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Synthesis of Chemical Models of Hydrolase Enzymes for Intramolecular Catalysis

A thesis

presented to

the faculty of the Department of Chemistry

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Chemistry

by

Cornelius N Ndi

December 2011

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Keywords: Intramolecular, Nucleophilic, General Acid/Base, Hydrolase

ABSTRACT

Synthesis of Chemical Models of Hydrolase Enzymes for Intramolecular Catalysis

by

Cornelius N. Ndi

Most nuclease enzymes can hydrolyze phosphoester bonds (in DNA and RNA) by using metal ions cofactors that coordinate and activate water molecules in the enzymes' active sites. However, there are some hydrolase enzymes (including nucleases) that can function without the aid of metal ions. 2,6-Di(1*H*-imidazol-2-yl)phenol, a model compound for hydrolase enzyme, was synthesized by the reaction between ethylenediamine and dimethyl-3-carboxysalicylate, initially resulting in the formation of diimidazoline. The diimidazoline was subsequently aromatized to the diimidazole by dehydrogenation over palladium. The overall reaction yield was low; therefore, other dehydrogenation transformation reactions were tried but all failed to improve the yield. Converting this diimidazolphenol into diimidazolphenyl monophosphate derivative was attempted but failed to give desired products.

Synthesis of 2,2'-anthracene-1,8-diylbis-1*H*-imidazole, another model compound for hydrolase enzymes, was attempted using dimethyl-1,8-anthracenedicarboxylate, but synthesis was unsuccessful due to solubility problem.

DEDICATION

In loving memory of my late grandfather Mr. Ndi Moses Mbah.

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Words are not sufficient to express my gratitude to my family, especially my mum Miss Ndi Roseline T., for their prayers and support. I am also thankful for having wonderful friends here at ETSU, Stanley Jing, Jude Lakbub, Leonard Fai, Thomas Simerly, Kelley Cross.

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CHAPTER 1

INTRODUCTION

Phosphate esters are common in nature. They exist in living organisms as DNA and RNA and are encountered in the environment as insecticides and pesticides. These esters are known to be very stable; DNA and RNA have half-lives of several hundreds of years. The key for successful hydrolysis of phosphodiester bonds is the delivery of a primary nucleophile at the reaction center (phosphorus atom).¹ Although most hydrolytic enzymes use metal ion cofactors, a few enzymes are known to do so without metal ions. Such enzymes and their model compounds have attracted increasing interest in the recent years due to their potential applications in pharmaceutical industry.

Intramolecular Catalysis

The acceleration of chemical transformation at one site of a molecular species through the involvement of a neighboring functional (catalytic) group in the same molecule has been reported in literature, using enzymes as well as model compounds². The use of the term autocatalysis (intramolecular catalysis) is restricted to chemical systems where the substrate and the catalyst are in the same compound.

Intramolecular catalysis, where the substrate and catalytic group are brought close together by specific spacers has the following advantages:

1. Increasing the effective concentration of substrate and catalytic group.
2. Enhancing the catalytic reaction rate due to the optimal molecular geometry that results in favorable interaction between the substrate and the catalytic site. Two to three orders of magnitude of rate enhancements have been observed in model compounds.
3. Although less effective than enzymes (with rate enhancements of five to nine orders of magnitude), these model compounds help understand mechanism of action of enzymes.

Enzymes

Enzymes are biological catalysts and are required by most metabolic reactions to occur at required rates. Each cell contains over 500 different enzymes to enable it to function properly. The type of enzyme required depends on the nature and function of the cell in the living organism. Over 2000 different enzymes are known and described in variable depths.

As proteins, enzymes have characteristic primary, secondary, and tertiary structures. Sequences of amino acids linked by peptide bonds form the primary structure. Only 20 amino acids are known to constitute all types of proteins that vary in sizes. The secondary and tertiary structures are involved in determining the three-dimensional nature of the enzyme.

Enzymes involved in cellular metabolic reactions often have quaternary structures as well. This means that these enzymes have more than one polypeptide subunits that cooperate in their functions. In some metabolic systems, more complex forms of enzyme organization are observed. An example of this level of organization is observed in the fatty acid synthetic pathway in plants and bacteria where seven different enzymes physically associate with each other to catalyze a series of reactions.³

Enzyme Catalysis

Enzymes, E, are very sensitive to temperature, pH, and solvents. Despite these disadvantages as proteins, they tend to be more efficient catalysts than synthetic catalysts which are less sensitive to such factors. As catalysts, they follow the normal rules for catalysis:

- 1) They do not catalyze thermodynamically unfavorable reactions.
- 2) They remain unchanged at the end of the reaction.
- 3) They do not change the direction of a reaction.
- 4) They do not change the equilibrium of a reaction but increase the rate at which the equilibrium is reached.

Enzymes lower the activation energy of metabolic reactions by forming an intermediate enzyme-metabolite complex, E – S, with one or more of the

metabolites (Figure 1). This complex subsequently breaks down to give product and enzyme. This process takes place in the most important catalytic region of the enzyme called the active site. The active site of an enzyme is a small crevice composed of specific amino acids that are directly involved in catalysis. Some of these are shown in Table 1.

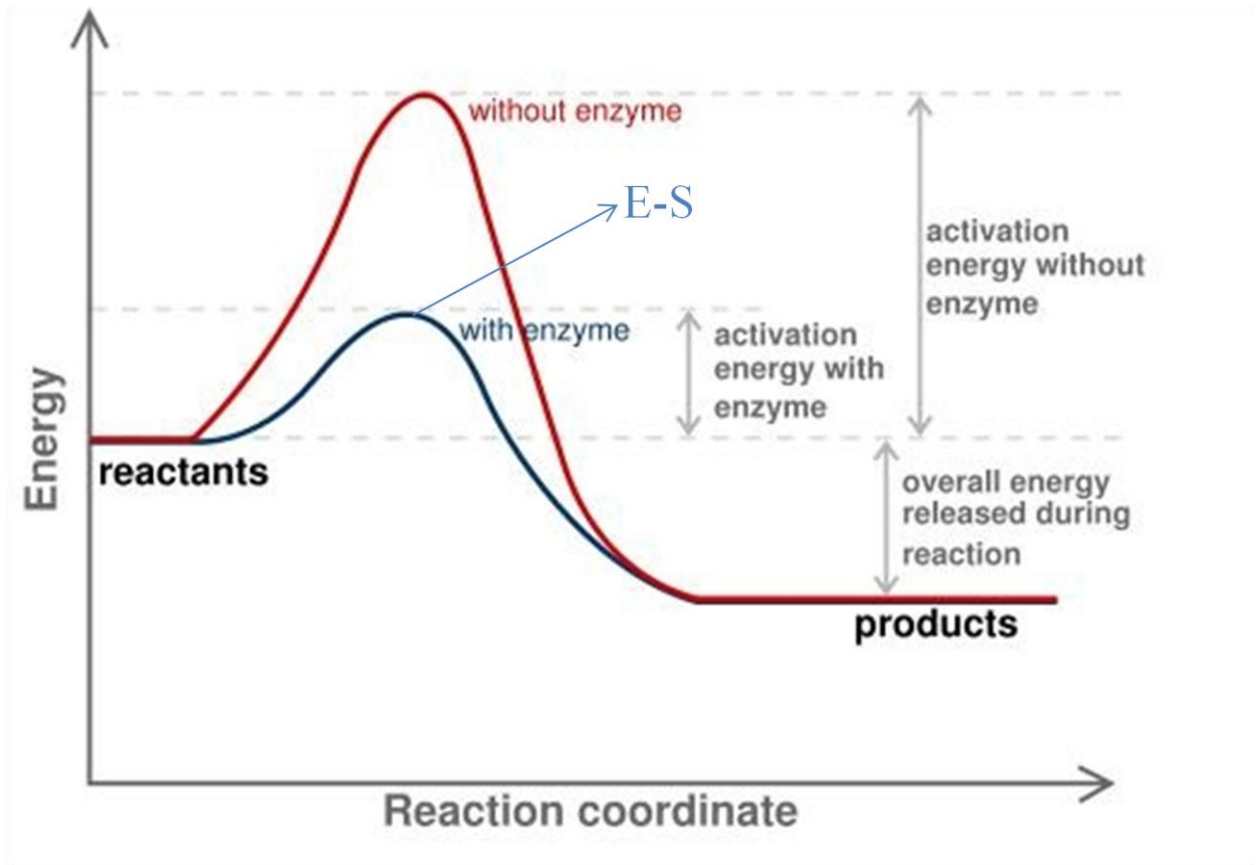


Figure 1. Energy diagram for a catalyzed versus non-catalyzed reaction⁴

(adapted)

Table 1. Examples of amino acids that are usually involved in the active sites of enzymes³

Amino acid	Side group`	Interaction with substrate
Serine	-OH	Hydrogen bonding
Cysteine	-SH	Hydrogen bonding/disulphide bridging.
Histidine	-Imidazole	Hydrogen bonding/electrostatic
Lysine	-NH ₃ ⁺	Electrostatic
Arginine	-NH ₃ ⁺	Electrostatic
Aspartic acid	-COO ⁻	Electrostatic
Glutamic acid	-COO ⁻	electrostatic

Mechanism of Enzyme Catalysis

Many different mechanisms by which enzymes bring about catalysis are known. Two such mechanisms are:

1. General acid-general base mechanism
2. Nucleophilic mechanism

General Acid-General Base mechanism.

A general acid or base is a compound that is weakly ionizable. At physiological pH, the protonated imidazole group of histidine acts as a general acid while the free imidazole acts as a general base.

The role of the imidazole groups is exemplified in the active site of Ribonuclease (RNase A)⁴. This enzyme is a pancreatic enzyme found in ruminants and is responsible for the breakdown of excess RNA produced in the stomach by bacteria and other microorganisms.

The hydrolytic mechanism of RNase A outlined in Figure 2 is a two-step process that involves the obligatory formation of a cyclic 2', 3'-phosphoribose nucleotide intermediate. In the first stage of the mechanism, His 119 protonates the phosphodiester, and in a concerted manner, His 12 acts as a general base by abstracting a proton from the ribose-3'-hydroxyl group forming a nucleophile. This nucleophile attacks the phosphate group forming the cyclic intermediate and breaking the RNA chain. The cyclic intermediate is broken down in the second step by a reversal of the first step with water replacing the leaving group. The histidines are reverted to their original states.

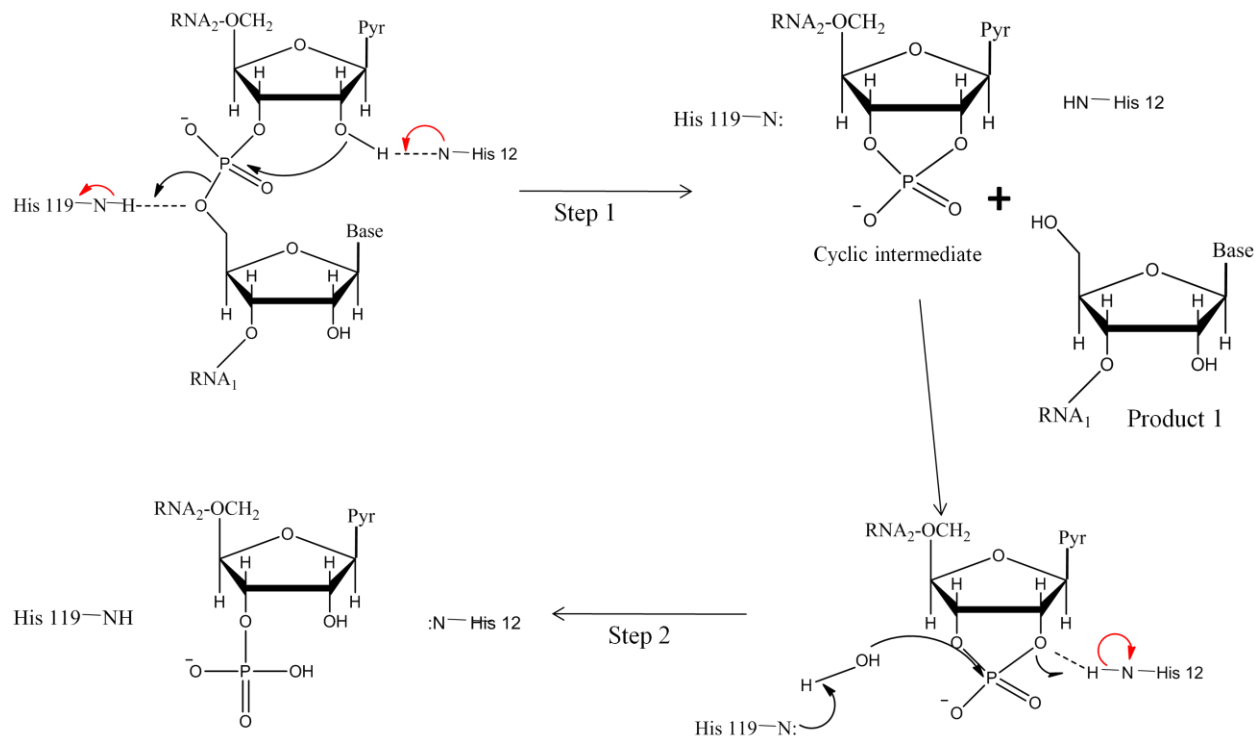


Figure 2. Role of Imidazole in the active site of RNaseA⁴

Nucleophilic Mechanism.

In this mechanism, the attack of a nucleophilic group on the substrate results in an enzyme-substrate intermediate that is more labile compared to the original substrate.

Trypsin, chymotrypsin, and thrombin are serine protease enzymes that use this mechanism. Another good example is the salicylic acid binding protein 2 (SABP2)⁵ that is responsible for the hydrolysis of methyl salicylate to salicylic acid during the defense mechanism against pathogens in the tobacco plant. In the above

enzymes, a catalytic triad is observed. This triad is made up of the -COO^- group of aspartic acid, the imidazole group of histidine, and the -OH group of serine.

The hydrolysis of a peptide bond by chymotrypsin is initiated by a nucleophilic attack by the alkoxide nucleophile of the Ser 195 residue. This nucleophile is formed by hydrogen transfer from the -OH group to the imidazole group. This transfer is stabilized by the -COO^- group (Figure 3). The alkoxide attack results in the release of the amino terminal of the protein and the formation of an acylated enzyme. The acylated enzyme is then subsequently broken down by a reversal of the reaction pathway with water acting as a nucleophile (Figure 3).

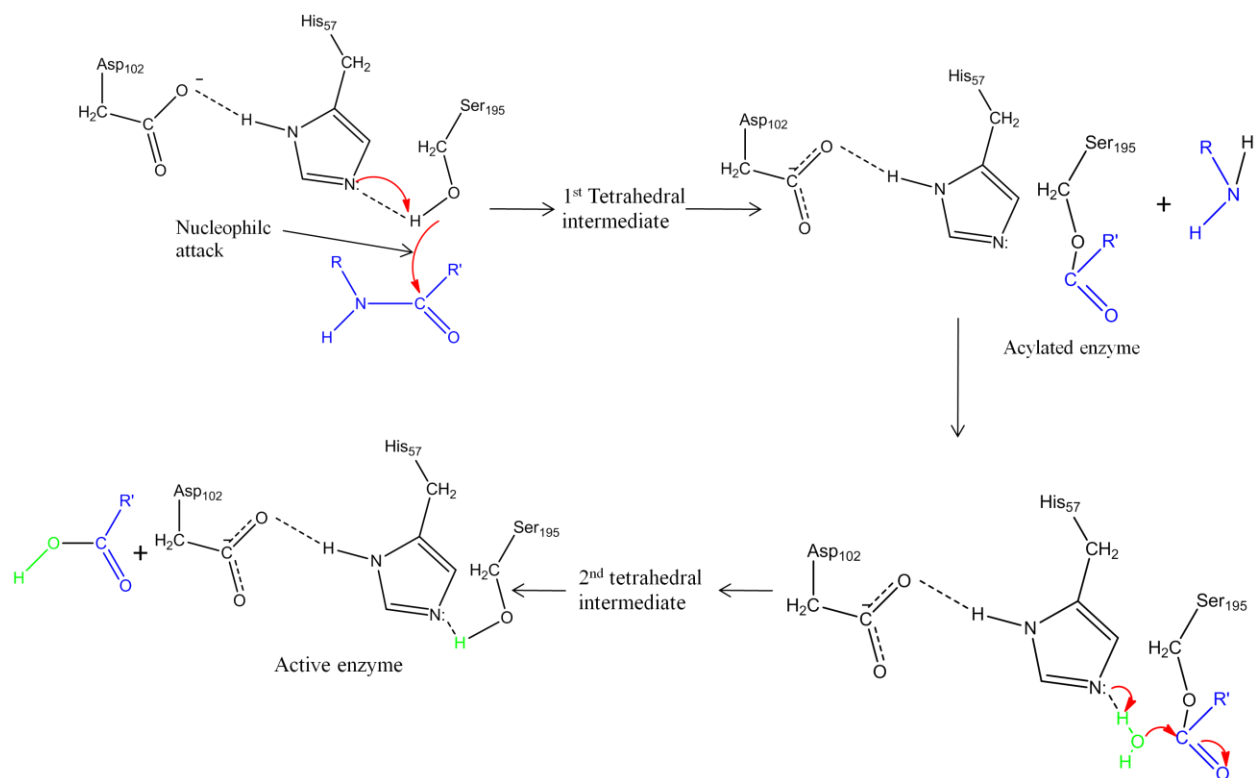


Figure 3. Nucleophilic catalysis in active site of chymotrypsin⁴

Previously Studied Models

We have shown so far that at the physiological pH of the body, some groups have very high catalytic activities and that these groups act as either general acids, bases, or nucleophiles. Many early scientists have explored the properties of these catalytic groups to study and shed more light on their mechanism of action.

As early as the 1950s, L. J Edwards studied in great details the hydrolysis of aspirin. In the last decade, his work has been adapted into a laboratory exercise for undergraduate students⁶. In his work, Edwards showed that the mechanism of hydrolysis of aspirin is pH dependent. The mechanism changes from specific acid to general base nucleophilic assisted to specific base as the pH was increased. The general base nucleophilic assisted mechanism occurred between pH of 4.5 and 8.5; within this range the carboxylic acid group exists in the deprotonated form as carboxylate.

The kinetics of aspirin hydrolysis was monitored using a UV spectrophotometer by measuring the absorbance of the product formed at 298 nm; the isosbestic point of the salicylic acid-salicylate ion pair.⁶ The observed rate was consistent with general base nucleophilic assisted mechanism. This mechanism involved the carboxylate ion deprotonating a water molecule, forming a strong nucleophile that attacks the ester, bringing about its own hydrolysis (Figure 4).

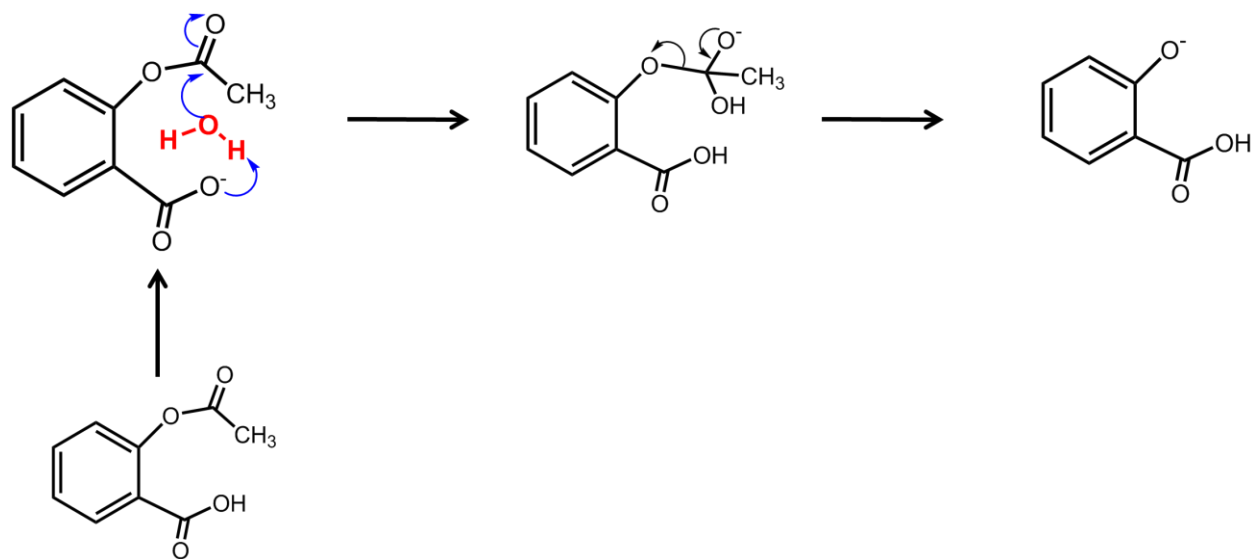


Figure 4. General base nucleophilic assisted hydrolysis of aspirin⁶

In 1968, Fresht and Kirby studied the intramolecular hydrolysis of the nitro-derivatives of aspirin^{7,8}. Unlike the case of aspirin, they observed that the rate of hydrolysis is independent of pH between 4 and 8. In contrast to the mechanism proposed earlier by Edwards (Figure 4), a mechanism involving a direct nucleophilic attack by the carboxylate group on the ester function resulting into an intermediate anhydride was proposed (Figure 5). The anhydride is subsequently broken down in a rate determining step by general base mechanism involving an alkoxide ion. It was observed also that the overall reaction rate was 50 times faster compared to hydrolysis of aspirin⁷. This rate enhancement was attributed to the increased stability of the phenoxide intermediate, which makes a good leaving group.

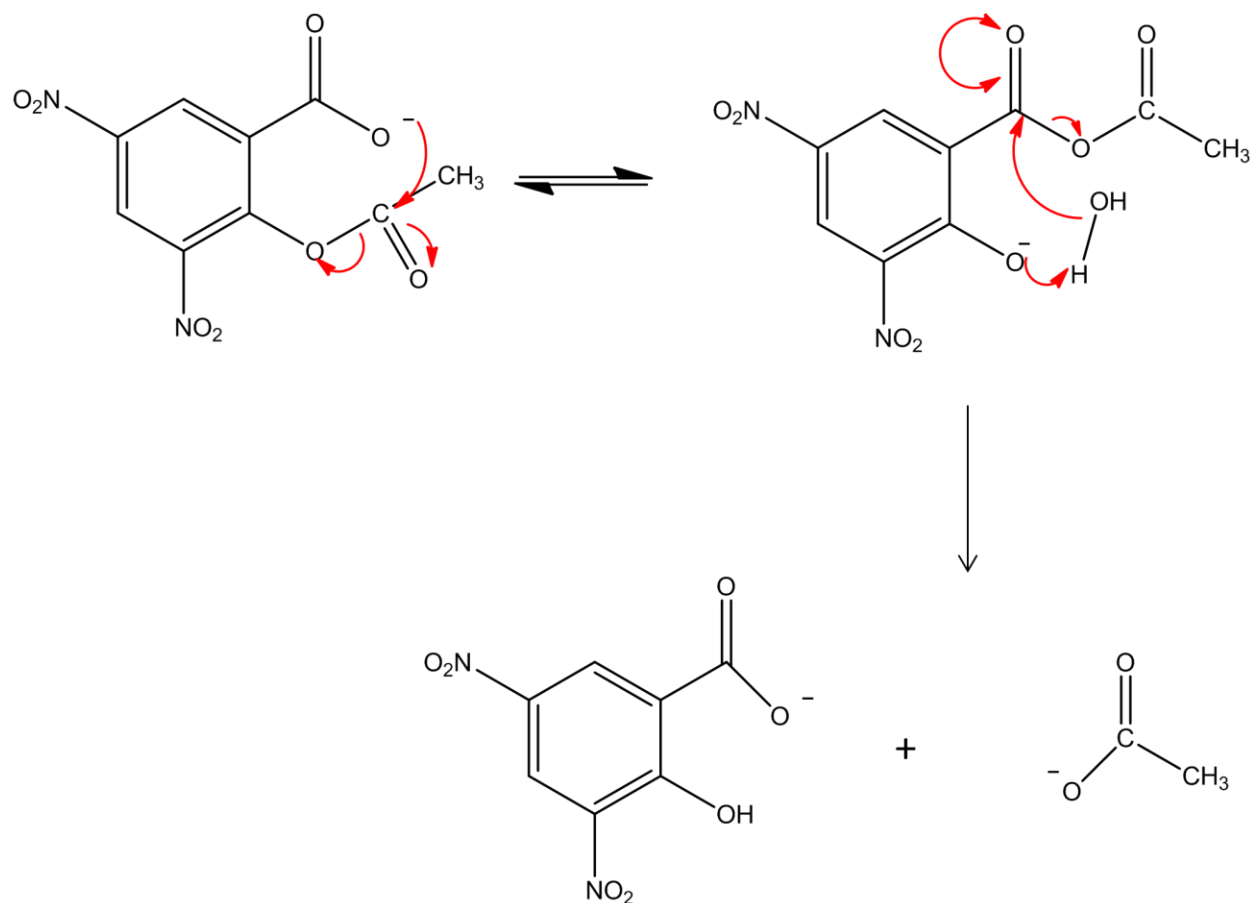


Figure 5. Nucleophilic hydrolysis of 3, 5- dinitroaspirin⁷

In 1983, Katherine and Kirby studied the hydrolysis of bis-2-carboxyphenyl phosphate⁹. Results showed that the hydrolysis was fastest around pH 4.5. At this pH, the molecule exists in the dianion form. A mechanism involving an initial nucleophilic attack by the carboxylate group to form a cyclic phosphate ester was proposed. This nucleophilic attack was followed by a rather inefficient general acid attack by the -COOH group (Figure 6).

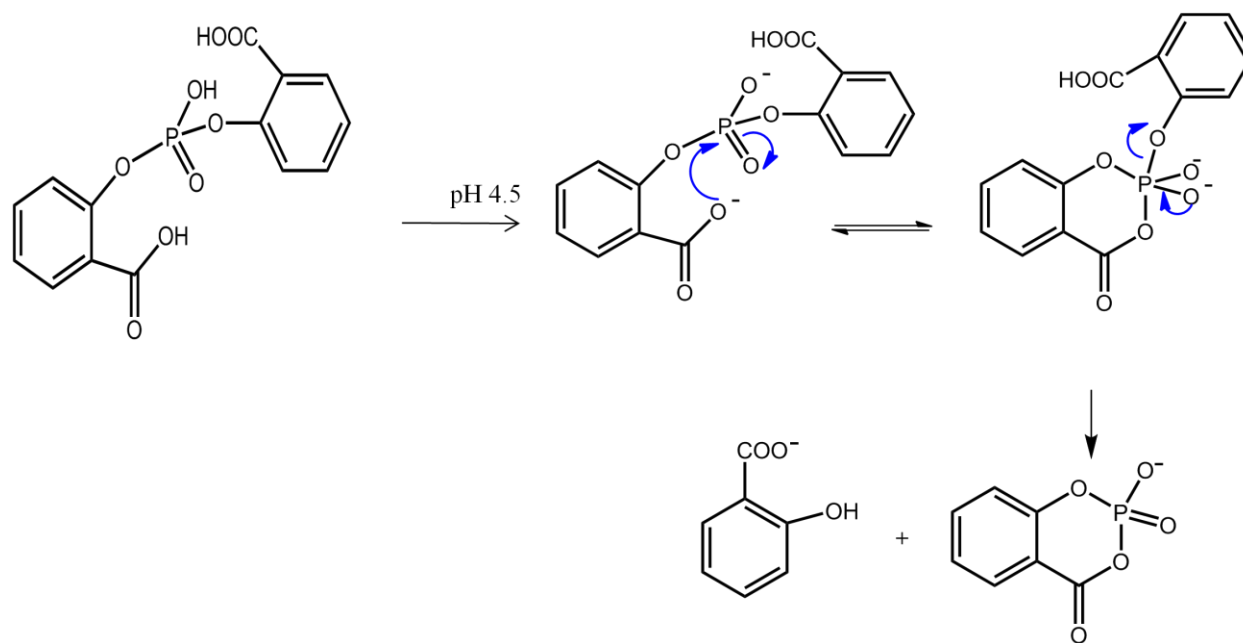
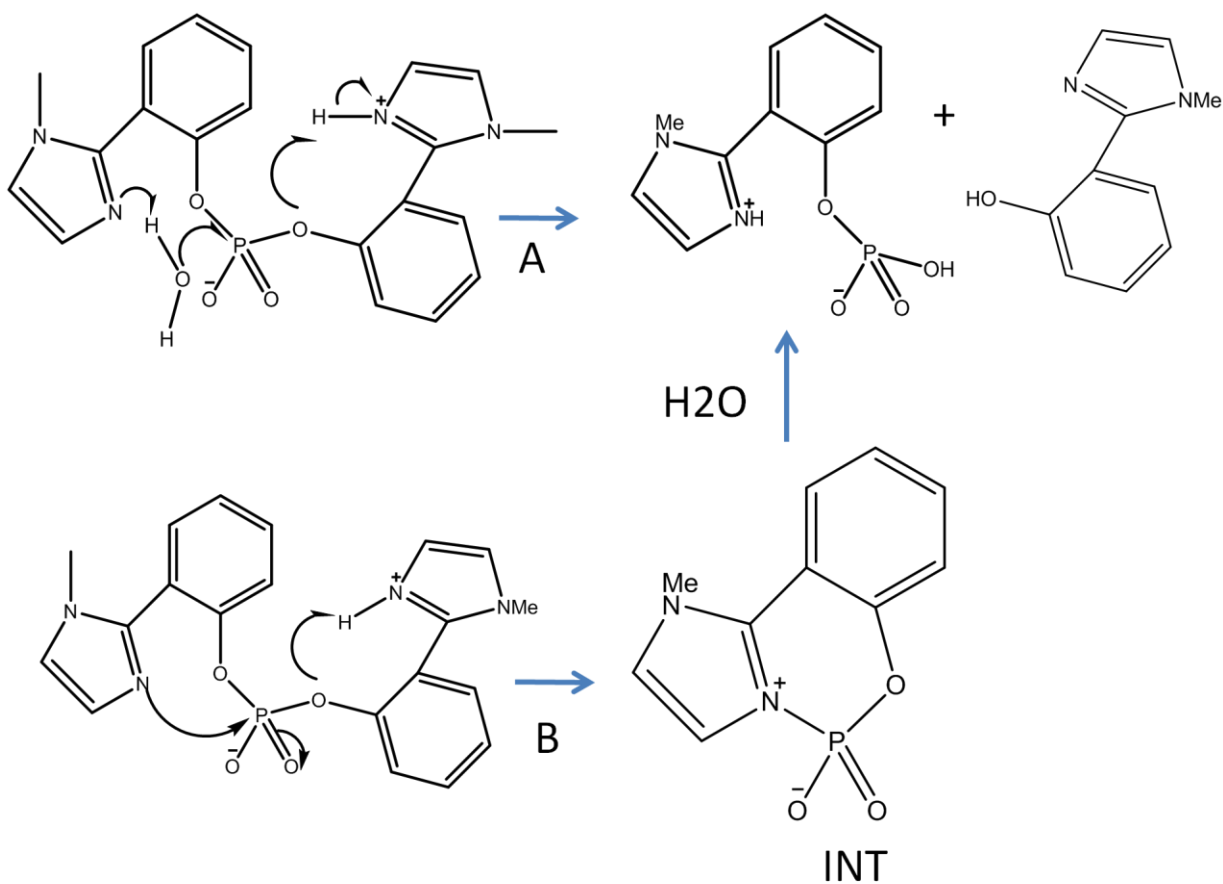


Figure 6. Hydrolysis of bis-2-carboxyphenyl phosphate⁹

We have so far looked at some model compounds bearing -COOH groups. Extensive investigation of imidazole catalysis dates back to 1973. Gary A. Rogers and Thomas C. Bruice inspired by the participation of an imidazolyl group in the catalytic action of serine esterases, synthesized and studied model compounds to investigate the influence of electronic and steric factors on the intramolecular imidazole catalysis¹⁰. Based on their findings, they concluded that three modes of intramolecular imidazole catalysis of ester hydrolysis can take place: (1) general acid assisted water attack, (2) general base assisted water attack, and (3) $\text{O} \rightarrow \text{N}$ acetyl transfer to imidazole anion followed by subsequent hydrolysis.

Recently, Elisa S. Orth et al. have shown that two imidazole group can assist in the hydrolysis of phosphodiester. They found that Bis(2-(1-methyl-1H-imidazolyl)phenyl) phosphate (BMIPP) auto catalyzes its own hydrolysis¹. Based on both hydrolysis experiments and computational evidence, they proposed a concerted intramolecular nucleophilic catalysis and intramolecular general acid catalysis (path B). Their conclusion was based on the detection of the short-lived intermediate INT (Scheme 1)



Scheme 1. Mechanism for the rapid hydrolysis of BMIPP¹

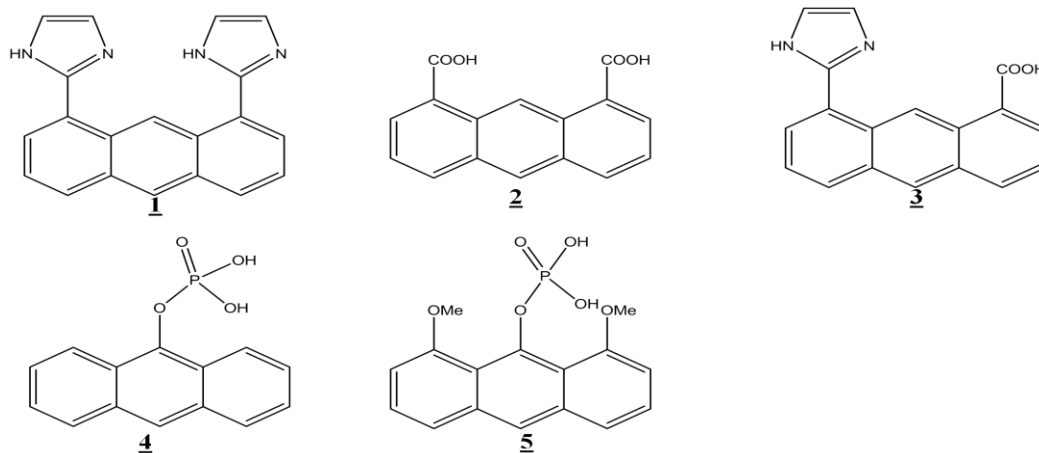
Research Prospective

Our initial research plan was to synthesize and study two model enzyme systems. Enzyme model compounds in model system 1 can recognize the substrate through $\pi - \pi$ stacking. This model system involves either two carboxylic groups or two imidazole rings anchored on an anthracene ring as a spacer (Scheme 2). The substrate for this model would be a 9 – anthylphosphate (**4**). The two anthracene rings, of both the catalyst and the substrate, would recognize each other in a highly ordered manner by $\pi - \pi$ interactions; such recognition allows both the catalytic groups and the phosphate ester of the substrate to be brought into close proximity, thus resulting in hydrolysis of the phosphate ester by general acid – base mechanism. Model system 2 involves attaching both the phosphate ester (substrate) and the catalytic groups (either carboxylic groups or imidazole rings) to the same anthracene spacer (Scheme 2).

Our synthetic approach for 9 – anthylphosphate was modified after facing synthetic problems. Maintaining the same initial idea of involving anthracene, we decided to use dimethoxyl-9-anthylphosphate substrate (**5**).

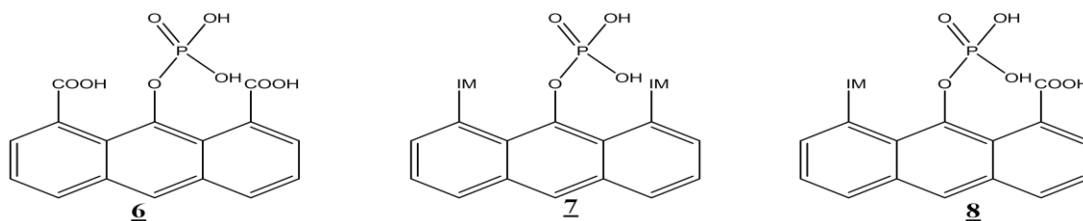
In related work, we later explored the synthesis of yet a third model system (model system 3), in which both the catalytic imidazole rings (or the carboxyl groups) and the phosphate ester substrate are attached to a benzene ring (Scheme 2).

Model system 1



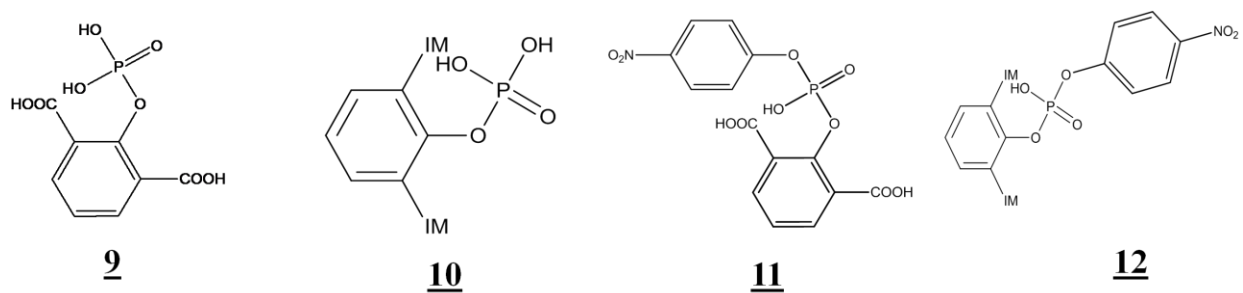
Substrates (**4** and **5**) for model compounds 1 through 3.

Model system 2



IM- Imidazole

Model 3



Scheme 2. Target enzyme model compounds planned for this research

CHAPTER 2

EXPERIMENTAL

General Method

All commercial reagents were purchased and used without further purification except where otherwise stated. Organic solvents were distilled prior to use, over proper drying agents.

Proton (^1H) and carbon-13 (^{13}C) (decoupled) NMR spectra were recorded on JEOL- NMR Eclipse spectrophotometer operating at 400MHz. The chemical shift values are recorded in parts per million (ppm) relative to TMS. The multiplicity of signals is recorded as such; singlet (s), doublet (d), triplet (t) and multiplet (m).

Thin layer chromatography (TLC) was carried out using silica gel plates and viewed under UV. Column chromatography separation of compounds was carried out using silica gel. Melting points were obtained using Cambridge Mel-Temp instrument and the values are uncorrected.

Synthesis of 9-Anthranol

Anthrone (3g, 15.3mmol) was dissolved in freshly prepared 10% NaOH (180mL). The mixture refluxed for 20 mins under nitrogen. Ice (300g) was added to the mixture followed by conc. H_2SO_4 (15 mL). Toluene (120mL) was used to

extract the organic compound from the aqueous mixture. After washing with water (200mL), the organic layer was dried over anhydrous Na_2SO_4 . The organic solvent was then evaporated and the resulting solid (1.02g) was dried under vacuum. Characterization of the resulting solid by NMR and melting point showed unreacted starting material. No further attempts to synthesize 9 – anthranol were made.

Synthesis of Anthracen-9-yl Hydrogen Phosphate

Anthrone (2.55mmol) was mixed with 150mg (2.67mmol) of KOH in 25 mL of THF. The mixture was refluxed under nitrogen until all solid dissolved.

Phosphorus oxychloride (POCl_3) (5.35mmol) was dissolved in ice-cold THF (10 mL). The anthrone/KOH solution was then added drop wise to the POCl_3 solution and left to react for 2 hours. Water (0.5mL) was then added to the mixture and stirred overnight.

Tetrahydrofuran, THF, was removed under reduced pressure. A mixture of methanol (5mL) and water (3mL) was added to the resulting solid and left standing overnight. The resulting solid (0.53g) was then filtered out and dried under vacuum. Characterization of this solid by melting point and then NMR was consistent with the structure of anthrone.

Synthesis of Anthraquinone-1,8-dicarboxylic Acid

1,8 –Dichloroanthraquinone (20g, 72.4 mmol) and cuprous cyanide (20g, 216 mmol) were stirred overnight under reflux in N,N-dimethylacetamide(150mL) under dry nitrogen gas. The hot solution was poured into a beaker containing 1L of water with stirring. The copper (I) complex precipitate was filtered and digested in 150mL of hot 3N HNO₃ to afford the 1,8 – anthraquinonedinitrile.

The crude dinitrile was hydrolyzed in 80% H₂SO₄ acid at 170°C for 4 hours. The hot solution was slowly poured into ice and the precipitate was filtered, washed with water, and redissolved in 10% NaOH. The resulting slurry was then filtered. The filtrate was acidified with 6M HCl to yield crude product. The product was recrystallized from water to yield fine-brown crystals (16 g, 77%, m.p. 297°C-300°C, lit 305°-311°).

Synthesis of Anthracene-1,8-dicarboxylic Acid (2)

Anthraquinone -1,8-dicarboxylic acid (7.5 g, 25.33mmol) was stirred with Zn dust and copper (II) sulfate at reflux in 300 mL aqueous ammonia solution (20%) overnight. The hot amber solution was filtered, cooled, and acidified with conc. HCl. Crude product was recrystallized from aqueous dioxane to yield anthracene-1,8-dicarboxylic acid (4 g, 75%, m.p. 297°C-300°C (decomposed)).

Synthesis of 9-Hydroxyanthracene-1,8-dicarboxylic Acid

Anthraquinone-1, 8-dicarboxylic acid (1 g, 3.62 mmol) was suspended in glacial acetic acid and freshly prepared solution of SnCl₂ and 37% HCl was added. The mixture was then stirred overnight at room temperature. The mixture was subsequently poured into 100 mL of water and stirred for 1 hour. The solution was extracted with methylene chloride (2×100 ml) and the organic layer was dried over anhydrous sodium sulfate and evaporated on the rotavap to yield 0.8 g of product. The NMR spectrum of the material was consistent with starting material.

Synthesis of 1,8-Dimethoxyanthracenedione

Dihydroanthraquinone (4 g, 16.65 mmol) and K₂CO₃ (5 g, 33.3 mmol) was dissolved in 100 mL of acetone and Me₂SO₄ (3.5 mL, 33.3 mmol) was added and stirred for 30 min at room temperature. The mixture was then refluxed for 20 hours. The solution was filtered and evaporated and the resulting solid was washed with excess water. The product was purified by column chromatography with methylene chloride as the mobile phase giving a yield of 3.71 g (83.07%), (m.p. 210-215⁰C)

Synthesis of 1,8-Dimethoxyl-9-hydroxyanthracene (5)

1, 8-Dimethoxyanthracenedione (0.5 g, 1.87mmol) was suspended in glacial acetic acid (50 mL). A solution of SnCl₂ (4 g, 15.6 mmol) in HCl (15 mL) was

added in one portion and the suspension was stirred for 30 mins at room temperature. The mixture was poured into water (500 mL) and stirred for additional 10 mins. The organic portion was then extracted with methylene chloride, and the extract was washed with water (3×100 mL), saturated NaHCO₃ (2×100 mL), and rinsed with water (100 mL). The methylene chloride solution was dried with sodium sulfate and purified by column chromatography to provide 0.1 g (20%) of product.

Synthesis of 2,2'-Anthracene-1,8-diylbis(1*H*-imidazole) (1)

1,8- Dimethoxycarbonylanthracene (514.2 mg, 17.47 mmol) was added to 1.5mL of freshly distilled ethylenediamine. The reaction mixture was then refluxed under nitrogen atmosphere for 8 hours. Excess ethylenediamine was distilled under reduced pressure leaving a brownish solid. Several attempts were made to characterize this product by NMR but all were unsuccessful as it was not soluble in any of the common NMR solvents. The product was also insoluble in hot phenyl ether and toluene and hence could not be used in subsequent oxidation steps.

Synthesis of 2-(2'- Hydroxyphenyl)imidazole

Wintergreen oil (4.23 mL, 33 mmol) was dissolved in 3 molar excess of ethylenediamine and the mixture was refluxed overnight under a stream of dry

nitrogen gas. The unreacted ethylenediamine was distilled under reduced pressure and the resulting solid was washed with methylene chloride to remove trace amounts of wintergreen oil and ethylenediamine. The remaining solid was then recrystallized from 95% ethanol to obtain pale yellow crystals of 2-(2'-hydroxyphenyl)-1H-imidazoline. (3.63 g, 67.2%, m.p. 190° – 195°)

The imidazoline (1.8 g, 11 mmol) was suspended in 12mL of phenylether and heated until it dissolved. Palladium supported on carbon (Pd/C) (153.6 mg) was added to the mixture while stirring and the mixture was refluxed for 5 hours. The Pd/C catalyst was filtered and filtrate was loaded on a silica gel column and eluted with benzene. The solid obtained was recrystallized from water. (900 mg, 50.64%, m.p. 131° – 133°, lit 133° – 134°)¹⁰.

Synthesis of 2-Hydroxyisophthalic acid

Potassium hydroxide (120 g, 2.35 mol) was dissolved in 50 mL of water. After the mixture cooled to room temperature, 3-methylsalicylic acid (20.00 g, 131.46 mmol) was added, and the slurry was stirred gently until all the acid dissolved. Lead dioxide (120.00 g, 501.67 mmol) was then stirred and the mixture was heated until the temperature reached 250°C. The mixture was kept at this temperature for 15 minutes then cooled to room temperature. Water (250 mL) was added to the mixture with stirring. The cold suspension was then filtered and the

solid was washed with 100 mL of water. The filtrate and washing were combined and the mixture was partially acidified using HCl (35%, 80 mL). Sodium sulfide was added to the mixture to precipitate any excess lead as PbS. The suspension was brought to gentle boiling and then allowed to cool to coagulate the lead sulfide, which was then filtered. The filtrate was cooled down to 10°C and acidified with conc. HCl (90 mL) to precipitate the diacid. The diacid was filtered out and recrystallized from water. (9.84 g, 47.33%, m.p. 238° – 240°)

Synthesis of Dimethyl-3-carboxysalicylate

2-Hydroxyisophthalic acid (5 g, 27.4 mmol) was dissolved in freshly distilled methanol (75 mL) and 10 drops of concentrated sulfuric acid was added to the mixture. The mixture was then refluxed for 6 hours. Excess methanol was removed on the rotavap. The obtained solid was suspended in 5% NaHCO₃ solution (150 mL) and extracted with methylene chloride. The methylene chloride layer was dried over anhydrous sodium sulfate and evaporated to give the diester. Recrystallization from ethylacetate – hexane mixture provided fine white crystals of the diester. (2.45 g, 57.63%, m.p. 65° – 68°)

The NaHCO₃ washing was acidified with HCl and the obtained solid was filtered. The solid was dissolved in methylene chloride and chromatographed on a silica gel column eluted with methylene chloride. The isolated mono-ester (2-

hydroxy-3-(methoxycarbonyl)benzoic acid) was recrystallized from a mixture of acetone-petroleum ether. (1.3 g, 40 %, m.p. 130° – 132°)

Synthesis of 2,6-Di(1*H*-imidazol-2-yl)phenol

Dimethyl-3-carboxysalicylate (500 mg, 2.37 mmol) was refluxed in 2 mL of ethylenediamine overnight under nitrogen. Excess ethylenediamine was distilled under vacuum. The residue was suspended in 12 mL of phenylether and 150 mg of Pd/C was added and the mixture was refluxed for 7 hrs. The charcoal was filtered and the product eluted from a silica gel column using a CH₂Cl₂/methanol mixture. (90 mg, 16.78%, m.p. 195°C – 200°C, ¹HNMR (400MHz, CD₃OD) δ 6.9 (1H, t), δ 7.2 (4H, s), δ 7.9 (2H, d) ¹³CNMR (100MHz, CD₃OD) δ 116, 122, 126, 146, 154)

Synthesis of 2-Hydroxy-3-(1*H*-imidazol-2-yl)benzoic acid

2-Hydroxy-3-(methoxycarbonyl)benzoic acid (300 mg, 1.53 mmol) was dissolved in 2mL of ethylenediamine and refluxed overnight under nitrogen. Excess ethylenediamine was distilled under reduced pressure. Traces of ethylenediamine were removed using a vacuum pump. The product obtained was then suspended in 4mL of phenylether and 300 mg of Pd/C added to the mixture. The mixture was then refluxed overnight and monitored by TLC. The product was purified by silica gel column eluted with CH₂Cl₂/methanol mixture to give 30 mg of product. (¹HNMR (400 MHz, CDCl₃, ppm) δ 6.83(1H, t), δ 7.04 (1H, d), δ 7.10

(2H, s), δ 7.22(t), δ 7.55 (1H, d) ^{13}C NMR (100MHz, CDCl_3 , ppm) δ 113.50, 117.50, 118.50, 124.0, 130.50, 146.50, 157.00)

Synthesis of Methyl 2-({hydroxy[2-(1*H*-imidazol-2-yl)phenoxy]phosphoryl}oxy)benzoate

Potassium salicylate (300 mg, 1.856 mmol) was dissolved in dry THF (15 mL) and PCl_5 (386 mg, 1.856 mmol) was added to the reaction mixture. The mixture stirred for 120 minutes under nitrogen. 2-(2'-Hydroxyphenyl)imidazole¹⁰ (300 mg, 1.856 mmol) was then added and the mixture was stirred for 120 more minutes. Water (0.5 mL) was added to the mixture and THF was evaporated in the hood. Saturated NaHCO_3 (1 mL) was added followed by methanol (5 mL) and the precipitate obtained was collected by filtration.

The above procedure was repeated in pyridine as solvent instead of THF. The product was obtained as a sodium salt. (^1H NMR (400MHz, DMSO, ppm) δ 3.70 (3H, s), δ 6.9(1H, t), δ 6.9(1H, d), δ 7.05(1H, t), δ 7.20(2H, s), δ 7.40 (1H, t), δ 7.55 (2H), δ 7.85(1H, d))

Synthesis of *N*-(2-aminoethyl)-3-{2-[(2-aminoethyl)amino]-2-oxoethyl}-2-hydroxybenzamide

Dimethyl-3-carboxysalicylate (1.5 g, 7.19 mmol) was dissolved in acetonitrile (15 mL). Ethylenediamine (1.0 mL) was added and the mixture was

refluxed until product precipitated out completely. The product was filtered and dried in vacuo. (m.p. $180^{\circ} - 183^{\circ}$), (^1H NMR (400 MHz, CD_3OD) δ 8.0 (2H, d), δ 6.50 (1H, t), δ 5.0 (4H, s), δ 3.6 (4H, t), δ 3.00 (4H, t), ^{13}C NMR (100MHz, CD_3OD) δ 170.0, 134.0, 120.0, 112.0, 40.0, 38.0)

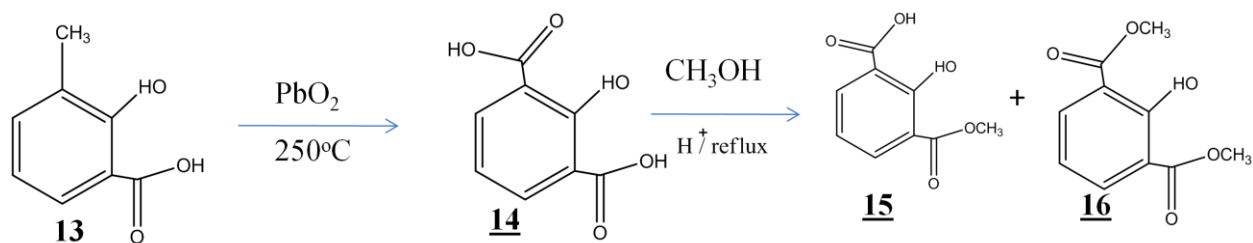
CHAPTER 3

RESULTS AND DISCUSSION

Synthesis of Compounds in Model System 3

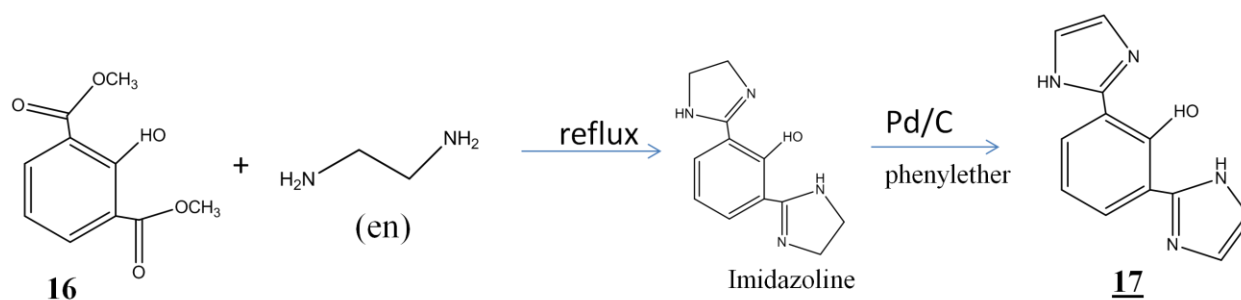
Synthesis of 2,6-Di(1*H*-imidazol-2-yl)phenol(17)

2,6-Di(1*H*-imidazol-2-yl)phenol was obtained as pale yellow solid from the synthetic pathway proposed in Scheme 4. The initial step involved the synthesis of dimethyl-3-carboxysalicylate (**16**) from 3-methylsalicylic acid (**13**) shown in Scheme 3. The scheme involves the oxidation of the methyl group of (**13**) using PbO_2 under high temperature followed by the esterification of the resulting diacid (**14**) yielding the diester (**16**) as the major product and the monoester (**15**) as a minor product¹¹. (Scheme 3)



Scheme 3. Synthesis of the esters **15** & **16**

The next step in the synthesis involves a reaction between the diester (**16**) and ethylenediamine to yield an imidazoline that is subsequently oxidized in the presence of Pd/C^{10} at very high temperatures to the imidazole (**17**). (Scheme 4)

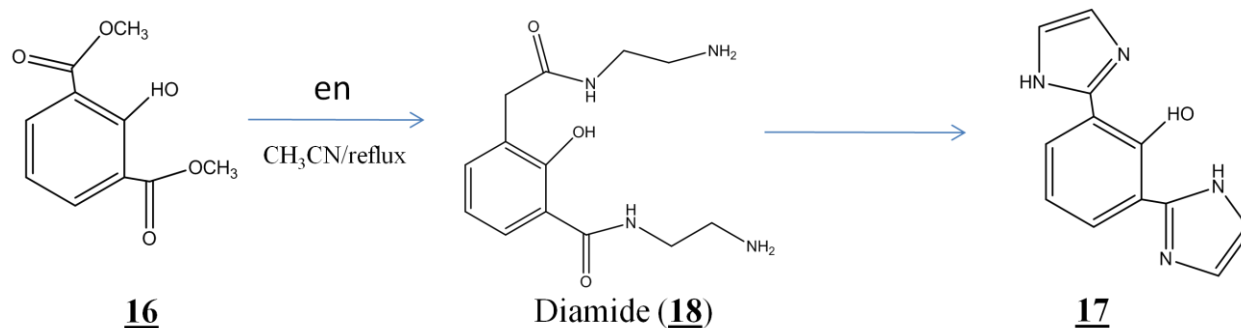


Scheme 4. Synthesis of 2,6-di(1*H*-imidazol-2-yl)phenol(**17**)

2,6-Di(1*H*-imidazol-2-yl)phenol (**17**) was purified by column chromatography using 5% methanol / CH₂Cl₂ mixture. The yield was very low (~16%). The structure of the compound was confirmed using NMR. The proton NMR showed three peaks; a doublet and triplet at δ 7.9 and δ 6.9 ppm respectively corresponding to aromatic protons of the benzene ring and a singlet at δ 7.2 ppm corresponding to the imidazole proton. To further correlate the proton NMR to structure, the integration of the individual peaks was calculated. The singlet corresponding to the imidazole showed 4 times the value for the triplet that corresponds to a single proton on the benzene ring. The doublet was twice as intense as the triplet corresponding to the other two protons on the ring that are equivalent. The ¹³C NMR showed 6 peaks in the aromatic region (δ 110-160 ppm) that corresponded to 5 pairs of equivalent carbons (2 pairs have same chemical shift) and 2 unequivalent carbons thus further confirming the structure.

Even though the NMR techniques confirmed the structure of **17**, the overall synthetic pathway is inefficient due to the resulting low yield. The low yield is thought to come about as a combined effect of insolubility and catalyst poisoning. During the reaction after the ethylenediamine has been distilled, the resulting product was observed to be thick brownish viscous oil that was insoluble in both cold and hot phenyl ether. When the Pd/C was added to the solution, it was observed to stick to the oil and settle at the bottom of the reaction flask rendering it inactive. Other dehydrogenation procedures found in literature¹² were attempted, but all failed due to similar solubility issues.

An alternative synthetic pathway was adopted via the formation of the diamide. (Scheme 5)



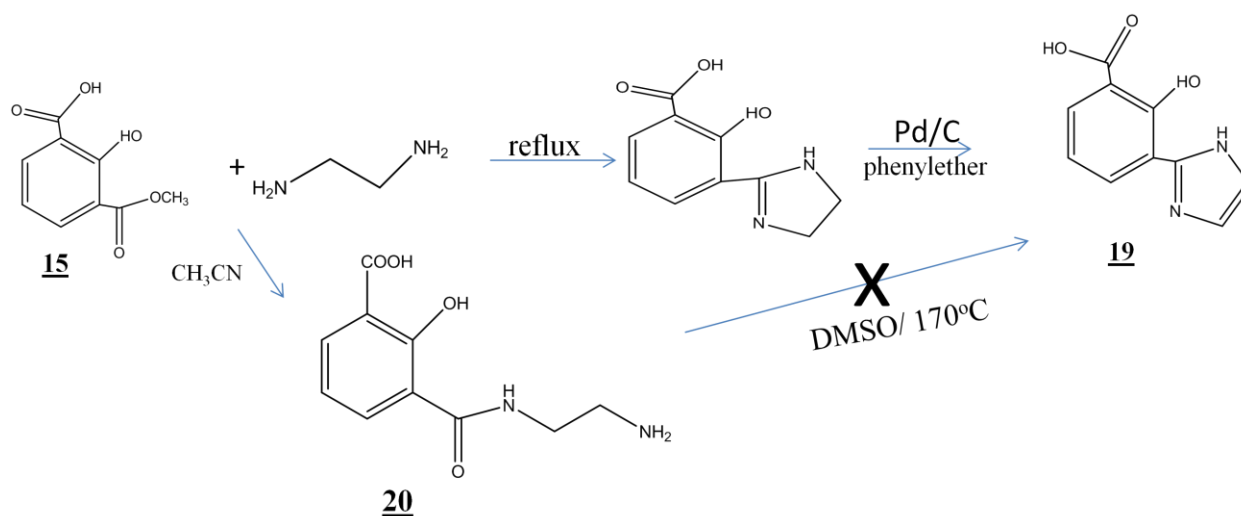
Scheme 5. Alternative proposed pathway for synthesis of **17**

In this pathway, the synthesis of compound **17** from the diamide was attempted in different solvents. The diamide (**18**) was insoluble in all attempted solvents except

DMSO¹³. The initial reaction is a dehydration reaction that should result in ring formation giving the imidazoline product. The following oxidation step of imidazoline to imidazole resulted in total decomposition to intractable product.

Synthesis of 2-Hydroxy-3-(1H-imidazol-2-yl)benzoic Acid(19)

2-Hydroxy-3-(methoxycarbonyl)benzoic acid (**15**) was isolated as a side product. Based on knowledge of the catalytic importance of both the –COOH group and the imidazole group, 2-hydroxy-3-(1H-imidazol-2-yl)benzoic acid would be a very interesting compound to study. It was also thought it would pose less solubility issues compared to previous models. A synthetic scheme (Scheme 6) was used based on literature procedures and methods already discussed earlier.

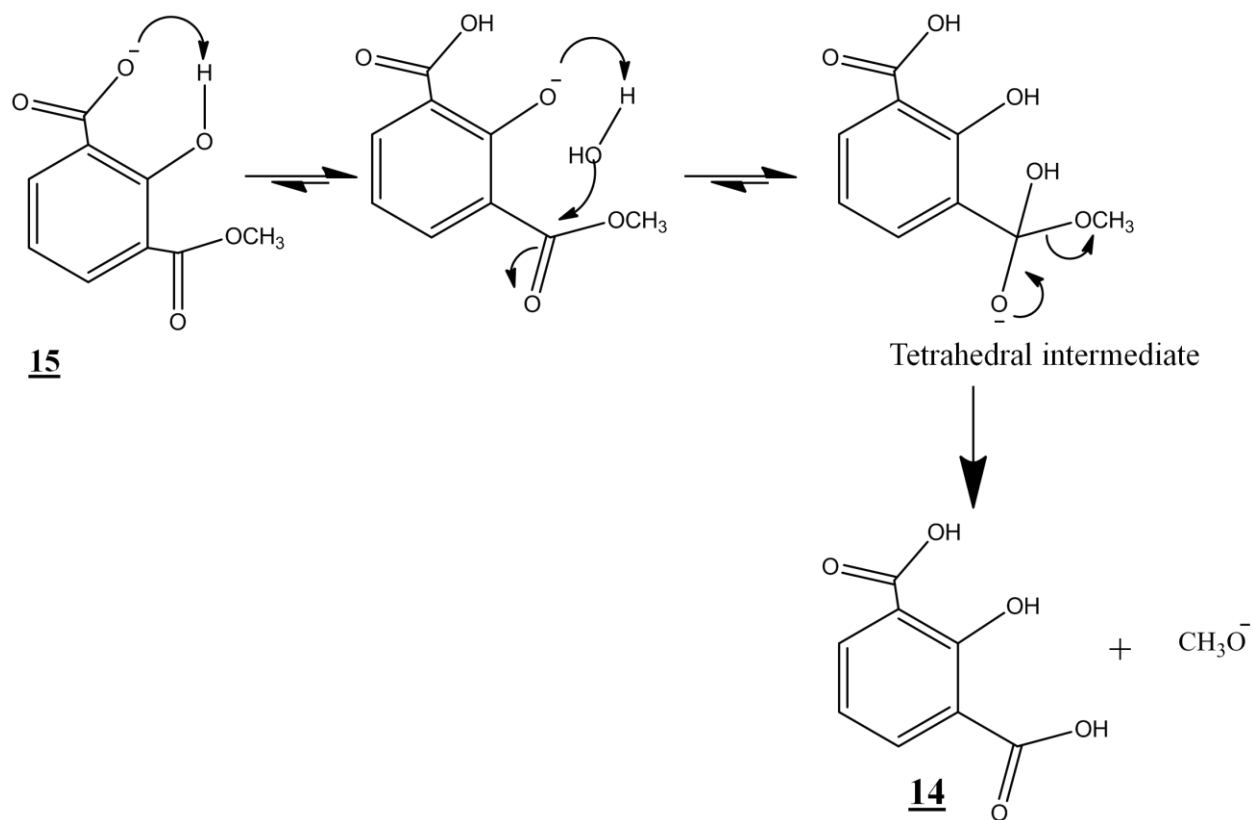


Scheme 6. Scheme for the synthesis of 2-hydroxy-3-(1H-imidazol-2-yl)benzoic acid (**19**)

The imidazole was eluted from a silica gel column using methanol/CH₂Cl₂ solvent system. Proton NMR showed the imidazole peak as a singlet at δ 7.1ppm and two doublets and a triplet at δ 7.55, δ 7.05, and δ 6.85 ppm respectively. A triplet observed at δ 7.20 ppm is associated with impurities that could not be removed by column chromatography. A controversy arose from the ¹³C NMR where only 7 peaks were observed instead of 9 peaks. Thus structure could not be accurately confirmed by NMR data.

Interestingly, when the monoester, 2-hydroxy-3-(methoxycarbonyl)benzoic acid (**15**) was allowed to stand in open atmosphere at room temperature, it was found to undergo complete auto hydrolysis in 48 hours as confirmed by TLC and NMR.

The auto hydrolysis of **15** is thought to be a nucleophilic general base assisted mechanism (Scheme 7). The initial step of the mechanism is the formation of the alkoxide nucleophile, a strong nucleophile. The alkoxide ion formed deprotonates a water molecule to form the OH⁻ ion, which subsequently attacks the ester to form the tetrahedral intermediate. The intermediate being unstable breaks down to the diacid and the methoxide ion.

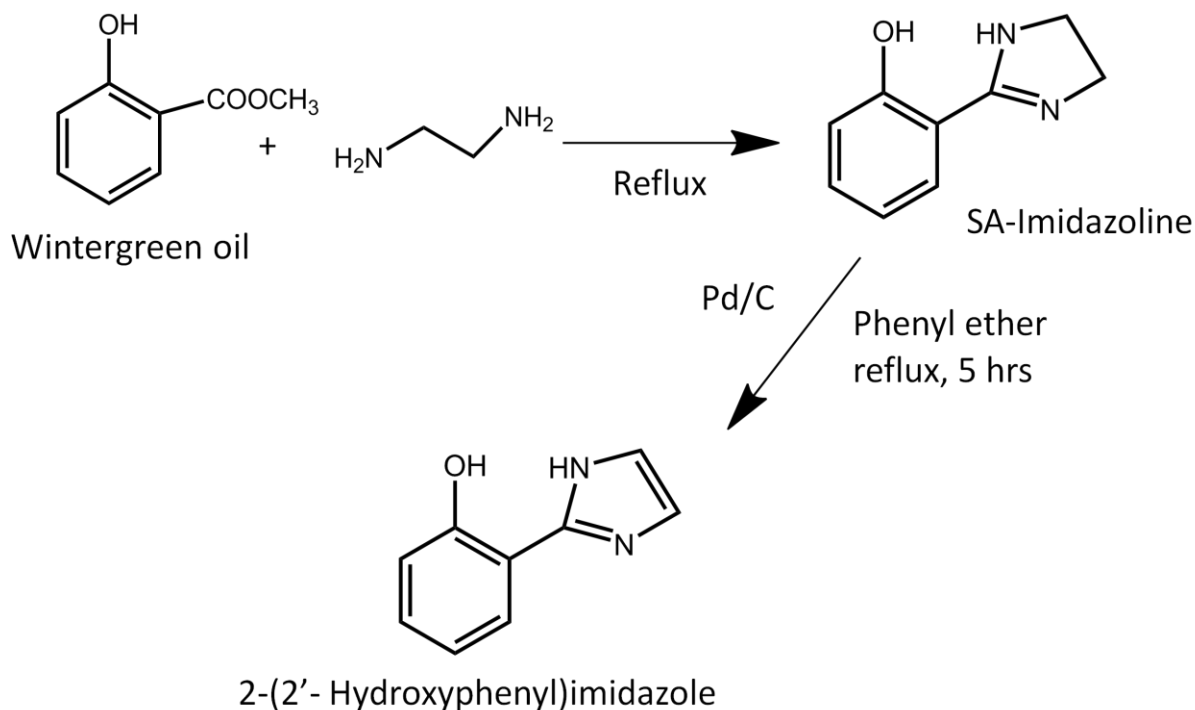


Scheme 7. Proposed mechanism for hydrolysis of 2-hydroxy-3-(methoxycarbonyl)benzoic acid (**15**)

Synthesis of Methyl 2-({hydroxy[2-(1H-imidazol-2-yl)phenoxy]phosphoryl}oxy)benzoate (**21**)

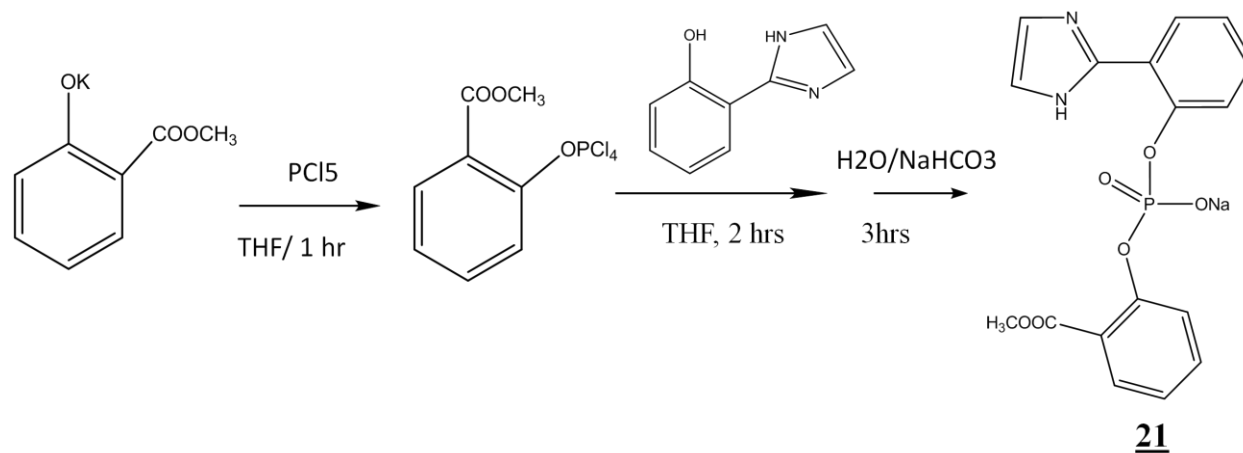
2-(2'- Hydroxyphenyl)imidazole¹⁰ was synthesized (Scheme 8), characterized, and used to confirm the structure of the imidazole group of the other synthesized compounds. Based on a similar compound reported in literature¹, attempts were made to synthesize methyl 2-({hydroxy[2-(1H-imidazol-2-yl)phenoxy]phosphoryl}oxy)benzoate (**21**) (Scheme 9).

2-(2'- Hydroxyphenyl)imidazole was synthesized based on procedure outlined by Thomas C. Bruice¹⁰ (Scheme 8).



Scheme 8. Scheme for the synthesis of 2 – (2'-hydroxyphenyl)imidazole

The initial attempt to carry out a reaction between salicylic acid and the imidazole was unsuccessful. The acid functional group of salicylic acid was then protected by a methyl ester. The potassium salt of wintergreen oil was then used and reacted with the 2 – (2'-hydroxyphenyl)imidazole to form the compound **21** in very low yield (Scheme 9). Compound **21** was isolated and NMR spectra obtained in DMSO were consistent with the assigned structure.



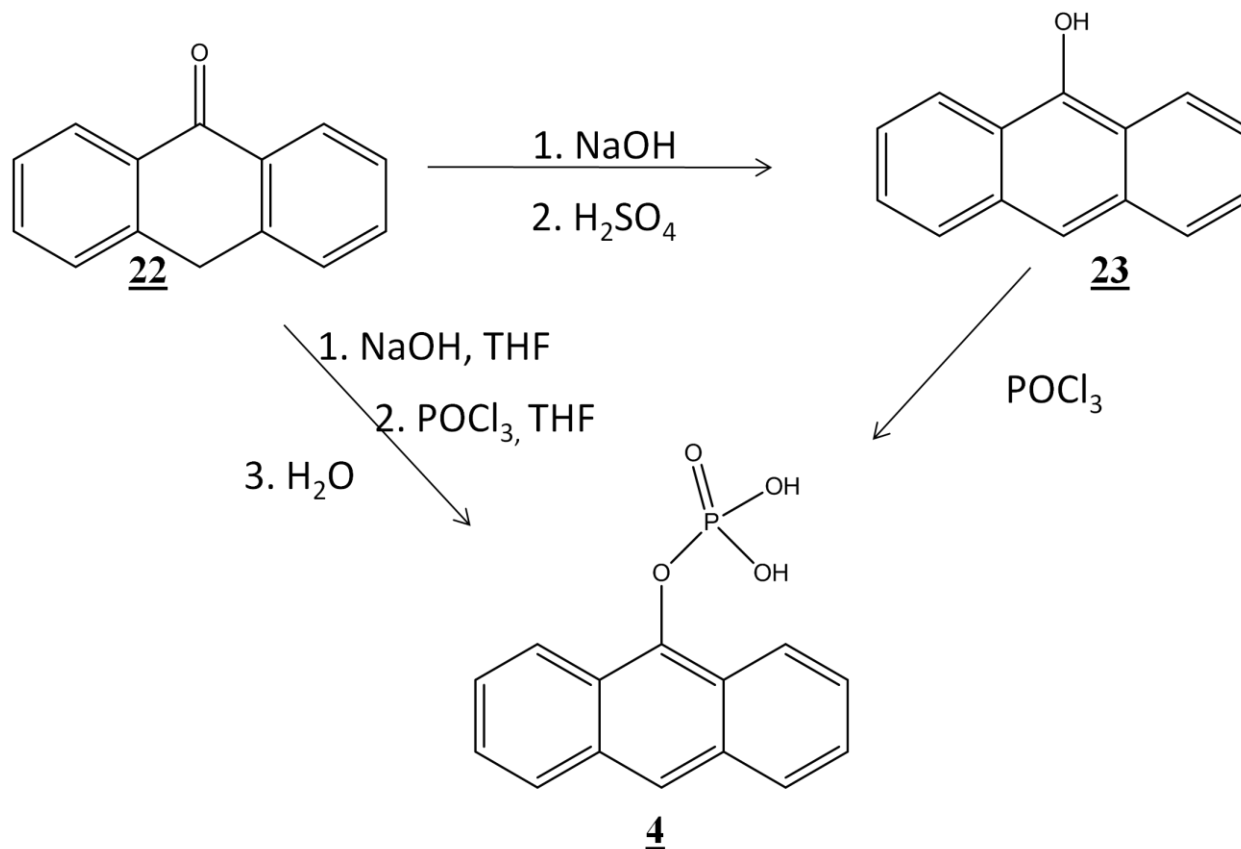
Scheme 9. Synthesis of methyl 2-({hydroxy[2-(1H-imidazol-2-yl)phenoxy]phosphoryl}oxy)benzoate (**21**)

Synthesis of Anthracene – Based Compounds for Model Systems 1 and 2

(Scheme 2)

Two synthetic routes (Scheme 10) were attempted for the synthesis of 9 – anthylphosphate (**4**). The first route involved the formation and isolation of the alcohol (**23**)¹⁴ and the later reaction of compound (**23**) with POCl₃ to form the phosphate. This route was flawed in that the alcohol rapidly oxidizes to the anthrone (starting material) under atmospheric conditions hence we were unable to isolate the alcohol. Facing this difficulty, we decided to attempt to synthesize the phosphate *in situ* without isolating the alcohol. After carrying out the reaction as presented in Scheme 10, we were only able to isolate the starting material. A

plausible explanation for this is that the conversion of the alcohol (**23**) to anthrone is faster than the reaction between the alcohol and POCl_3 .

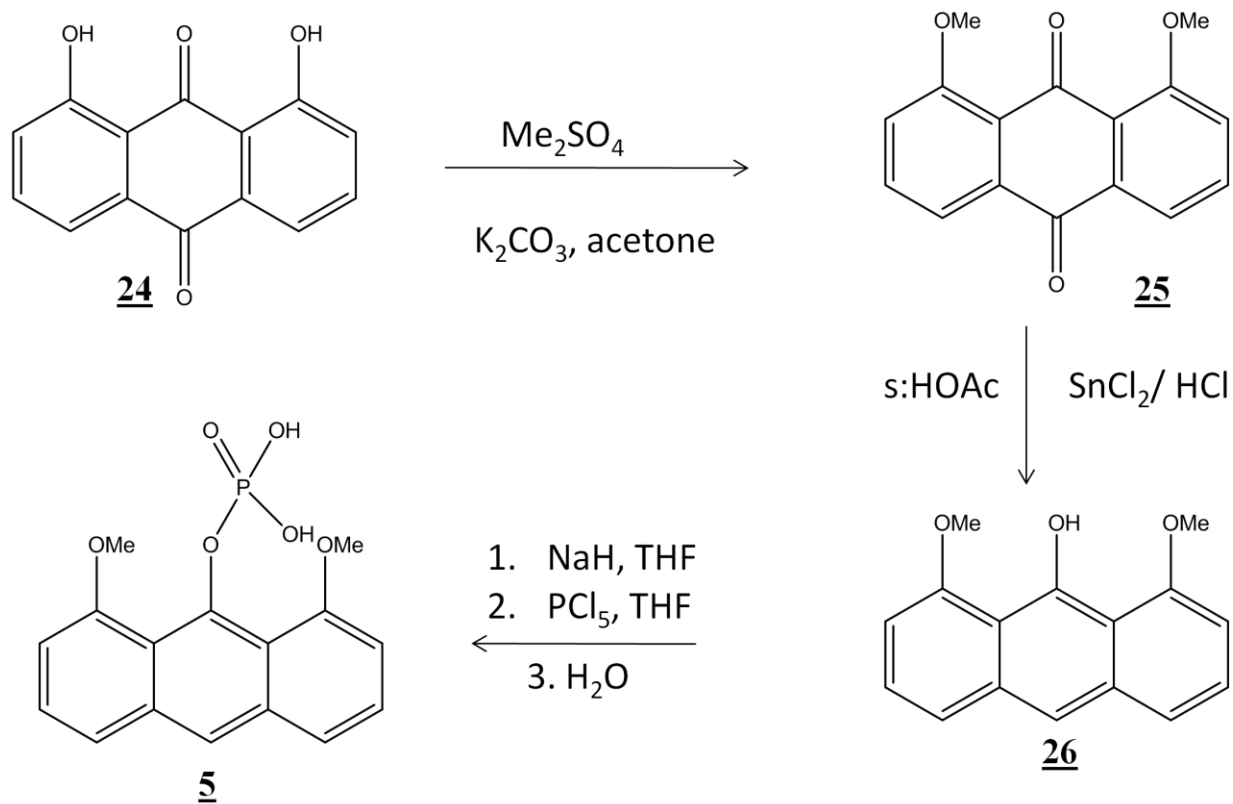


Scheme 10. Synthetic scheme used for 9 – anthylphosphate (**4**)

In an attempt to circumvent this problem, we modified our substrate from compound **4** to compound **5** (Scheme 2). To synthesize compound **5**, we needed to synthesize 1, 8 – dimethoxy- 9- hydroxyanthracene¹⁵ (**26**) as an important intermediate compound (Scheme 11).

Despite the similarities of compound **23** (Scheme 10) to compound **26**, the latter was synthesized successfully. This was possible because the alcohol

function formed was stabilized through intramolecular hydrogen bonds (Figure 7) as opposed to compound **23** that had no such stability factor. Compound **26** was isolated in good yield and purified by silica gel column chromatography.



Scheme 11. Synthetic scheme for model compound **5**

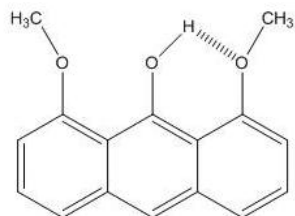
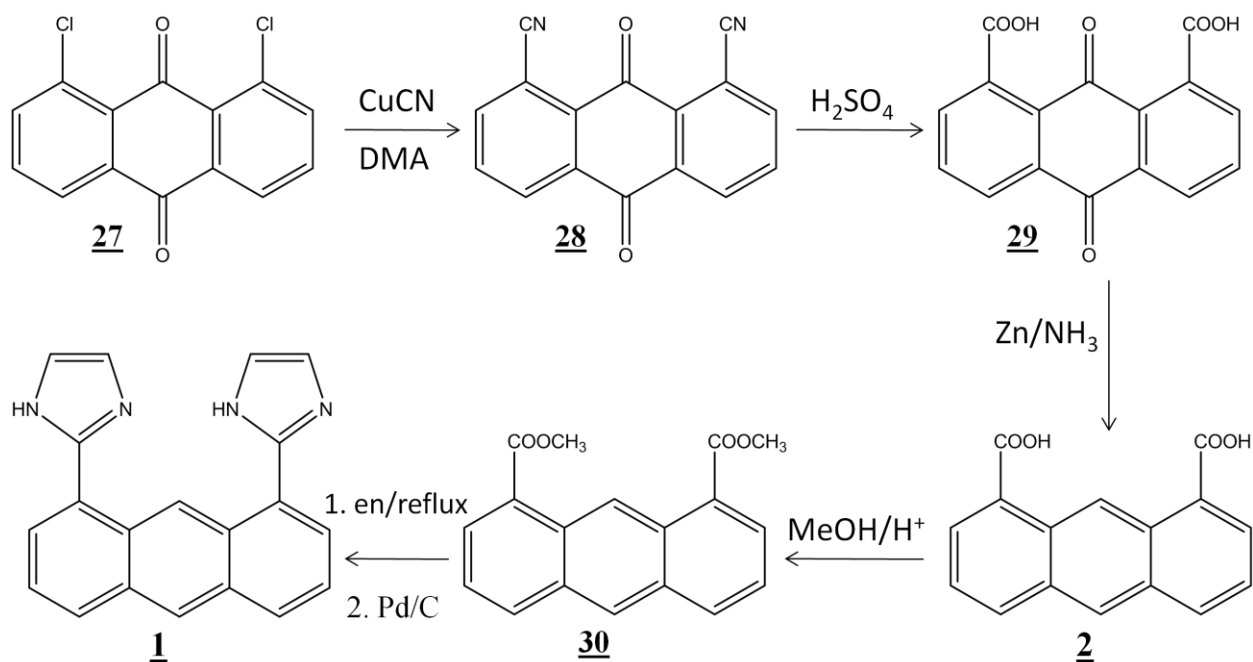


Figure 7. Hydrogen bond stabilization of 1, 8 – dimethoxy- 9- hydroxyanthracene
(26)

Scheme 10 was adopted to synthesize enzyme models **1** and **2**. These compounds are expected to recognize substrates such as compounds **4** and **5** by $\pi - \pi$ stacking. Such interaction would bring the catalytic groups in close proximity to the phosphate ester leading to catalysis either by general acid – base, nucleophilic, or a combination of both.



Scheme 12. Scheme for synthesis of 2,2'-anthracene-1,8-diylbis-1H-imidazole (**1**)

Anthracene-1, 8-dicarboxylic acid, compound **2** was synthesized according to literature¹⁶. It was isolated in good yield and its data consistent with literature. Synthesis of compound **1** from compound **2** as shown in Scheme 12 is presumably

involving an imidazoline intermediate through reaction with ethylenediamine. The reaction gave a product that we were unable to adequately characterize and confirm it as the imidazoline intermediate due to solubility problems. The synthesis was however continued based on the assumption that the product was the imidazoline. Oxidation over Pd/C gave a product that was insoluble in all common solvent, and was not pursued any further.

Conclusion

2,6-Di(1*H*-imidazol-2-yl)phenol has been successfully prepared by a two-step pathway involving reaction of ethylenediamine and dimethyl-3-carboxysalicylate followed by dehydrogenation (oxidation) of the imidazoline intermediate to the imidazole. The yield was low, about 16 %. Other dehydrogenation aromatization reactions^{12, 17,13} were attempted to improve the yield but were unsuccessful despite their reported successes for similar reactions. Another synthetic pathway involving the diamide intermediate was attempted but failed due to solubility problems. Similarly, all attempts to synthesize 2,2'-anthracene-1,8-diylbis-1*H*-imidazole were unsuccessful.

Throughout all attempted synthetic pathways to obtain the imidazole – based model compounds, the imidazoline intermediate could not be isolated and confirmed. Syntheses were continued based on the assumption that the imidazoline intermediates were formed. Oxidative aromatization reactions of

imidazolines^{10, 12, 17} have been reported in literature. We believe that in order to improve the yield of the desired imidazole products, major improvement in the synthesis of the imidazoline intermediates are needed.

Synthesis of methyl 2-({hydroxy[2-(1H-imidazol-2-yl)phenoxy]phosphoryl}oxy)benzoate was accomplish although in low yield. After initial attempts failed, it was realized that the order of addition of the reactants is very important and that converting the phenols to their salts makes synthesis easier. The phenolate salts are more basic and more nucleophilic than neutral phenols and thus more reactive with phosphorus pentachloride. We thus recommend the use of the salts of all phenols in the synthesis of methy- 2-({hydroxy[2-(1H-imidazol-2-yl)phenoxy]phosphoryl}oxy)benzoate.

The mono ester, 2-hydroxy-3-(methoxycarbonyl)benzoic acid was found to undergo auto hydrolysis to produce 2-hydroxyisophthalic acid when exposed to open atmosphere. A nucleophilic, general base assisted mechanism was proposed for the hydrolysis process. Further studies will follow to verify the proposed mechanism for this hydrolysis reaction.

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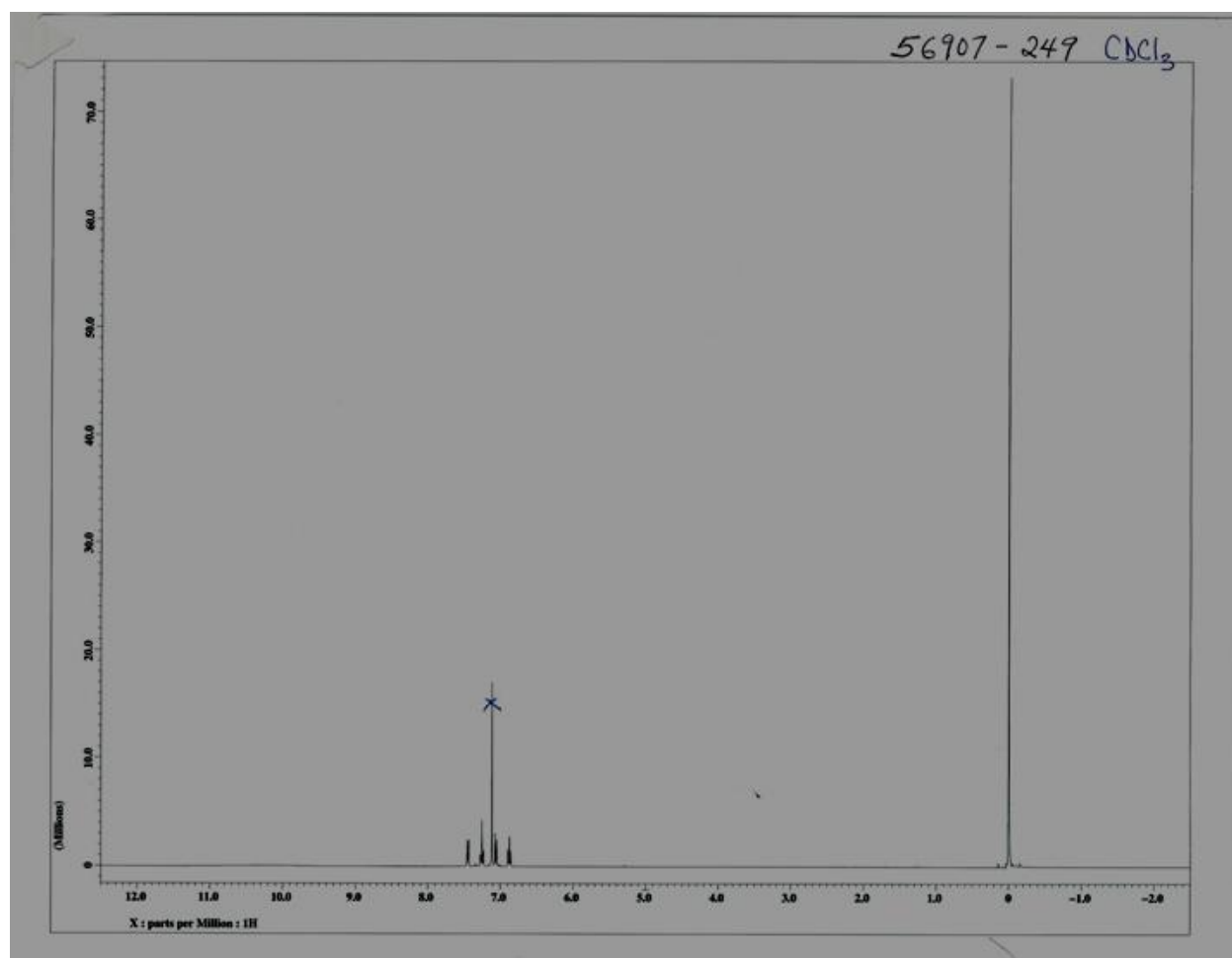
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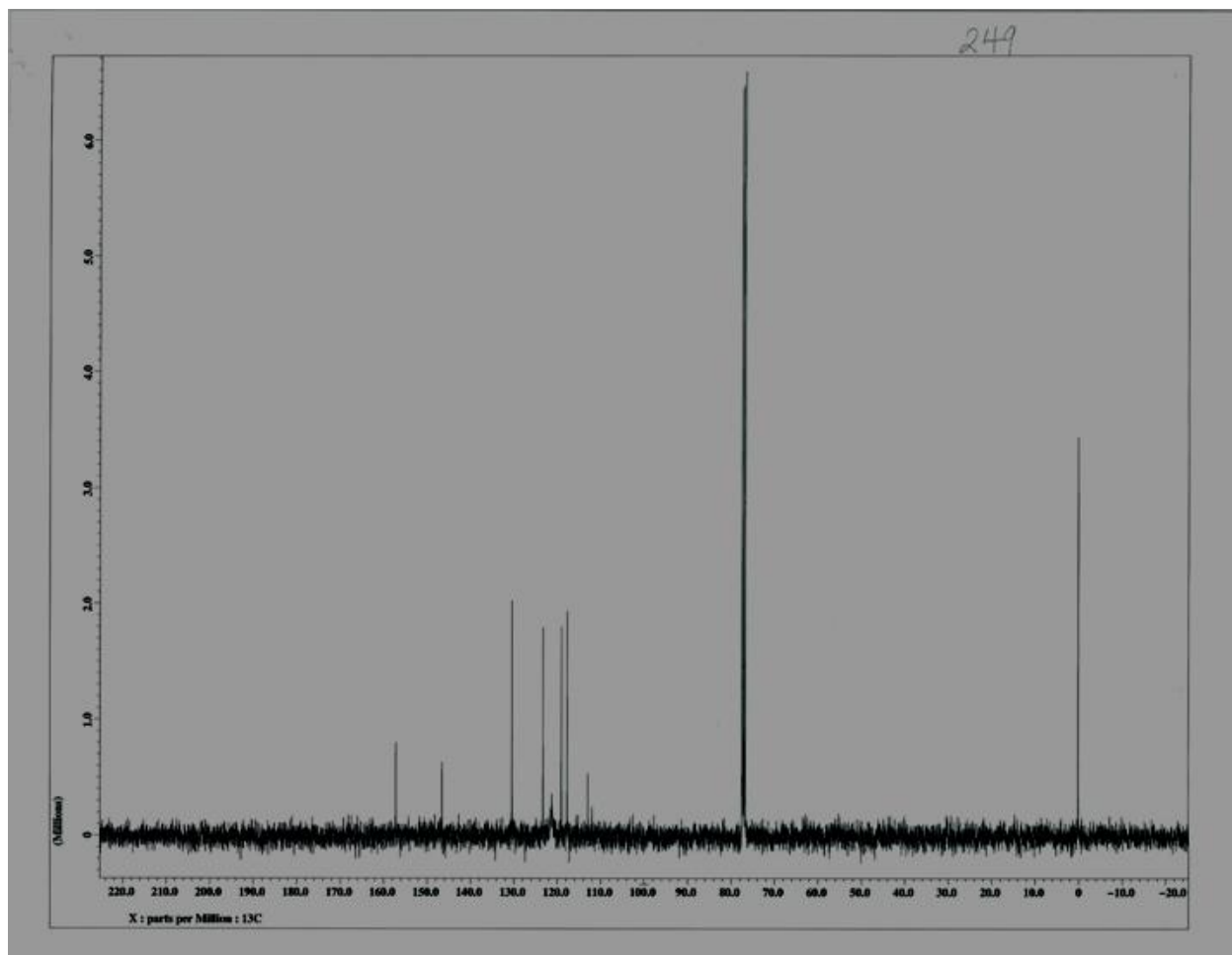
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APPENDICES

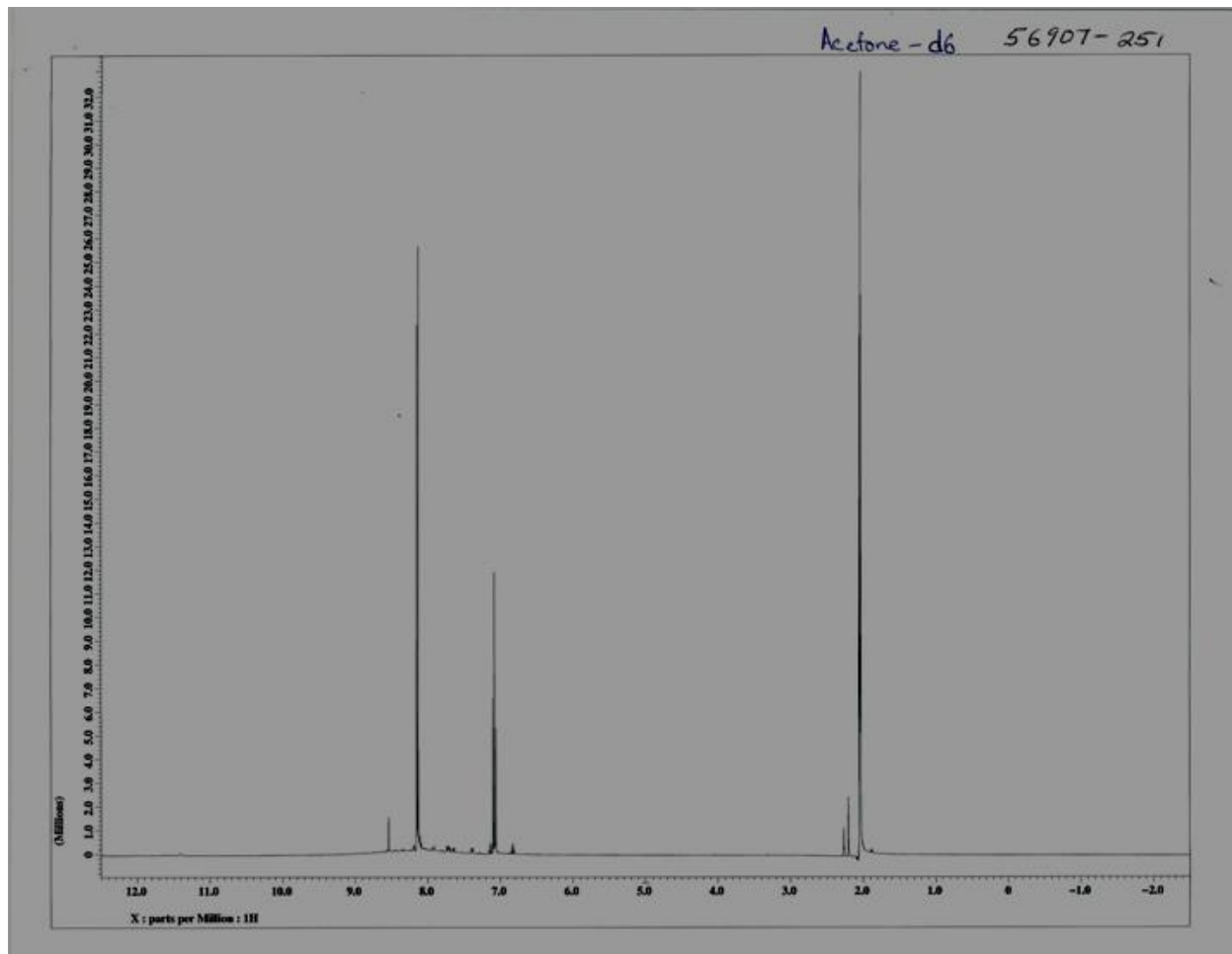
Appendix A ^1H NMR of 2 – (2'-hydroxyphenyl)imidazole in CDCl_3



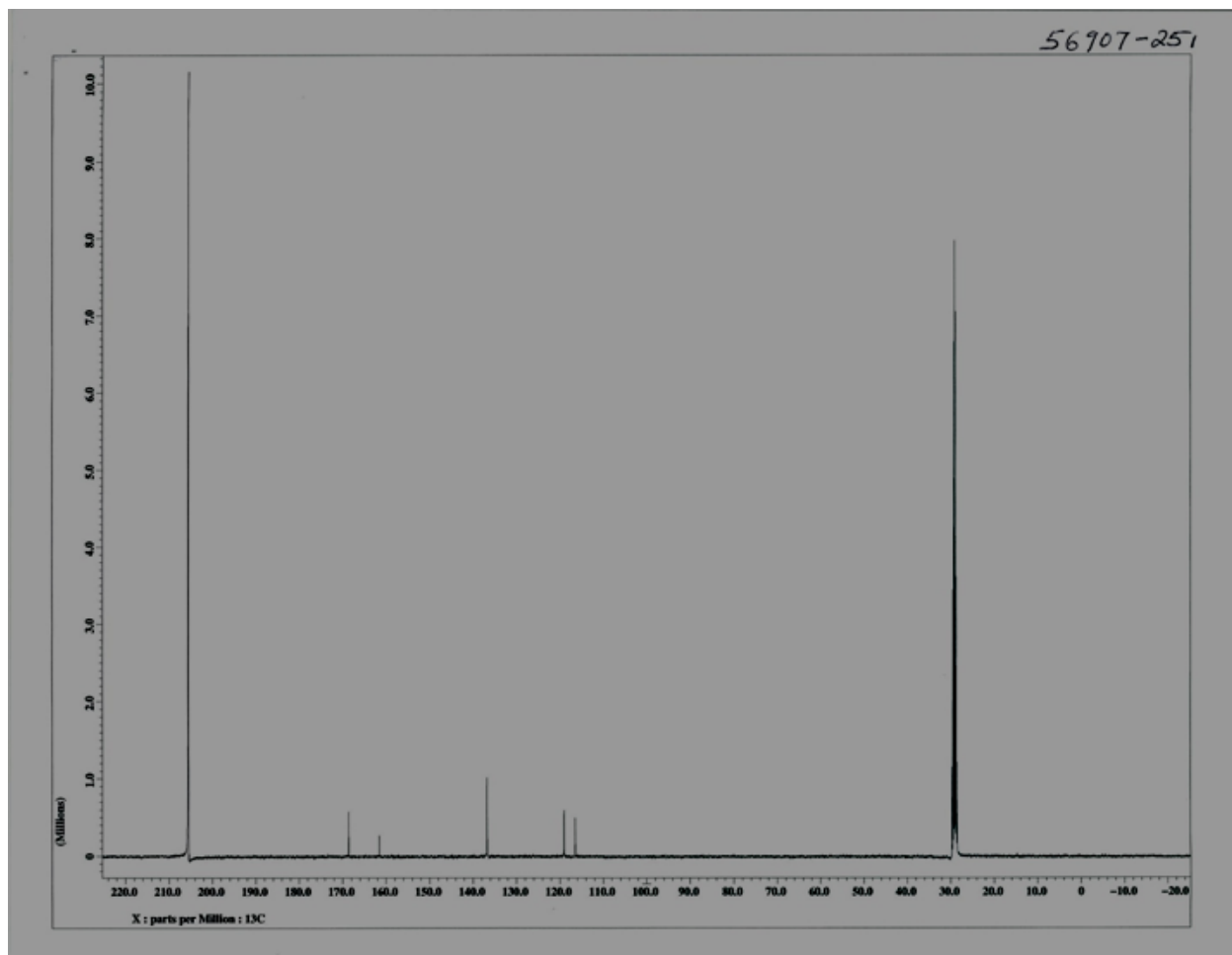
Appendix B ^{13}C NMR of 2 – (2'-hydroxyphenyl)imidazole in CDCl_3



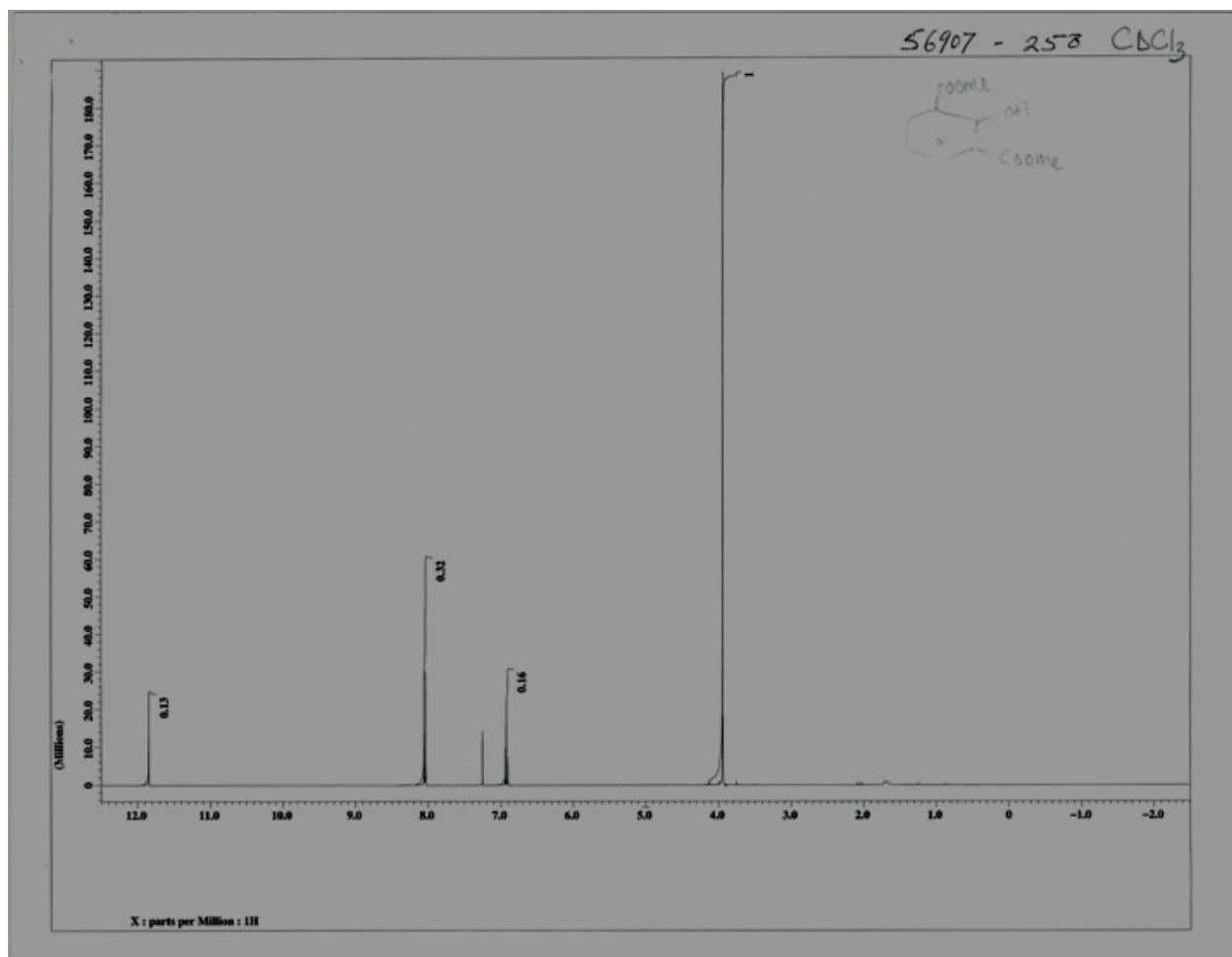
Appendix C ^1H NMR of 2-hydroxyisophthalic acid (**14**) in acetone - d_6



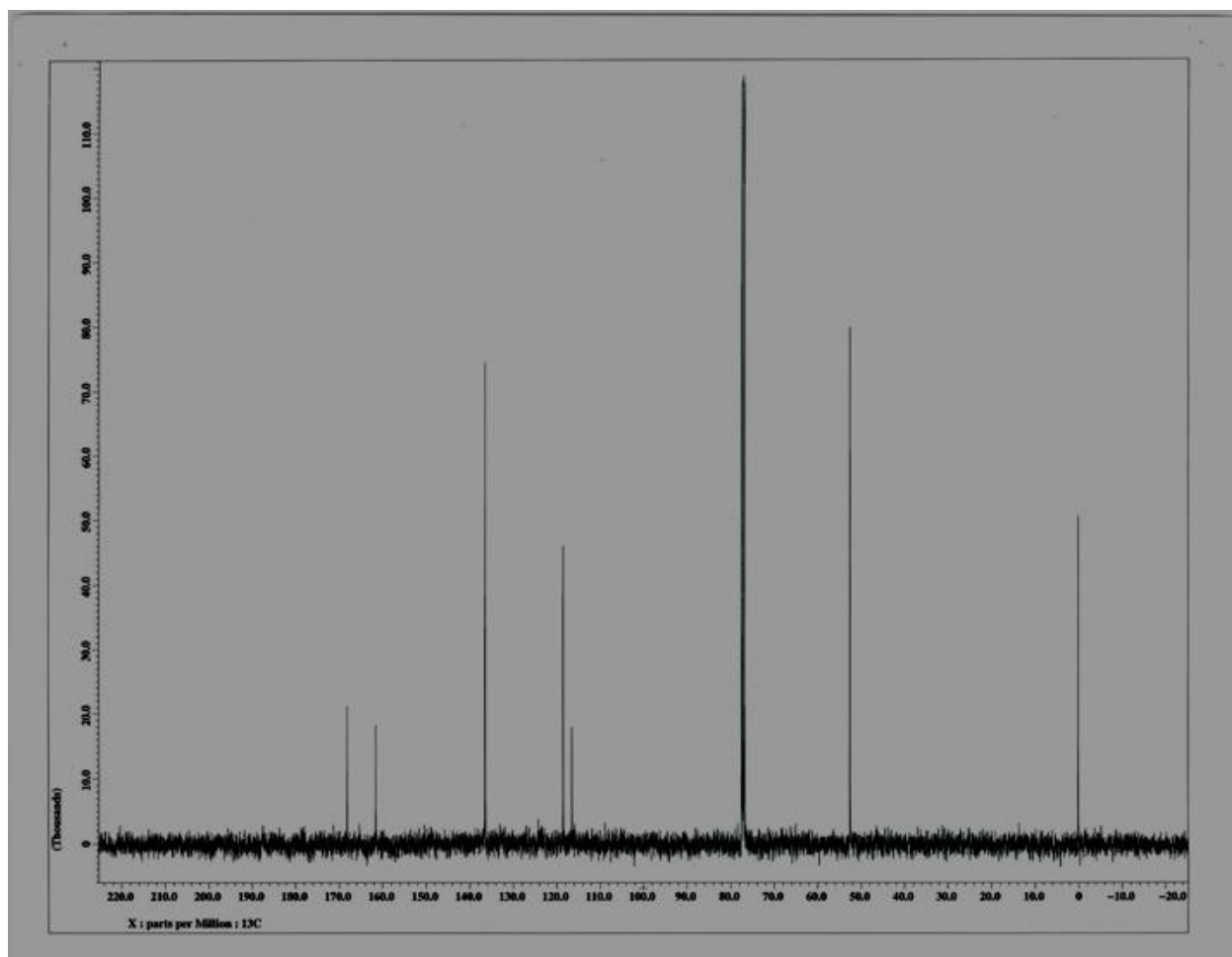
Appendix D ^{13}C NMR of 2 – hydroxyisophthalic acid (**14**) in acetone - d_6



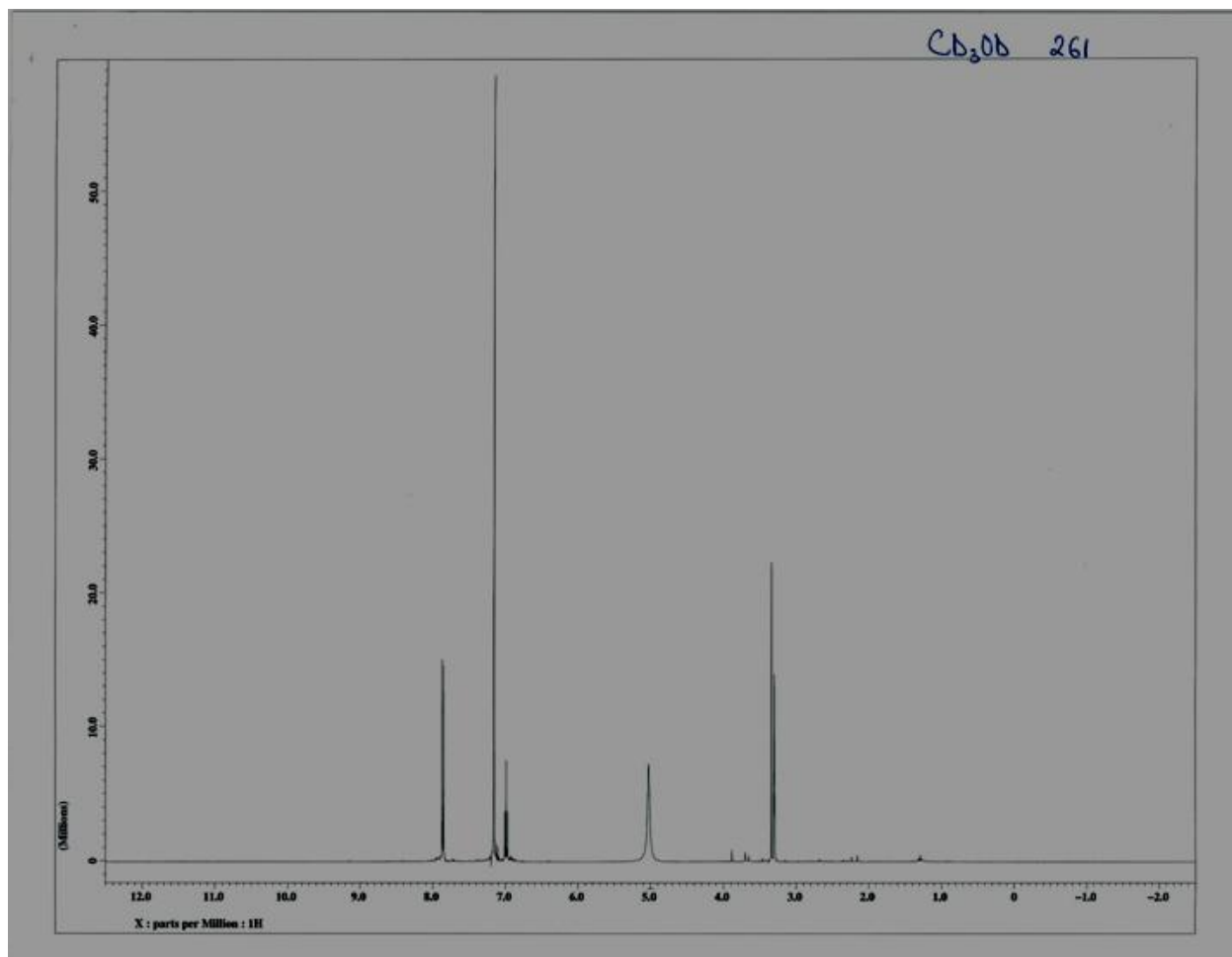
Appendix E ^1H NMR of dimethyl-3-carboxylsalicylate (**16**) in CDCl_3



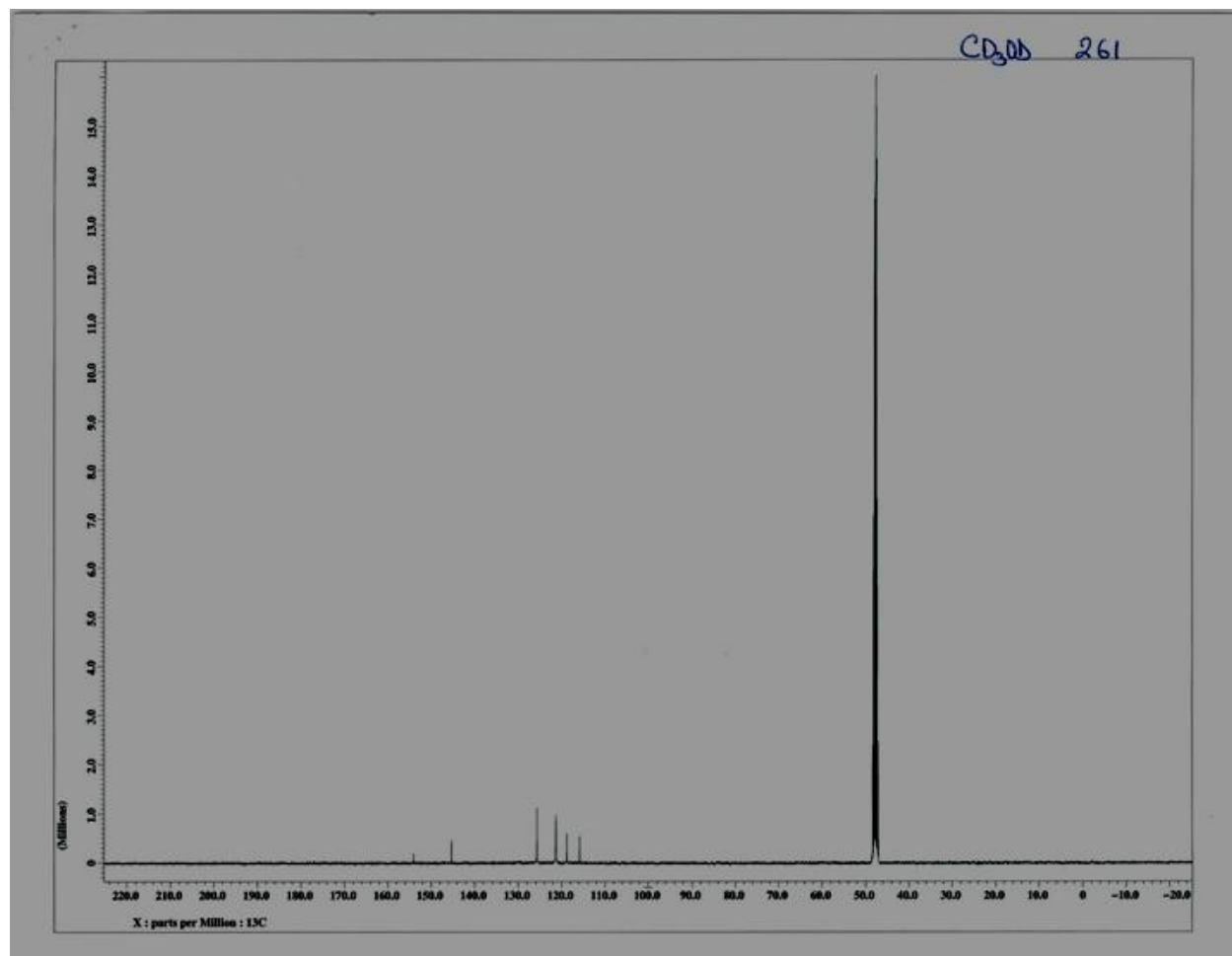
Appendix F ^{13}C NMR of dimethyl-3-carboxylsalicylate (**16**) in CDCl_3



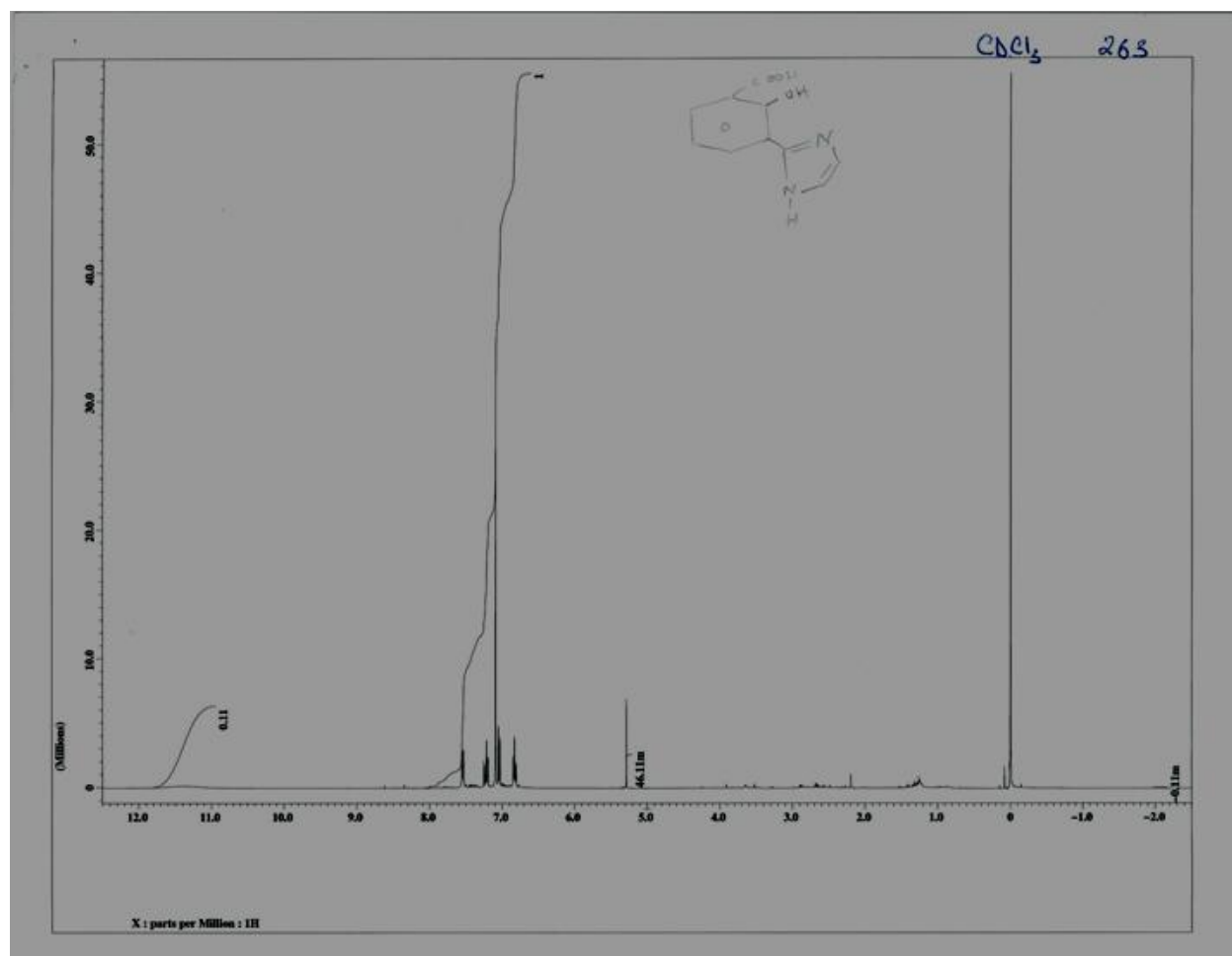
Appendix G ^1H NMR of 2,6-di(1*H*-imidazol-2-yl)phenol (**17**) in CD_3OD



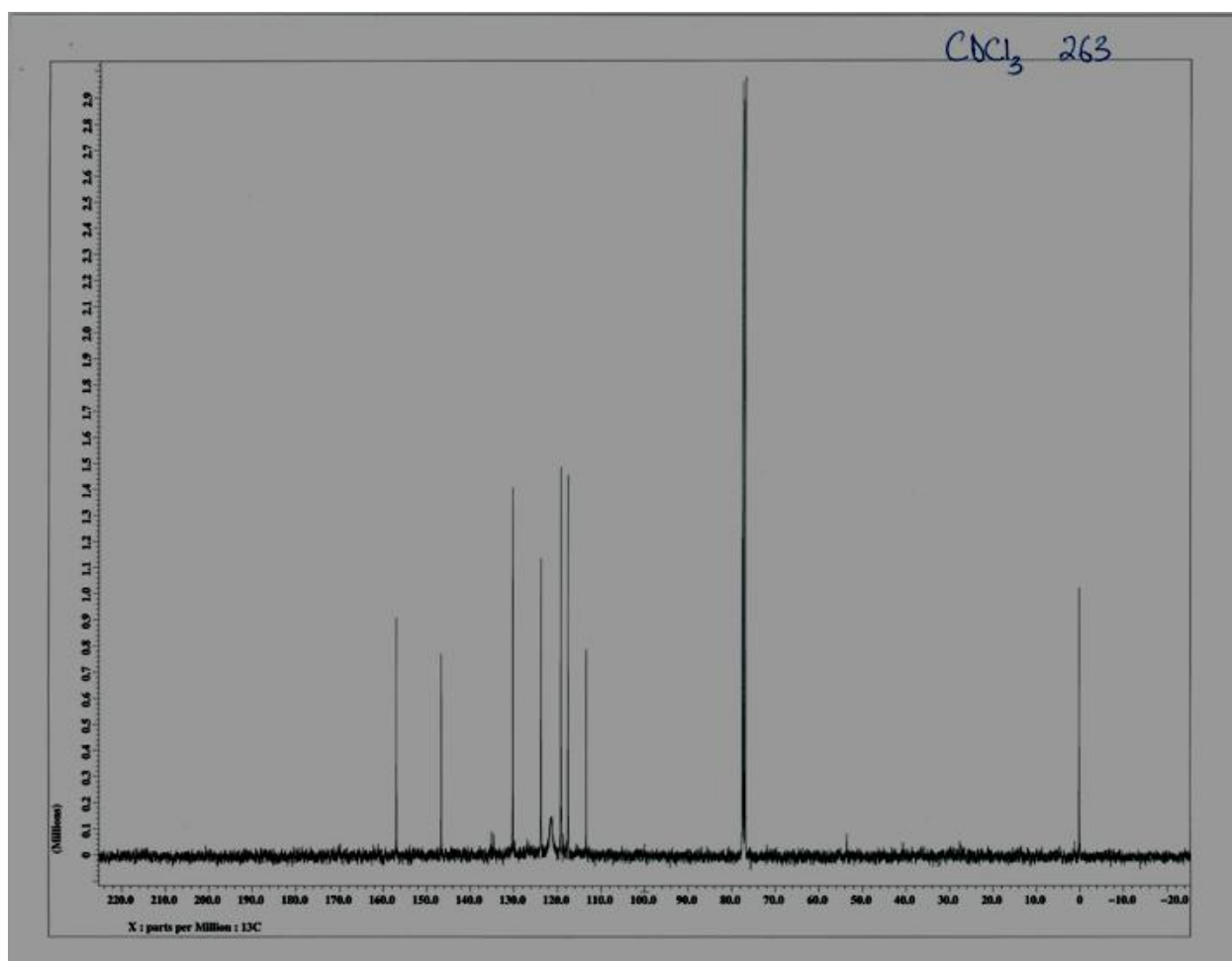
Appendix H ^{13}C NMR of 2,6-di(1*H*-imidazol-2-yl)phenol (**17**) in CD_3OD



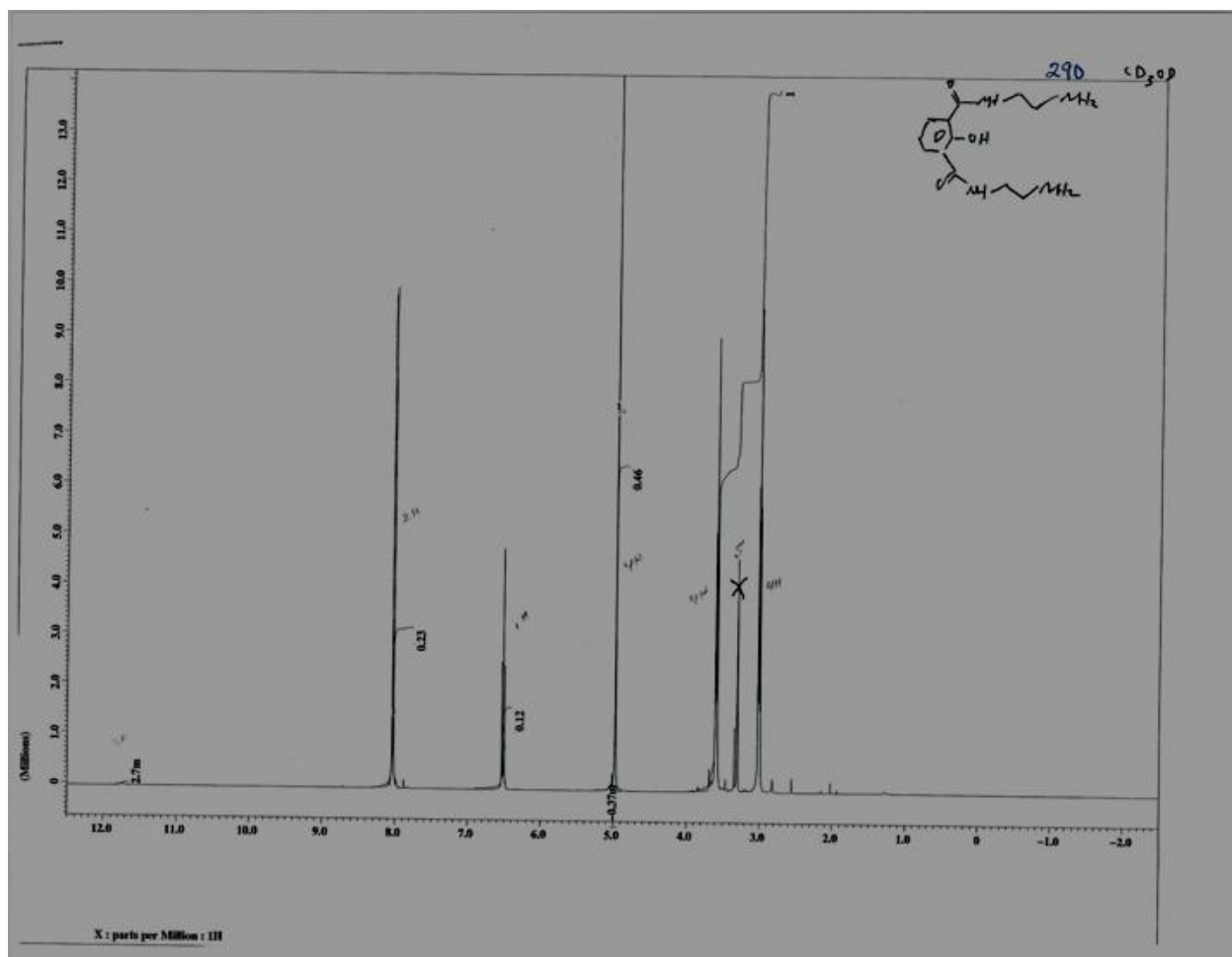
Appendix I ^1H NMR of 2-hydroxy-3-(1H-imidazol-2-yl)benzoic acid (**19**) in CDCl_3



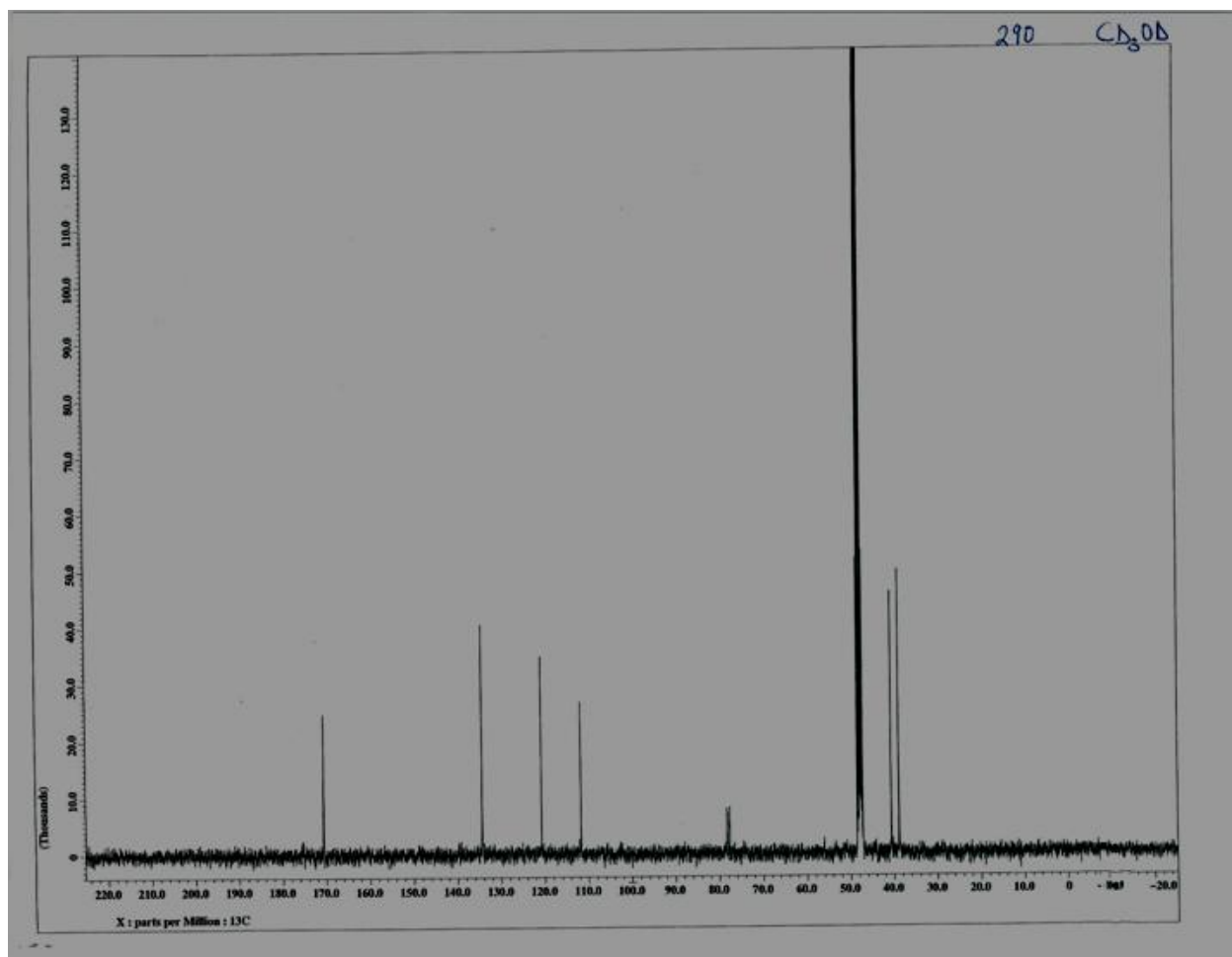
Appendix J ^{13}C NMR of 2-hydroxy-3-(1H-imidazol-2-yl)benzoic acid (**19**) in CDCl_3



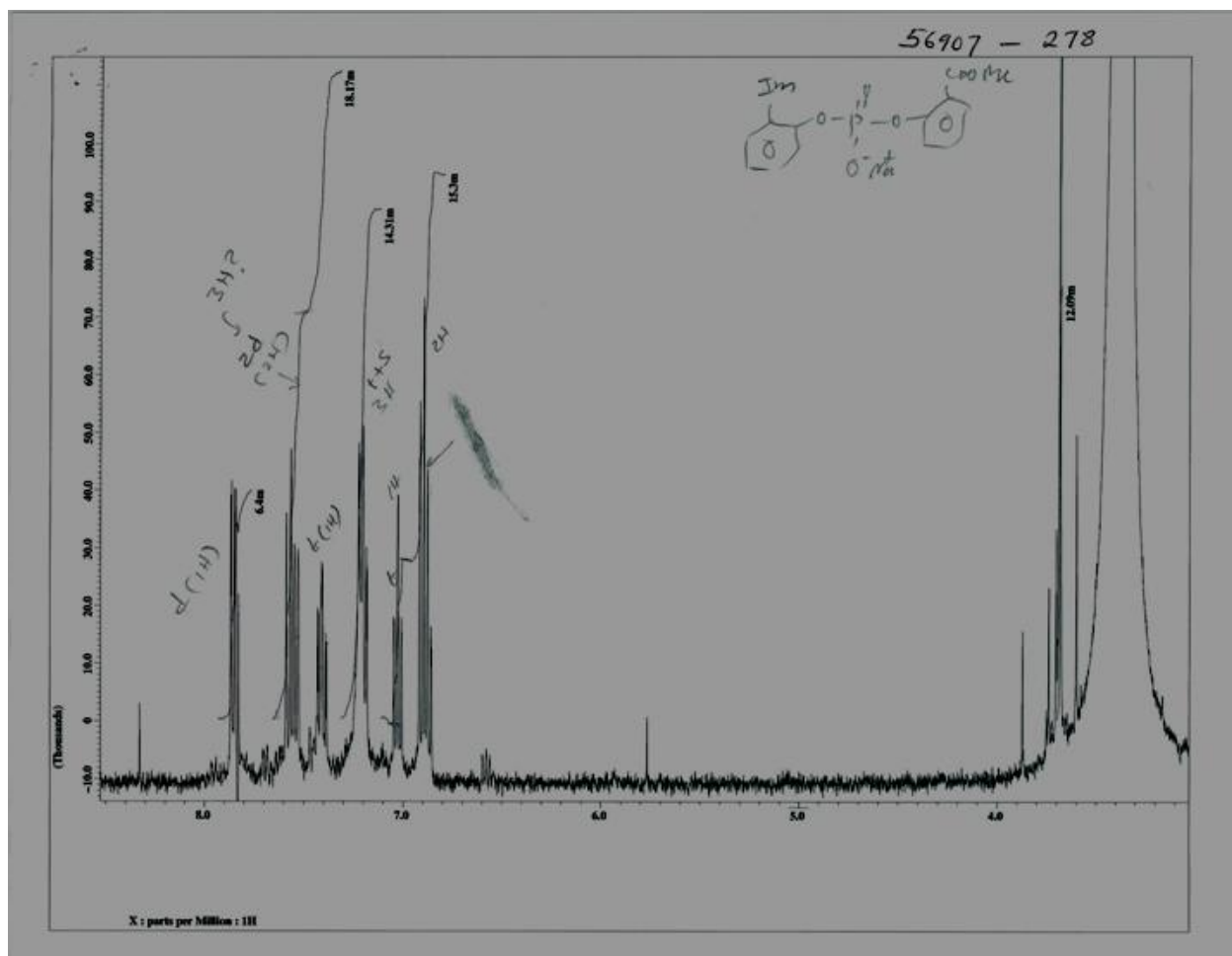
Appendix K ^1H NMR of Compound **18** in CD_3OD



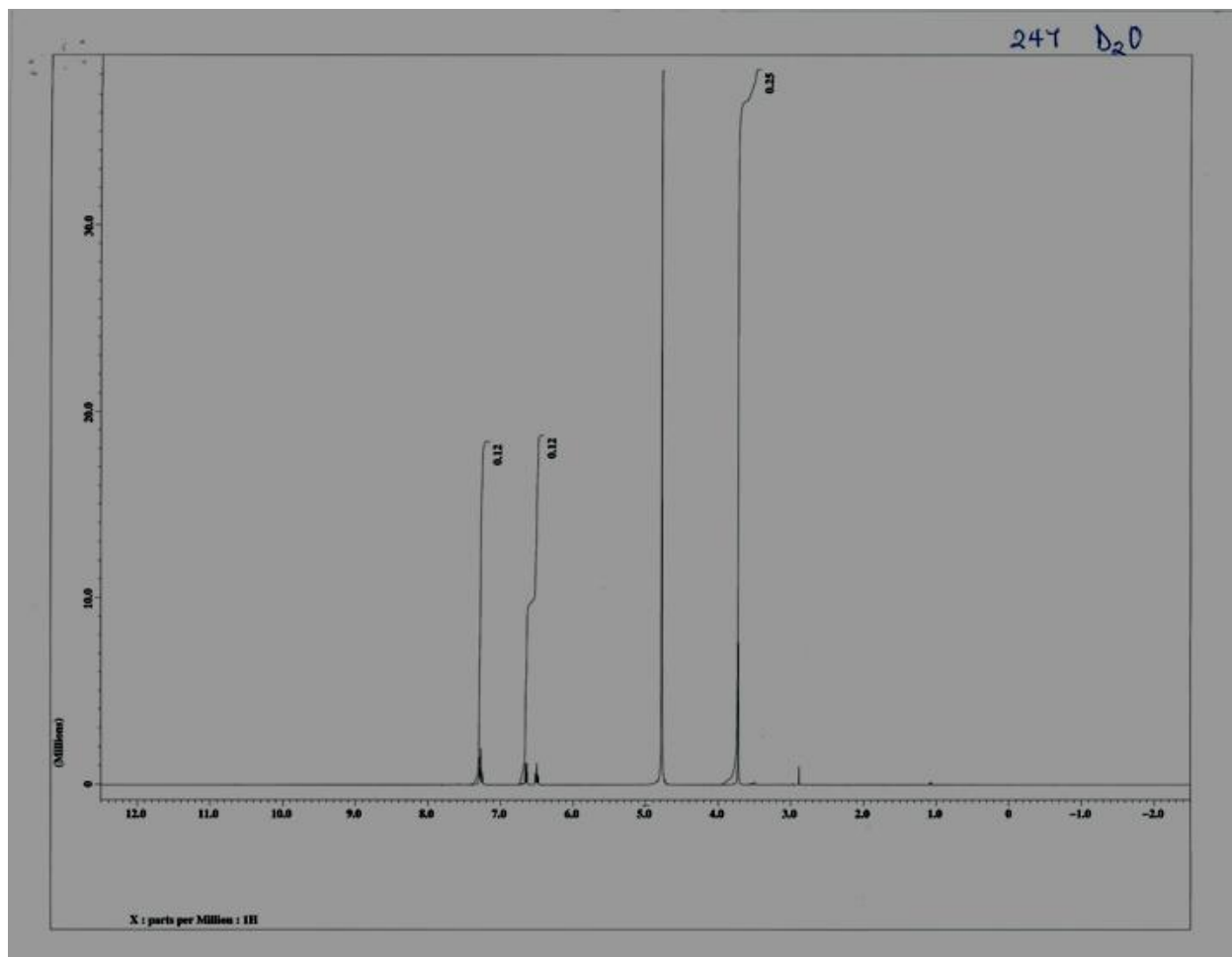
Appendix L ^{13}C NMR of Compound **18** in CD_3OD



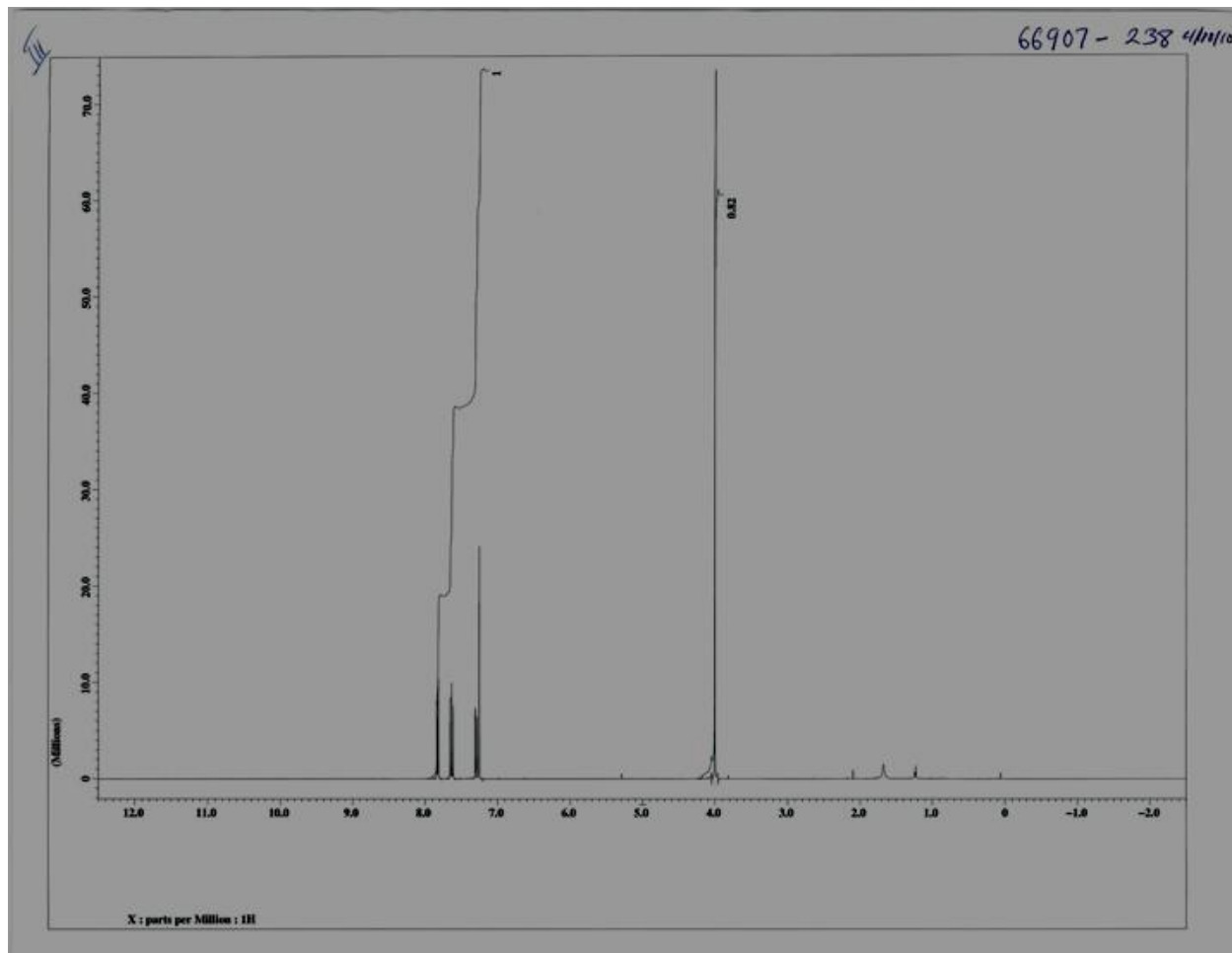
Appendix M ^1H NMR of methyl 2-((hydroxy[2-(1H-imidazol-2-yl)phenoxy]phosphoryl)oxy)benzoate (**21**) in DMSO- d_3



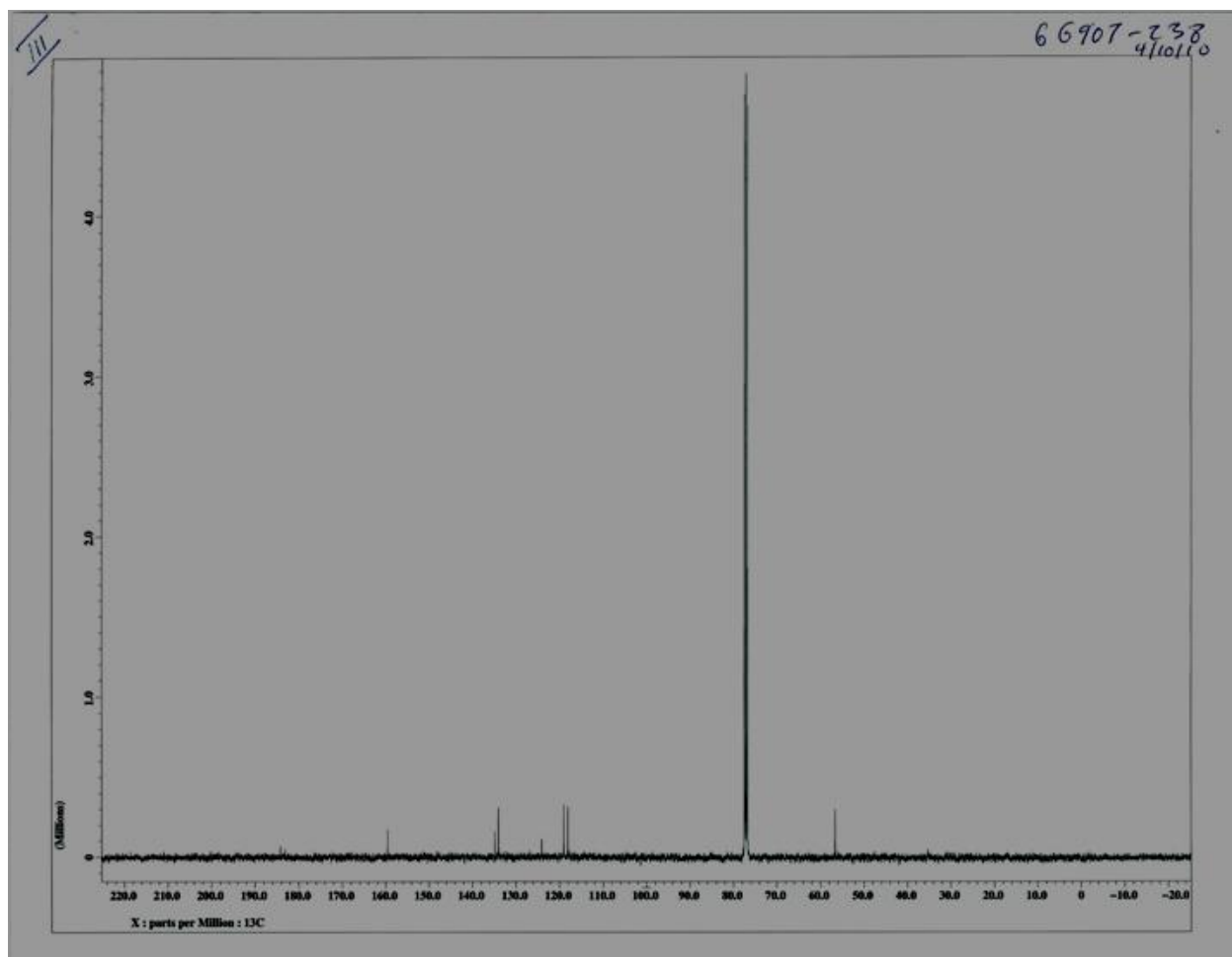
Appendix N ^1H NMR of 2-(2'-hydroxyphenyl) -1H-imidazoline in D_2O



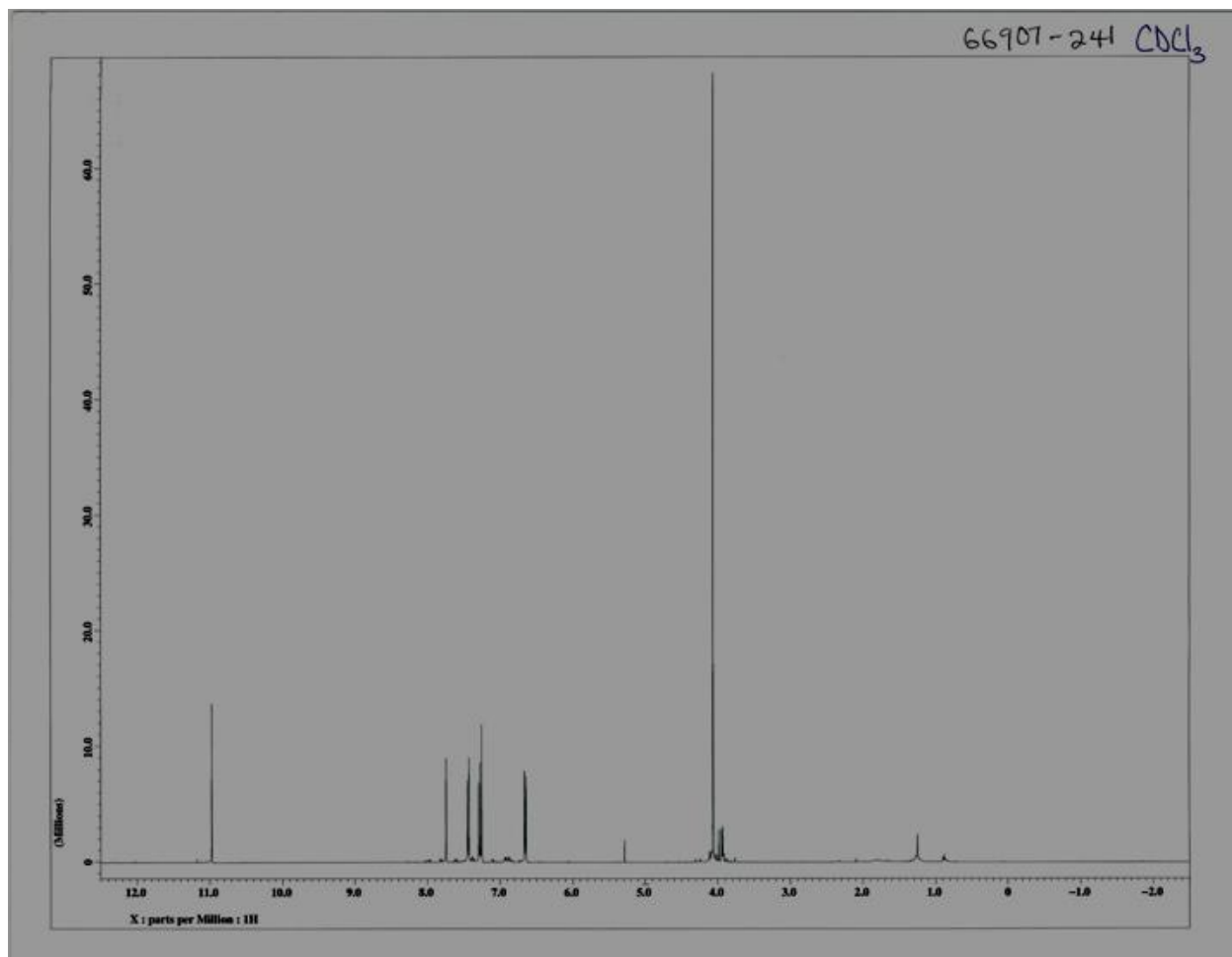
Appendix O ^1H NMR of 1,8-dimethoxyanthracedione (**25**) in CDCl_3



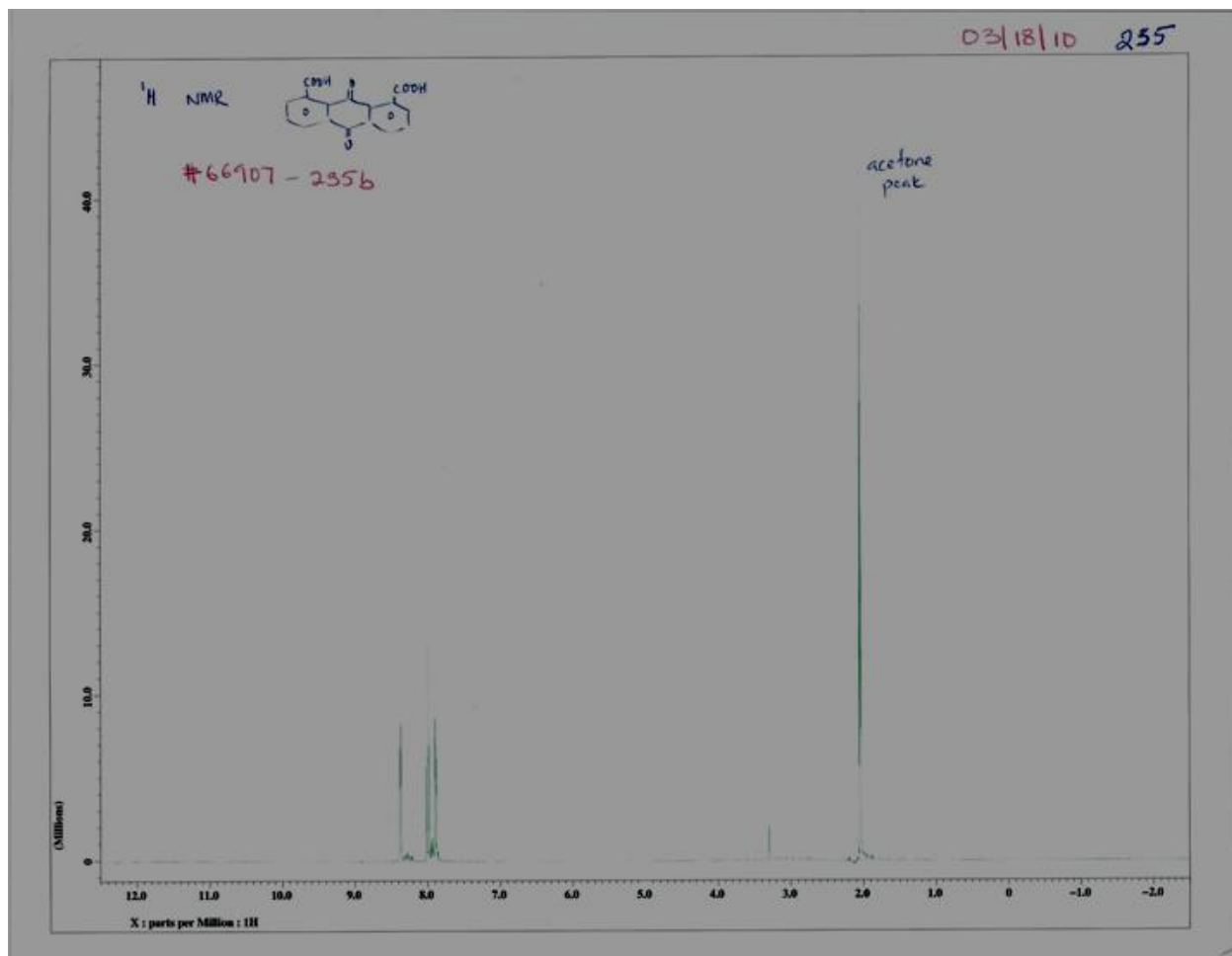
Appendix P ^{13}C NMR of 1,8-dimethoxyanthracedione (**25**) in CDCl_3



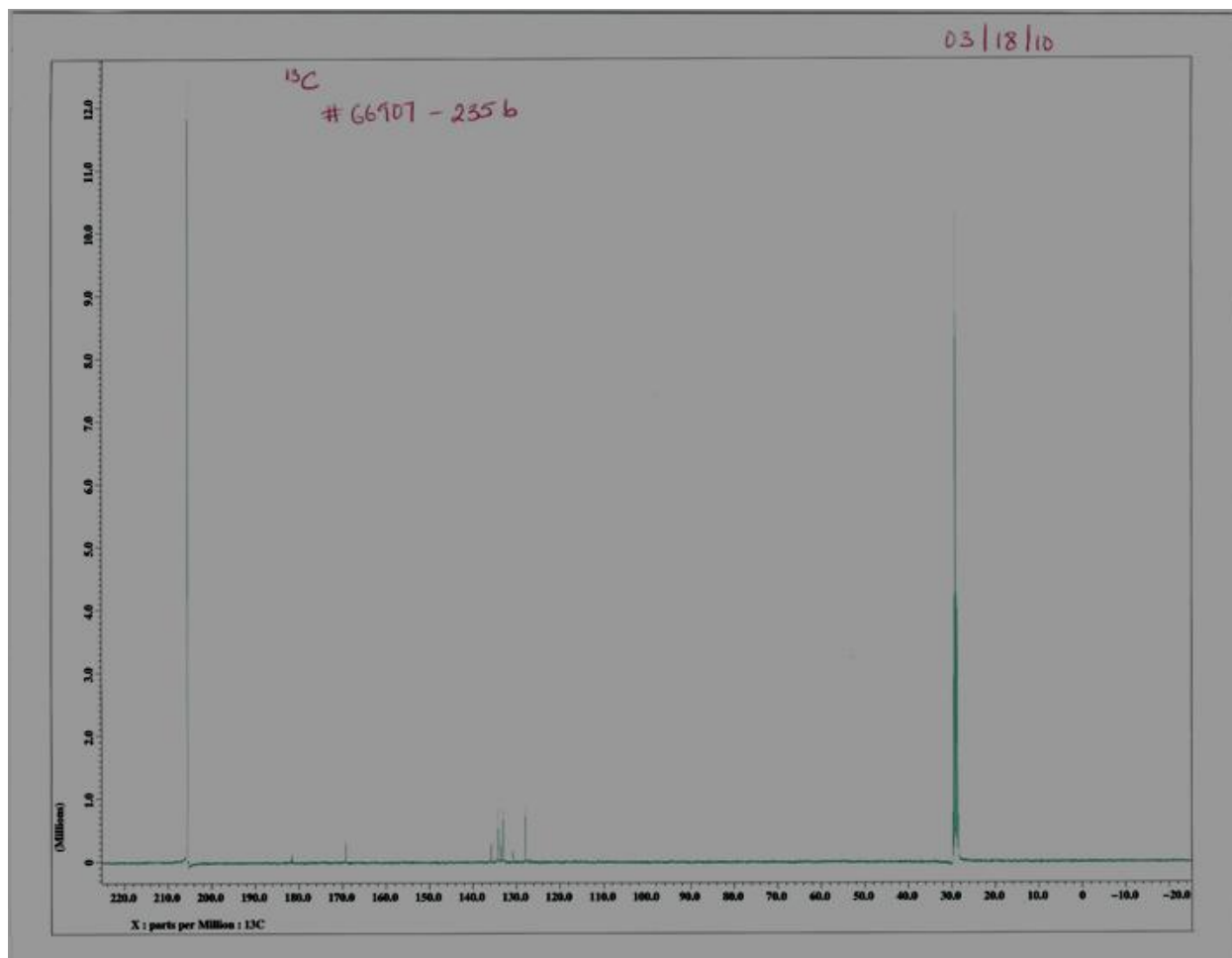
Appendix Q ^1H NMR of 1, 8 – dimethoxy- 9- hydroxyanthracene (**26**) in CDCl_3



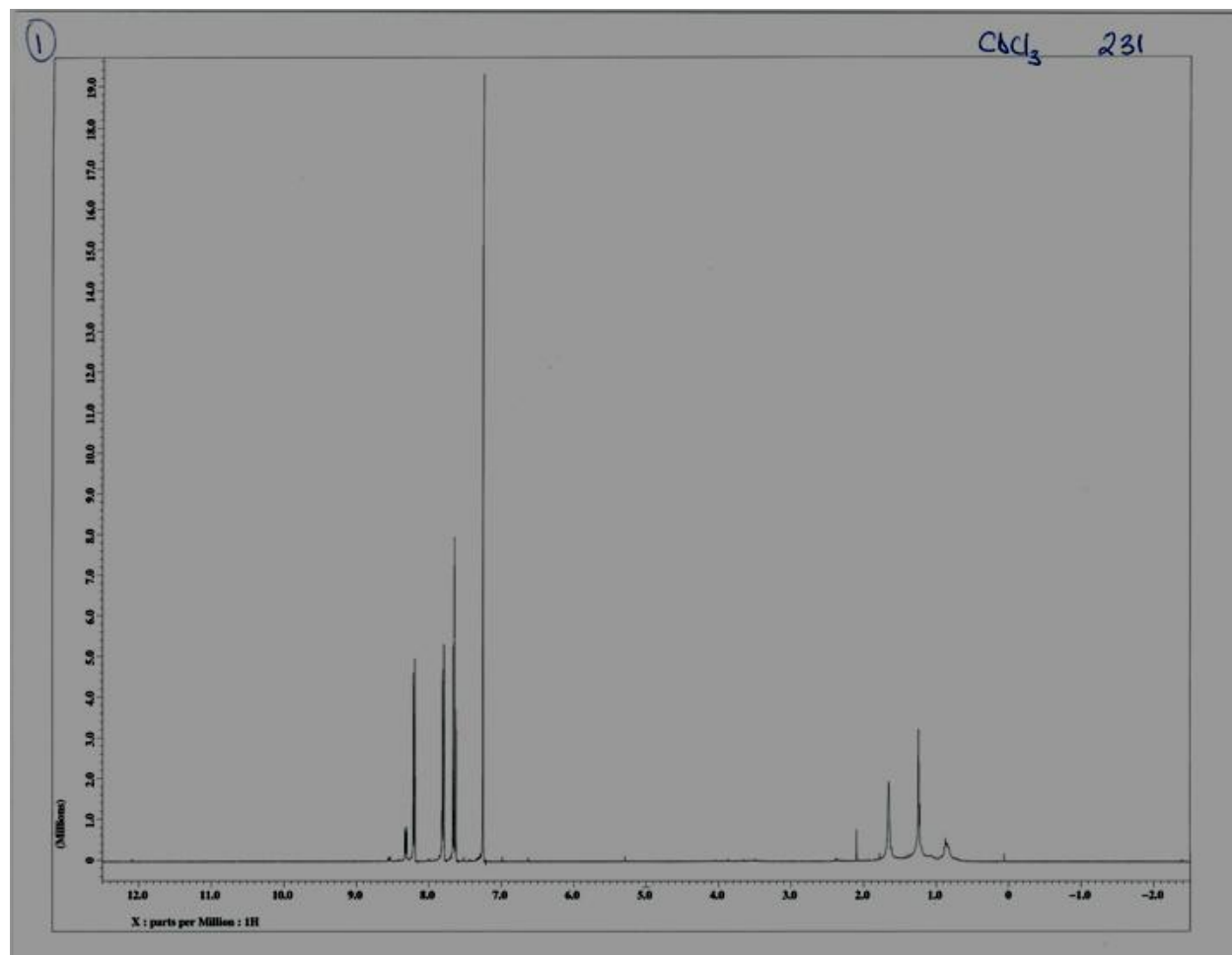
Appendix R ^1H NMR of anthraquinone-1,8- dicarboxylic acid (**29**) in acetone- d_6



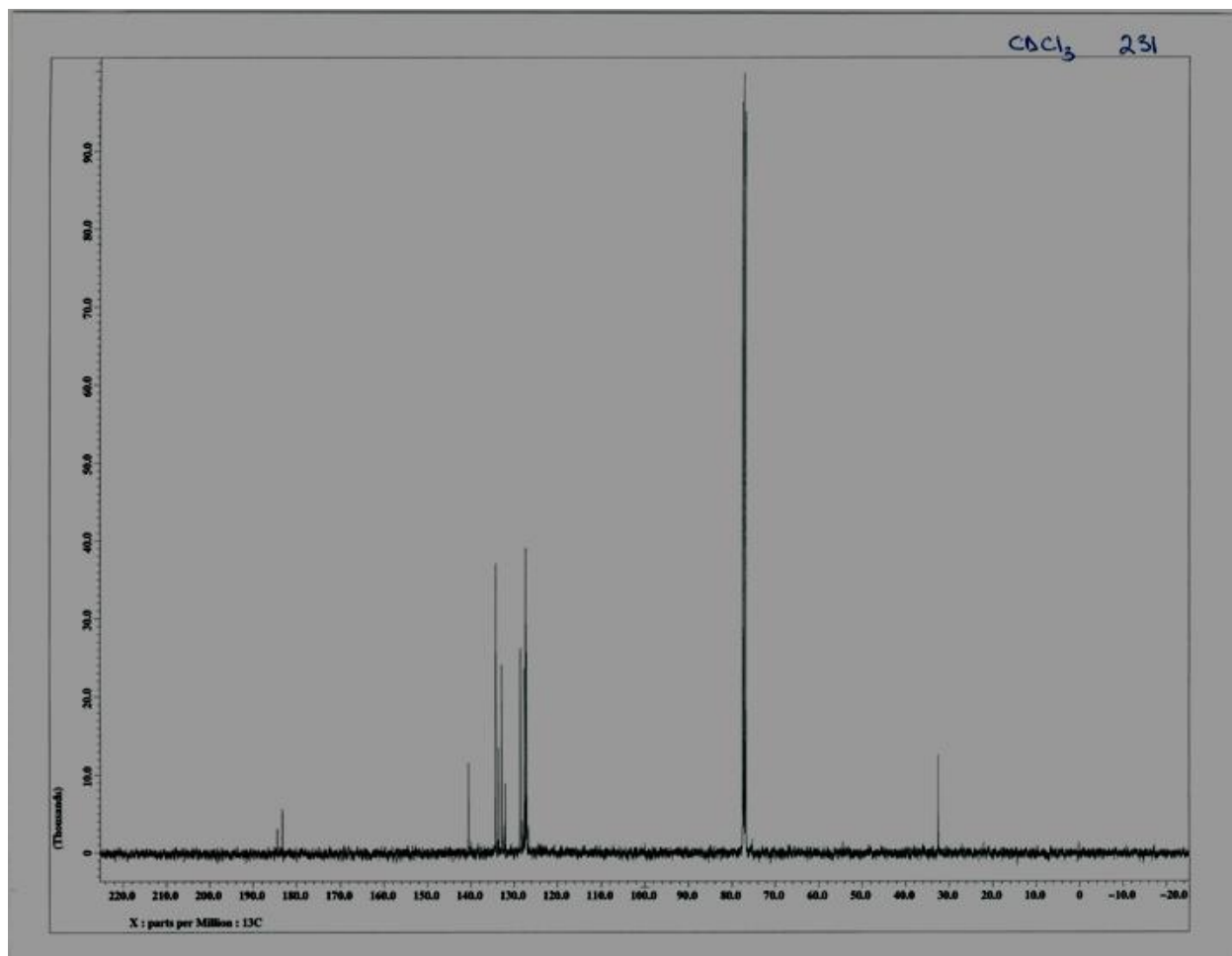
Appendix S ^{13}C NMR of anthraquinone-1,8- dicarboxylic acid (**29**) in acetone- d_6



Appendix T ^1H NMR of product obtained from converting anthrone to 9-anthranol
(23) in CDCl_3



Appendix U ^{13}C NMR of product obtained from converting anthrone to 9-anthranol (**23**) in CDCl_3



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