriers used in this method are synthetic resins and ion exchangers. Due to simplicity and easiness in coupling process, ionic binding method was used for first time for the immobilization of enzymes for large-scale purposes.

The main problem in this method is the leakage of enzyme may take place if there are high ionic strength and pH fluctuations. Biocatalyst proteins are ampholytes (containing both acidic and basic groups). Depending on the pH of the medium, biocatalysts can behave as neutral, negatively charged by dissociation, or positively charged by protonation. The carboxyate group (COO^-) is responsible for negative charge while protonated amine group (NH_3^+) is responsible for the positive charge on the enzyme. The binding can take place through either of these groups [27].

1.3.3 Covalent Binding

Enyzmes can also be immobilized chemically. In covalent bindig there is sharing of electrons between functional groups of the enzymes and the surface functional groups of the substarte. The biocatalysts are firmly bound by covalent bonding. Therefore, there is less chance of biocatalyst leakage from the surface. On ther hand, this method is complex and costly.

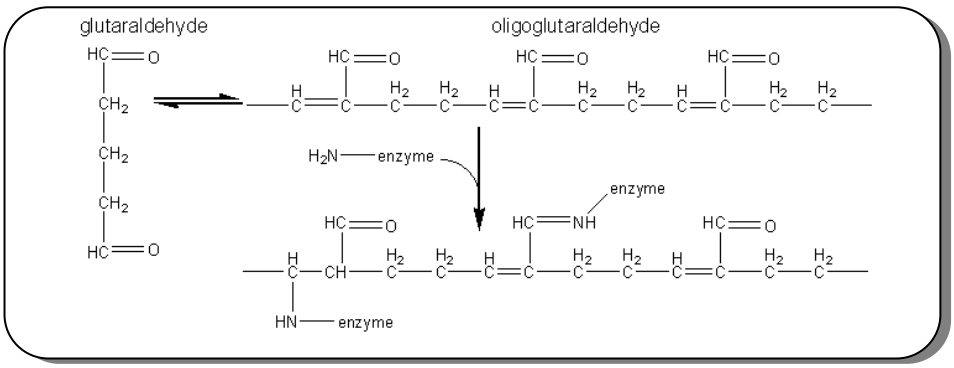


Figure 21: Coupling of glutaraldehyde with enzyme

Enzyme (Figure 21) is covalently bonded with glutaraldehyde through one of the aldehyde groups. The second aldehyde on the glutaraldehyde can be used to bind to any surface (electrode, glass) containing amine ($-NH_2$) functional groups for the enzyme immobilization [27].

1.3.4 Matrix Entrapment

The entrapment technique of immobilization is based on trapping the enzyme in synthetic or natural polymers. These gel-like matrixes should allow the entrance and leaving of the substrates and products, and keeping the enzyme inside the matrix [27].

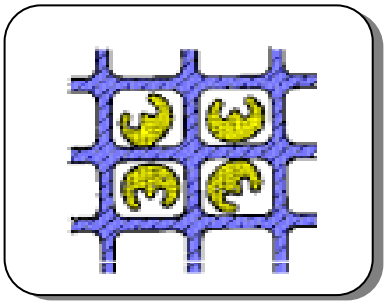


Figure 22: Enzyme trapped in matrix

1.3.5 Membrane Confinement

Semi-permeable membranes can also be used for traping the big molecules (enzymes) while leaving the small molecules to pass through the membrane. Membrane confinement can be achieved by three different techniques: by microencapsulation, by the liposome process, and by using biocatalyst in membrane reactors. In all three methods the substrates and products are crossed while keeping enzyme confined inside the membrane [27].

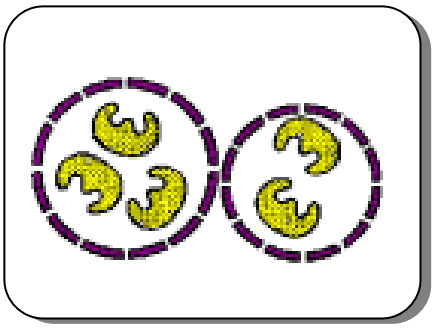


Figure 23: Enzmye confined in membrane

Table 2: Generalised comparison of different enzyme immobilisation techniques [27]

Characteristics	Adsorption	Covalent binding	Entrapment	Membrane confinement
Preparation	Simple	Difficult	Difficult	Simple
Cost	Low	High	Moderate	High
Binding force	Variable	Strong	Weak	Strong
Enzyme leakage	Yes	No	Yes	No
Applicability	Wide	Selective	Wide	Very wide
Running Problems	High	Low	High	High
Matrix effects	Yes	Yes	Yes	No
Large diffusional barriers	No	No	Yes	Yes
Microbial protection	No	No	Yes	Yes

1.4 Aim of the Work

The main objective is the waste-free production of enantiopure fine chemicals. Using the Dehydrogenases, we will develop an innovative electrochemical reactor, where all catalytically active substances are immobilized with spacers or in special surface layers so that, ideally, the end-product will not have to be purified.

As a part of this work, some metal complex mediators will be synnthesized for the regeneration of the cofactor. The activities of these mediators will be measured toward the reduction of the NAD^+. Moreover, suitable linker and conductive monomer will be prepared for the immoblization of the galctitol dehydrogenase.

CHAPTER 2

RESULTS AND DISCUSSION

2.1 Perspective of the Work

Redox reactions have important applications 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 this work we will try to develop an electrochemical reactor by using dehydrogenases for enantiopure synthon preparation (Figure 24).

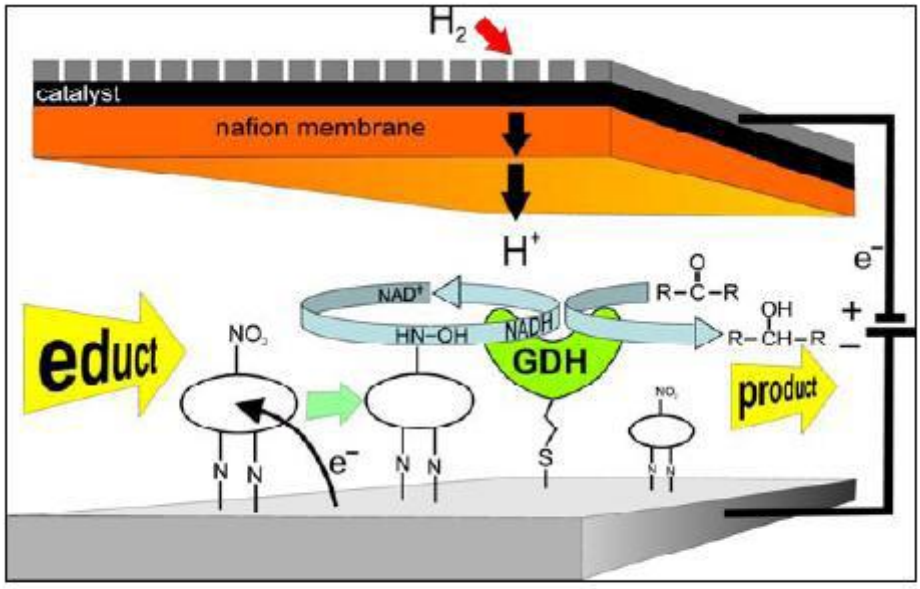


Figure 24: Model for electrochemical reactor

As nicely discussed in the Steckhan review [18], this is a not so easy task as such mediator should react with NAD(P) $^{+}$ and not transferring directly the electrons (or hydride ion) to the substrate, and the potential window for electrochemical activation of the catalysts is rather narrow. The mediator must indeed operate at potentials less cathodic than -0.9 V (otherwise direct electrochemical reduction of NAD (P) $^{+}$ will lead to dimmer formation) and more cathodic than the standard potential of the cofactor redox couple (i.e., -0.59 V vs. SCE for NAD^{+}/NADH) to make the reduction reaction thermodynamically feasible [28]. The best systems to date fulfilling these requirements are tris(2,2'-bipyridyl)rhodium complexes [29-31] and substituted or nonsubstituted (2,2'-bipyridyl)(pentamethylcyclopentadienyl)-rhodium complexes [32] and some others [33]. Among the most effective is $[Cp^{*}Rh(bpy)]^{2+}$ (where "Cp*" means pentamethylcyclopentadienyl and "bpy" 2,2'-bipyridyl), which has been extensively studied in solution [34-36].

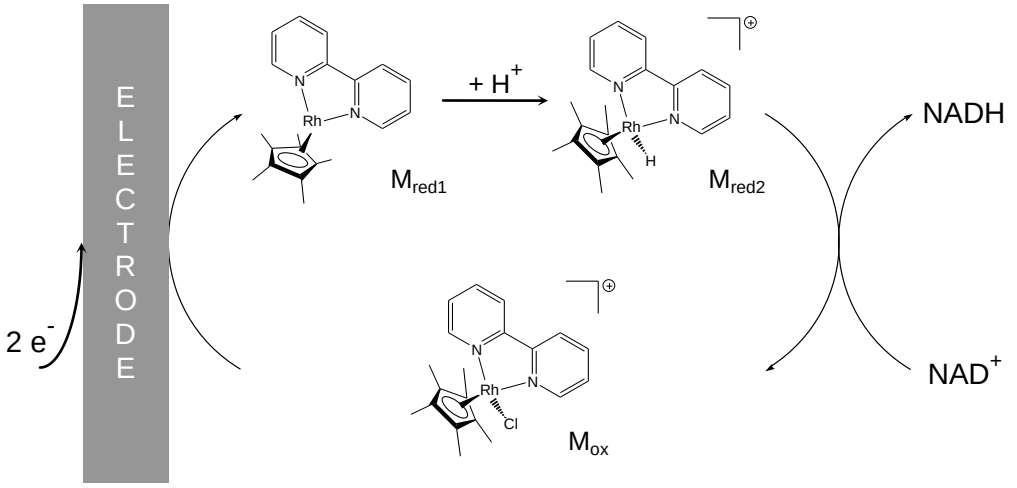


Figure 25: Mechanism of NAD^{+} Reduction

As illustrated in Figure 25, its electrocatalytic behaviour is rather complicated, involving first a two-electron electrochemical reduction of Rh^{III} (M_{ox}) into transient Rh^{I} species (M_{red1}; this reaction occurring itself in several steps [18]) that can be transformed upon protonation into a rhodium hydride complex (M_{red2}), which is then likely to transfer the hydride to NAD(P) $^{+}$ under formation of only 1,4-NAD(P)H.

It was reported that the chloro complex $[Cp^*Rh(bpy)Cl]^+$ was the most efficient form of the mediator [36].

In this work, linkers for immobilization of mediators, enzyme and cofactor will be synthesized and compounds participating to the processes 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.

The working electrode will be prepared from one partner and immobilization of mediator, enzyme and cofactor will be carried out by silica sol- gel method and covalent binding. Gold nanoparticles will present in silica sol-gel film in order to increase the electroactive surface of the electrode.

Our part in this project is to synthesize suitable linkers for enzyme, cofactor and mediator immobilization in addition to mediator synthesis.

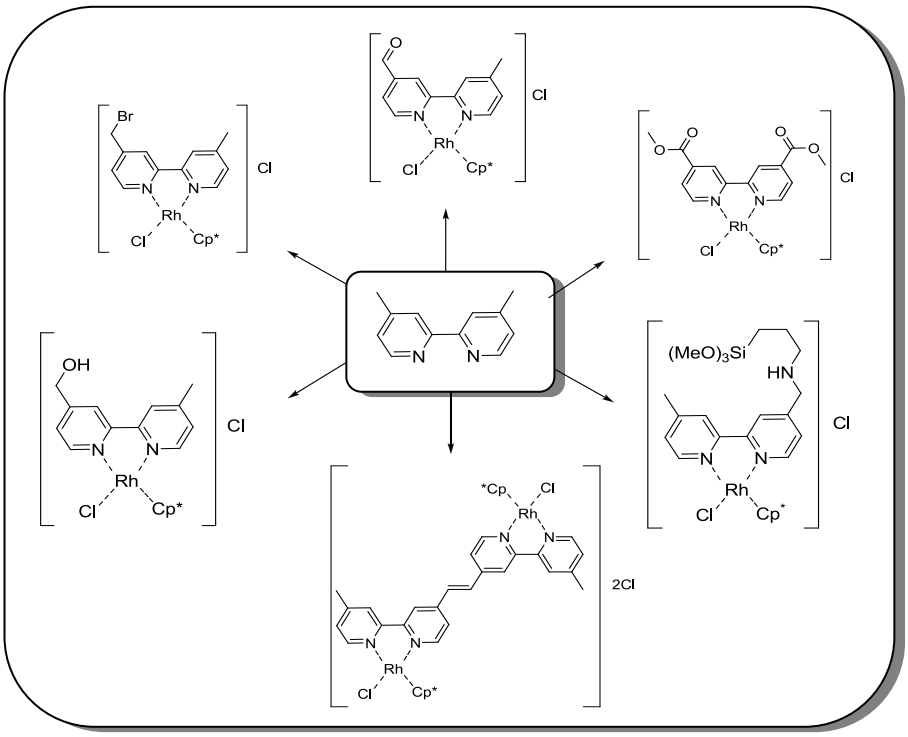


Figure 26: Synthesized pentamethylcycopentadienyl rhodium bipyridine derivatives as mediators

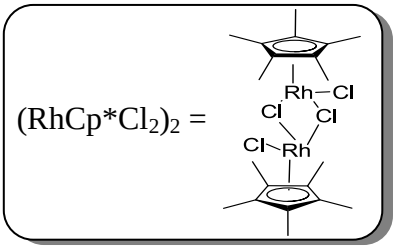


Figure 27: Rodium complex

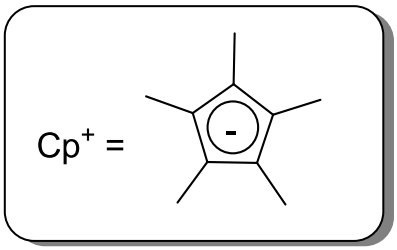


Figure 28: Pentamethylcyclopentadienyl anion

2.2 Synthesis of Mediators

2.2.1 Synthesis of mediator (2)

The biypridine **0** was brominated **1** selectively using NBS, and AIBN as radicalic initiator. The yield is low around 30% [38]. It is difficult to brominate selectively with high yield. The reason for this low yield is the formation of many side products. Bromination of both methyl groups is possible, on the other hand, two bromines can replace two protons of the methyl groups. It is almost impossible to prevent these side reactions and increase the yield. The proton NMR of the molecule is shown in Figure A. 1. The – CH_3 and –CH_2 resonate at 2.45 and 4.48 ppm respectively. The six different aromatic protons resonate between 7-9 ppm.

In the second step, the monobromo derivative **1** was reacted with rhodium complex dimmer to obtain the mediator **2.**

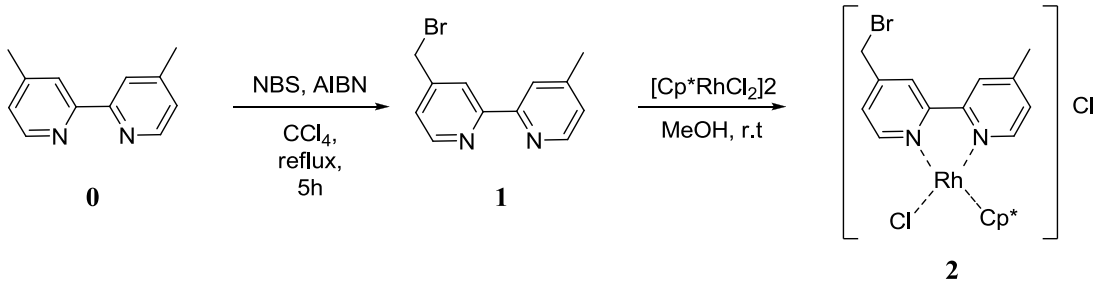


Figure 29: Synthesis of mediator **2**

While characterization of the mediator **2** (monobromo complex), it was observed that the peaks are splitted. Normally, protons (15H) from pentamethylcyclopentadienyl, - CH_3, and – CH_2 protons should resonate as singlets, and the aromatic protons should resonate as doublets. But the singlets are converted to doublets, and aromatic doublets are converted to unresolved triplets (Figure A. 3).

Form these observations, it is concluded that there are two isomers synthesized. The unresolved triplets are due to the overlap of the two doublets. Also in the carbon NMR (Figure A. 4), there are two close peaks arising from the five methyl carbons of the pentamethylcyclopentadienyl at 9.3 and 9.6 ppm.

2.2.2 Synthesis of mediator (4)

The bipyridine **0** was oxidized into monoaldehyde **3** by selenium dioxide and then it was reduced into monoalcohol **5** by $NaBH_4$ as shown in Figure 30 [37]. After purification of the alcohol derivative **5** by column chromatography, it was again oxidized into monoaldeyhde in the presence of MnO_2. After purification, the product was obtained with a yield of 47%. Since the bipyridine has two equal methyl groups, it is difficult to make allylic oxidation of one the methyl groups with high yield. The other reason for low yield is that some of the starting molecule was oxidized into acid rather aldehyde.

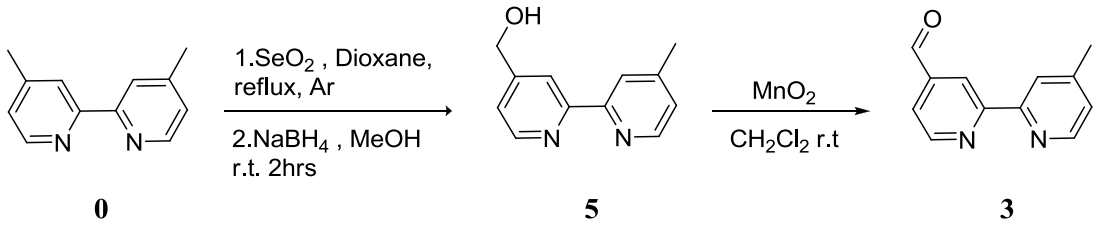


Figure 30: Synthesis of molecule **3**

In order to increase the yield and find an easier method, the above procedure was modified. Selective oxidation of one of the methyl groups of the bipyridine in the presence of the selenium dioxide was performed. Cooling the reaction mixture caused the precipitaion of the acid derivative leaving the monoaldehyde **3** in the solvent. After evaporation, the product was obtained with a 50% yield [38].

This method was perferred for synthesis of the monoladehyde because the product was obtained in a single step reaction and without any time consuming purification methods. The proton NMR of this compound clearly shows the presence of the monoaldehyde derivative of bipyridine in Figure A. 5

The peak resonating around 2.3 ppm shows the presence of the methyl group, likewise, resonance of six different peaks between the 7-9 ppm shows the presence of aromatic pyridine protons. Lastly, the aldehyde proton resonates around 10.1 ppm. (Figure A. 5).

In the second step, the monoaldeyhyde was reacted with $(RhCp^*Cl_2)_2$ to obtain the mediator **4** in high yield as shown in Figure 31.

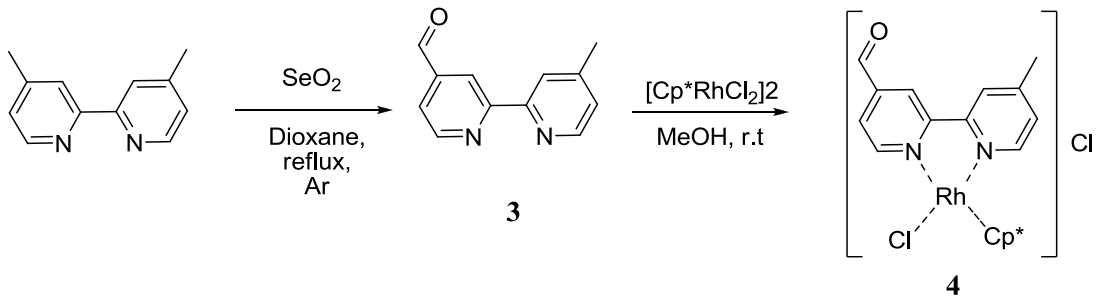


Figure 31: Synthesis of mediator **4**

For the characterization of the the mediator **4** both proton and carbon NMR were employed (Figure A. 7 and Figure A. 8).

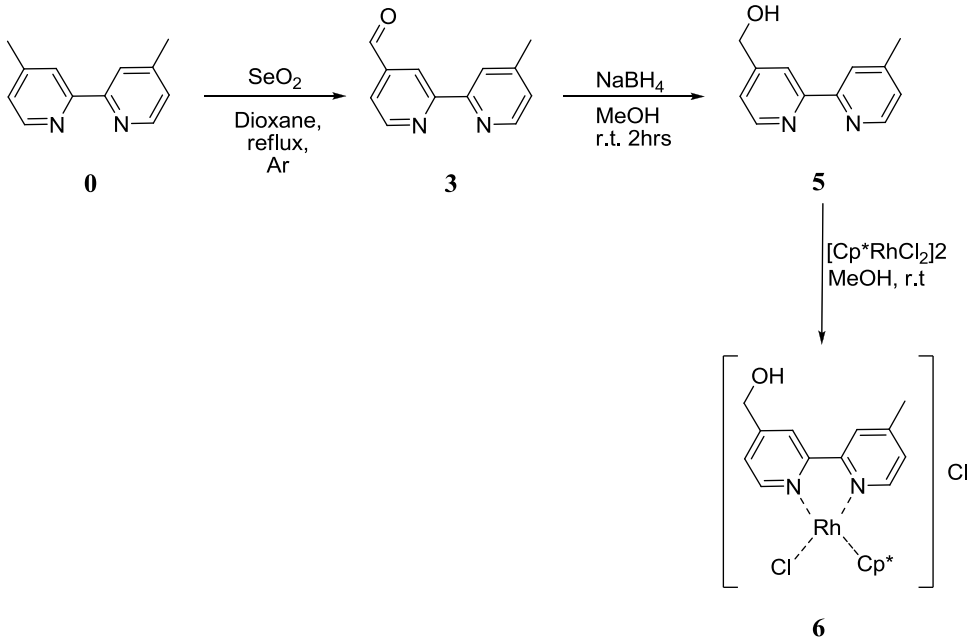
The tall peak (15H) resonating around 1.8 ppm which arises from the pentamethyl cyclopentadienyl five methyl protons. This means that we have the rohdium complex present but this does not prove that it is cordinated through the nitrogens of the bipyridine. The proof for the complex formation is the shift of the peaks to low field. After coordination, the complex formation decreases the electron density of the bipyridine, as a result, the peaks chemical shifts change.

For instance, the methyl protons resonates at 2.4 ppm before complexation. But after the coordination, the methyl protons resonate at 2.67 ppm. The same case is true for all other peaks. Also in the carbon NMR (Figure A.8) of the complex, there is a peak around 97 ppm which is arising from the five quaternary carbons present in the structure of pentamethycyclodienyl. All carbon peaks are also shifte to the left or low field (Figure A. 7 and Figure A. 8).

2.2.3 Synthesis of mediator (6)

Bipyridine **0** was oxidized to monoaldehyde **3** with same way as above, then it was reduced to monoalcohol **5** in the presence of $NaBH_4$ [Figure 32][39].

In the proton NMR, all the monoalcohol peaks are seen clearly. The methyl protons resonates around 2.32 ppm and the $–CH_2$ protons connected to –OH group give a single peak at low field at 4.64 ppm. Just beside the $–CH_2$, a broad peak is observed whic is belong to –OH group. Six different protons are resonating between 7-9 ppm arising from aromatic bipyridine (Figure A. 9).



0 **3** **5** **6**

Figure 32: Synthesis of mediator **6**

In the next step, monoalcohol **5** was reacted with rohdium complex to give expected mediator **6**. The characterization of the mediator **6** (monoalcohol complex) was done by NMR spectroscopy.

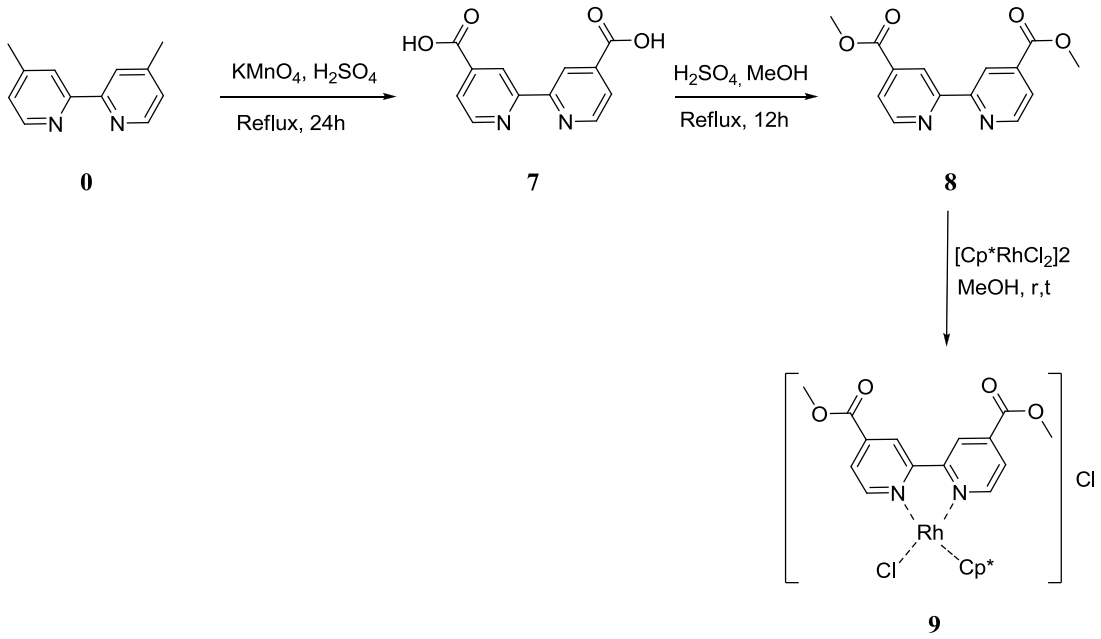
The shifts in the peaks positions show that the rohdium complex is coordinated to the monoalcohol. These shifts to lower field are due to the lowering of the electron density in the molecule. The long singlet at 1.59 ppm is arising from the penta ethylcyclopentadienyl. The $-CH_3$ protons are shifted from 2.32 to 2.47 ppm and the $-CH_2$ is shifted from 4.64 to 4.80 ppm. The most interesting point is the broad, –OH peak. As it is seen clealy above that the broad singlet is changed to a sharp singlet as the electron density over the molecule decreases (Figure A. 11).

In the carbon NMR (Figure A. 12), a tall peak is seen at 8.3 ppm which is arising from the 5 methyl carbons of pentamethylcyclopentadienyl. The peak around 97 ppm is due the quaternary carbons of pentamethylcyclopentadienyl. All other peaks are shifted to the lower field.

2.2.4 Synthesis of mediator (9)

Again starting from bipyridine **0**, methyl groups were oxidized to acid groups (**7**) in the presence of chromium(VI) oxide [40]. A second method was attempted to get much better results using $KMnO_4$ instead of chromium(VI) oxide [41]. The second method with $KMnO_4$ gave beter result and it was preferred for oxidation. The diacid derivative **7** of bipyridine has poor solubility almost in all solvents, therefore, its NMR could not be recorded and it was used for next step for esterification. The diacid derivative was reacted with concentrated H_2SO_4 and methanol to give the methyl ester **8** derivative in excellent yield.

The proton NMR data clearly shows the peaks that are belong to the methyl ester bipyridine derivative. The singlet peak at 4.0 ppm is arising from the two methyl groups. Since the molecule is symmetric, there are three different aromatic protons instead of six and as a result three peaks are seen arising from six aromatic protons (Figure A. 13).



0 **7** **8** **9**

Figure 33: Synthesis of mediator **9**

In the third step, the ester derivative **8** was reacted with rohdium complex dimmer to get the expected mediator **9** as shown in Figure 33. The mediator **9** (ester complex) was identified by NMR spectroscopy.

The tall peak at 1.9 ppm is due to the 15 protons of pentamethylcyclopentadienyl. The methyl ester protons are shifted from 4.0 from 4.07 ppm and similarly, the six (6H) aromatic protons are shifted to the left (Figure A. 15). In carbon NMR (Figure A.16), the peaks at 9.7 and 97 ppm are arising from pentamethylcyclopentadienyl. And all other peaks are shifted to the lower field.

2.2.5 Synthesis of mediator (11)

For the synthesis of alkoxysilane linked mediators which can be used for sol-gel process, bipyridine **0** was brominated selectively as above procedure and then it was reacted with 3-aminopropyltrimethoxysilane (**01**) in the presence of triethylamine to get resulting intermediate **10** for complexation [42]. In the last step, the resulting intermediate was converted into the desired mediator **11** by reaction with $(RhCp^*Cl_2)_2$, as illustrated in Figure 34.

The mediator **11** was characterized using NMR spectroscopy, as it is seen clearly from Figure A. 19 that the three $-CH_2$ groups between the –NH and methoxysilane resonate between 0.6- 2.6 ppm and the other $-CH_2$ gorup connected to pyridine and the three methoxy groups resonate at about 3.75 ppm and 3.55 ppm respectively. The protons arising from the bipyridine resonate at the aromatic range as its expected.and all the expected peaks are shifted to lower field. Similarly, the carbon shifts (Figure A. 20) give the expected results. The methyl and quarternary carbons from pentamethyl cyclopentadienyl resonate at 9.5 and 98 ppm respectively.

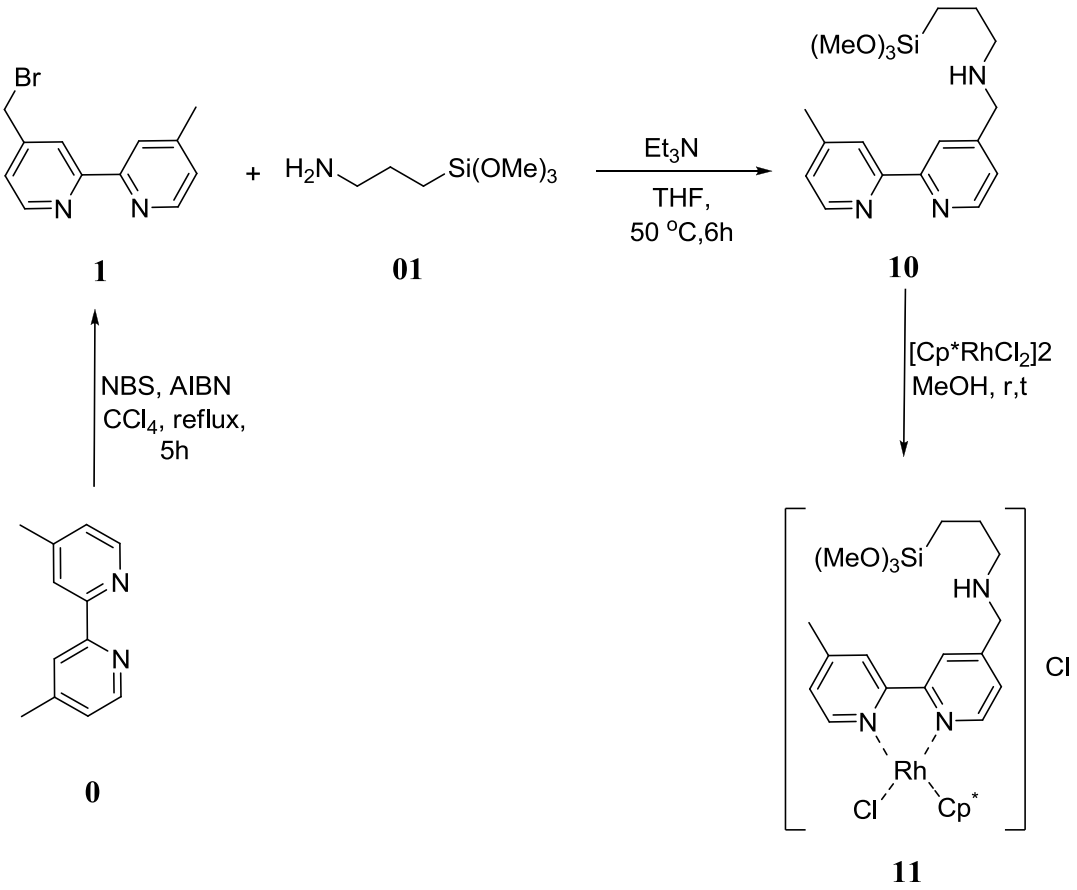


Figure 34: Synthesis of mediator **11**

Mediators containing alkoxysilane groups can be immobilized or trapped through sol-gel process. The alkoxysilane group can make chain-like polymer networks as shown in Figure 35. This process gives the advantage of recyclying of the mediator. Since the mediator is be trapped in sol-gel, it can not escape and mix with the reaction mixture, therefore, it can be reused without any purification.

The sol-gel process is a wet-chemical technique used for the fabrication of both glassy and ceramic materials. In this process, the sol (or solution) evolves gradually towards the formation of a gel-like network containing both a liquid phase and a solid phase. Typical precursors are metal alkoxides and metal chlorides, which undergo hydrolysis and polycondensation reactions to form a colloid. The basic structure or morphology of the solid phase can range anywhere from discrete colloidal particles to continuous chain-like polymer networks (Figure 35)

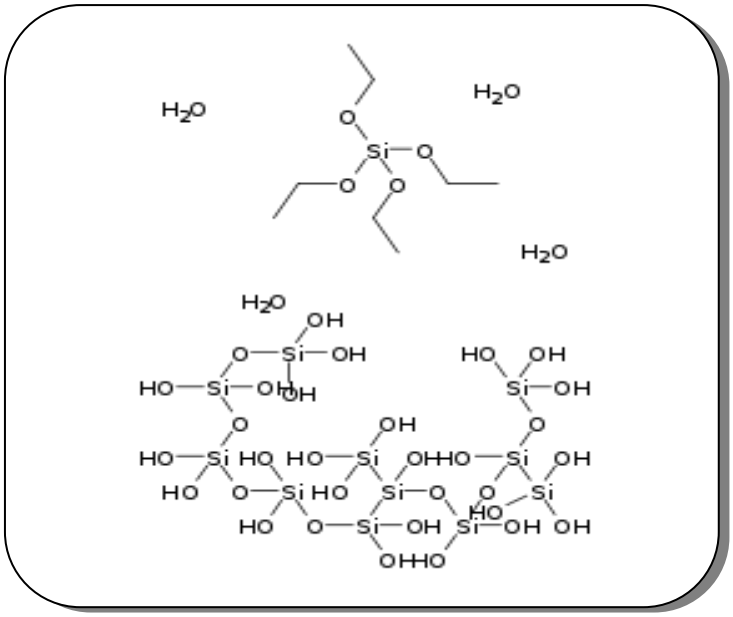


Figure 35: TEOS, water and polymerized Sol-gel

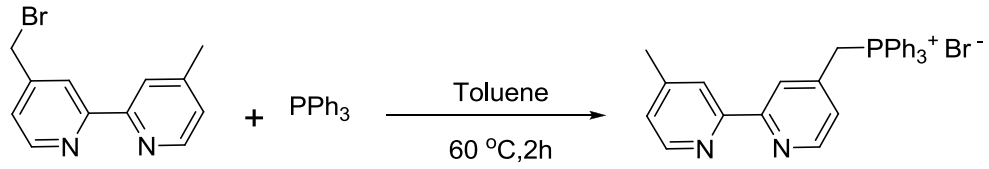
A well studied alkoxide is silicon tetraethoxide, or tetraethyl orthosilicate (TEOS). The chemical formula for TEOS is given by: $Si(OC_2H_5)_4$, or $Si(OR)_4$ where the alkyl group R = C_2H_5. Alkoxides are ideal chemical precursors for sol-gel synthesis because they react readily with water [57]. The reaction is called hydrolysis, because a hydroxyl ion becomes attached to the silicon atom as follows:

$$Si(OR)_4 + H_2O \rightarrow HO\text{-}Si(OR)_3 + R\text{-}OH$$

2.2.6 Synthesis of mediator (14)

Mediators are electron transporters in electrochemical method. They are electron rich or contain some conjuagted units. Inspirising from this idea, we thought that synthesizing a molecule with a richer electron density and having some conjugation would increaase the efficiency of the mediator. In order to this, the two bipyridine unites were connected through a double bond to maintain the conjugation. To get the intermediate **13,** the below procedure was applied successfully and the then it was converted to the expected mediator **14.**

The monobromo bipyridine derivative **1** was reacted with tirphenylphosphine to give the bromide salt **12.** The bromide salt was used for the next step without characterization, because its proton NMR (Figure A. 01) is vague and the peaks are broad. It's difficult to comment on its NMR spectrum.

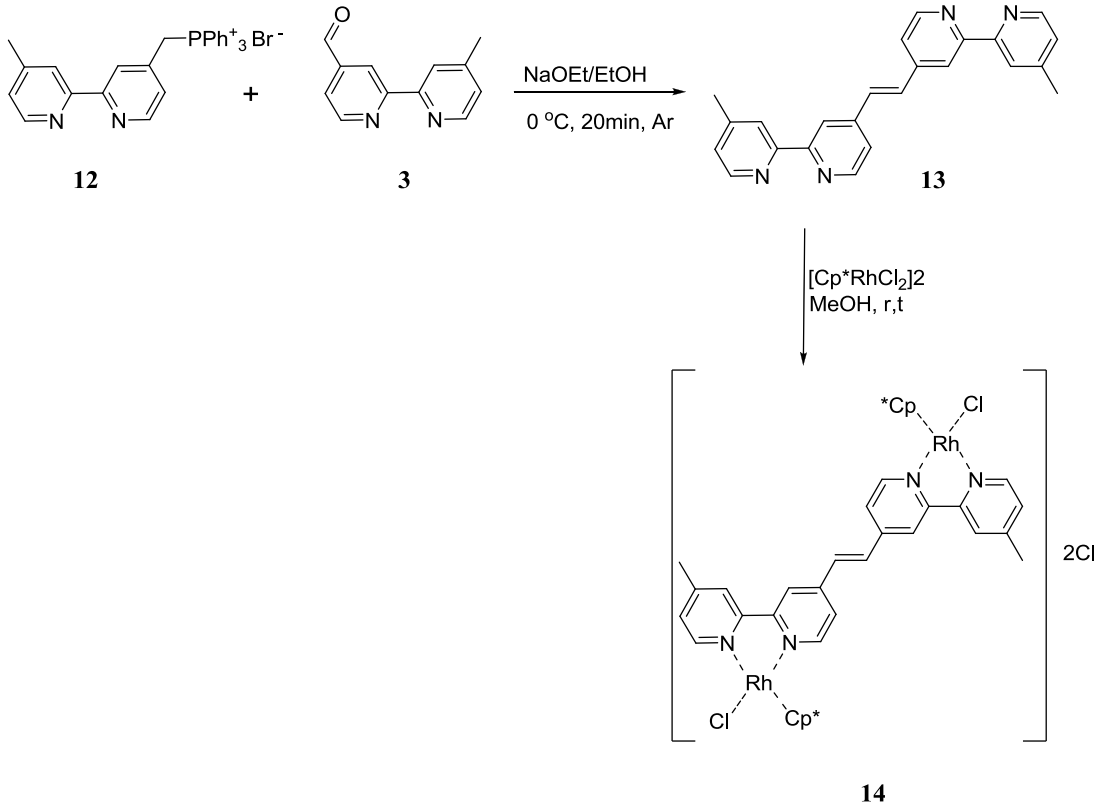


1 **12**

Figure 36: Synthesis of intermediate **12**

In the second step, using the Wittig reaction, the bromide salt **12** was reacted with the monaldehyde **3** in presence of a base to give the expected intermediate **13** [38]**.**

The proton NMR of the intermediate **13** clearly demonstrates the belonging peaks of the molecule. The first singlet (6H) peak at 2.39 ppm is arising from two equal methyl groups connected to the two bipyridine units. The two olefinic protons resonate at 7.11 ppm as doublets. The large coupling constant of olefinic protons shows that the molecule is in the *trans*-form.



12 **3** **13** **14**

Figure 37: Synthesis of mediator **14**

The resulting intermediate **13** was converted into the desired mediator **14** by reaction with $(RhCp^*Cl_2)_2$, as illustrated in Figure 37. The mediator was identified using NMR spectroscopy.

The NMR spectrum clearly shows the formation for the mediator **14.** The tall singlet (15H) at 1.63 ppm arises from pentamethylcyclopentadienyl protons and the methyl protons are shifted to the lower field from 2.39 to 2.41 ppm. The aromatic and olefinic protons are also shifted to the left. In carbon NMR (Figure A. 24), there are two peaks at 9.1 and 96.8 ppm which are arising from pentamethylcyclopentadienyl. The rest of the carbons are also shifted to the lower field due to the decrease in electron density of the molecule.

2.2.7 Cyclic voltammetry analysis of mediators

A series of rhodium complexes (**1**-**12**) [Figure 38] have been tested in cyclic voltammetry. All mediators gave rise to well-defined signals in the absence of cofactor. The cathodic peak potential values were found to be dependent on the nature of the substituents present on the bipyridine ligand. The presence of electron-donating groups increases the electron density at the rhodium centre and make it more difficult to be reduced (cathodic shift) whereas electron-withdrawing groups tended to facilitate the reduction (anodic shift), in agreement with previous observations [43]. For example, the reduction of the compound bearing electron-donating amino groups in 4-position on the bipyridine ligand (**3**) occurred at -0.89 V. Alkyl-substituted complexes (**2**, **10**) as well as methoxy species (**8**) did not have significant influence on reduction potentials.

On the other hand, the nature of the substituents on the bipyridine had a dramatic impact on the electrocatalytic properties of the rhodium complexes. Actually, the complexes which were active with respect to NAD^+ reduction are compounds **1**, **2**, **4**, **5**, **8**, **10**, while the others (**3**, **6**, **7**, **9**, **11**, **12**) did not exhibit any noticeable electrocatalytic activity. It is not straightforward to rationalize these data but a trend seems to appear as inactive compounds are those bearing amine or thiol groups or carbonyl moieties. In particular, derivatives functionalized with thiol groups are attractive for immobilization of reagents in the form of, e.g., self-assembled monolayers (SAMs) on gold electrodes [44,45] and the amine-functionalized ones are good precursors to form, e.g., organosilane reagents likely to be grafted onto metal oxides or incorporated within sol-gel matrices then deposited onto electrode surfaces [45-48].

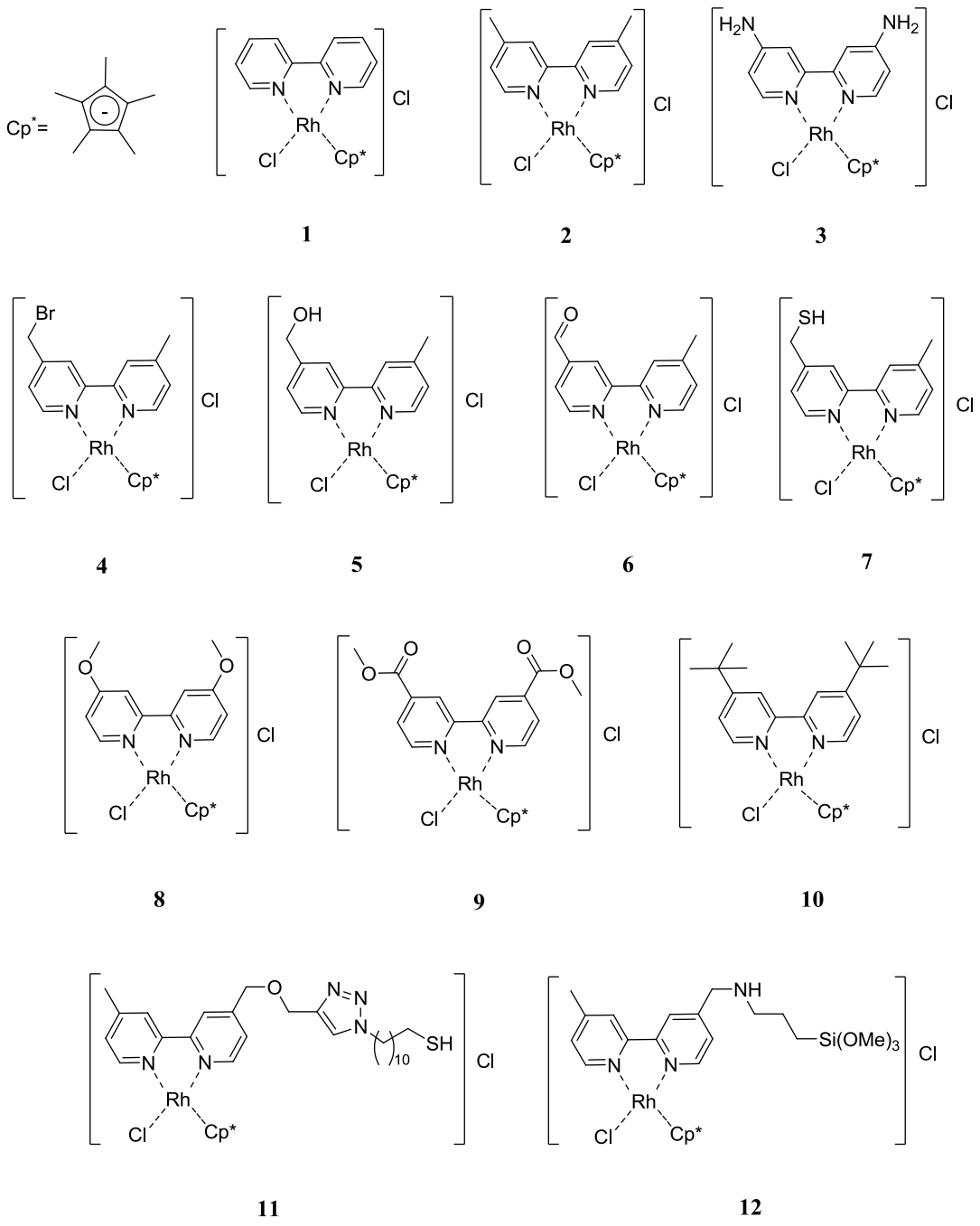


Figure 38: Bipyridine complex mediators

All the mediators from **1** to **12** (Figure 38) were synthesized and their activities toward the NAD^+ reduction were determined by the partners who are involved in this Project.

2.3 Synthesis of linkers

Synthesizing suitable linkers for enzyme, cofactor and mediator immobilization is very important. Orgonosulfur compounds which include dialkyl sulfides, dialkyl disulfides and thiols have been investigated on gold. These groups interact with the gold surface strongly. One 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 [49, 50][Figure 39].

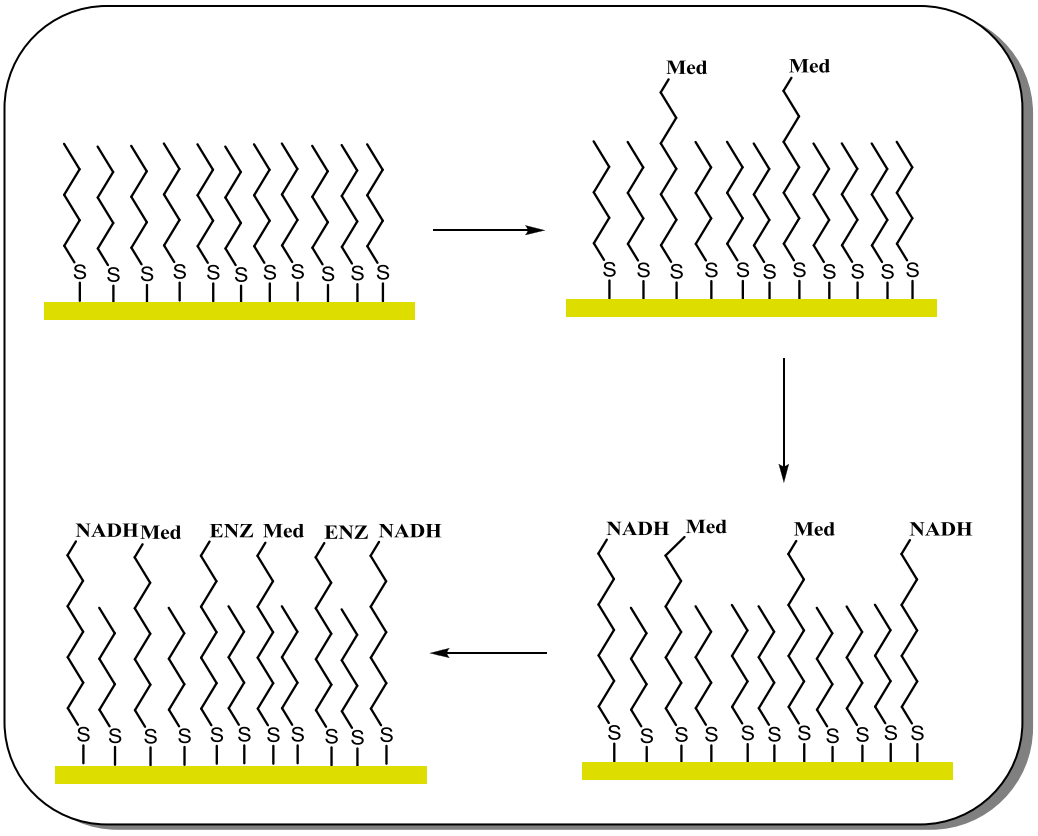


Figure 39: Immobilization of Mediator, NADH, and Enzyme on gold nano surface via Linkers

Another approach for the immobilization of biomolecules is the polymerization of a conductive monomer- having long chain (linker) with –COOH groups- over an electrode. The –COOH can react with $–NH_2$ groups of enzyme, cofactor, and mediator in the presence of water soluble carbidiimides (CMC, EDC and DCC) to form amide. The length of the linker is very important for the flexibility of the enzmye, cofactor, and mediator. If the flexibility of these immobilized biomolecules are disturbed, their activities may decrease or totally diminished (Figure 40).

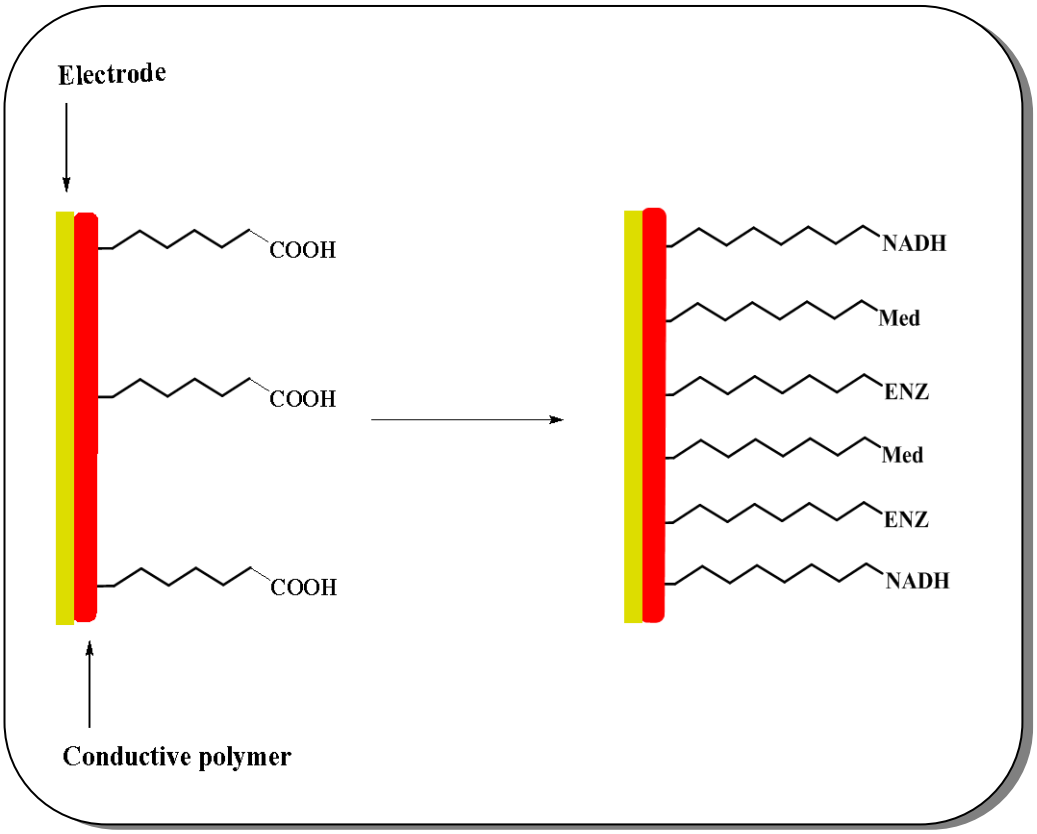


Figure 40: Immobilization of Mediator, NADH, and Enzyme on electrode surface containing conductive polymer (SNS) via Linkers

Two different immobilization methods are shown in Figure 39 and Figure 40. The aim of these immobilization methods are to resuse or recycle the Enzyme, Medaitor, and the Cofactor. In this study the second method will be used for the immobilization of the galactitol dehydrogenase.

A conductive SNS monomer will be prepared which contains a linker with acid group (-COOH) as shown in Figure 41. There are two ways of immobilization. First, the monomer will be synthesized and then it will be polymerized over the electrode. Then the eletrode is soaked to a solution containing the enzyme. After washing the electrode, the enzyme will be bound to the electrode surface through the linker.

In the second way, after preparation of the monomer, it will be reacted with the enzyme and then it will be polymerized over the electrode.

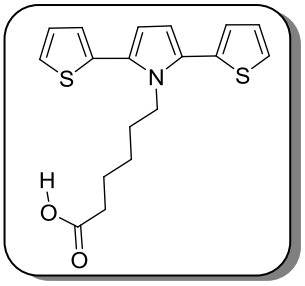


Figure 41: Conductive SNS monomer

The SNS monomer will not only be used for the immobilization of the enzyme but also it will be used for immobiliztion of mediator and cofactor. As it shown in Figure 42 and 43 that mediator and enzyme are bound to SNS monomer. After the polymerization of these species on electrode, they will be immobilized.

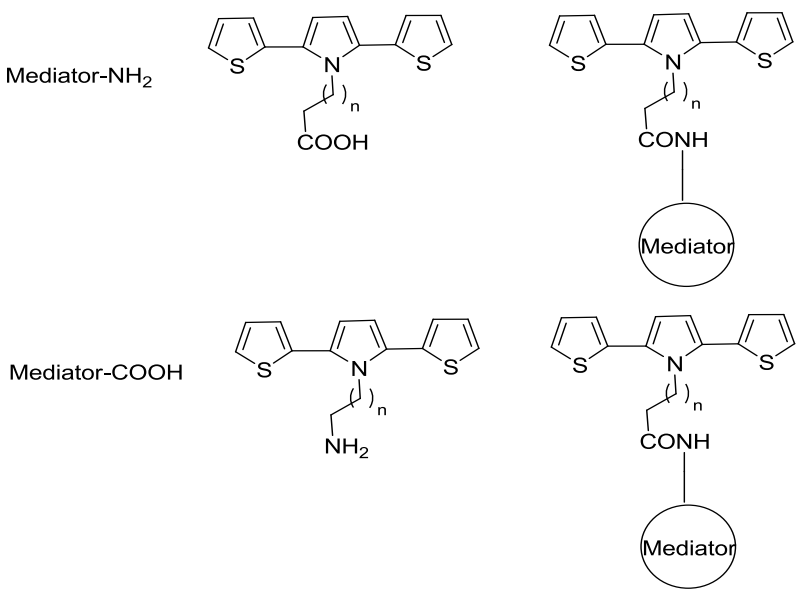


Figure 42: Reaction of the mediators with SNS monormer

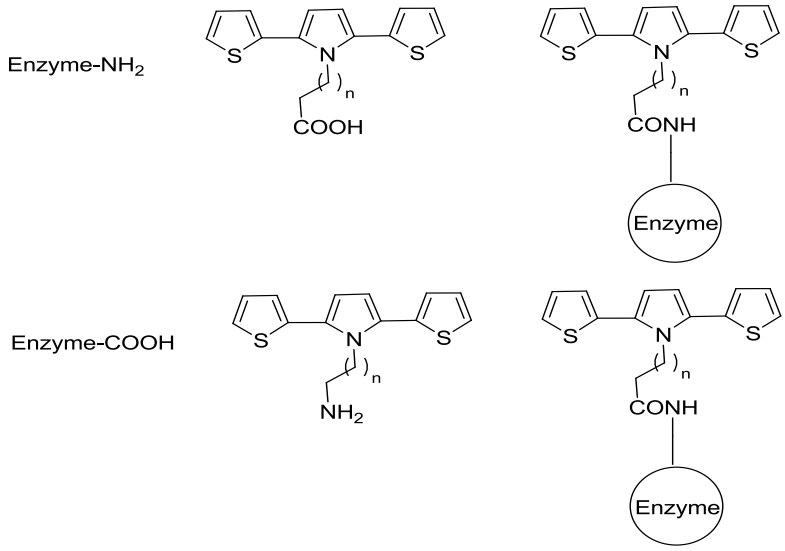


Figure 43: Reaction of the Enzymes with SNS monormer

2.3.1 Synthesis of 6-Aminocaproic acid methyl ester linker

Ring-opening of ε-caprolactam **02** using hydrochloric acid gives the amino acid salt intermediate **15** [51]. This intermediate was characterized with NMR spectroscopy (Figure A. 25). Since amino acid salt has poor solubility, it was converted to a more soluble intermediate **16** by estirification in the presence of TMSCl [52]. This intermediate was also characterized by NMR spectroscopy (Figure A. 27). In order to get rid of the hydrochloride, ammonia gas was passed through amino acid salt ester **16** [53]. Ammoniun chloride precipitated and the linker **17** formed. The product **17** was used immediately for the next step because it is not stable and recyclization takes place.

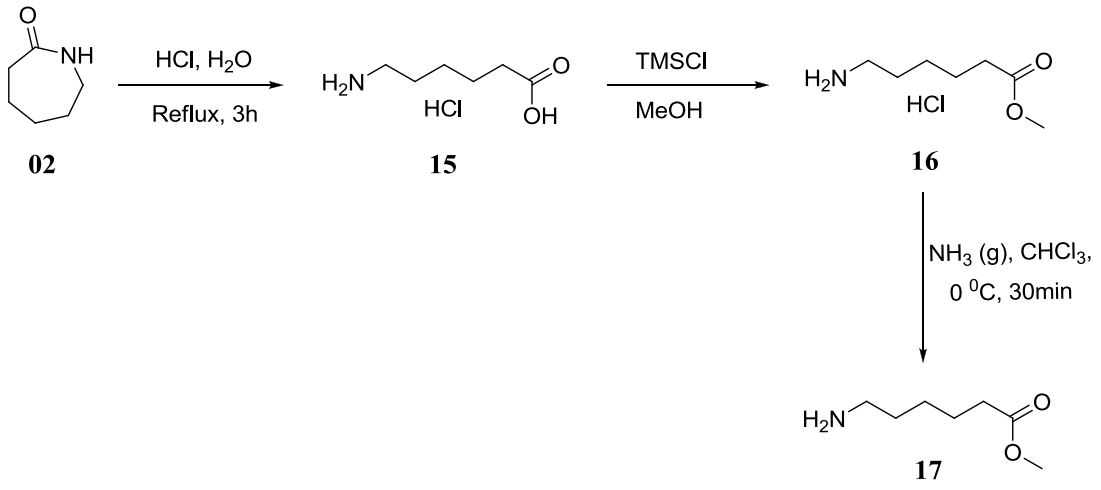


Figure 44: Synthesis of linker **17**

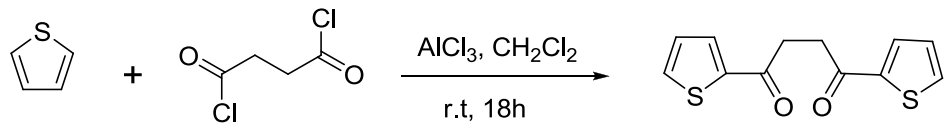
Both proton and carbon NMR spectra (Figure A. 29) clearly show the formation of the linker **17.** In the proton NMR, the three multiplets between 1-1.8 ppm are arising from the three $–CH_2$ groups. A singlet around 1.9 ppm is arising from two protons of $–NH_2$. The two triplets between 2-3 ppm are arising from the two $–CH_2$ protons. Finally, the long singlet at 3.7 ppm belongs to methyl group (3H) coonected to oxygen.

As it is seen clearly from carbon NMR (Figure A. 30) that there are seven different carbons which is consistent with the structure of the linker **17.** The five $–CH_2$ carbons resonate between 20-40 ppm and the $–CH_3$ carbon just resonates at around 52 ppm due to the oxygen bonded directly to it and shifting to much lower field. Finally, quarternary ester carbon resonates at 174 ppm which is the expected result.

As mentioned above, the linker **17** is not stable and it should be used immediately, kept in cold or kept in its salt form so that recyclization is prevented or slowed down to some extent. Also its NMR should be recorded as soon as it is synthesized in order to get a better spectrum.

2.3.2 Synthesis of monomer (SNS) connected to linker

The thiophene **03** and succinyl chloride **04** were reacted in the presence of aluminium chloride to get the intermediate **18** [54]. It was characterized by NMR spectroscopy (Figure A. 31).



03 **04** **18**

Figure 45: Synthesis of intermediate **18**

The intermediate **19** was hydrolyzed by lithium hydroxide to convert the ester to acid group [56] to obtain the expected SNS-monomer containing the linker **20**. In order to synthesize intermediate **19,** the intermediate **18** was reacted with the linker **17** in the presence of the acetic acid as a catalyst to obtain the molecule **19** (SNS-linker) in low yield [55]. The reason for the low yield is the cyclization of the linker **17** during 50 hours of reflux.

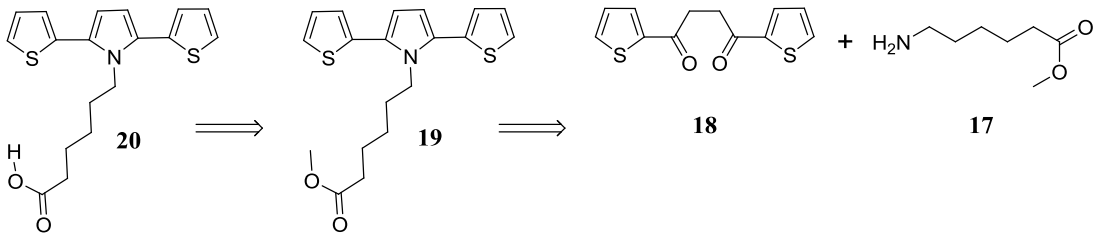


Figure 46: Retrosynthetic pathway for the synthesis of conductive SNS monomer **20**

The proton NMR of intermediate **19** explicitly shows the expected chemical shifts. All the $–CH_2$ groups from linker **17** are present, only the $–CH_2$ at 4.2 ppm connected to the pyrole shifted to lower field. It is clearly seen that the two protons coming from $–NH_2$ of linker **17** are lost because pyrole is formed. The singlet (3H) at 3.7 ppm is arising from the ester of the linker as expected. And all other pyrole and thiophene protons are present in the aromatic region. The presence of carbon peak around 174 ppm shows the presence of ester (Figure A. 34).

The proton NMR of SNS-linker **20** explicitly shows that ester group is lost and the rest of the peaks are present. Acidic proton around 11 ppm is not seen but, in carbon spectrum (Figure A. 36), the carbon peak around 178 ppm shows the presence of acid group.

2.3.3 Reaction of SNS-linker with galactitol deydrogenase

The SNS monomer was reacted with galactitol dehydregenase in the presence of a water soluble carbodiimide (EDC or CMC) to get the expected product **21** which can be polymerized for immobilization of the enzyme [58].

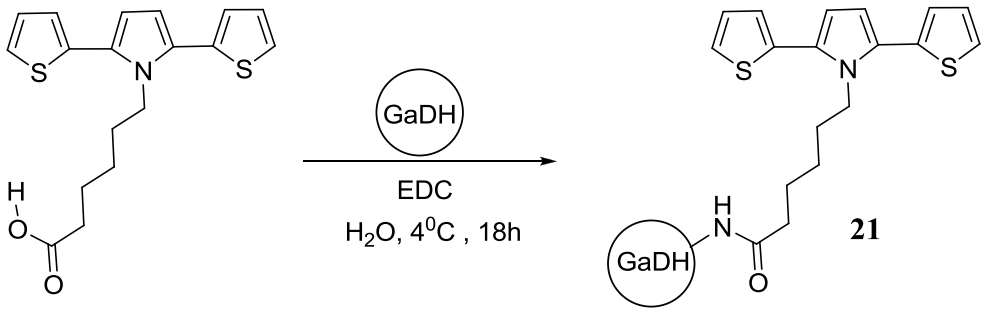


Figure 47: Rection of Galactitol dehydrogenase (GADH) with SNS

Characterizations of immobilized or functionalized enzymes are performed using UV spectrometry [58]. The product **21** was also identified using UV. The reaction mixture was extracted with chloroform and then the aqueous extract UV was recorded. In order to compare the result, pure enzyme dissolved in water UV was also recorded. The pure enzyme UV spectrum shows maxima at 205 nm while the aqueous extract UV shows maxima at 215 nm (Figure 37). The positive shift in UV spectrum demonstrates the formation of product **21.** The SNS-enzyme will be polymerized over an electrode for immobilization and the enzyme activity will be determined.

CHAPTER 3

EXPERIMENTAL

3.1 Materials and Methods

Melting points are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded at 25 °C in $CDCl_3$ solutions at 300 MHz or 400 MHz and 75 MHz or 100MHz, respectively, with Me_4Si as internal standard. Chemical shifts (δ) and coupling constants (*J*) are given in ppm and in Hz, respectively. All reactions were analyzed by TLC on silica gel 60 F_{254}. TLC was carried out on aluminum sheets precoated with silica gel 60F254 (Merck), and the spots were visualized with UV light (l = 254 nm). Column chromatography was performed on silica gel 60 (70-230 mesh). Evaporation refers to the removal of solvent under reduced pressure.

3.2 General Procedure for Preparation of Mediators, Linker, and SNS

3.2.1 Synthesis of 4-(bromomethyl)-4'-methyl-2, 2'-bipyridine (1)

4-(bromomethyl)-4'-methyl-2, 2'-bipyridine (1.8 g, mmol) is dissolved in 50 mL of CCl_4 in a 100 mL balon. To this solution, N-bromosuccinimide (1,8 g, 10,1 mmol), and azobis(isobutyronitrile) (0.05 g) are added respectively. The resulting solution is refluxed at 80 °C under argon atmosphere for 5 h. After cooling the mixture to room temperature, it is filtered and the solvent is evaporated using Rotary Evaporator. After evaporation of the solvent the residue is dissolved in a minimum amount of CH_2Cl_2 for column chromatography on silica gel.

The clomun is eluted by Ethylacetate: Acetone (98: 2) and the residue is collected from the **second band.** After the combination of all parts, 0.75 g (30%) of product (pinkish crystals) is obtained after drying. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.45 (s, 3H), 4.48 (s, 2H), 7.16 (d, *J*=4.9 Hz, 1H), 7.33 (dd, J_1=5.0 Hz, J_2=1.7 Hz, 1H), 8.24 (s, 1 H), 8.41 (s, 1H), 8.54 (d, *J*=4.9 Hz, 1H), 8.66 (d, *J*=5.0 Hz, 1H); ^{13}C NMR (400 MHz, $CDCl_3$): δ 21.21, 30.76, 120.98, 122.06, 123.52, 125.01, 147.18, 148.27, 149.04, 149.67, 155.30, 156.97 ppm

3.2.2 Synthesis of 4-(bromomethyl)-4'-methyl-2, 2'-bipyridine rhodium complex (2)

20 mg 4-(bromomethyl)-4'-methyl-2, 2'-bipyridine (0.076 mmol) of was mixed with 23, 5 mg (0.038 mmol) of penta methylcyclopentadienylrhodium (III) chloride in 15ml of dichloromethane and was stirred for 7- 8 hours. After evaporating dichloromethane, 15 mL of ether was added and stirred over night. After decantation of ether, 40 mg of yellowish solid was obtained. Two isomers; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.73 and 1.77 (two s, 15 H), 2.64 (two s, 3 H), 4.99 (two s, 2 H), 7.66 (t, *J*=6.0 Hz, 1H), 7.99 (t, J=5.9 Hz, 1H), 8.70 (dd, J1=14.8 Hz, J2=5.7 Hz, 1H), 8.83-8.90 (m, 2H), 9.26 (d, J=10.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 9.3, 9.6, 21.5, 43.3, 97.0, 97.1, 97.3, 97.4, 124.2, 124.3, 126.0, 126.1, 129.5, 129.6, 150.6, 151.1, 151.5, 151.9, 153.3 ppm

3.2.3 Synthesis of 4'-methyl-[2, 2'-bipyridine]-4-carbaldehyde (3)

4,4'-dimethyl-2',2'-bipyridyl (1,8 g, 9.7 mmol) was dissolved in 50 mL of 1,4-dioxane and then SeO_2 (1,1 g, 9,8 mmol) was added. The mixture was refluxed at 101°C under argon atmosphere for 24 h. The hot solution was filtered in order to remove the precipitated selenium.

After cooling the solution, it was allowed to stay for 1 hour at room temperature. A pale yellow precipitate was formed and the mixture was filtered for the second time to remove 4'-methyl-2, 2'-bipyridine-4- carboxylic acid. The 1, 4-dioxan was evaporated and was well dried under vacuum and the residue was redissolved in 500 mL of ethyl acetate. Aqueous Na_2CO_3 (0,1 M, 20 mL) was added to the solution to remove the remaining 4'-methyl-2,2'-bipyridine-4- carboxylic acid. After extraction with 0.2 M aqueous solution of sodium bisulfite (3 x 30 mL), the aqueous extracts were gathered and were set to a pH of 9 by the addition of Na_2CO_3. Final extraction was performed with CH_2C1_2 (3 x 30 mL). The CH_2C1_2 extracts were gathered and evaporated to dryness under vacuum to give the product **(**0.82 g., 43% yield) as a white powder. ^{1}H NMR (400 MHz,$CDCl_3$) δ 2.40 (3H, s) 7.13 (d, lH); 7.6 (dd, lH); 8.22 (s, lH); 8.52 (d, lH); 8.77 (s, lH); 8.83 (d, lH); 10.12 (s, lH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.22, 120.58, 121.93, 122.09, 125.40, 142.67, 148.42, 149.22, 150.31, 154.75, 158.34, 191.77 ppm.

3.2.4 Synthesis of 4'-methyl-[2, 2'-bipyridine]-4-carbaldehyde rhodium complex (4)

20 mg 4'-methyl-[2, 2'-bipyridine]-4-carbaldehyde (0.1 mmol) of was mixed with 31.2 mg (0.05 mmol) of penta methylcyclopentadienylrhodium (III) chloride in 15 mL of dichloromethane and was stirred for 8-10 hours. After evaporating dichloromethane,

15 mL of ether was added and stirred over night. After decantation of ether, 50 mg of yellowish solid was obtained.^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.76 (s, 15 H), 2.67 (s, 3H), 7.62 (d, J=5.5 Hz, 1H), 8.29 (s, 1H), 8.68 (d, J=5.6 Hz, 1H), 9.12-9.17 (m, 2H), 9.94 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 9.3, 21.6, 97.4, 125.7, 127.1, 129.6, 144.3, 148.5, 150.3, 152.8, 153.6, 153.8, 156.5, 190.6 ppm

3.2.5 Synthesis of 4-Hydroxylmethyl-4'-methyl-2, 2'-bipyridine (5)

To a cold (0 °C) solution of 4'-methyl-[2, 2'-bipyridine]-4-carbaldehyde (70 mg, 0.353 mmol) in MeOH (5 mL) was added sodium borohydride (13.4 mg, 0.353 mmol). The solution was stirred for 2-3 hours at 0 °C and then methanol was evaporated and the residue was dissolved in H_2O (5 mL). The product was extracted with CH_2Cl_2 (20 mL × 3). The combined organic parts was extracted with saturated aqueous $NaHCO_3$ (5 mL), and saturated aqueous NaCl (5 mL) respectively. After drying over $MgSO_4$, the solvent was evaporated to give the 4-hydroxylmethyl-4'-methyl-2, 2'-bipyridine as a white solid (30mg).^{1}H NMR (400 MHz,$CDCl_3$) δ 2.32 (s, 3H); 4.64 (s, 2H); 4.81 (brs, -OH, 1H); 7.03 (d, lH); 7,16 (d, lH); 8.06 (s, lH) 8.10 (s, lH); 8.37 (d, lH); 8.45 (d, lH);^{13}C NMR (100 MHz, $CDCl_3$): δ 20.16, 62.13, 117.71, 1201.4, 121.33,123.78, 147.46, 147.71, 148,10, 150,74, 154.79, 154.98 ppm.

3.2.6 Synthesis of 4-Hydroxylmethyl-4'-methyl-2, 2'-bipyridine rhodium complex (6)

30 mg 4-hydroxylmethyl-4'-methyl-2, 2'-bipyridine (0.15 mmol) of was mixed with 46.3 mg (0.075 mmol) of penta methylcyclopentadienylrhodium (III) chloride in 15 mL of dichloromethane and was stirred for 8-10 hours. After evaporating dichloromethane, 15 mL of ether was added and stirred over night. After decantation of ether, 72 mg of yellowish solid was obtained. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.59 (s, 15H), 2.47 (s, 3H), 4.80 (s, 2H), 4.84 (-OH, 1H), 7.53 (d, *J*=5.0, lH); 7.68 (d, *J*=5.0, lH), 8.32 (s, lH), 8.60 (t, J=4.9 Hz, 2H), 8.67 (s, lH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 8.3, 20.6, 62.0, 95.6, 117.8, 120.7, 121.2,123.8, 148.0, 148.2, 149.7, 152.0, 155.0, 157.3 ppm.

3.2.7 Synthesis of [2, 2'-bipyridine]-4, 4'-dicarboxylic acid (7)

4, 4'-dimethyl-2, 2'-bipyridine (2.0 g, 10.86 mmol) in 100 mL beaker in an ice bath was poured into 50% sulfuric acid (36 mL). This cooled solution was poured to a round bottom flask containing potassium permanganate (4.044 g, 25.5 mmol) in an ice bath. The reaction mixture was stirred for 50 minutes and then was allowed to be warm to the room temperature. Again the reaction mixture was cooled in an ice bath and a second portion of potassium permanganate (4.044 g, 25.5 mmol) was added. The reaction mixture was refluxed till the color of the the reaction changed from purple to white. After cooling the reaction to room temperature, it was poured in a beaker contained 40 mL of distilled cold water. Then it was filtered by a Büchner funnel and the white solid product was obtained. Yield: 1.2 g (50%).

3.2.8 Synthesis of dimethyl [2, 2'-bipyridine]-4, 4'-dicarboxylate (8)

80 mL of methanol was poured to a round bottom flask containing [2, 2'-bipyridine]-4, 4'-dicarboxylic acid (0.98 g, 4.01 mmol) and to this suspension concentrated sulfuric acid (10 mL) was poured very carefully (dropwise).The reaction mixture was refluxed at 80^{o}C for 12 h. After cooling, distilled H_2O (20 mL) was added and slurry formed. $NaHCO_3$ (30 g approx.) was added very slowly until the pH of the reaction reached to 7. Then extraction was performed with chloroform (15 mL * 3). Using UV light the layers were seen well. The chloroform extracts were gathered and was dried over MgSO4 and evaporated to dryness to give dimethyl [2, 2'-bipyridine]-4, 4'-dicarboxylate as a white solid. Yield: 0.90 g (82 %). ^{1}H NMR (400 MHz,$CDCl_3$) δ'ppm = 4.00 (s, 6H), 7.91 (d, 2H), 8.86 (d, 2H),8.96 (s, 2H); ^{3}C NMR (400 MHz, $CDCl_3$): δ 53.34, 121.15, 123.83, 139.20,150.74, 157.01, 166.22 ppm.

3.2.9 Synthesis of dimethyl [2, 2'-bipyridine]-4, 4'-dicarboxylate rhodium complex (9)

30 mg of dimethyl [2,2'-bipyridine]-4,4'-dicarboxylate (0.11 mmol) was mixed with 34 mg (0.055 mmol) of penta methylcyclopentadienylrhodium(III) chloride in 100ml of methanol and was stirred for 2-3 hours. After evaporating methanol, 15 mL of ether was added and stirred over night. After decantaioın of ether, 61 mg of yellowish solid was obtained**:** ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.88 (s, 15H), 4.07 (s, 6H), 8.51 (d, *J*=5.1, 2H), 8.83 (s, 2H), 9.36 (d, *J*=5.1, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 9.5, 53.7, 97.0, 122.8, 128.3, 140.7, 153.6, 154.4, 163.4 ppm.

3.2.10 Synthesis of N-((4'-methyl-[2, 2'-bipyridin]-4-yl) methyl)-3-(Trimethoxysilyl) propan-1-amine (10)

4-(Bromomethyl)-4'-methyl-2, 2'-bipyridine (131.6 mg, 0.5 mmol) was dissolved in 15 mL of THF. Then triethylamine (0.55 mL, 5 mmol) and 3-aminopropyltrimethoxysilane (0.87 mL, 5 mmol) were added respectively. The reaction mixture was heated at 50°C for 6h. After cooling the reaction mixture, 15 mL of hexane poured and was filtered by Bush funnel containg $MgSO_4$. Then the solvent was evaporated and residue was vacuumed for 24h for the purification. An oily yellow product(135 mg) obtained.

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.57-0.62 (m, 2H), 1.51-1.61 (m, 2H), 2.38 (s, 3H), 2.58 (t, J=7.1 Hz, 2H), 3.48 (s, 9H), 3.82 (s, 2H), 7.04 (d, J=5.0 Hz, 1H), 7.23-7.26 (m, 1H), 8.17 (s, 1H), 8.25 (s, 1H), 8.45 (d, J=4.9 Hz, 1H), 8.52 (d, J=5.0 Hz, 1H); ^{13}CNMR ($CDCl_3$, 100MHz) δ: 5.7, 20.1, 22.1, 49.4, 51.0, 51.8, 119.4, 120.9, 121.8, 123.5, 146.8, 147.8, 148.1, 149.6, 155.2 ppm.

3.2.11 Syntheis of N-((4'-methyl-[2, 2'-bipyridin]-4-yl) methyl)-3-(Trimethoxysilyl) propan-1-amine rhodium complex (11)

135 mg (0.373 mmol) of N-((4'-methyl-[2, 2'-bipyridin]-4-yl) methyl)-3-(trimethoxysilyl) propan-1-amine was mixed with 115 mg (0.187 mmol) of penta methyl cyclopentadienylrhodium (III) chloride in 15ml of methanol and was stirred for 2-3 hours. After evaporating methanol, 15ml of ether was added and stirred over night. After decantation of ether, 250 mg of yellowish solid was obtained. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.59-0.64 (m, 2H), 1.51-1.61 (m, 2H), 1.92 (s, 15H), 2.40 (s, 3H), 2.61 (t, J=7.1 Hz, 2H), 3.51 (s, 9H), 3.83 (s, 2H), 7.08 (d, J=5.0 Hz, 1H), 7.26-7.29 (m, 1H), 8.19 (s, 1H), 8.27 (s, 1H), 8.48 (d, J=4.9 Hz, 1H), 8.55 (d, J=5.0 Hz, 1H); ^{13}CNMR ($CDCl_3$, 100MHz) δ: 5.9, 9.5 20.3, 22.4, 49.6, 51.3, 52.0, 96.7 119.6, 121.1, 121.9, 123.7, 146.9, 147.9, 148.4, 149.8, 155.4 ppm.

3.2.12 Synthesis of 4-(4'-Methyl)-2, 2'-bipyridyl) methyl) triphenylphosphonium Bromide (12)

Triphenylphosphine (5 g, 19 mmol) was dissolved in toluene (20 mL) and 4-(bromomethyl)-4'-methyl-2, 2'-bipyridin (0.5 g, 1.9 mmol) was poured to this solution. The solution was heated at 60°C for 3 h. After cooling to room temperarure, it was filtered and dried. 0.9 g of the triphenylphosphonium bromide salt was obtained.^{1}H NMR (DMSO) δ 2.61 (3H, s); 5.51 (lH, s); 5.58 (lH, s); 7.16 (lH, d); 7.38 (lH, d); 7.86 (12H, m); 8.00 (3H, d); 8.19 (lH, s); 8.26 (lH, s); 8.52 (lH, d); 8.63 (lH, d).

3.2.13 Synthesis of 1, 2-Bis (4-(4'-methyI)-2, 2'-bipyridyl) ethene (13)

4'-Methyl-[2, 2'-bipyridine]-4-carbaldehyde (0. 1 10 g, 0.56 mmol) and 4-(4'-methyl)-2,2'-bipyridyl)methyl)triphenylphosphonium bromide (0.335 g, 0.64 m o l) were added in 100 mL round bottom flask containing magnetic bar. The balon opening was wrapped with a rubber septum. Argon was passed through the balon for a few minutes and then EtOH (20 mL) was injected and the solution was stirred under argon at 0 ^{o}C for 30 min. A fresh solution of 0.3 M NaOEt/EtOH (2 mL) was prepared and injected dropwise over a 2 min period. After cooling to the room temperature, the reaction mixture was waited for 5 h and then the volume was reduced to ca**.** 10 mL by evaporation. Distilled water (5 mL) was added and then it was filtered and washed with 1: l (H_2O: EtOH) to obtain the product as a white powder (93 mg, 45 % yield). ^{1}H NMR (400 MHz,$CDCl_3$)δ: 2.39 (s, 6H); 7.11 (d, *J*=4.17 H, 2H); 7.34 (d, *J*=1.65 Hz, 1H); 7.35 (d, *J*=1.64 Hz, 1H); 7.40 (s, 2H); 8.20 (s, 2H); 8.56 (s, 4H); 8.70 (d, *J*=5.10 H, 2H); ^{13}C NMR (400 MHz, $CDCl_3$): δ 20.2, 117.6, 120.3, 121.0,123.9, 129.8, 143.6, 147.2, 148.0, 148.7, 154.7, 156.0 ppm.

3.2.14 Synthesis of 1, 2-bis (4-(4'-methyI)-2, 2'-bipyridyl) ethene rhodium complex (14)

1, 2-Bis (4-(4'-methyI)-2, 2'-bipyridyl) (23 mg, 0.0631 mmol) was mixed with (19.5 mg, 0.0316 mmol) penta methylcyclopentadienylrhodium (III) chloride in 15 mL of methanol and was stirred for 2-3 hours. After evaporating methanol, 15 mL of ether was added and stirred over night. After filtration of ether, 40 mg of yellowish solid was obtained. ^{1}H NMR (400 MHz,$CDCl_3$)δ: 1.63 (s, 15H); 2.41 (s, 6H); 7.13 (d, *J*=4.76 Hz, 2H); 7.37 (d, *J*=4.71 Hz, 2H); 7.42 (s, 2H); 8.22 (s, 2H); 8.54 (s, 4H); 8.64 (d, *J*=4.93 Hz, 2H); ^{13}C NMR (400 MHz, $CDCl_3$): δ 9.1, 21.2, 96.8, 118.5, 121.3, 122.0,124.9, 130.8, 144.6, 148.2, 149.0, 149.7, 155.6, 156.9 ppm.

3.2.15 Synthesis of 6-aminocaproic acid hydrochloride (15)

E-caprolactam (1.13 g, 1 mole) was dissolved in water (1.5 ml.) and then concentrated hydrochloric acid (1.5 ml.) was added. After refluxing at 120 °C for **3** hours, the reaction mixture was cooled and acetone (500 ml.) was added. The *e*-Aminocaproate hydrochloride precipitated and collected by filtration as a white product (1.06 g, 64%).
e -aminocaproic acid hydrochloride (m.p. 132-133"). ^{1}H NMR (400 MHz, DMSO): δ 1.37 (s, 2H); 1.61 (d, 4H); 2.23 (s, 2H); 2.79 (s, 2H); 8.14 (s, 3H, -NH_3); 11.5- 12.3 (br. S, 1H, -OH) ^{13}C-NMR: δ ^{13}C-NMR: δ 23.8, 25.4, 26.6, 33.3, 39.5, 174.2 ppm.

3.2.16 Synthesis of 6-aminocaproic acid methyl ester hydrochloride (16)

6-Aminocaproic acid hydrochloride (167.63 mg, 1 mmol) and fresh chlorotrimethylsilane (0.3 mL, 2 mmol) in a round bottom flask were stirred for a while and then methanol (10 mL) was added. The solution was stirred for 12h at room temperature. Then the methanol was evaporated to dryness to obtain 6-aminocaproic acid methyl ester hydrochloride as a wide powder in quantitative yield. ^{1}H NMR (400 MHz, D_2O) δ: 1.33 (m, 2H); 1.61- 1.55 (m, 4H); 2.34 (t, *J*=7.36 Hz, 2H); 2.90 (t, *J*=7.06 Hz, 2H); 3.60 (s, 3H) ^{13}C-NMR: δ 23.7, 25.1, 26.4, 33.4, 39.3, 52.2, 177.4 ppm.

3.2.17 Synthesis of 6-aminocaproic acid methyl ester (17)

6-Aminocaproic acid hydrochloride (0.60 g, 3.9 mmol) dissolved in $CHCl_3$ (50 ml) at 0°C NH_3 gas was passed through for 30 min. The precipitate (NH_4Cl) was filtered off and the solvent was evaporated to get 6-aminocaproic acid methyl ester as an oily pale yellow liqiuid in quantitative yield. It was used immediately for the next step. ^{1}H NMR (400 MHz,CDCl) δ: 1.32- 1.42 (m, 2H); 1.44- 1.54 (m, 2H); 1.6- 1.70 (m, 2H); 1.91 (s, 2H, -NH_2); 2.32 (t, *J*=7.45 Hz, 2H); 2.71 (t, *J*=7.01 Hz, 2H); 3.67 (s, 3H) ^{13}C-NMR: δ 24.7, 26.3, 33.0, 33.9, 41.8, 51.4, 174.0 ppm.

3.2.18 Synthesis of 1, 4-bis (2-thienyl) butane-1, 4-dione (18)

To a suspension of $AlCl_3$ (16 g, 0.12 mol) in CH_2Cl_2 (15 ml) a solution of thiophene (9.61 ml, 0.12 mol) and succinyl chloride (5.51 ml, 0.05 mol) in CH_2Cl_2 was added dropwise. The red mixture was stirred at r.t. for 18 h. This was then quenched with ice and conc. HCl (5 ml). After stirring for 2 h the dark green organic phase was separated, washed with 2 M HCl, H_2O, and $NaHCO_3$ solution and then active carbon was added and dried over $MgSO_4$. After evaporation of the solvent a blue-green solid was obtained. The product was purified by Column chromatography (SiO_2, CH_2Cl_2-Hexane) (1:1) to afford a white solid (9.98g, 39.81mmol; 80%). ^{1}H NMR (400 MHz,CDCl) δ: ^{1}H-NMR: δ 3.40 (s, 4H); 7.15 (dd, *J*=3.84, J=4.88 Hz, 2H); 7.82 (d, *J*=1.07, 2H); 7.83 (d, *J*=1.07, 2H); ^{13}C-NMR: δ 33.2, 128.1, 132.1, 133.6, 143.8, 191.4 ppm.

3.2.19 Synthesis of methyl 6-(2, 5-di (thiophen-2-yl)-1H-pyrrol-1-yl) hexanoate (19)

1, 4-Bis (2-thienyl) butane-1, 4-dione (50 mg, 0.0002 mmol) and acetic acid (0.24 mL) in benzene (10 mL) were stirred for 1h. Then 6-aminocaproic acid methyl ester (0.0004 mmol) was added and the resulting solution was refluxed for 50 hours. Benzene was evaporated and the crude was purified by column chromatography, Hexane: ethyl acetate (2:1) was used as eluent. The product was collected from the first band as an oily liquid (20 mg). ^{1}H NMR (400 MHz,$CDCl_3$ δ: ^{1}H-NMR: δ 1.0- 1.40 (m, 2H); 1.50- 1.62 (m, 2H); 1.6- 1.70 (m, 2H); 2.17 (t, *J*=7.51 Hz, 2H); 3.63 (s, 3H) 4.13 (t, *J*=7.86 Hz, 2H); 6.33 (s, 2H); 7.04- 7.10 (m, 4H); 7.30 (d, *J*=1.29 Hz, 1H)); 7.32(d, *J*=1.29 Hz, 1H) ^{13}C-NMR: δ 24.3, 25.9, 30.7, 33.7, 44.9, 51.5, 110.9, 125.3, 126.0, 127.3, 128.3, 135.0, 174.0 ppm.

3.2.20 Synthesis of 6-(2, 5-di (thiophen-2-yl)-1H-pyrrol-1-yl) hexanoic acid (20)

A solution of methyl 6-(2, 5-di (thiophen-2-yl)-1H-pyrrol-1-yl) hexanoate (26.8 mg, 9.21 mmol) and LiOH (5 mL, 1N aqueous) in THF (10 mL) was refluxed for 8h. The reaction mixture was quenched with 1N HCl aqueous (pH ~2-3) and H_2O (7 mL). The mixture was extracted with CH_2Cl_2. The combined organic extracts was dried ($MgSO_4$) and concentrated. The crude material was purified by column chromatography (silica gel. EtOAc/hexane: 1/1) to give the title compound (24.4 mg, 94%) as pale yellow liquid. . ^{1}H NMR (400 MHz,$CDCl_3$ δ: ^{1}H-NMR: δ 1.0- 1.15 (m, 2H); 1.35- 1.44 (m, 2H); 1.45- 1.55 (m, 2H); 2.14 (t, *J*=7.51 Hz, 2H); 4.06 (t, *J*=7.75 Hz, 2H); 6.25 (s, 2H); 6.97-7.03 (m, 4H); 7.23 (d, *J*=1.33 Hz, 1H)); 7.24(d, *J*=1.35 Hz, 1H) ^{13}C-NMR: δ 22.9, 24.7, 29.6.7, 32.6, 43.7, 109.8, 124.3, 125.0, 126.2, 127.2, 133.8, 178.1 ppm.

3.2.21 Reaction of 6-(2, 5-di (thiophen-2-yl)-1H-pyrrol-1-yl) hexanoic acid with galactitol dehydrogenase (26)

27 mg of galactitol dehydrogenase and 30 mg of EDC were dissolved in 2 mL of water distilled water, and the solution was kept at 4°C. Then 16 mg of 6-(2, 5-di (thiophen-2-yl)-1H-pyrrol-1-yl) hexanoic acid was added to this solution and the reaction mixture was kept for 24 hours at 4°C.

CHAPTER 4

CONCLUSION

In this work, six different mediators were synthesized for the regeneration of the cofactor. The starting molecule for all mediator synthesis is 4-(4'-methyI)-2, 2'-bipyridyl. First, it was functionalized into different molecules and then it was converted to the expected mediators by reacting with rhodium complex ($RhCp^*Cl_2$)2. The activities of these mediators toward the NAD^+ reduction were determined by the one of the partners. All mediators gave rise to well-defined signals in the absence of cofactor. The cathodic peak potential values were found to be dependent on the nature of the substituents present on the bipyridine ligand. The presence of electron-donating groups increases the electron density at the rhodium centre and make it more difficult to be reduced (cathodic shift) whereas electron-withdrawing groups tended to facilitate the reduction (anodic shift), in agreement with previous observations [43].

In the second part of the study, 6-aminocaproic acid methyl ester was synthesized which was used as a linker for the immobilization of galactitol dehydrogenase. The linker was reacted with 4-Bis(2-thienyl butane-1,4-dione to form the SNS conductive monomer having a long chain (6-aminocaproic acid methyl ester). The ester group in SNS was hydrolyzed to acid. The –COOH group of the SNS could react with $–NH_2$ group of the galactitol dehydrogenase in the presence of soluble carbadiimides like CMC or EDC. After this reaction the enzyme galactitol deydrogenase was binded covalently to SNS monomer which was characterized by UV spectrometry.

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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 CCl4 (^{13}C: δ=96.4) as internal standards.

^1H and ^{13}C NMR spectra of products are given below.

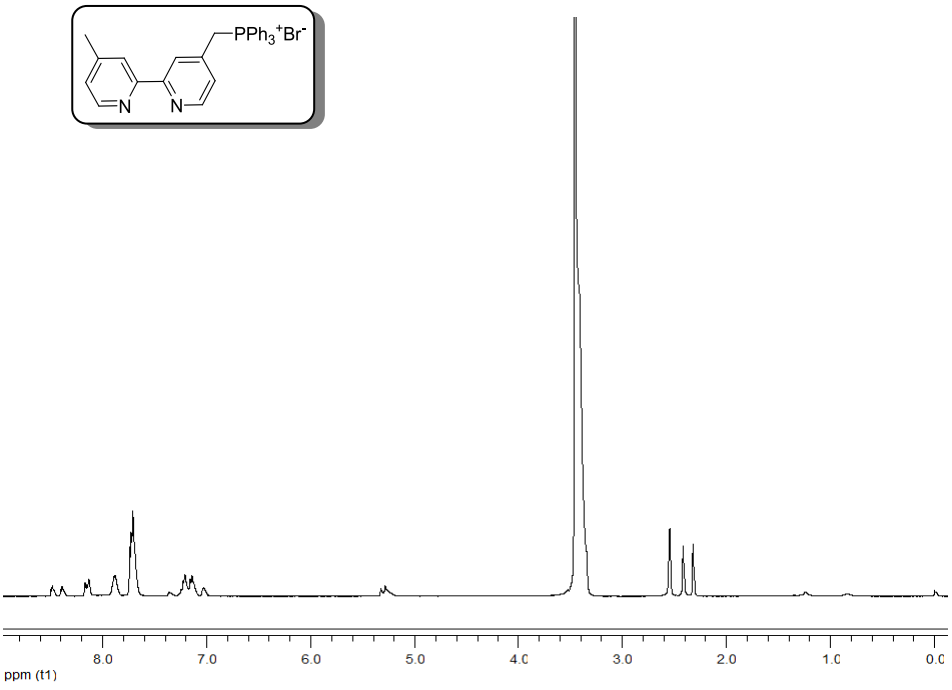

Figure A. 0 ^1H-NMR spectrum of **(12)**

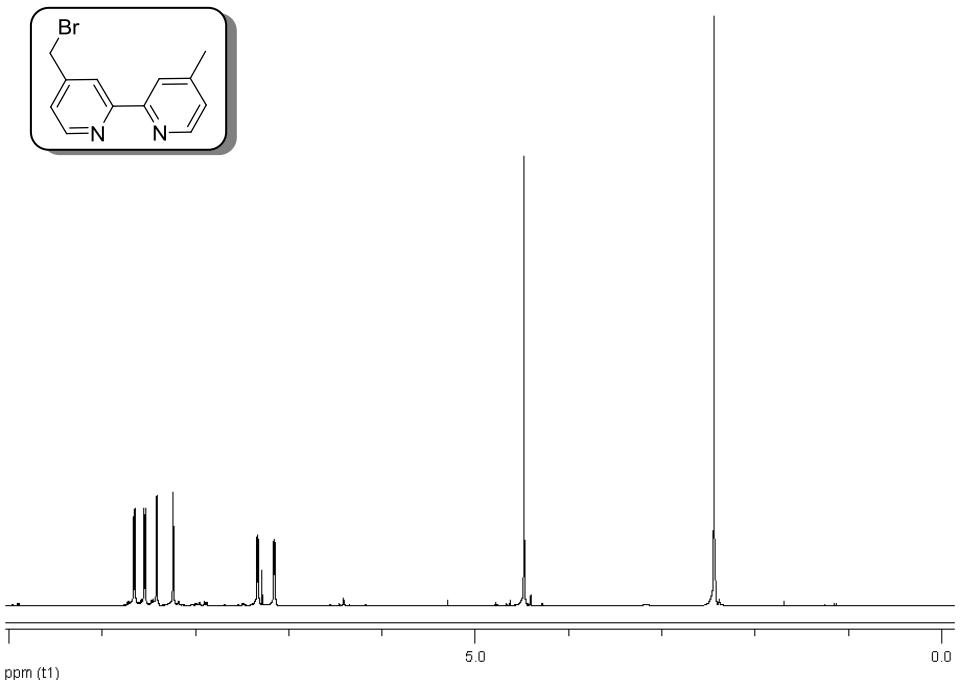

Figure A. 1 ^{1}H-NMR spectrum of **(1)**

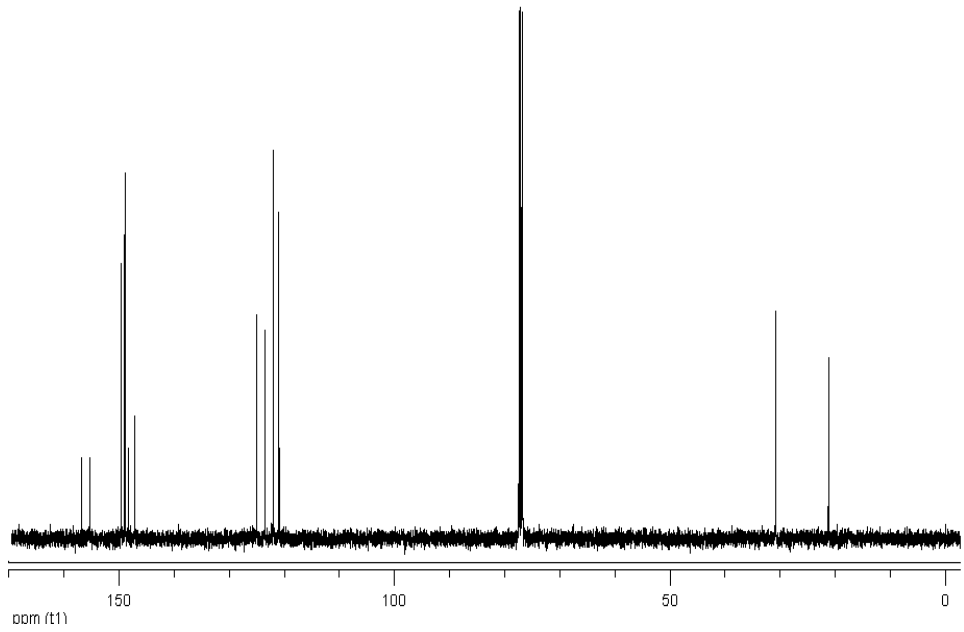

Figure A. 2 ^{13}C-NMR spectrum of **(1)**

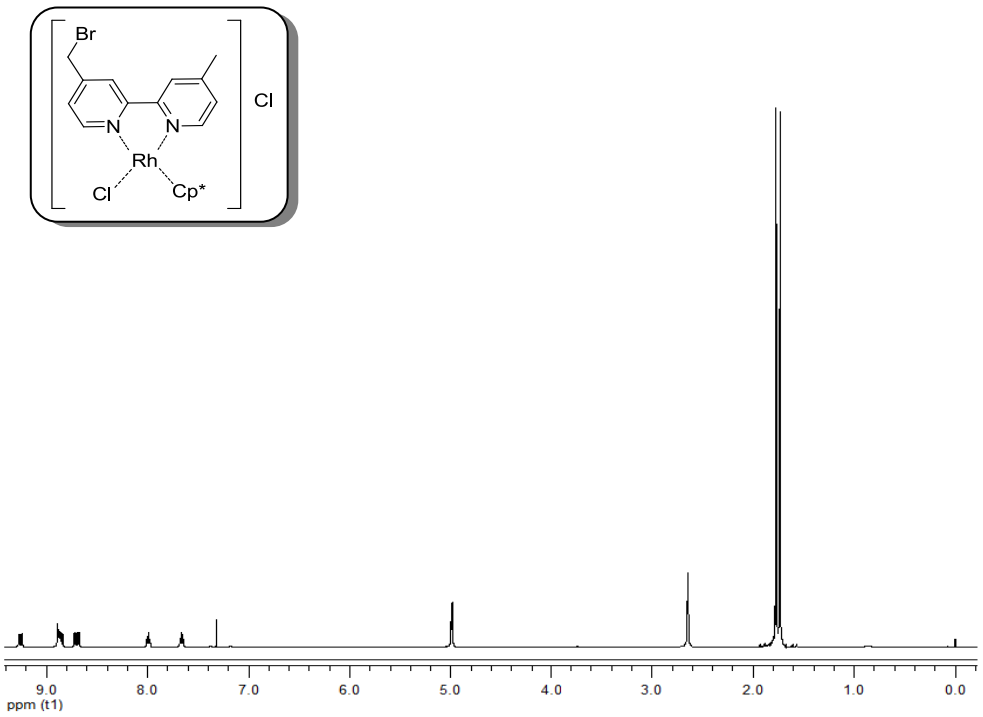

Figure A. 3 ^{1}H-NMR spectrum of **(2)**

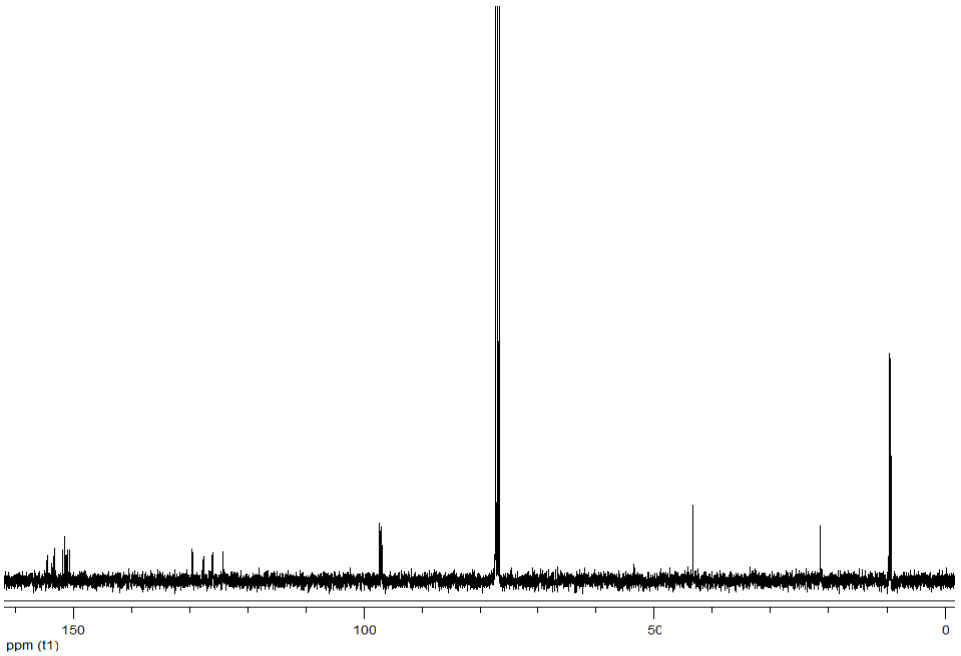

Figure A. 4 ^{13}C-NMR spectrum of **(2)**

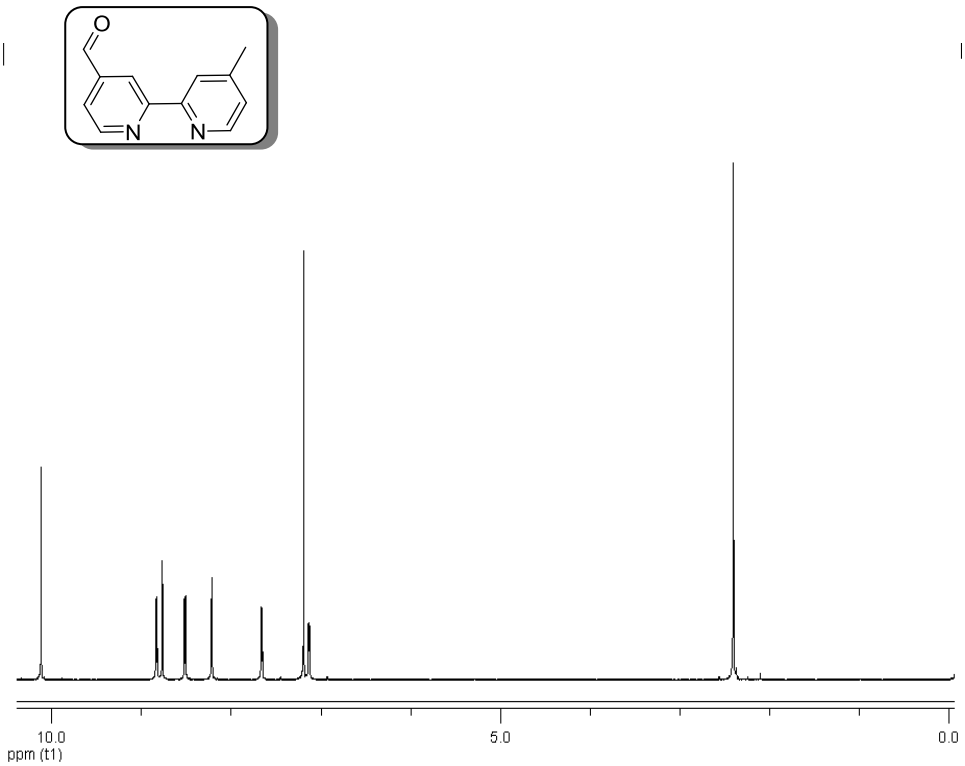

Figure A. 5 ^{1}H-NMR spectrum of **(3)**

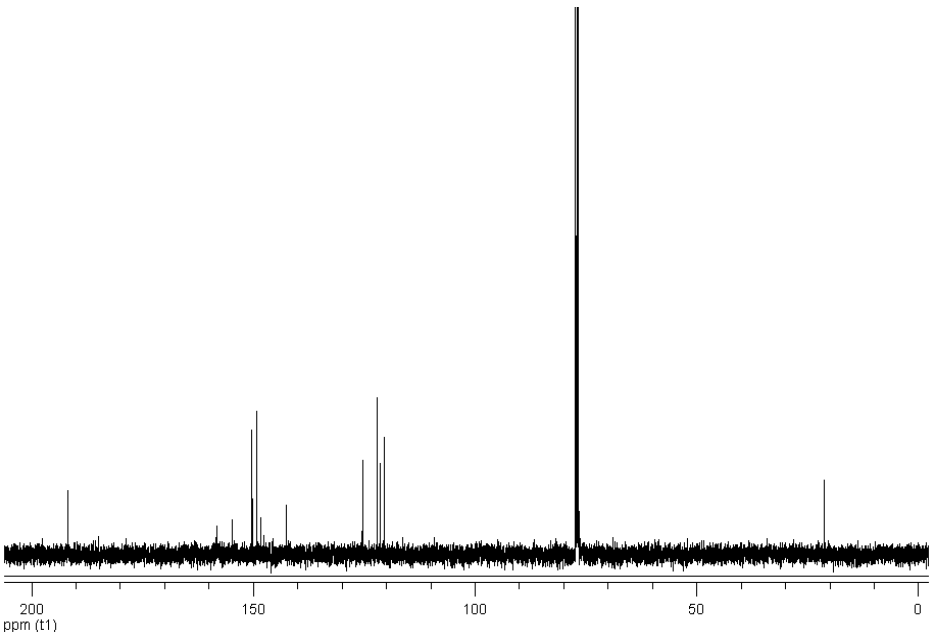

Figure A. 6 ^{13}C-NMR spectrum of **(3)**

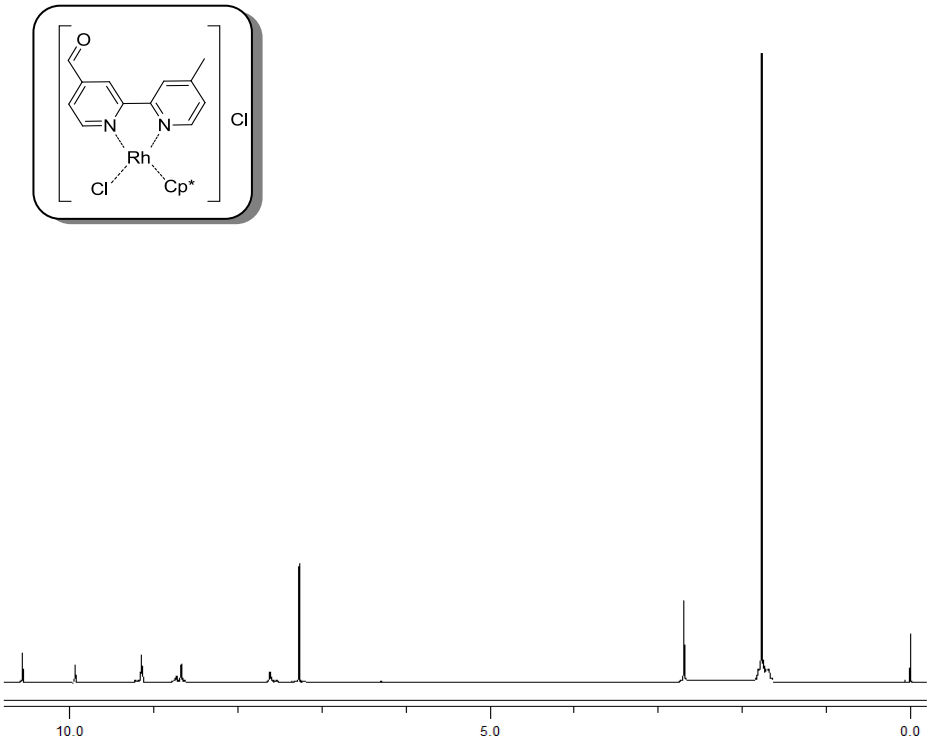

Figure A. 7 ^{1}H-NMR spectrum of **(4)**

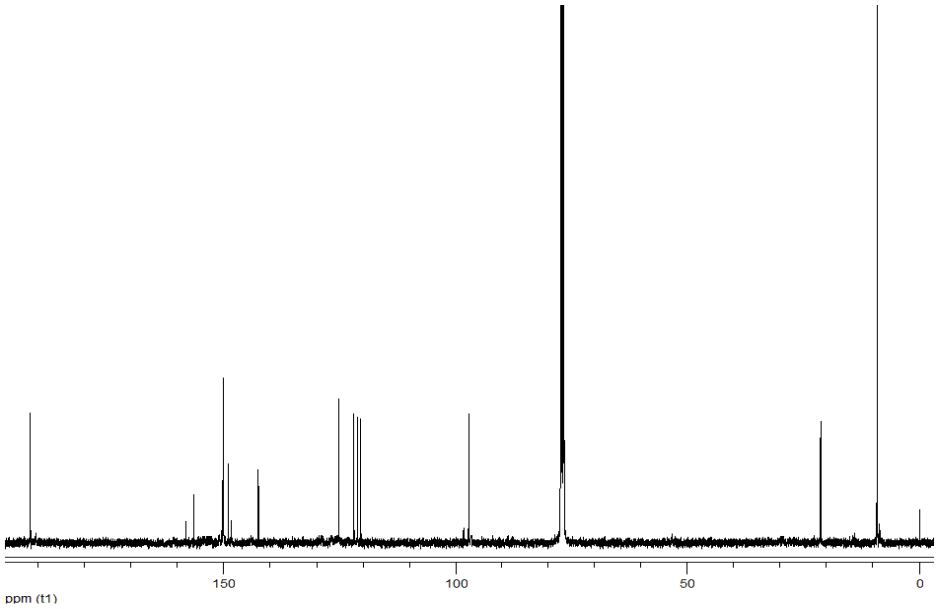

Figure A. 8 ^{13}C-NMR Spectrum of **(4)**

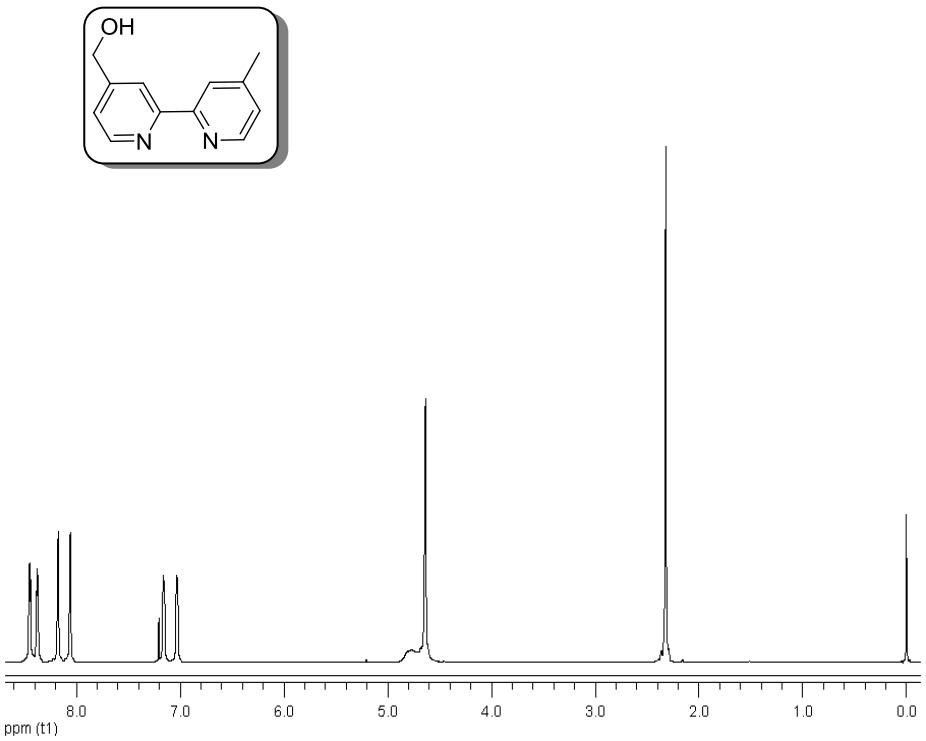

Figure A. 9 ^{1}H-NMR spectrum of **(5)**

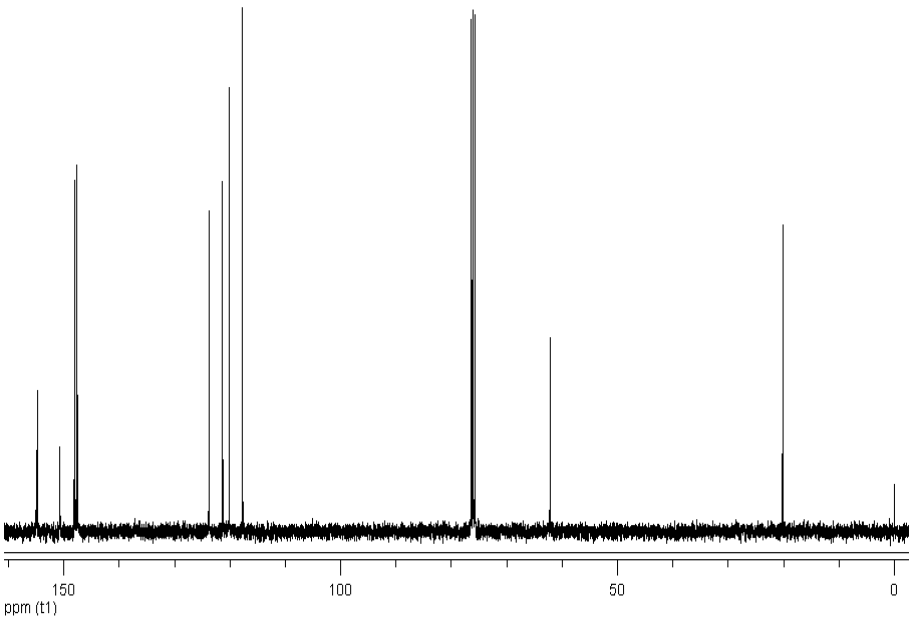

Figure A. 10 ^{13}C-NMR spectrum of **(5)**

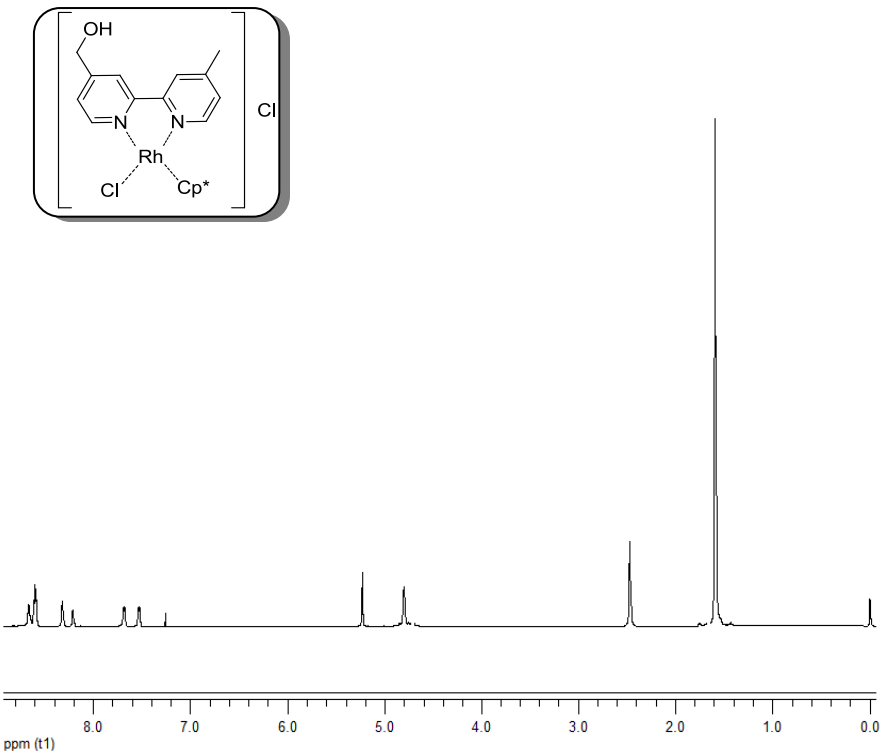

Figure A. 11 ^{1}H-NMR spectrum of **(6)**

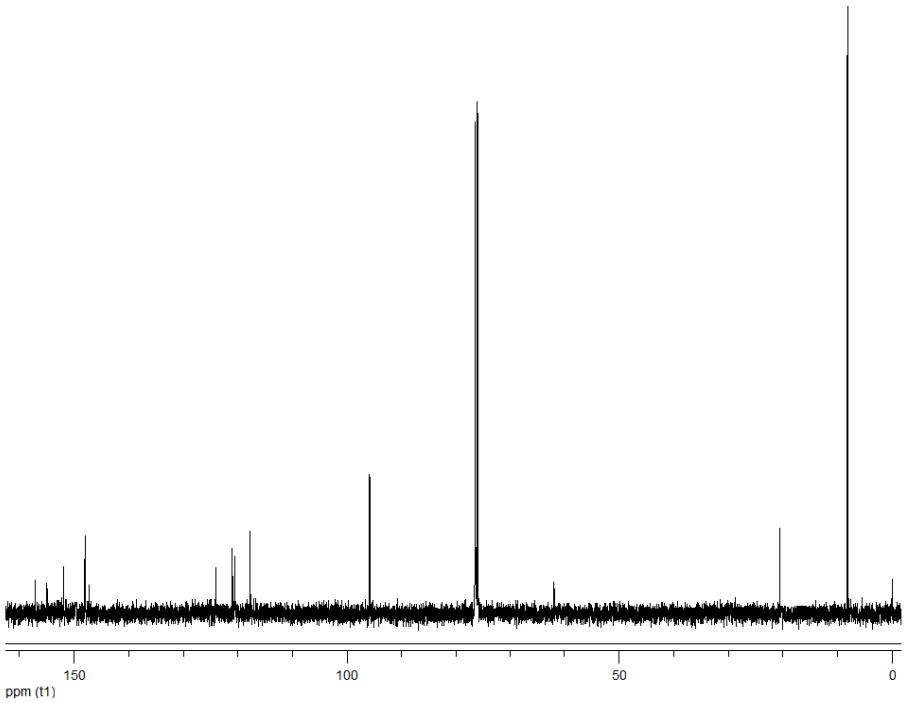

Figure A. 12 ^{13}C-NMR spectrum of **(6)**

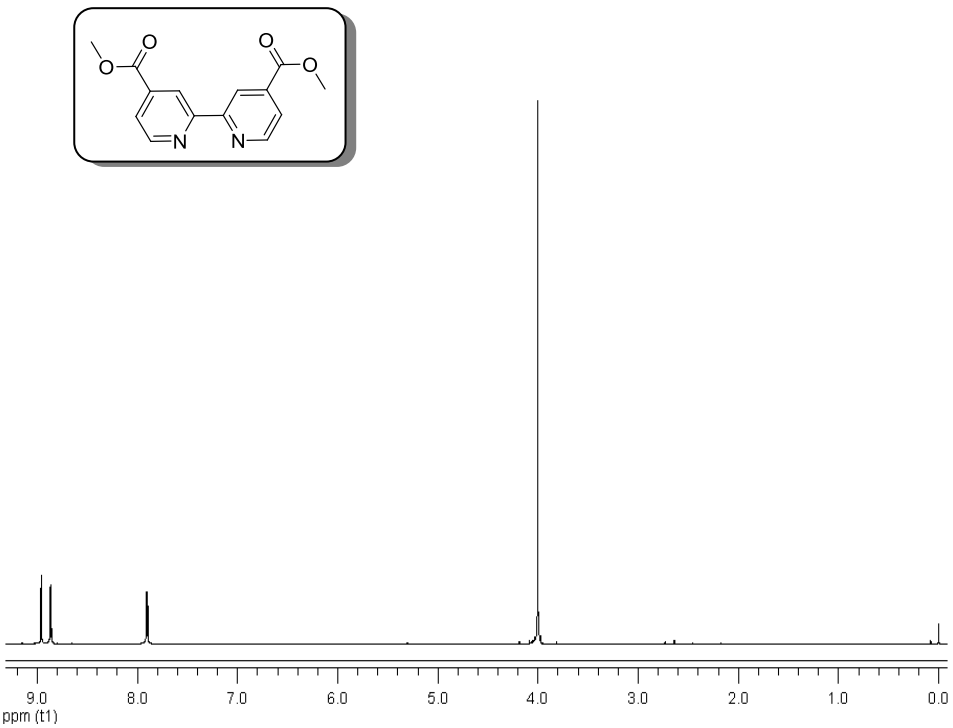

Figure A. 13 ^{1}H-NMR spectrum of **(8)**

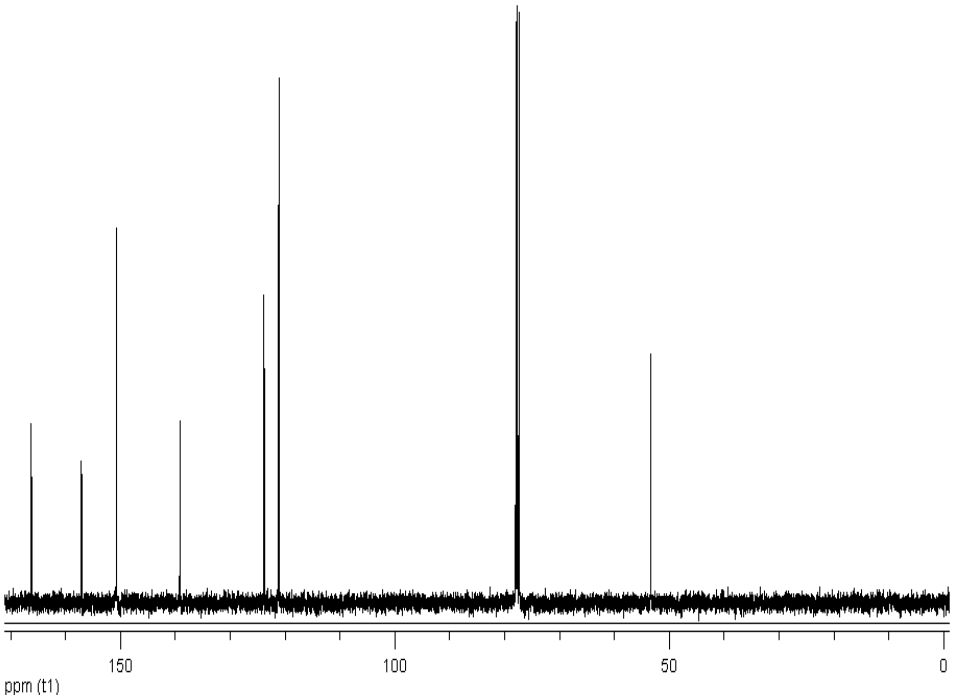

Figure A. 14 ^{13}C-NMR Spectrum of **(8)**

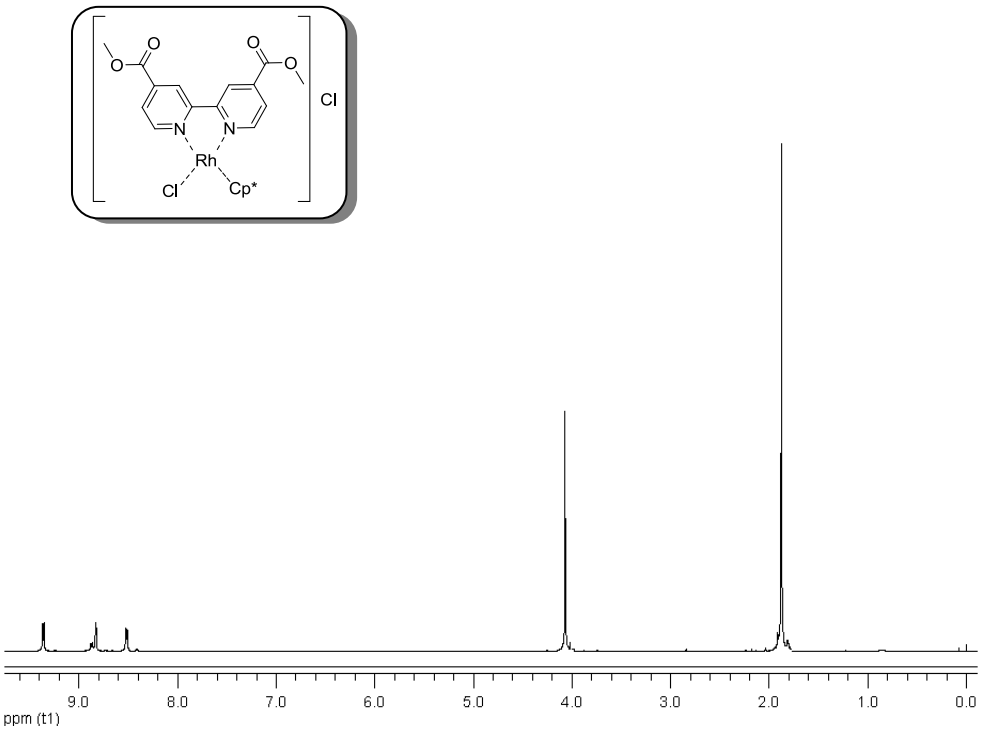

Figure A. 15 ^{1}H-NMR spectrum of **(9)**

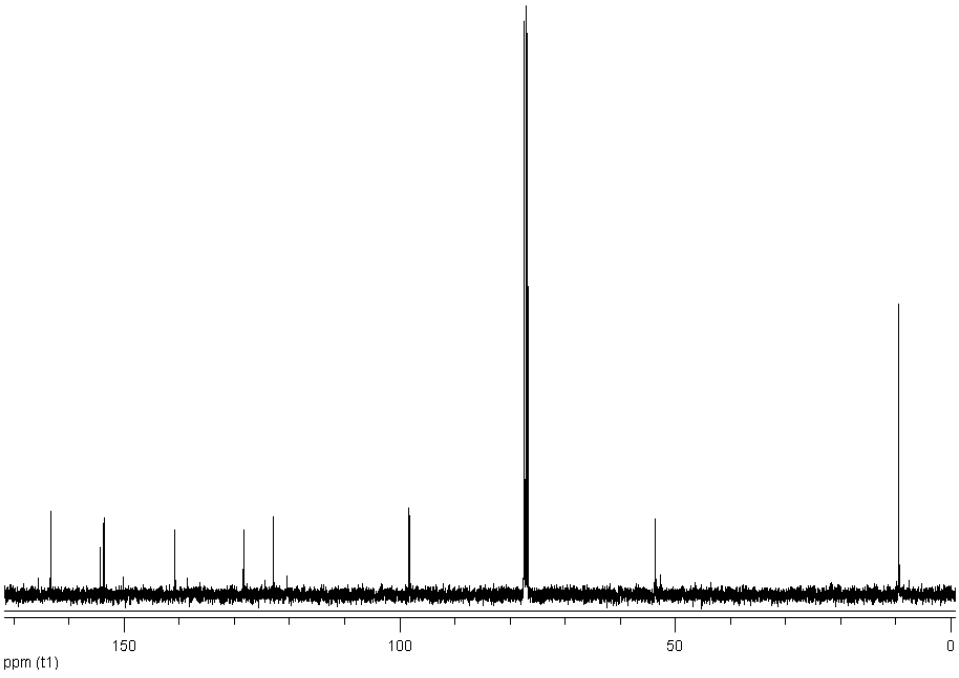

Figure A. 16 ^{13}C-NMR Spectrum of (**9**)

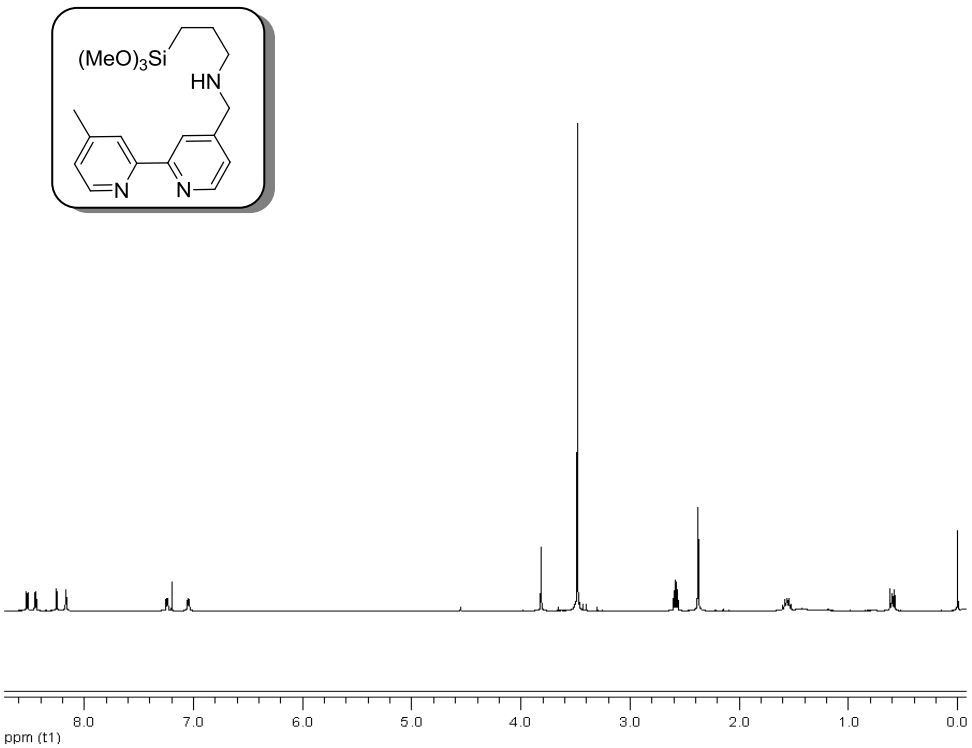

Figure A. 17 ^{1}H-NMR spectrum of **(10)**

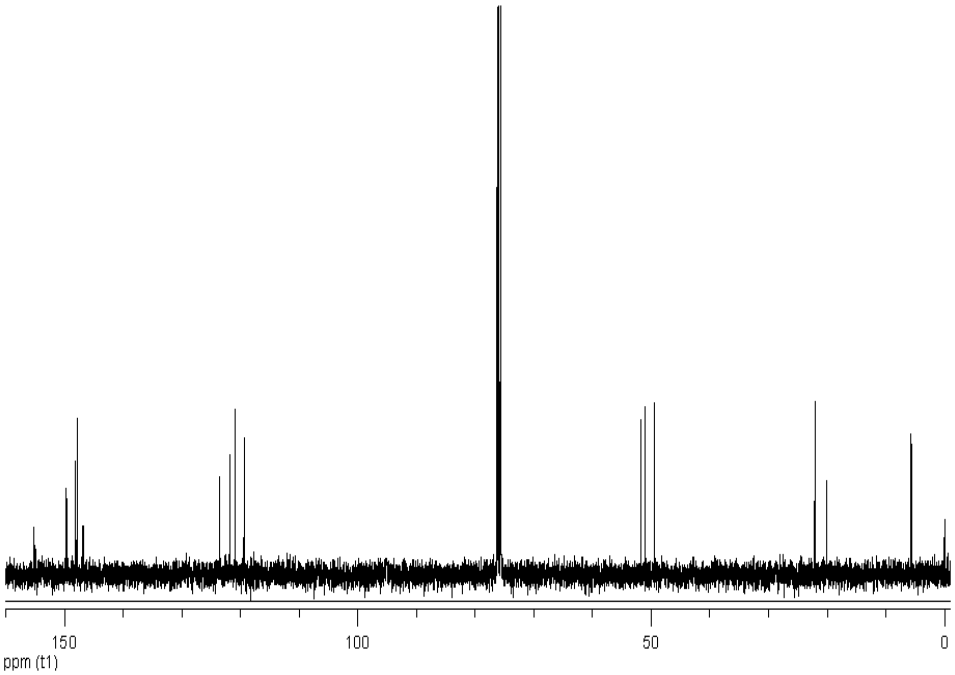

Figure A. 18 ^{13}C-NMR Spectrum of **(10)**

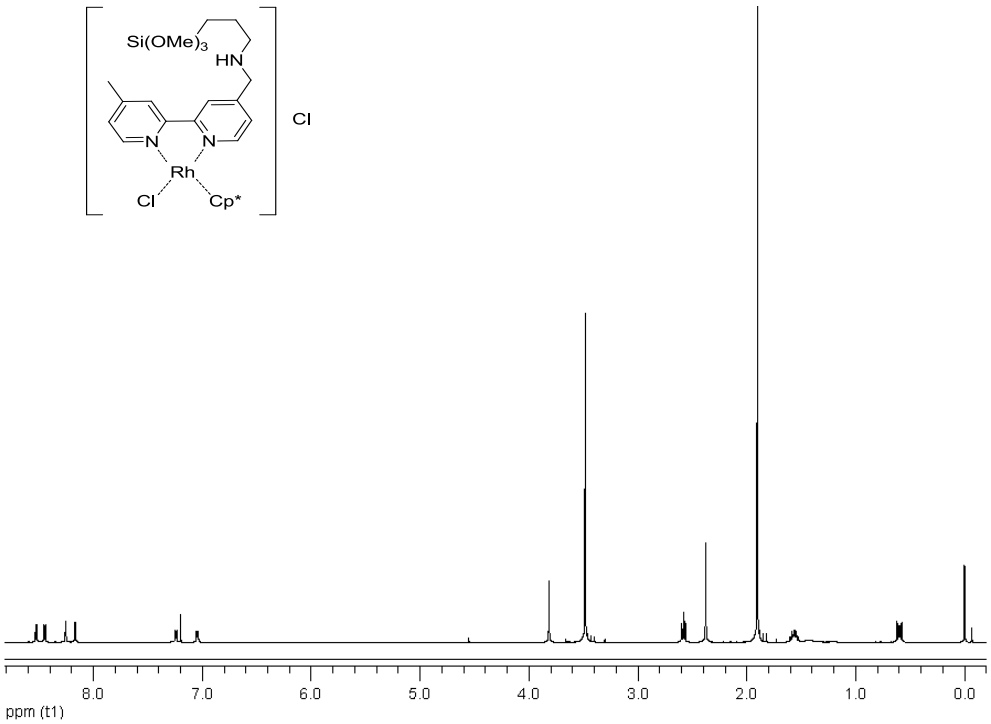

Figure A. 19 ^{1}H-NMR spectrum of **(11)**

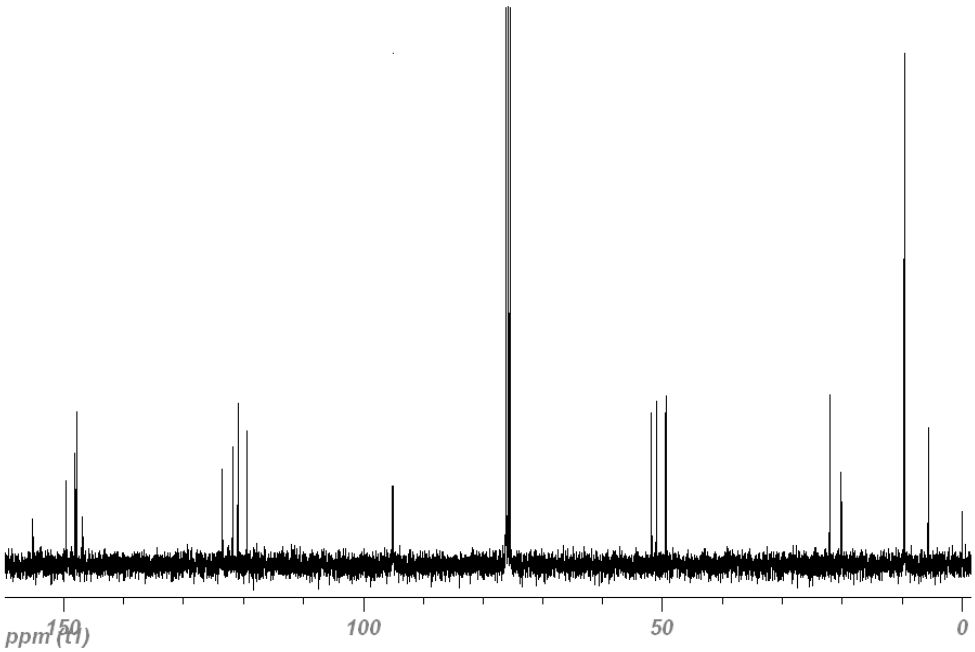

Figure A. 20 ^{13}C-NMR Spectrum of **(11)**

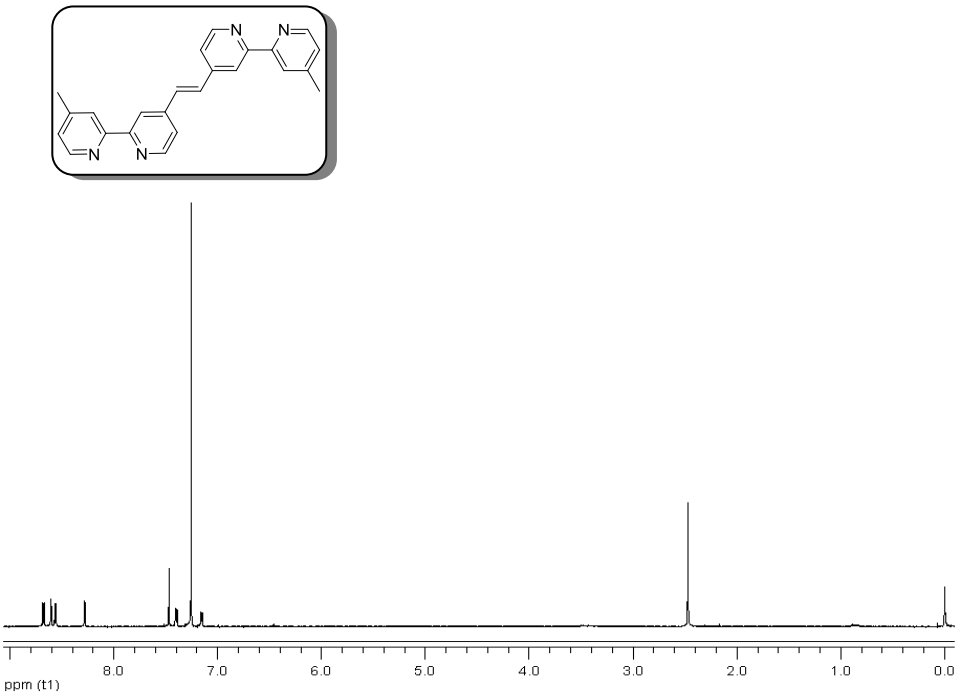

Figure A. 21 ^{1}H-NMR spectrum of **(13)**

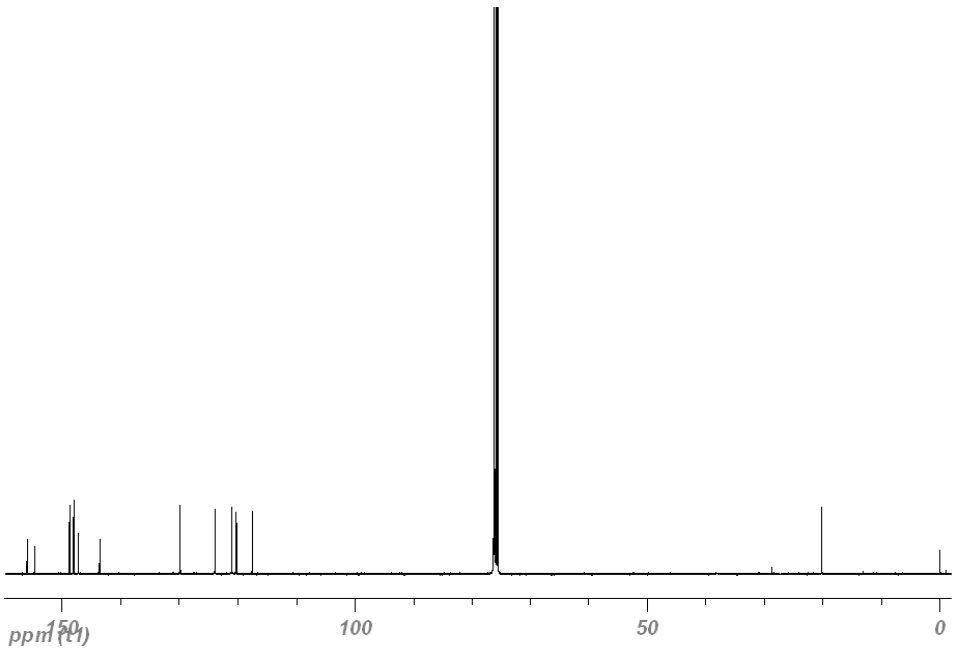

Figure A. 22 ^{13}C-NMR Spectrum of **(13)**

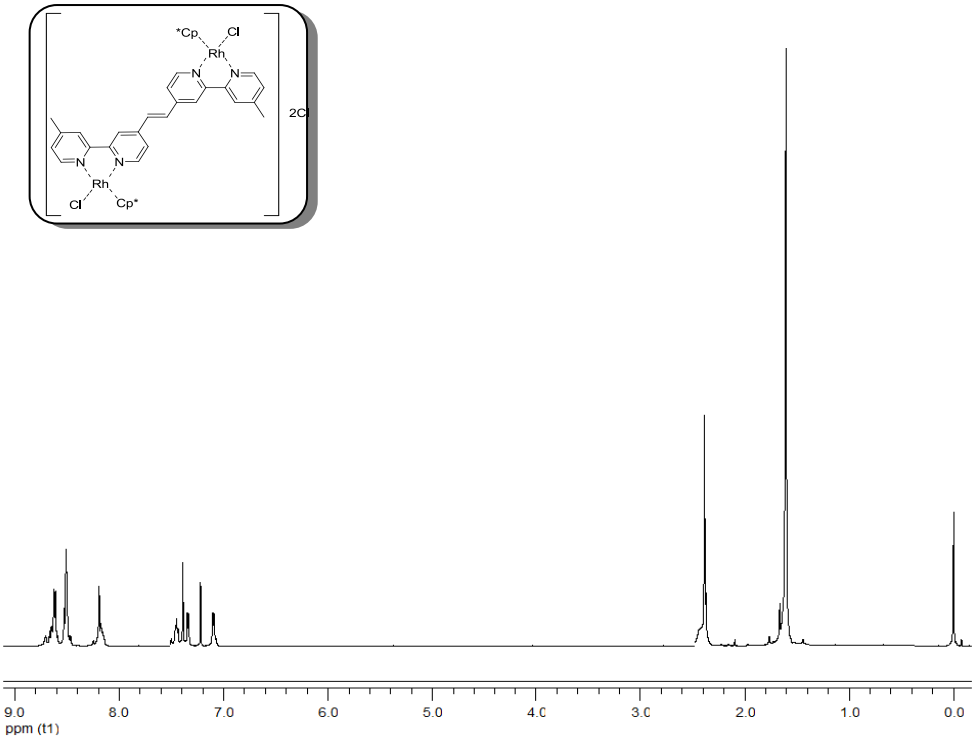

Figure A. 23 ^{1}H-NMR spectrum of **(14)**

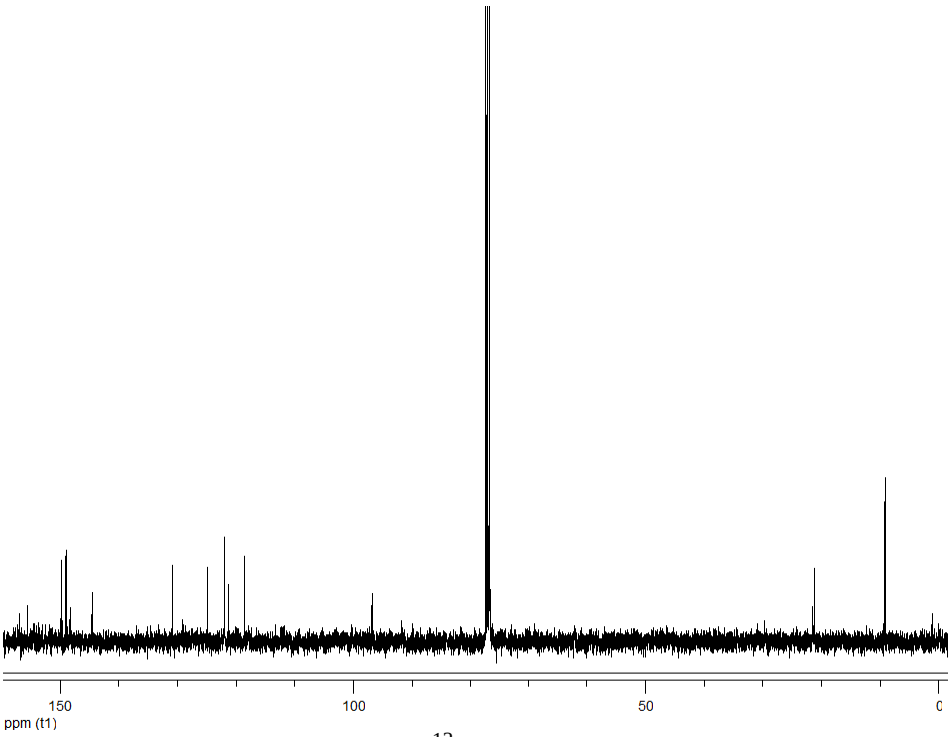

Figure A. 24 ^{13}C-NMR Spectrum of **(14)**

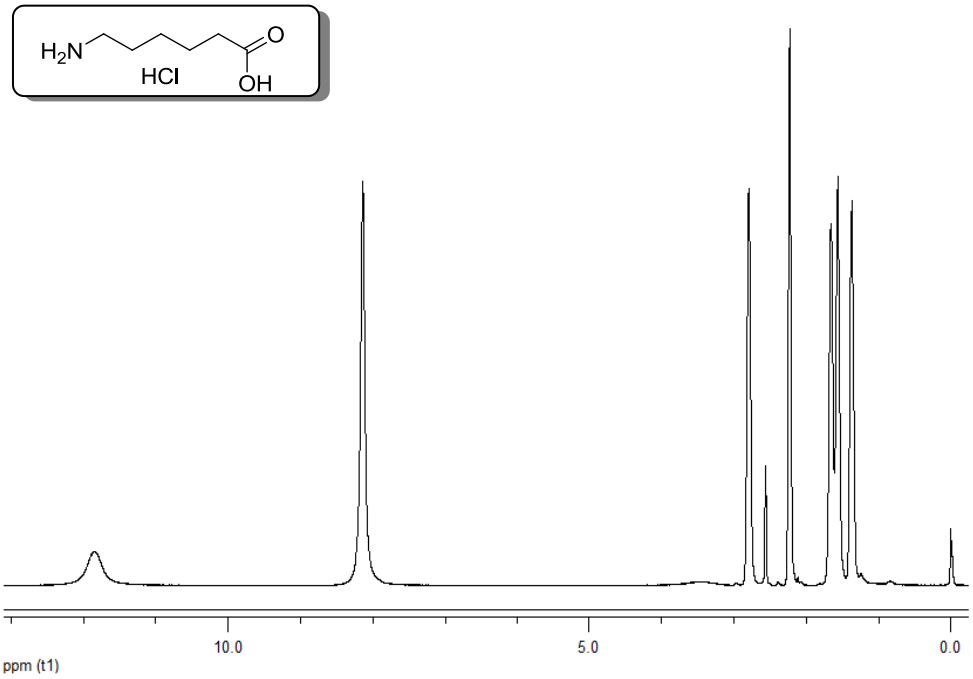

Figure A. 25 ^{1}H-NMR spectrum of **(15)**

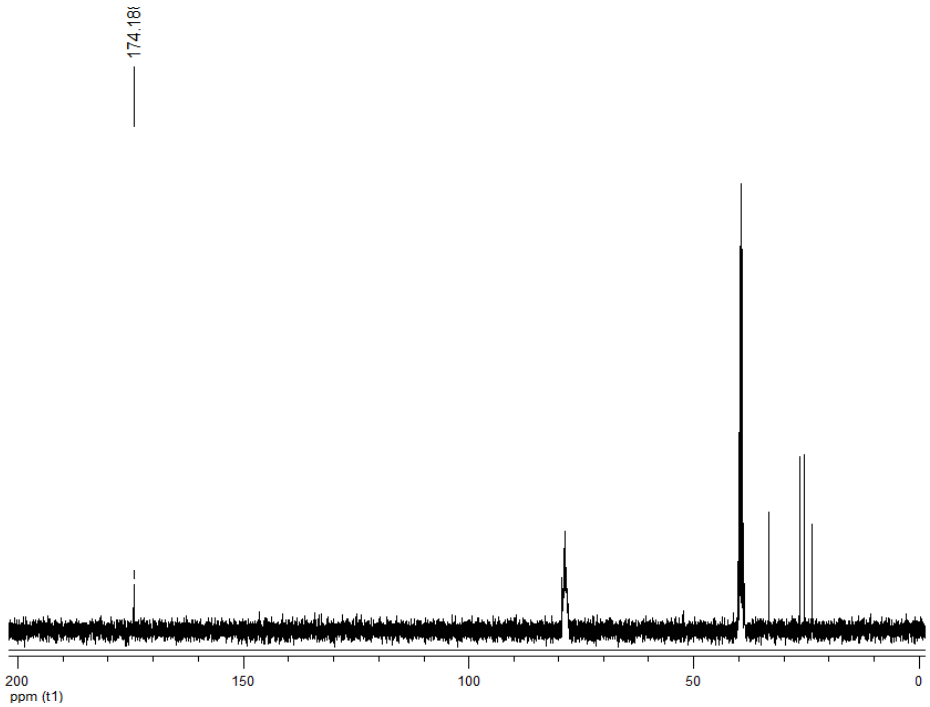

Figure A. 26 ^{13}C-NMR Spectrum of **(15)**

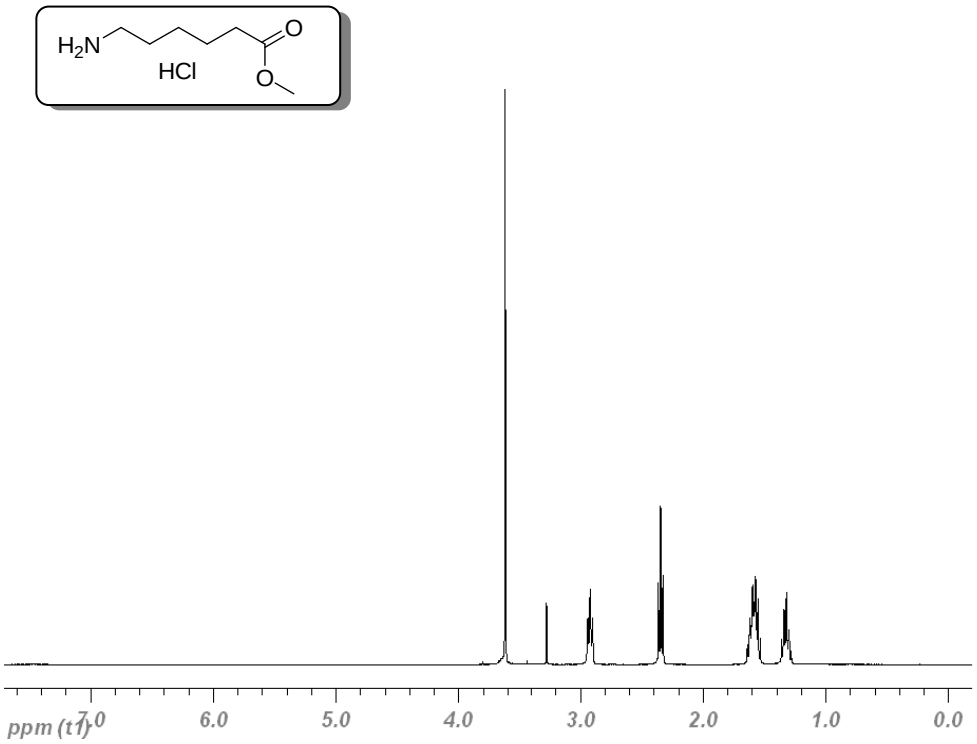

Figure A. 27 ^{1}H-NMR spectrum of **(16)**

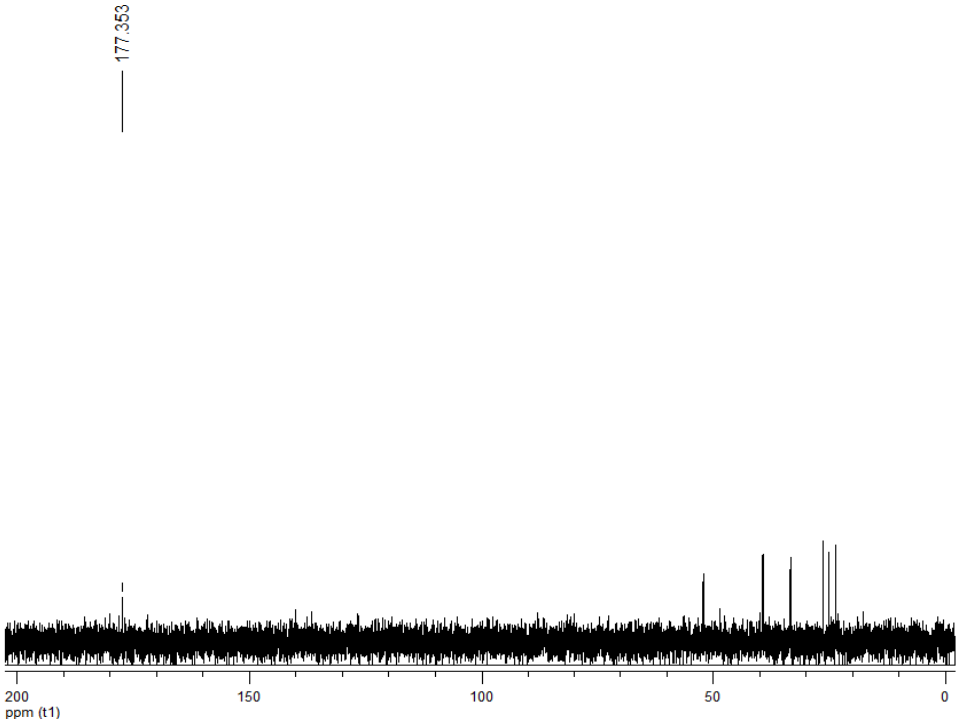

Figure A. 28 ^{13}C-NMR Spectrum of **(16)**

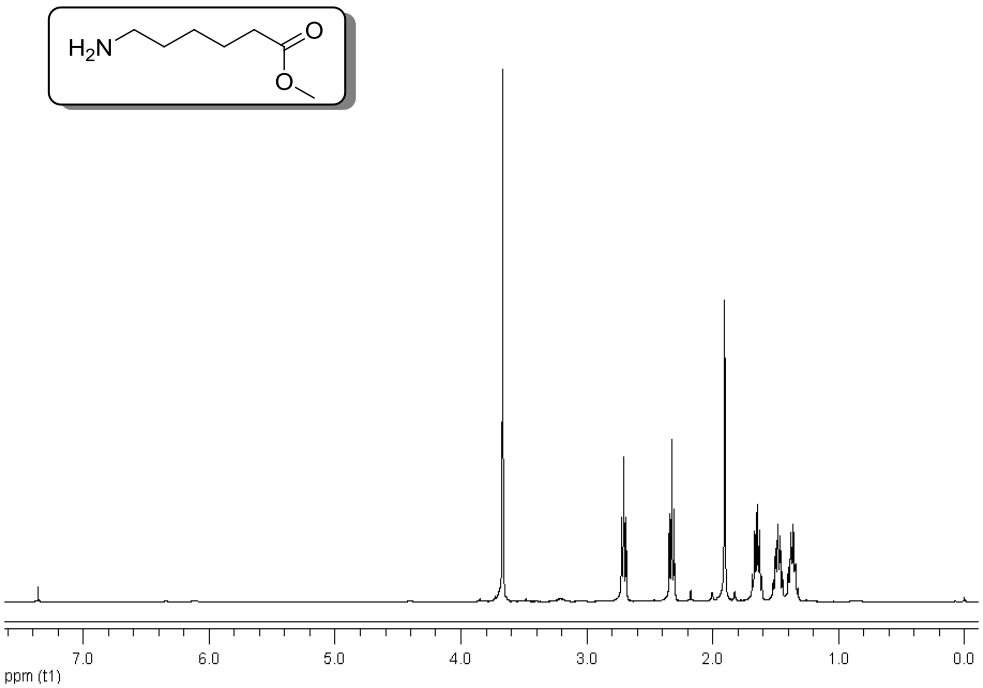

Figure A. 29 ^{1}H-NMR spectrum of **(17)**

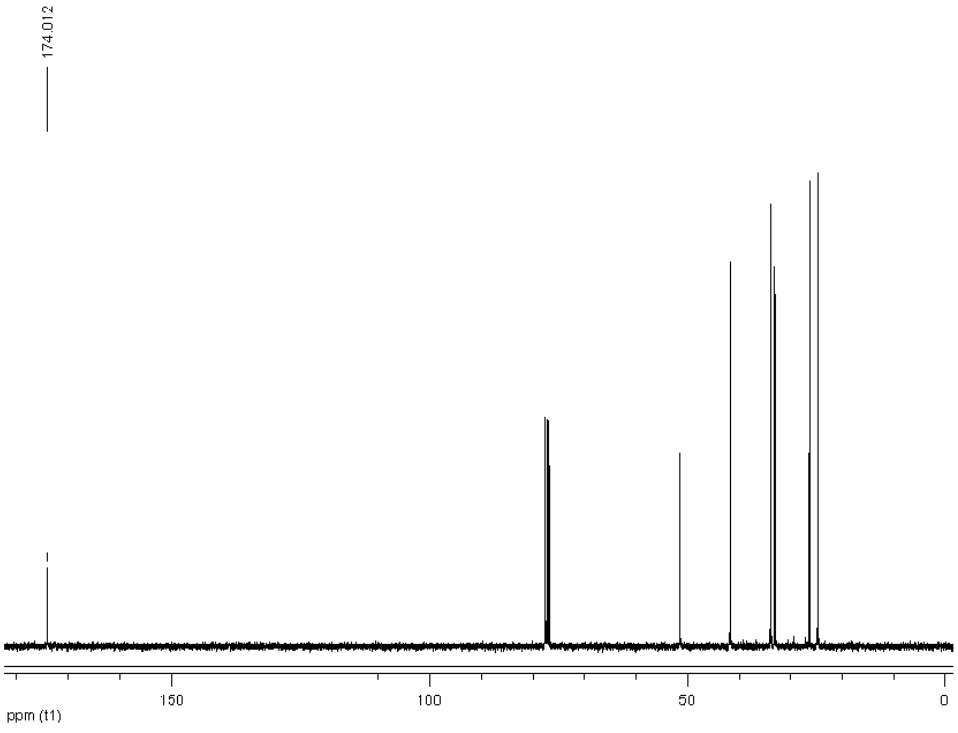

Figure A. 30 ^{13}C-NMR Spectrum of **(17)**

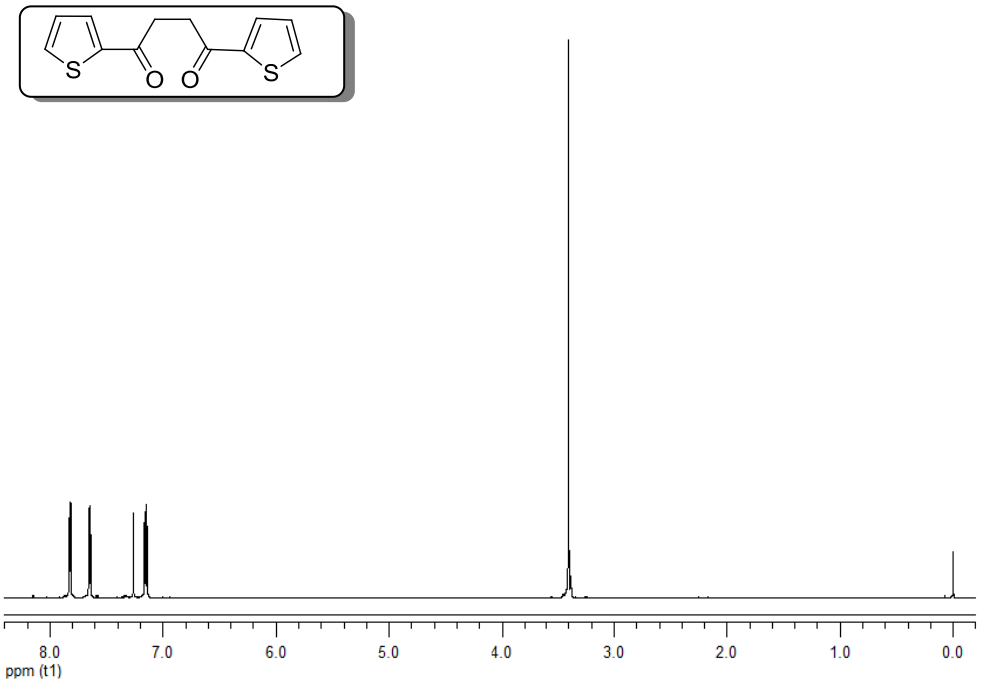

Figure A. 31 ^{1}H-NMR spectrum of **(18)**

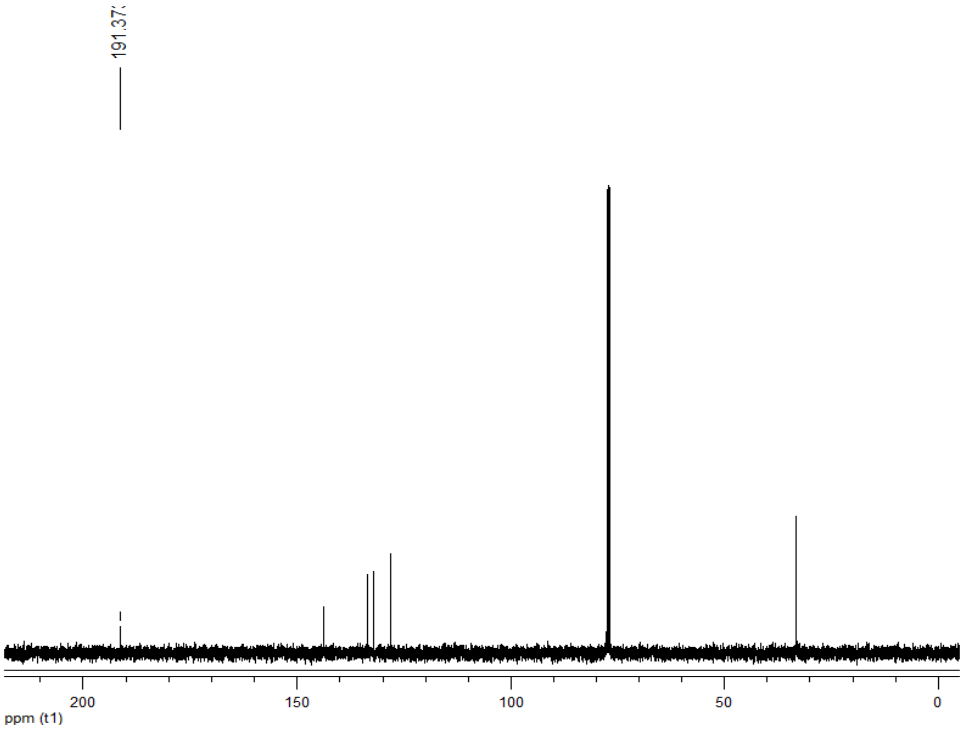

Figure A. 32 ^{13}C-NMR Spectrum of **(18)**

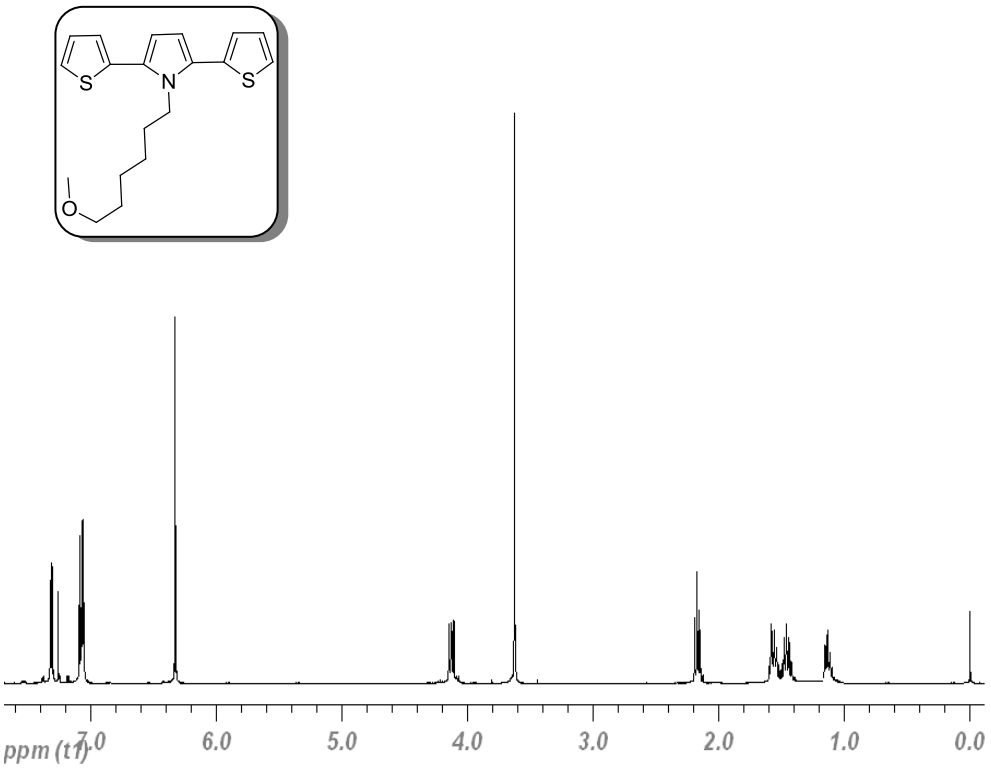

Figure A. 33 ^{1}H-NMR spectrum of **(19)**

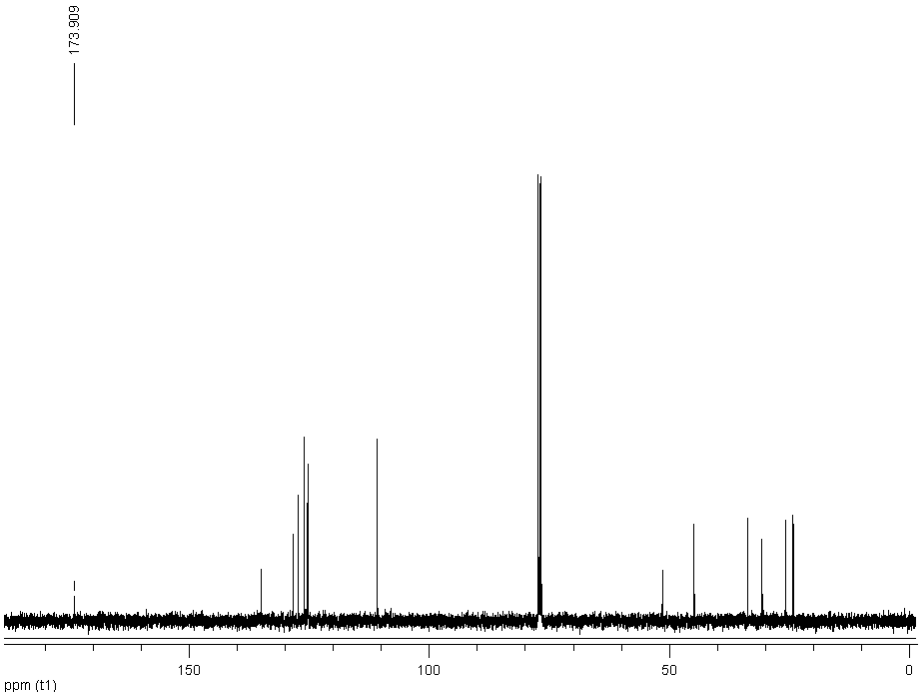

Figure A. 34 ^{13}C-NMR Spectrum of **(19)**

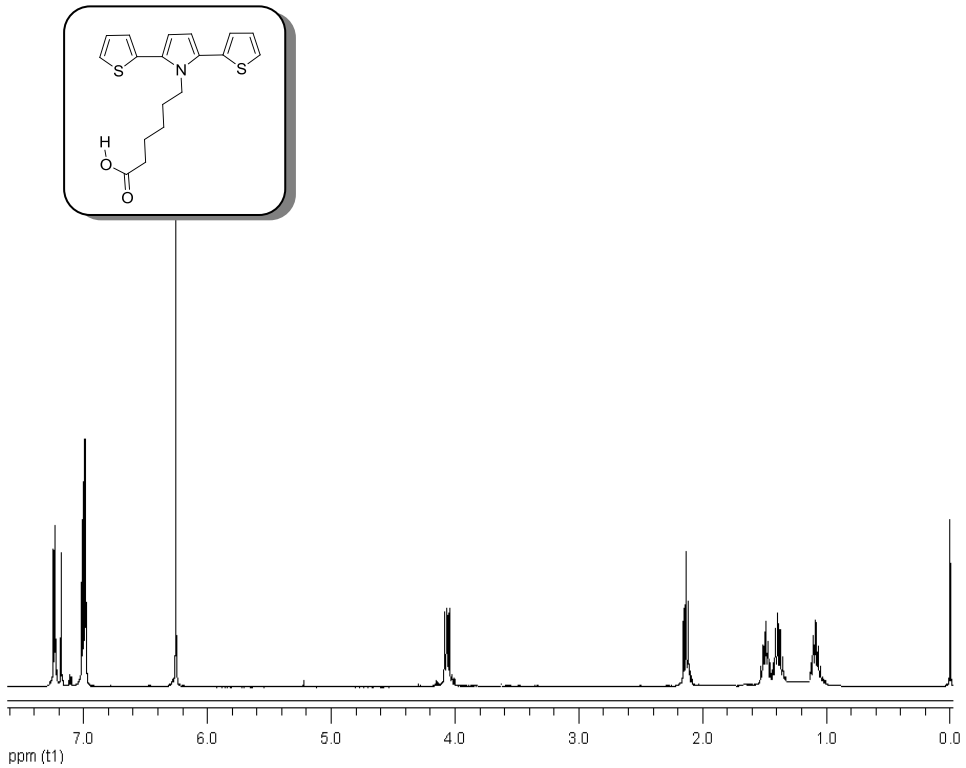

Figure A. 35 ^{1}H-NMR spectrum of **(20)**

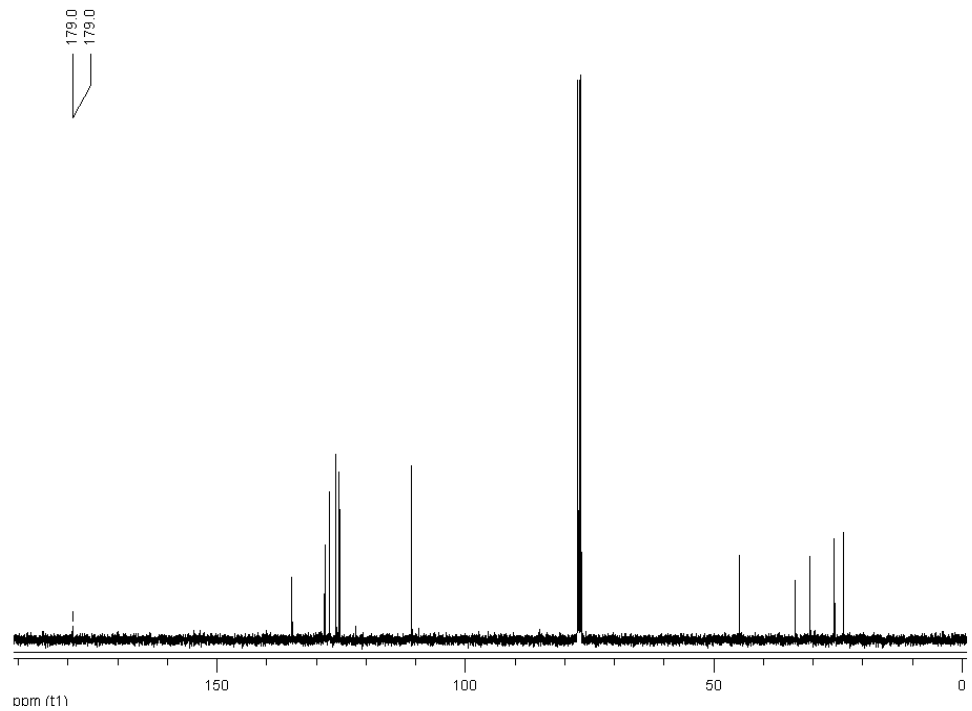

Figure A. 36 ^{13}C-NMR Spectrum of **(20)**

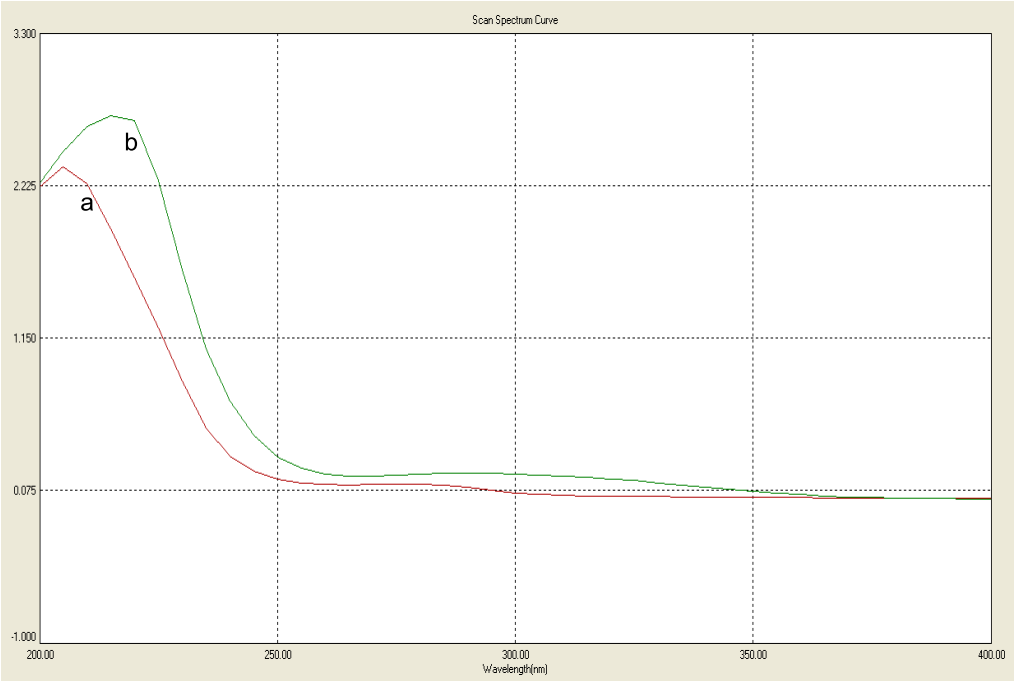

Figure A. 37 UV- Spectrum of **(21)**

(a) Pure enzyme in water Peak 205.00 nm Absorbance:2.363

(b) Water extract Peak 215.00 nm Absorbance:2.722