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Synthesis and Study of Hydroxytyrosol Derivatives

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Synthesis and Study of Hydroxytyrosol Derivatives

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by

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ABSTRACT

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Hydroxytyrosol is one of the most powerful known antioxidants. It is a naturally occurring polyphenol, most commonly produced in olive trees, (*Olea europaea*). The remarkable antioxidant and pharmacological properties of hydroxytyrosol has made it an outstanding compound in the polyphenol family and of great interest to many researchers. Hydroxytyrosol can scavenge free radicals produced during cellular oxidative stress and helps to protect the integrity of cells in living systems. Despite its numerous biological and pharmacological uses, it is found in very low concentration in olive oil, this limits its availability for biomedical applications. This work reports a novel and effective method for synthesizing hydroxytyrosol from the readily available precursor catechol. The cellular uptake of hydroxytyrosol is slow due to its high hydrophilicity. Therefore, this research aimed at synthesizing less hydrophilic derivatives of hydroxytyrosol by introducing some selected hydrophobic groups (such as alkyl, acyl, …) to its molecular skeleton.

DEDICATION

This work is dedicated to the Nyarambi family for their prayers and support.

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TABLE OF CONTENTS

LIST OF SCHEMES

Scheme Page

LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
DCM	Dichloromethane
DPPH	2,2-diphenyl-1-picryldrazyl
FRAP	Ferric reducing antioxidant power
Hr	Hour
HT	Hydroxytyrosol
LDL	Low-density lipoproteins
Min	Minutes
Mmol	Millimoles
mL	Milliliter
NMR	Nuclear Magnetic Resonance
ROS	Reactive oxygen species
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography

CHAPTER 1

INTRODUCTION

The relationship between lifestyle and metabolic diseases has been closely related to the production of free radicals and oxidative stress.[1] Several biological processes in our bodies generate free radicals.[2] However, when their production exceeds the body's natural antioxidant production, it leads to oxidative stress.[3] Some reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$) and some reactive nitrogen species such as nitric oxide (NO) have been identified to be the leading cause of oxidative stress.[4,5] Oxidative stress caused by these free radicals have been implicated in protein, lipid and DNA damage.[6,7,8] Damage of these biomolecules result in aging, cell death, neurodegenerative and cardiovascular diseases.[9]

Antioxidants are chemical substances that inhibit oxidation and protect the body and other biomolecules from damages caused by free radicals.[2,10] Plants and animals use antioxidants as one of the major lines of defense against the harmful effects of free radicals thereby reducing or preventing oxidative stress.[10] Several researchers have carried out studies to understand the mechanism of action and properties [2, 4,11]

Hydroxytyrosol is one of the most powerful antioxidants currently known worldwide.[12, 13] It is a naturally occurring polyphenol produced by the olive tree (*Olea europaea*).[14] The remarkable antioxidant and pharmacological properties of hydroxytyrosol have made it an outstanding compound in the polyphenol family.[15] Even though hydroxytyrosol has many biological properties its concentration is very low reducing its biomedical application. Also, the bioavailability of the less amount of hydroxytyrosol in the olive oil is compromised due to its high hydrophilicity. In view of this, novel methods for synthesizing pure derivatives of hydroxytyrosol from a

commercially less expensive precursor; catechol has become very attractive. The principal aim of this research is to synthesize less hydrophilic derivatives of hydroxytyrosol by introducing some hydrophobic groups like; alkyl, acyl, halogens to the molecular skeleton of hydroxytyrosol.

Free Radicals

Uncharged molecules, ions or atoms with unpaired valence electrons generated from various chemical reactions are short-lived and extremely reactive if not complexed with metals.[16] They are unstable and very reactive towards compounds or stable molecules.[5] These species are known as free radicals.[5] Free radicals, for example; reactive oxygen species (ROS) abstract electrons from stable biomolecules and produce free radicals.[17,7] This subsequently results in a cascade of reactions which will possibly cause destructions to a lot of biologically relevant molecules and structures including; carbohydrates, nucleic acids, proteins, cell membrane or living cells.[16,18]

Free radicals stem from either internal or external sources. Among the internal source include metabolic processes occurring in living things. For instance, the immune system produces ROS in the process of nullifying pathogens; the respiratory chain from the metabolic pathway also spill out a myriad of ROS in the body.[19,20] Though they are produced during metabolism and exposure to some type of radiations, their production increases drastically during disease conditions.[21] Prostaglandin synthesis and cytochrome P450 systems also form a couple of ROS in the body.[21] The external source of radicals encompasses exposure to X-ray, air pollutants, chemicals from industries, and cigarette smoking among others.[22,23] It is also important to note that ionizing and non-enzymatic reactions of organic complexes and oxygen form ROS.[17] Some reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), hydroxyl

radical (HO•) and some reactive nitrogen species (RNS) such as nitric oxide (NO) are the major cause of oxidative stress.[22,23,24]

Oxidative Stress

Oxidative stress is used to describe a state in which harmful ROS and derivatives produced in biological systems oxidatively damages molecular entities including lipids, carbohydrates and proteins.[23] Homeostasis in living cells is compromised in such cases because there is a disproportion between the generation of free radicals and the body's capacity to use antioxidants to scavenge or neutralize these radicals.[26] Consequently, the structure and role of certain molecular species are altered.[25,27] There is also an interruption of the respiratory chain which happens to be a key metabolic pathway in the oxidative phosphorylation process. All these factors enhance the creation of abundant ROS.[23]

Oxidative Stress - Related Disease

A host of diseases are hastened and progressed by an excessive amount of free radicals in the body.[19] Among such diseases include cardiovascular disease, oxidative damage to proteins and nucleic acids, cancer, diabetes mellitus, Parkinson's disease, stroke, atherosclerosis, hypertension, muscular dystrophy, aging process and many others.[22,23]

Cardiovascular diseases are known to be liable for many death cases reported annually.[17] Oxidative events are thought to affect the heart tremendously. This is because polyunsaturated fatty acids, which happen to be a major constituent of low-density lipoproteins (LDL) in the blood normally undergo peroxidation in the presence of an excess ROS.[16] The highly reactive products formed after that has been linked to atherosclerosis.[26] Excessive amounts of oxidized lipids tend to accumulate unusually in the artery walls of the body. The plaque formed also plays a crucial role in stroke.[23]

Carcinogenesis is thought to stem from RNS, ROS and their metabolites produced from biochemical reactions.[25, 29] Countless researchers have explicitly indicated that ROS react with DNA and damage it by breaking up the various bonds holding the strands together.[3] The structure and functions of such DNA are modified or impaired.[30] This goes a long way to affect their corresponding proteins and other molecular species. Mutations and cancer are thus inevitable in this case.[28, 31] Initiation of genetic mutations and carcinogenesis activation in biosystems caused by radiation is greatly linked to HO• radicals.[32] HO• radicals do not only abstract hydrogen from the sugar moiety but also add on to the alkene functional groups of pyrimidine bases of DNA.[23] These mainly result in a deleterious series of interconnected reactions of DNA.[17,32]

The body has mechanisms to minimize the aging process in humans.[24] Several studies have linked aging process to ROS. When there is an increased in oxidative stress these destructive molecules interfere with DNA activities and cause a couple of cellular damage.[16] This, therefore, results in extreme variations exemplified by aging.[23] Oxidative damage to proteins, lipids and nucleic acids are unavoidable in the presence of excess free radicals.[16] Proteins are thought to be oxidatively transformed via breakage of an amide bond under the influence of ROS, oxidative transformation of amino acids and creation of protein cross-linkage.[5] Mostly, proteins containing basic amino acids, for example; histidine, arginine are liable to oxidation.[5] Sulfhydryl - containing amino acid (methionine) and thiol containing amino acid (cysteine) has also been reported to be prone to oxidation.[16,33] Proteins that have been predisposed to oxidative damage produce very reactive products. These products do normally affect enzyme activities, membrane transport, heat stability, cellular actions and receptors.[23] Lipids also react with free radicals through a process called lipid peroxidation.[29,34] Some of the products formed afterward serve as a source of secondary free radical (lipid radical).[29] Membrane lipids containing polyunsaturated fatty acid are normally susceptible to lipid peroxidation and radical chain reaction.[24] This suggests that the

possibility of cell membrane integrity being compromised is high. A couple of diseases such as diabetes, neurodegenerative ailments, and ischemic - reperfusion damage are attributed to lipid peroxidation. Fortunately, oxidative stress and its associated disease of humans can be reduced or eliminated with functional foods and antioxidants as well.[23]

Antioxidants

Antioxidants are stable molecules that have the propensity to provide an electron to a free radical in to nullify it, therefore, the damaging ability of free radicals is hampered.[10] Antioxidants play very crucial roles in the well-being of an individual in that they protect cells and organs from damage by either deactivating or stabilizing free radicals.[35] The body, therefore, uses antioxidants as one of the major lines of defense against the harmful effects of free radicals thereby reducing or preventing oxidative stress or damage to biomolecules.[23] Donation of an electron by an antioxidant to a free radical in the body to break a cascade reaction mechanism mediated by ROS, and elimination of ROS/RNS creators by quenching chain initiating substances such as metals serve as the two main mechanisms underlying antioxidant activity.[19,21] This suppresses the creation of free radicals. For example; they prevent the conversion of lipid or hydrogen peroxide into lipid or hydroxyl radical (Fenton's reaction).[19] And they do this by forming harmless molecules such as alcohols, water, and oxygen respectively from the lipid or hydrogen peroxide without any radical formation.[20] Radical scavenging antioxidants nullify active radicals to prevent them from initiating or propagating any chain reactions. These endogenous radical scavengers could either be lipophilic example; ubiquinol, and vitamin E or hydrophilic. Among the most potent hydrophilic antioxidants include albumin, thiols, uric acids and vitamin C.[23]

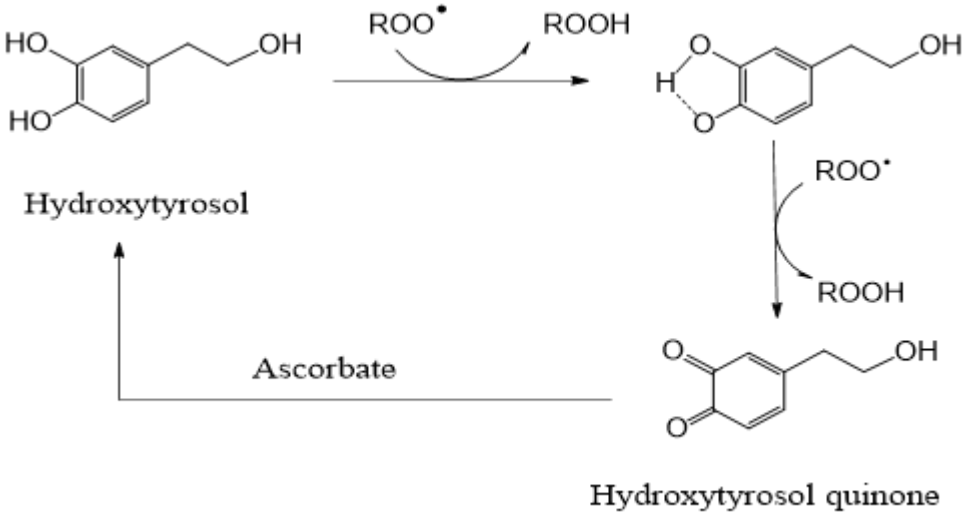
Polyphenols are also thought to exhibit numerous biological properties. Among such properties include protection of DNA from damaging, prevention of cancer, delay aging process,

anti-inflammatory, anti-histamine, metal chelating ability and prevention of cardiovascular diseases.[6] Most plant species that are used traditionally for tonic preparations have been reported to have remarkable pharmacological and antioxidant properties.[36] It has also been established that most naturally occurring food antioxidants made up of phenols. These secondary metabolites also serve as a defense mechanism for plants and organisms that produce them.[37] Several studies have been done to investigate the possible health and antioxidant benefits of various polyphenols in plants.[37] Polyphenols and other naturally occurring antioxidants obtained from plants have been reported little or no side effects compared to synthetic ones.[38]

Several mechanisms have been suggested for phenolic antioxidants by many researchers. Their hydroxy groups are been shown to be excellent hydrogen donors.[35,36] These hydrogen atoms react with the radicals to form stable molecules in the termination step of the free radical reaction.[36] It has also been established by many researchers that polyphenols have the capacity to chelate metal ions which are responsible for free radical production.[39] Again, the benzenoid rings of phenolic compounds are hydrophobic which increases their ability to bind with different types of proteins.[40] This also increases their antioxidant capacity due to their ability to inhibit some enzymes that generate free radicals. Most phenolic compounds have also been reported to have synergistic effects with other antioxidants. Countless polyphenols have been discovered in plants so far. Quercetin, catechin, rutin, flavanols, and hydroxytyrosol, are among the most powerful polyphenol antioxidants known.[40]

Hydroxytyrosol is one of the most powerful antioxidants currently known in the world. It is a naturally occurring polyphenol and most commonly produced by olive tree (*Olea europaea*). The remarkable antioxidant and pharmacological properties of hydroxytyrosol have made it an outstanding compound in the polyphenol family and of great interest to many researchers.[15]

The high antioxidant activity of hydroxytyrosol has been attributed to the presence of the *ortho*-dihydroxyphenyl (catechol) moiety. During oxidation, they form intramolecular hydrogen bonds with the free radicals thereby preventing these radicals from attacking valuable cells and organs (Scheme 1).[43] Several reaction mechanisms have been proposed by different researchers for the free radical scavenging activity of hydroxytyrosol both in vivo and in vitro. It is very clear that most polyphenols like hydroxytyrosol have several other biological activities apart from their free radical scavenging activity.[44]



Scheme 1. Mechanism of free radical scavenging by hydroxytyrosol.[43]

Several researchers have reported that hydroxytyrosol is also implicated in anticancer, antibacterial and anti-inflammatory activities.[44] Hydroxytyrosol also plays a major role in enzyme regulations. A wide range of biological and pharmacological activity has been associated with hydroxytyrosol.[45] Its high and effective antioxidant capacity has made it perform several other biological roles.[43]

Biological Activity of Hydroxytyrosol

Several researchers have reported the pharmacological and antioxidant properties of hydroxytyrosol.[43] Perez-Jimenez *et al.,* 2005 established a strong correlation between olive oil consumption and cancer prevention.[46] Hydroxytyrosol has also been found to be one of the principal antioxidants present in olive oil.[46] Siriani *et al,* 2010 also suggested that the proliferation of human MCF-7 breast cancer cells is inhibited by hydroxytyrosol.[48] Several researchers have reported that hydroxytyrosol protects cell integrity by activating several antioxidant defense mechanisms of cells before oxidative stress.[49]

In vitro studies of the antimicrobial properties of hydroxytyrosol have also been reported by Belmonte *et al.*[50] Hydroxytyrosol was shown to have a greater cytotoxic effect on most of the clinically isolated bacterial.[51,52] It has also been established that hydroxytyrosol has antimicrobial activity for both gram positive and negative bacterial.[52] Studies have also shown that hydroxytyrosol and other phenolic antioxidants penetrate different bacterial structures and destroy the peptidoglycan of the cell.[48]

The inhibition of low-density lipoproteins (LDA) oxidation has been shown to increase with increasing concentration of hydroxytyrosol.[43] Hydroxytyrosol prevents oxidative stress induced by *tert-butyl* hydroperoxide.[18,43] Hydroxytyrosol therefore, enhance the lipid profile of many living cells, thus protecting cell integrity.[43, 50]

Hydroxytyrosol Derivatives

Chemical modification of organic compounds has been shown to improve their biological activities.[51] There has been a recent report that suggests a possible enhancement of biological and pharmacological activities of hydroxytyrosol derivatives with respect to hydroxytyrosol itself.[43] Derivatives and some analogs of hydroxytyrosol have been synthesized and their antioxidant

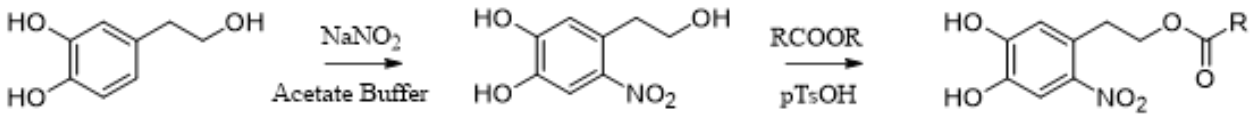
activities have been tested in different ways.[52] Some these derivatives were found to have increased antioxidant and other biological activity compared to the parent hydroxytyrosol.[53]

Nitrohydroxytyrosol and its Analogues

Hydroxytyrosol was reported to have higher antinitrosating properties than most phenols. At about pH 3, hydroxytyrosol has been found to react with sodium nitrite to produce 2-nitrohydroxytyrosol.[54] Hydroxytyrosol, therefore, acts as an excellent nitrosating scavenger.[43]

The relationship between a higher intake of nitrite and some type of cancer has been established by some researchers.[48] Nitrite has been reported to originate from consumption meat and certain vegetables like cabbages, radishes, celery etc.[15,55] They are mostly found to associate with nitrite which is reduced to nitrate by microorganisms in the oral cavity. Reactive nitrogen species that produce nitrite affects the integrity of cells and exposes the body to some pathogenic diseases.[56]

Trujillo *et al*.,[56] also synthesized 2-nitrohydroxytyrosol from hydroxytyrosol. The compound was found to have an excellent antioxidant activity and enhanced bioavailability when the Ferric reducing antioxidant power (FRAP) was tested.[56]

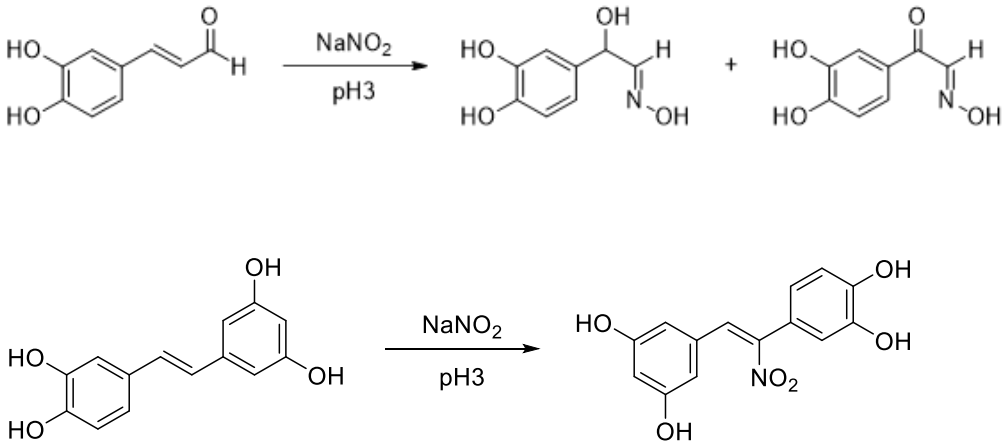


Scheme 2. Chemical structures of nitrohydroxytyrosol and nitrohydroxytyrosol ester.[56]

Nitrohydroxytyrosol and nitrohydroxytyrosol esters have also been synthesized and their antioxidant properties were studied using FRAP Assay.[56] Again, nitohydroxytyrosol was found to have a higher reducing activity than hydroxytyrosol.[57] However, nitrohydroxytyrosol esters

exhibited varying antioxidant activity compared to their nitrohydroxytyrosol depending on the chain length of the esters.[54] Nitrohydroxytyrosol acetate showed higher antioxidant activity than nitohydroxytyrosol while hexanoate and octanoate derivatives presented a lower activity.[54]

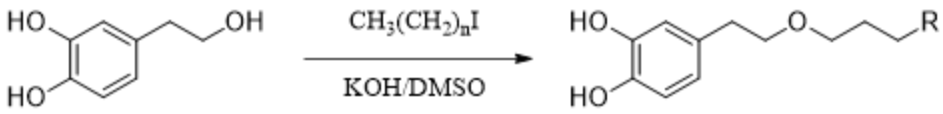
The improved antioxidant power of the nitro derivatives was also linked with the stabilization of ortho substitution of the nitrogen on the benzene ring of hydroxytyrosol. This substitution tends to make phenoxyl radical more stable.[58]



Scheme 3. Reactions of hydroxytyrosol analogs with nitrite.[54]

Lipohilic Hydroxytyrosol Alkyl Ethers

Madrona *et al*.[59] synthesized alkyl ether derivatives of hydroxytyrosol from waste olive water. The results from the synthesis showed a corresponding increase in yields of the alky derivative with an increasing chain length of the alkyl group due to the stability of the alkyl intermediate.



Scheme 4. Synthesis of hydroxytyrosol alkyl ethers.[59]

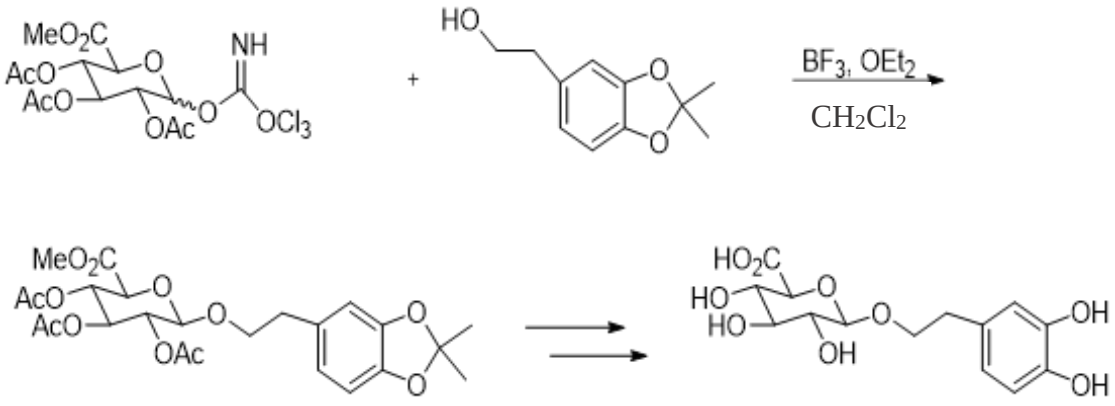
The Rancimate method was used to measure the oxidative stability of the various derivatives and it was found to have similar activity with the parent compound; hydrozytyrosol Pereica-Caro *et al.* [60] also reported the antioxidant activities of these alkyl ether derivatives with varying alkyl chain length with hydroxytyrosol using FRAP and DPPH assay. The lipophilic hydroxytyrosol ethers were found to have higher antioxidant activity in hydrophilic media compared to hydroxytyrosol. The transport, absorption, and cellular metabolism of these derivatives were also studied. The result showed a strong correlation between the lipophilic nature of the compounds and the degree of their metabolism. The longer the alkyl chain length, the longer the time it takes for the biotransformation.[59,60]

Some researchers have also reported that attachment of fluorine atoms into the molecular skeleton of some polyphenols has increases their lipophilicity greatly enhance their ability to complex with proteins and other ligands.[61] Halogenation has therefore been proven to be one of the useful methods to obtain highly lipophilic derivatives of organic compounds.[61,62]

Hydroxytyrosol Glucuronide Derivatives

Glucuronidation has been described as one of the pathways hydroxytyrosol and other phenolic compounds go through during metabolism.[43] Lucas et al.[63] developed a method for the synthesis of hydroxytyrosol glucuronide derivatives. (Scheme 5) An evaluation of their antioxidant activity, however, did not show any significant difference compared to hydroxytyrosol.

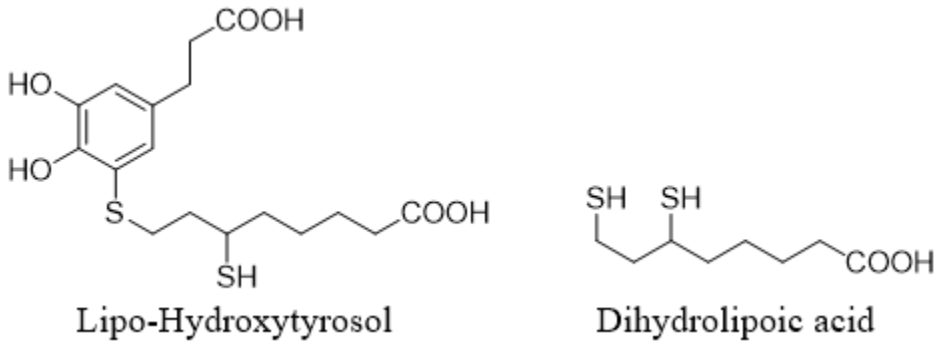
Antimicrobial activities of some hydroxytyrosol β-D-glucopyranosides isomers have also shown to be negative.[63]



Scheme 5. Synthesis of hydroxytyrosol glucuronide.[63]

Lipoyl Hydroxytyrosol (Lipo-HT)

Another derivative of hydroxytyrosol that has shown an excellent improvement in its antimicrobial and antioxidant activity is 5-S-Lipoyl hydroxytyrosol. This derivative with an enhanced activity was synthesized by conjugation between thiol-dihydrolipoic acid and hydroxytyrosol.[11]



Scheme 6. Lipoyl hydroxytyrosol derivative and dihydrolipoic acid

Lipo-HT was found to have higher antioxidant activity compared to hydroxytyrosol. The ability of the Lipo derivative to efficiently protect cells against several reactive radicals was linked to its sulfur-containing side chain.[11] This group was found to increase the lipophilicity of the compound, resulting in greater antioxidant activity.[11,60] In vitro analysis of the antioxidant capacity of hydroxytyrosol and the Lipo-HT derivative on Hg-induced oxidative stress also proved that Lipo hydroxytyrosol has a greater potential to protect cell damage.[11]

Research Objectives

The biological and pharmacological role of hydroxytyrosol has increased its use in food supplement and pharmacological industries. However, it is only available in low concentration in olive oil extract. Some researchers have extracted hydroxytyrosol from olive oil wastewater through industrial processes with very low yield. Again, most of the synthetic methods described in literature for the synthesis of hydroxytyrosol are not economically viable. This has made alternative method for the synthesis of hydroxytyrosol and its derivatives from a commercially less expensive precursor very attractive. It is widely believed that the limited cellular uptake of hydroxytyrosol is due to its high hydrophilicity. The aim of this research is to synthesize various less hydrophilic derivatives of hydroxytyrosol, in hope to improve its cellular uptake.

CHAPTER 2

EXPERIMENTAL

Materials

Catechol served as the major precursor (starting material) in this research. All solvents and reagents were used without further purification unless it is mentioned otherwise. Dichloromethane, acetyl chloride, lithium aluminum hydride, ethyl acetate, aluminum chloride, acetone, boron trifluoride, hydrochloric acid and sodium hydroxide were obtained from commercial sources and used without further purification. Separation and purification of products were performed either by silica gel column chromatography or recrystallization or both.

Proton and ^{13}C nuclear magnetic resonance (NMR) were recorded on a JEOL-NMR Eclipse-400 MHz spectrophotometer. Chemical shifts were recorded in parts per million (ppm) and referenced to $CDCl_3$. (δ_H/δ_C 7.25/76.8 ppm). The NMR signals description is reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet; the coupling constants are reported in Hz. Melting points were measured om Cambridge Melt-Temp device and reported without correction.

Synthesis of 3,4-Dihydroxyacetophenone **2**

A round-bottomed flask containing (0.98 g, 7.4 mmol) of aluminum chloride in 4 mL 1,2-dibromoethane at 10 ℃ was stirred for 30 minutes. Powdered catechol **1**, (0.5 g, 4.5 mmol) was then added to the reaction mixture in three portions over 4 minutes. The reaction was further stirred at 10 ℃ for 1 hour, after which a solution of acetyl chloride (0.26 mL, 3.2 mmol) in 0.5 mL dibromoethane was added dropwise. The reaction mixture was allowed to warm to room temperature and stirred for an additional 24 hrs. After the reaction was complete, the mixture was set on ice and cooled to about 6 ℃ before quenching with 1 M HCl (10 mL). The mixture was

stirred for two hrs, after which it was extracted with CH_2Cl_2 (20 mL) and subsequently with EtOAc (4x20 mL); and the combined organic layer was washed with saturated sodium chloride (30 mL) and dried over anhydrous sodium sulfate. Evaporation of the solvent in vacuo gave **2** as a dark-brown solid (0.63 g, 95%) with m.p value of 115-116 ℃.The crude product was purified by recrystallization using dichloromethane. ^{1}H-NMR ($CDCl_3$): δ 7.66-7.66 (s,1H), 7.50-7.48 (d,1H), 6.92-6.925 (s,1H), 2.56 (s, 3H). ^{13}C-NMR: δ 169.93 (C=O),134.50 (ArC), 132.30 (ArC), 127.33 (ArC), 119.50 (ArC), 112.30 (ArC), 111.15 (ArC), 27.06 (CH_3).

Synthesis of 3,4-Diacetoxyacetophenone **3**

To a round-bottomed flask containing 0.5 g (3.27 mmol) of 3,4-dihydroxyacetophenone **2**, 10 mL of acetyl chloride was added, and the solution was refluxed for 3 hours. After the reaction was complete (based on TLC) the solution was poured into 20 g of ice with stirring.

The aqueous solution was repeatedly extracted with dichloromethane (3x20 mL). The combined organic layer was washed with 10 mL of saturated sodium bicarbonate. After evaporation of solvent, the product was purified by recrystallization from 1:3 dichloromethane/hexane (v/v) to give 0.77 g (98.5 %) of the desired product **3** with melting point of 91.0-91.8 ℃. ^{1}H-NMR ($CDCl_3$): δ 7.86-7.783 (dd,1H), 7.78-7.76 (s,1H), 7.29-7.25 (d,1H), 2.58-2.56 (s,3H), 2.34-2.30 (d,6H). ^{13}C-NMR: δ 196.08 (C=O), 168.20 (ROC=O), 168.84 (ROC=O), 146.10 (ArC), 142.31 (ArC), 135.68 (ArC), 126.99 (ArC), 123.81 (ArC), 108.96 (ArC), 26.69 (CH_3), 20.80-20.70 (CH_3).

Synthesis of methyl (3,4-Diacetoxyphenyl) acetate

To a round-bottomed flask containing 0.10 g (0.42 mmol) of 3,4-diacetoxyacetophenone **3** dissolved in 0.5 mL methanol was added to a solution containing 0.4 g (2.78 mmol) boron trifluoride etherate ($BF_3.Et_2O$) and 0.25 g (0.56 mmol) of lead tetraacetate in 2 mL of benzene. The

reaction mixture was then stirred at room temperature for 8 hrs, after which the reaction was quenched by adding 5 mL cold water. The product was extracted 3 times with ethyl acetate (3x10 mL). The combined organic layer was washed with saturated NaHCO3 until the pH is around 7, followed by a second wash with an equal volume of saturated NaCl, and the organic layer was dried over anhydrous sodium sulfate. After evaporation of the solvent, 0.107 g of a dark brown viscous oily crude product was obtained. Further purified by column chromatography (dichloromethane/ethyl acetate 3: 1, v/v) gave a yellow viscous oil 4 (0.092g, 81% yield). 1H-NMR (CDCl3): δ 7.18-7.07 (m, 3H), 3.70-3.63 (s,3H), 3.63-3.55 (s,2H), 2.28-2.22 (s, 6H). 13C-NMR: δ 189.46 (ROC=O), 168.39 (ROC=O), 168.32 (ROC=O), 142.01 (ArC), 141.25 (ArC), 132.71 (ArC), 127.61 (ArC), 124.41 (ArC), 123.50 (ArC), 52.29 (CH3), 40.48 (CH2), 20.74 (CH3).

Synthesis of Hydroxytyrosol **5**

To a round bottom flask containing methyl (3,4-diacetoxyphenyl) acetate **4** (0.092 g, 0.346 mmol) dissolved in anhydrous THF (10 mL), was added, lithium aluminum hydride (361 mg, 9.52 mmol). After flushing with a stream of nitrogen, the mixture was refluxed for 90 min. The mixture was acidified with 2 M H_2SO_4 and extracted with ethyl acetate (3x20 mL). The combined organic layer was dried, and the solvent was evaporated. The product was purified by column chromatography using dichloromethane/ethyl acetate 80:20 (v/v) to obtain hydroxytyrosol as light yellow oil (0.045g, 83% yield). ^{1}H-NMR (CD_3O_D): δ 6.76-6.67 (d, 1H), 6.67-6.62 (s, 1H), 6.56-6.47 (d, 1H), 4.21-4.20 (t, 2H), 2.66-2.61 (t, 2H), 2.10-2.0. ^{13}C-NMR: δ 144.86 (ArC), 143.28 (ArC), 130.42 (ArC), 119.75 (ArC), 115.72 (ArC), 114.66 (ArC), 63.42 (CH_2), 38.10 (CH_2).

Hydroxytyrosol Acetate **6**

To a round-bottom flask containing 0.1 g (0.649 mmol) of hydroxytyrosol **5** dissolved in 2 mL of a mixture of ethyl acetate/THF (1:1v/v), was added *p*-toluenesulfonic acid (100 mg). The mixture was stirred at 60-70 ℃ overnight. Distilled water (0.5 mL) was added and the product was extracted with dichloromethane (3x10 mL). The combined organic layer was washed with 10 mL concentrated $NaHCO_3$ dried over anhydrous sodium sulfate and the solvent was evaporated. The crude product was purified by column chromatography using dichloromethane to obtain a viscous liquid (0.092 g, 92% yield). ^{1}H-NMR ($CDCl_3$): δ 6.76-6.67 (d, 1H), 6.67-6.62 (s, 1H), 6.56-6.47 (d, 1H), 4.21-4,20 (t, 2H), 2.81-2.80 (t, 2H), 2.10-2.0(s,3H). ^{13}C-NMR: δ 171.63 (C=O), 144.62(ArC), 144.36(ArC), 129.36(ArC), 119.82(ArC), 115.63 (ArC), 115.01(ArC), 65.30 (CH_2), 34.09 (CH_2), 19.50 (CH_3).

Synthesis of 6-Bromohydroxytyrosol Acetate **7**

To a solution of 0.06 g of hydroxytyrosol acetate **6** in 1mL dichloromethane, stirred under nitrogen atmosphere at 0 ℃, was added dropwise (at a rate of 10 drops/min) a solution of 0.145 g bromine in 1 mL dichloromethane. The reaction mixture was allowed to warm to room temperature then stirred for four more hours. The reaction was quenched with 1 mL of distilled water and then extracted three times with 2 mL dichloromethane; the combined organic layer was washed with saturated sodium chloride. The combined organic layer was dried over anhydrous sodium sulfate and solvent was evaporated. The product was purified by column chromatography using dichloromethane to obtain viscous liquid (0.070 g, 83% yield) m.p.83.4 -84.2 ℃. ^{1}H-NMR ($CDCl_3$): δ7.00-7.10 (s,1H), 6.67-6.62 (s, 1H), 4.19-4.00 (t, 2H), 2.85-2.55 (t, 2H), 2.10-2.0(s,3H). ^{13}C-NMR: δ 171.73 (C=O), 143.39(ArC), 141.36(ArC), 128.85(ArC), 121.12(ArC), 114.53 (ArC), 112.78(ArC), 64.97 (CH_2), 34.24 (CH_2), 20.7(CH_3).

Synthesis of Hydroxytyrosol Tri-acetate **8**

To a round bottom flask containing 0.1g of HT, 5 mL of acetyl chloride was added and refluxed for 3 hrs. After the reaction was over the resulting solution was poured with mixing into 20g of ice. The aqueous solution was repeatedly extracted with dichloromethane (3x20 mL). The combined organic layer was washed with 10 mL of saturated sodium bicarbonate and dried over anhydrous sodium sulfate. After evaporation of the solvent, the product was purified by chromatography using dichloromethane to obtain a viscous liquid, (0.16 g, 89% yield). ^{1}H-NMR ($CDCl_3$): δ 7.10-7.09 (d, 1H), 6.67-6.62 (s, 1H), 6.56-6.47 (d, 1H), 4.32-4.22 (t, 2H), 2.92-2.90 (t, 2H), 2.27(s, 6H), 2.03 (s, 3H). ^{13}C-NMR: δ 171.63 (C=O), 168.50 (ROC=O), 141.99 (ArC), 141.60 (ArC), 139.35 (ArC), 136.82(ArC), 127.10 (ArC), 123-123.44 (ArC), 64.30 (CH_2), 33.54 (CH_2), 20.75 (CH_3).

Synthesis of 6-Tert-butylyhdroxytyrosol **9**

To a round-bottomed flask containing 0.102g of HT dissolved in 5 mL of acetic acid, 1 mL of *tert*-butyl alcohol was added, and the solution was cooled in ice bath. Ice cold concentrated H_2SO_4 (0.5mL) was added dropwise with continuous stirring. The reaction was allowed to warm to room temperature and stirred for 6 hrs. Distilled water 0.5 mL was added and the reaction mixture was extracted with dichloromethane (3x10 mL). The combined organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated. The crude product was purified by gravity column chromatography using dichloromethane to obtain a gray solid m.p. 102-10 ℃, (0.114 g, 82% yield). ^{1}H-NMR ($CDCl_3$): δ 6.65-6.64 (s, 1H), 6.58-6.62 (s, 1H), 3.82-3.80 (t, 2H), 2.72-2.71 (t, 2H), 1.38-1.30(s, 9H). ^{13}C-NMR: δ 143.18 (ArC), 142.18 (ArC), 136.65 (ArC), 128.59 (ArC), 119.42 (ArC), 113.66 (ArC), 63.99 (CH_2), 38.61 (CH_2), 34.72 (C-C), 29.47-29.59(CH_3).

Synthesis of 6-Nitrohydroxytyrosol **10**

To a round-bottom flask containing 5ml of 0.1M acetate buffer (pH 3.8) was added to 0.1 g of hydroxytyrosol followed by addition of sodium nitrite (140 mg, 2.09 mmol). And the reaction was stirred at room temperature for 5 hours. The product was extracted using ethyl acetate (3x10 mL), the combined organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated to produce yellow-solid (0.129 g). The product was purified by column chromatography using ethyl acetate and hexane (3:1 v/v) to give a white solid, m.p. 194-194.8 ℃ (0.112 g, 92.6%). ^{1}H-NMR (CD_3OD): δ 7.65-7.57 (s, 1H), 6.77-6.75 (s, 1H), 3.76-3.73 (t, 2H), 3.05-3.04(t, 2H). ^{13}C-NMR: δ 144.96 (ArC), 142.48 (ArC), 130.46 (ArC), 115.54 (ArC), 114.72 (ArC), 110.66 (ArC), 63.42 (CH_2), 38.10 (CH_2).

Synthesis of 6-Nitrohydroxytyrosol Acetate (**11)**

To a round bottom flask containing 0.05 g of 6-nitrohydroxytyrosol in 2ml of a mixture of ethyl acetate/THF (1:1, v/v), p-toluenesulfonic acid (10 mg) was added and the mixture was stirred at 60-70 ℃) for overnight. Distilled water (0.5 mL) was added and the product was extracted with 5ml of dichloromethane. The organic layer was dried over anhydrous sodium sulfate. The crude product was purified by column chromatography using dichloromethane and methanol (3:1, v/v) to obtain a dark brown solid, m,p 105-106 ℃ (0.045 g, 83% yield) . ^{1}H-NMR (CD_3OD): δ 7.51 (s,1H), 6.67-6.62 (s,1H), 6.56-6.47 (d,1H), 4.30-4.27 (t, 2H), 3.15-3.14 (t, 2H), 2.10-2.0 (s,3H). ^{13}C-NMR: δ 172.01 (C=O) 144.96 (ArC), 142.48 (ArC), 130.46 (ArC), 115.54 (ArC), 114.72 (ArC), 109.36 (ArC), 63.42 (CH_2), 38.10 (CH_2), 20.09(CH_3).

CHAPTER 3

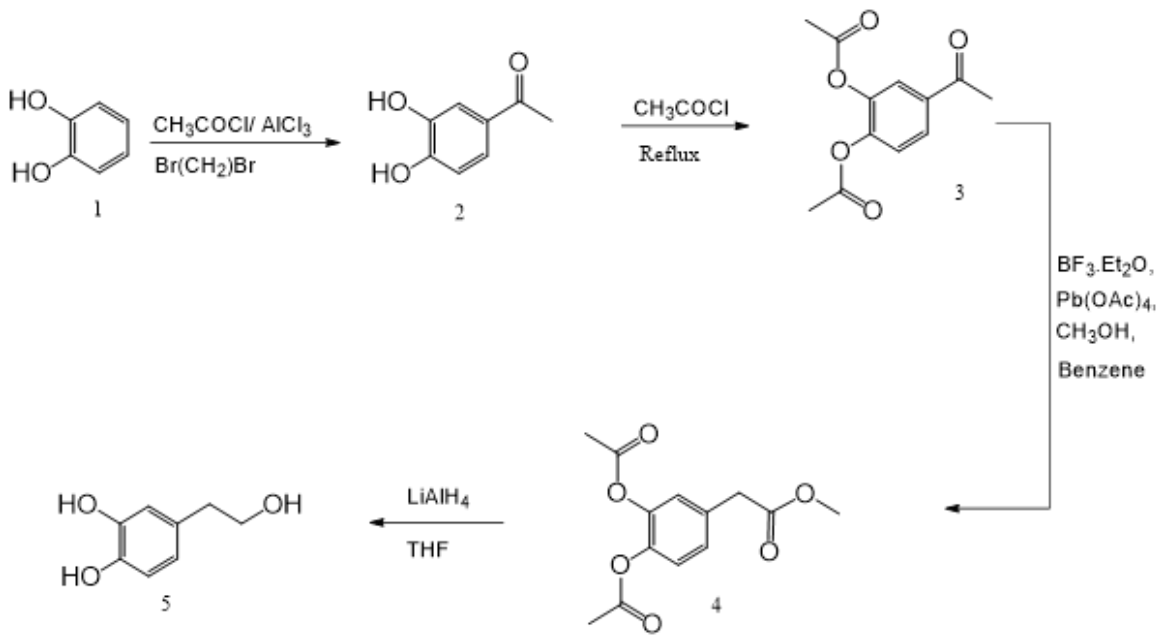
RESULTS AND DISCUSSION

Synthesis of Hydroxytyrosol

Various procedures used in the synthesis of hydroxytyrosol from catechol were adopted from methods previously described in literature, with modification.[63, 64,65,66] The four major steps involved are cost-effective and can be carried out under mild conditions.

Friedel-Craft Acylation

Electrophilic aromatic substitution can be achieved by through Friedel-Craft acylation reaction. It is a substitution reaction that introduces acyl groups on aromatic rings.[67] Acylium ion is the electrophile which is normally generated from acyl halide upon reaction with a Lewis acid such as aluminum chloride.[68,69] Electron donating groups on the aromatic ring increase the rate of the reaction. Many researchers have synthesized many aromatic ketones and its derivatives by this method.[69] The method involves the initial reaction of the Lewis acid with 1,2-dibromoethane for 30 to 45 minutes before introducing catechol. The initial reaction of the 1,2-dibromoethane protects the hydroxyl groups on the catechol to prevent O-acylation which might reduce the nucleophilicity of the ring for the acylium ion. The reaction mixture was stirred for another 45 minutes before the acetyl chloride was added. This allows sufficient time for the catechol to react with the 1,2-dibromoetane before introducing the acylating agent reducing the chances of esterification of the hydroxyl groups.3,4-Dihydroxyacetophenone **2** was produced from catechol using a previously described procedure as shown in scheme 7 below.[66]



Scheme 7. Synthesis of HT from catechol

Hydroxyl groups on benzene rings can undergo both O-acylation and C-acylation with acyl chlorides or anhydrides in the presence of Lewis acids, although the C-acylation product is found to be the major product under these conditions.[70] Therefore, the initial reaction of the catechol with 1,2-dibromometane protects the aromatic hydroxyl group of catechol which prohibits O-acylation. O-acylation (i.e., esterification) has been reported to reduce the nucleophilicity of the aromatic rings for C-acylation.[67,70] **2** was isolated in high yield (95%). ^{1}H-NMR analysis (Appendix A) of **2** showed three aromatic protons at δ 7.66 (s,1H), 7.49 (s,1H), 6.92 (s,1H), and three protons of the ketone at 2.56 (s, 3H). ^{13}C NMR also showed eight non-equivalent carbons with a peak at δ 169.93 ppm which corresponds to the carbonyl carbon. Both ^{1}H-NMR and ^{13}C NMR confirm the proposed structure of **2**.

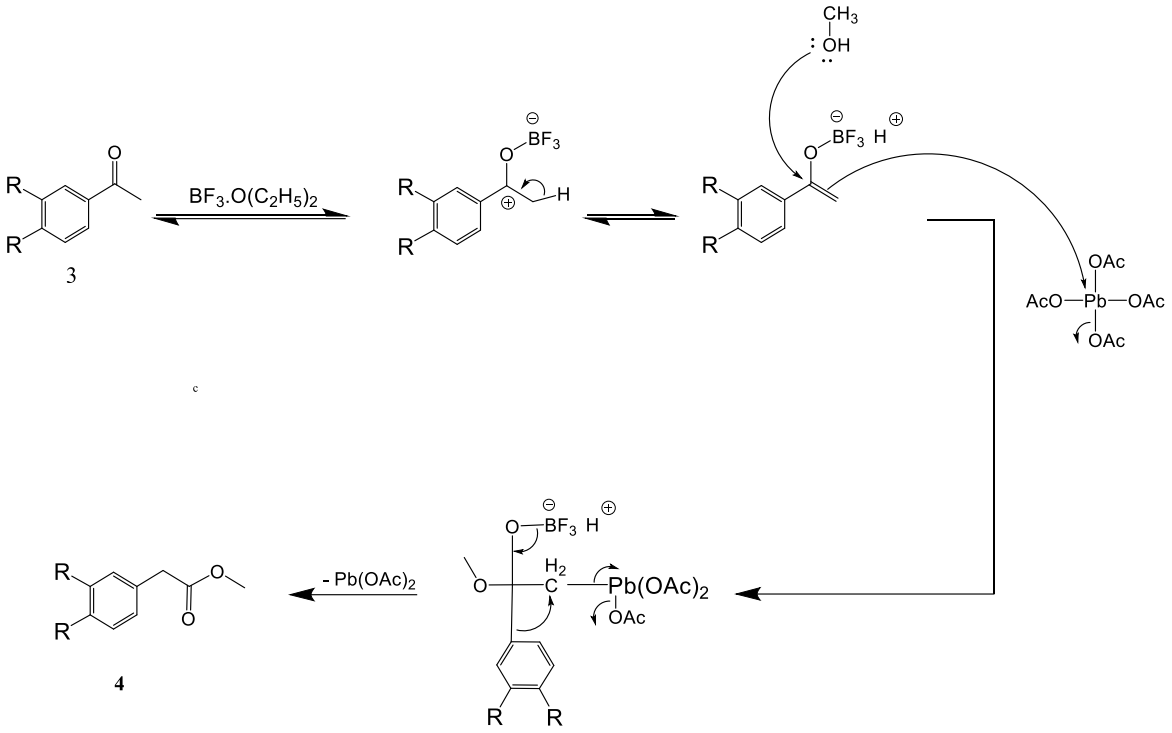
The next step involves esterification of two hydroxyl groups of **2** to produce 3,4-diacetoxyacetophenone **3**. This was accomplished by refluxing **2** in excess amount of acetyl

chloride.[69] The purified product melts sharply at 91.0-91.8 ˚C which agrees with literature.[69] Both ^{1}H-NMR and ^{13}C-NMR analysis of the product (Appendix B) confirms the structure of **3**. The three aromatic protons show at δ 7.86-7.78 (dd,1H), 7.78-7.76 (s,1H), 7.29-7.25 (d,1H) ;the peak at 2.58-2.56 (s,3H) represents the protons of the (CH_3) of the ketone, and the signal at 2.34-2.30 (d,6H) corresponds to the protons on the diester group. A total of ten (10) non-equivalent carbons were observed by the ^{13}C-NMR with peak at 196.08 ppm confirming the presence of (C=O) group. The ester groups also appeared at 168.20 and 188.4.

Initial attempts to synthesize 3,4-diacetoxyacetophenone **3** directly from catechol, using acetyl chloride and aluminum chloride resulted in a low yield and gave other intractable side products. This could be due to incompatibility of the unprotected hydroxyl groups of catechol and aluminum chloride. This problem was overcome when the reaction was conducted in 1,2-dibromoethane which is believed to provide protection of the hydroxyl groups.

A synthetic route has been proposed for synthesis of aryl acetates from acetophenone in high yield based on Favorskii-type rearrangement.[71,72] This protocol was used with little modification to synthesize methyl (3,4-diacetoxyphenyl) acetate **4** from **3.** The structure of **4** was confirmed by NMR. ^{1}H-NMR (Appendix D) of **4** showed three aromatic protons at δ 7.18-7.07 ppm. Two singlets at 3.70-3.63 and 3.63-3.55 correspond to methyl ester (-$COOCH_3$) and (-CH_2-) protons. The singlet at 2.28-2.22 is for the six protons on the diester groups. ^{13}C NMR spectrum of **4** also showed eleven signals, the peak at 189.46 and 168.39 ppm corresponds to the ester and ketone.

The underlying mechanism of this reaction depends on the presence of boron trifluoride; etherate which acts as a Lewis acid to cause enolization of the methyl ketone and subsequent migration of the aryl group as shown in Scheme 8 below.[68]

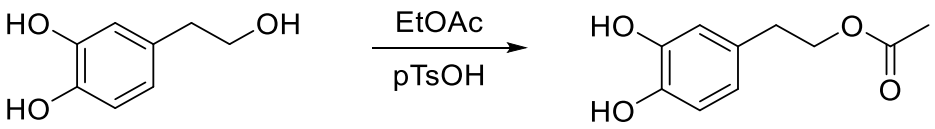


Scheme 8 Mechanism of the rearrangement of 3,4-diacetoxyacetophenone[72]

The final step involves reduction of **4** using lithium aluminum hydride in THF. Hydroxytyrosol **5** was isolated as light-yellow oil. ^{1}H-NMR analysis (Appendix D) of **5** showed three aromatic protons at δ 6.76-6.67 (d, 1H), 6.67-6.62 (s, 1H), 6.56-6.47 (d, 1H),two triplets at 3.68-3.64 (t, 2H) and 2.66-2.61 (t, 2H) for the two -CH_2- protons. ^{13}C-NMR shows a total of eight non-equivalent carbons made up of six aromatic and two aliphatic carbons. Both the NMR data and the physical appearance of the product agrees with the proposed structure of **5**.[65]

Synthesis of Hydroxytyrosol Acetate **6**

Hydroxytyrosol acetate was synthesized from hydroxytyrosol using a previously described method (Scheme 8). This method is selective for aliphatic hydroxyl groups and does not require the protection of phenolic hydroxyl groups prior to the esterification.[73,74,75] The transesterification utilizes *p*-toluenesulfonic acid as a catalyst. Heating HT and ethyl acetate in presence of catalytic amount of *p*-toluenesulfonic acid produced HT acetate **6** in 92% yield

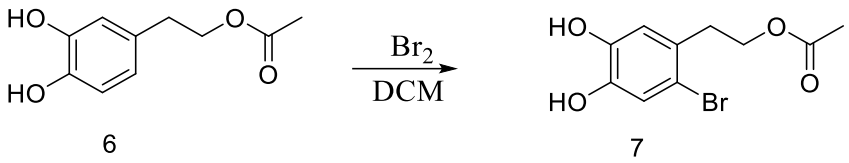


Scheme 9. Synthesis of hydroxytyrosol acetate **6.**

The structure of **6** was confirmed by NMR. ^{1}H-NMR (Appendix E) of **6** showed three aromatic protons at δ 6.76-6.67 (d, 1H), 6.67-6.62 (s, 1H), 6.56-6.47 (d, 1H), two triplets at 3.82-3.65 and 2.69-2.55 for the two sets of methylene (-CH_2-) protons. The singlet at 2.10-2.0 is for the methyl ester (-$COOCH_3$). ^{13}C NMR spectrum of **6** also showed ten signals, the peak at 170.21 ppm corresponds to the ester.

Synthesis of 6-Bromohydroxytyrosol Acetate **7**

6-Bromohydroxytyrosol acetate was successfully synthesized from hydroxytyrosol acetate (Scheme 9). Monobromination of hydroxytyrosol acetate was achieved by reacting hydroxytyrosol acetate with one equivalent of bromine in dichloromethane; Compound **7** was obtained in 83% yield after column chromatography purification. ^{1}H-NMR (Appendix F) indicated that only monobromination took place, and that di-bromination and tri-bromination did not occur. The only two singlets in the aromatic region at δ7.00-7.10(s,1H), 6.67-6.62 (s,1H) indicated monobromination of the benzene ring. Bromine was assigned position six (6) on the benzene ring due to the *ortho-para* directing substituents (hydroxy and ester) on the benzene ring which makes the position six the most likely point of attachment. Tert-butyl and nitro groups were also place at position six of the benzene ring of HT for the same reason.

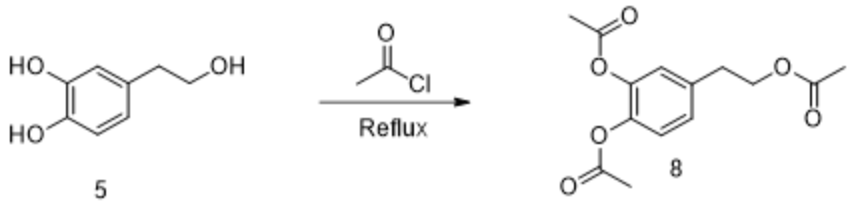


Scheme 10 Synthesis of 6-bromohydroxytyrosol acetate **7**

Previous attempts to brominate hydroxytyrosol with excess bromine in dichloromethane produced a light-brown solid **12** which melts at 80-83℃ after recrystallization from ethyl acetate:dichloromethane(1:3, v/v). ^{1}H-NMR of the product showed no proton on the benzene ring which suggests that tri-bromination took place. The two triplets for both -CH_2- groups appear at 3.68-3.64 (t, 2H) and 2.66-2.61(t, 2H). However, the singlet at 2.0-1.9 ppm which integrates to 2 protons could not be verified because no peak was expected to appear there. X-ray crystallography of the product also showed that the aromatic ring was tri-brominated but also indicated a methyl ester group (Appendix L). However, both ^{1}H-NMR and ^{13}C NMR do not support the presence of any ester functional group. The singlet at 2.0-1.9 ppm was initially believed to be due to impurities; attempts to remove impurity (including recrystallization and column chromatography) were unsuccessful. Investigations are still going on to verify the structure of this product.

Synthesis of Hydroxytyrosol Triester **8**

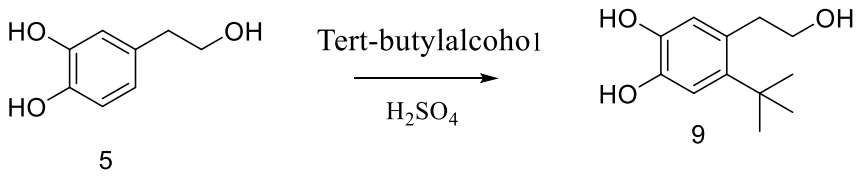
Esterification of phenols and alcohols with acyl chlorides is a well-known reaction.[76,77] Compound **8** was synthesized in 89% yield by refluxing HT in acetyl chloride for 3 hours scheme 10). Proton NMR (Appendix G) showed two singlets, one at 2.03 ppm (s,3H) for the aliphatic methyl ester and one at 2.27 ppm (s, 6H) for the two methyl esters on the ring. ^{13}C NMR also showed two esters peaks at 171.02 ppm and 168.5 ppm.



Scheme 11. Synthesis of hydroxytyrosol triester **8.**

Synthesis of 6-Tert-butylhydroxytyrosol **9**

There are several methods that describe how to introduce alkyl groups to aromatic rings.[78] Tert-butylhydroxytyrosol was obtained by reacting HT with tert-butyl alcohol in the presence of concentrated H_2SO_4. Compound **9** was obtained as a gray solid (m.p 102-103 ℃). ^{1}H-NMR analysis (Appendix H) of **9** showed a singlet at 1.38-1.30 (s, 9H) ppm (for tert-butyl). The peaks of the aromatic protons appeared at δ 6.65-6.64 (s, 1H), 6.58-6.62 (s,1H) ppm as singlets with 1:1 ratio, confirming a successful alkylation of the aromatic ring. ^{13}C NMR also showed a total of 10 non-equivalent carbons. Several attempts were made to alkylate catechol with 1-butanol and isoamyl alcohol, but they were unsuccessful. This may be due to the instability of the primary carbocations intermediates.

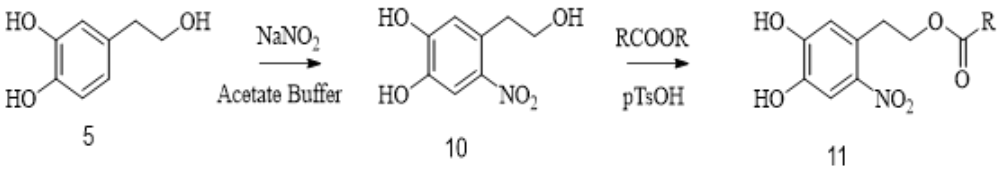


Scheme 12. Synthesis of 6-tert-butylhydroxytyrosol.

Synthesis of 6-Nitrohydroxytyrosol **10** and 6-Nitrohydroxytyrosol Acetate **11**

Nitration of the HT was done following the method of Gallardo *et al*.[79] After purification by column chromatography (ethyl acetate and hexane 3:1 v/v), 6-nitrohydroxytyrosol **10** was

isolated as a light-yellow solid, m.p. 194-194.8 ℃ (Scheme 13). 1H-NMR (Appendix I) showed two singlets for the two aromatic protons (7.65-7.57 and 6.77-6.6.75) versus three aromatic protons in the starting material.



Scheme 13. Synthesis of 6-nitrohydroxytyrosol and 6-nitrohydroxytyrosol acetate.

6-Nitrohydroxytyrosol acetate **11** was synthesized by transesterification of 6-nitrohydroxytyrosol. The product was isolated as a dark-brown solid m.p.106-108 ℃, consistent with literature.[79] ^{1}H-NMR (Appendix J) showed a singlet at 2.10-2.0(s,3H) which corresponds to the 3H of the ester. All the other peaks are similar to that of 6-nitrohydroxytyrosol. Attempts to reduce the nitro groups in **10** and **11** to their amino derivatives by Zn/conc. HCl were unsuccessful. This reaction is still under further investigation.

CHAPTER 4

CONCLUSION

Hydroxytyrosol **5** was successfully synthesized from the commercially available and inexpensive catechol through a four-step synthetic route. These steps include: Friedel Craft's acylation, esterification, rearrangement of methyl ketone, and finally reduction of the tri-ester. Additionally, various derivatives of hydroxytyrosol were synthesized these include hydroxytyrosol acetate, 6-bromohydroxytyrosol acetate, hydroxytyrosol triester, 6-tert-butylhydroxytyrosol, 6-nitrohydroxytyrosol, and 6-nitrohydroxytyrosol acetate. Purifications of all products were performed by column chromatography and/or recrystallization. Structures of all derivatives were confirmed by ^{1}H- and ^{13}C-NMR spectroscopy. Future work will involve assessment of biological activities of these derivatives compared to hydroxytyrosol.

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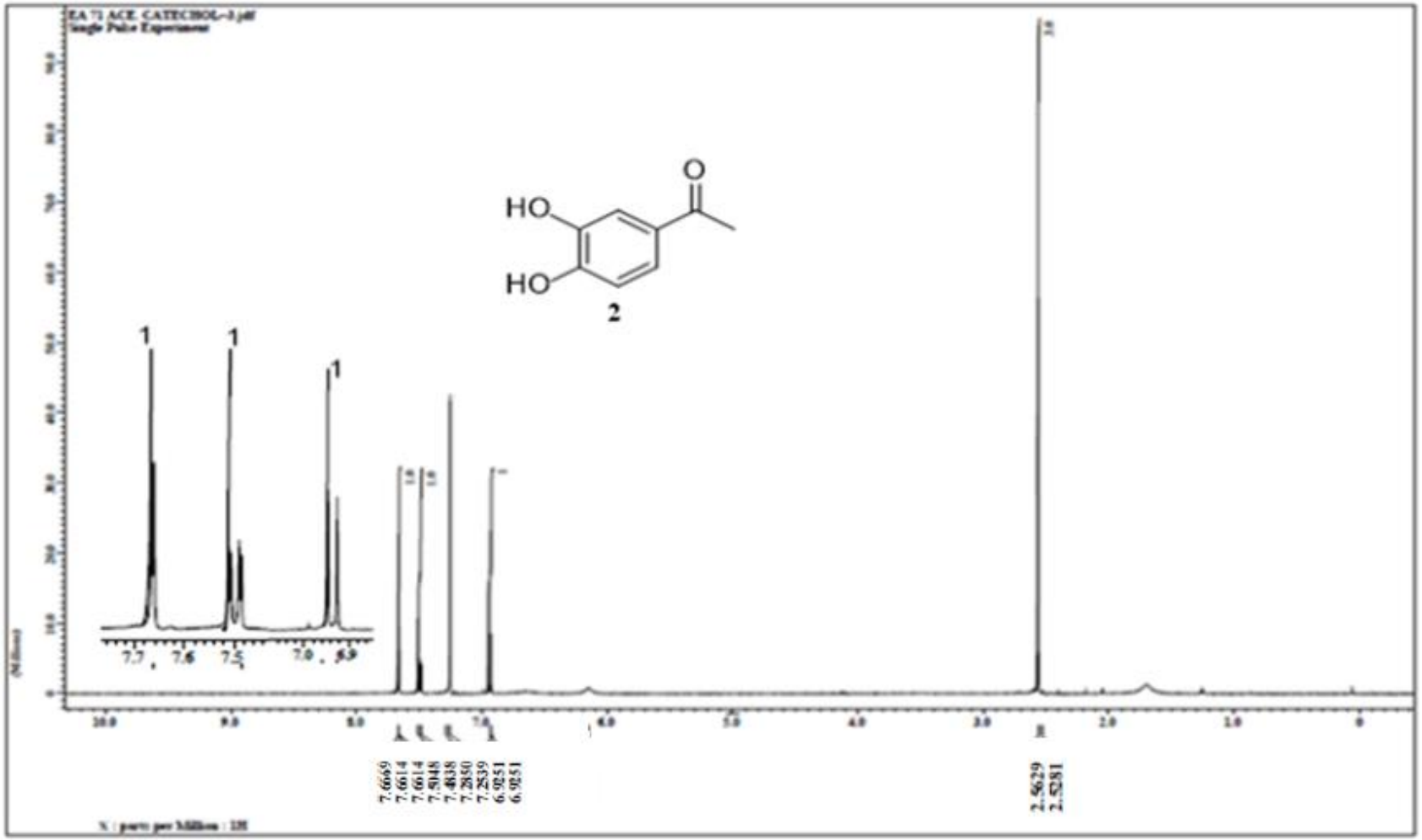
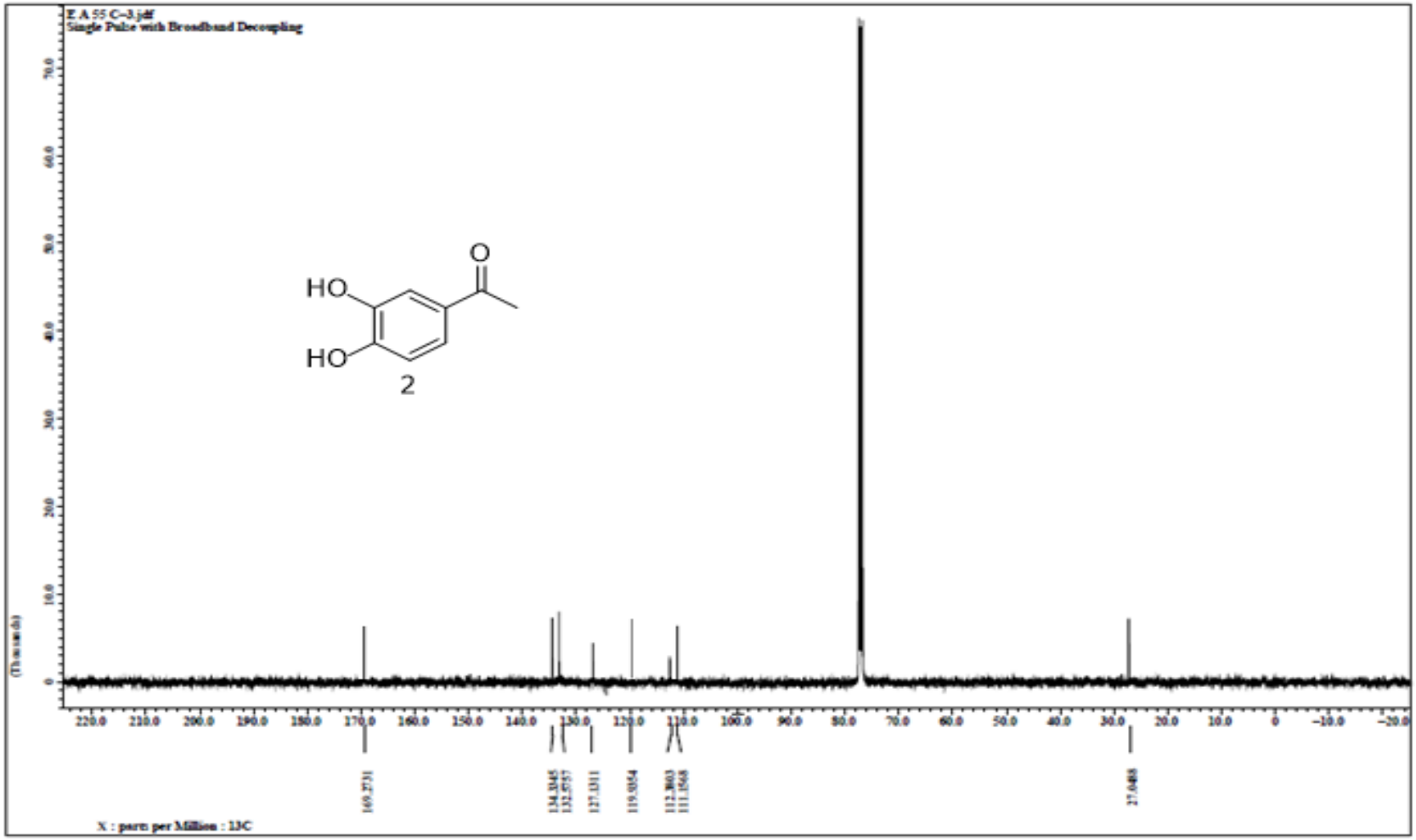
APPENDICES

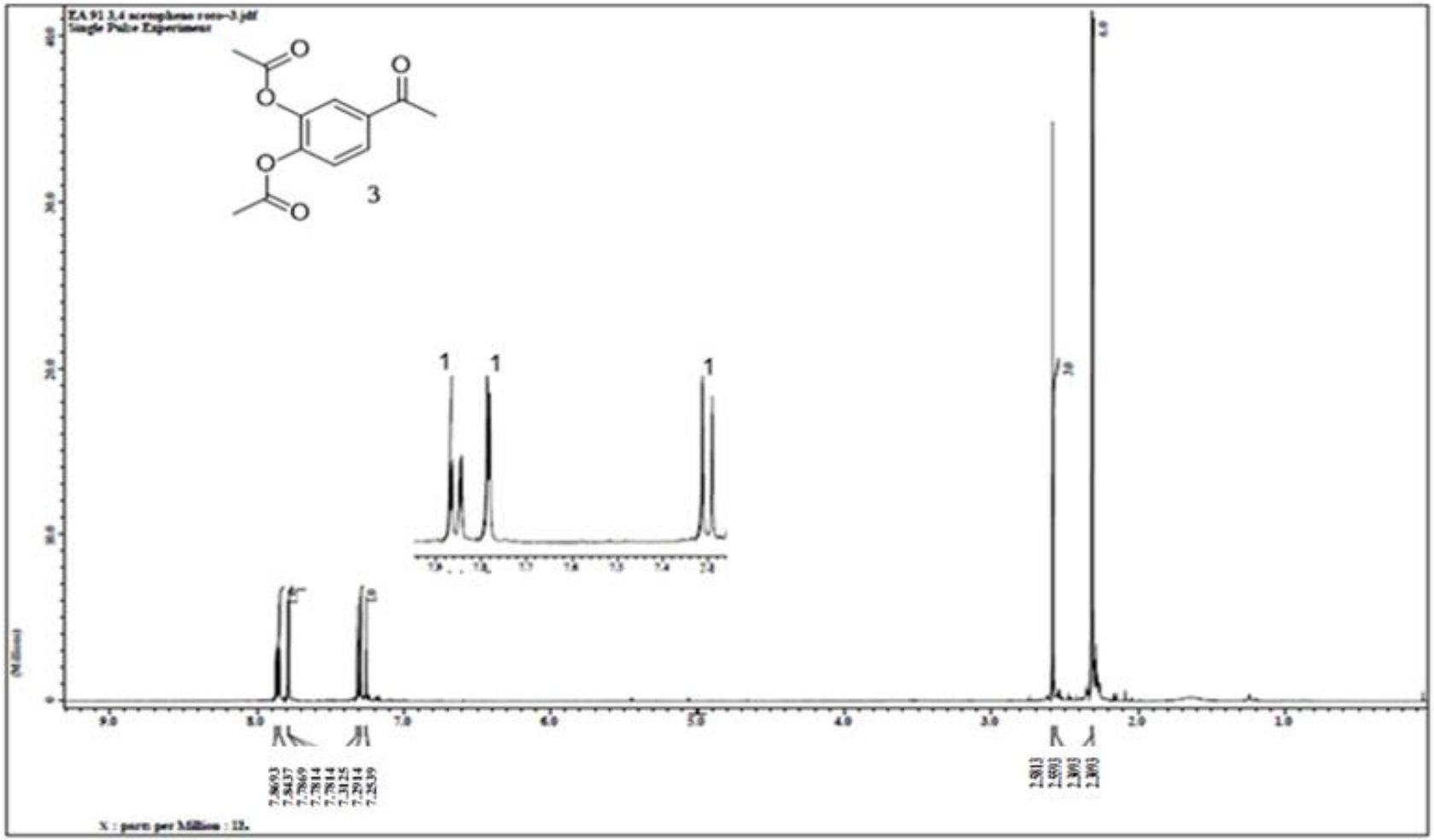
Appendix A1: ^{1}H NMR Spectrum for Compound **2** in $CDCl_3$



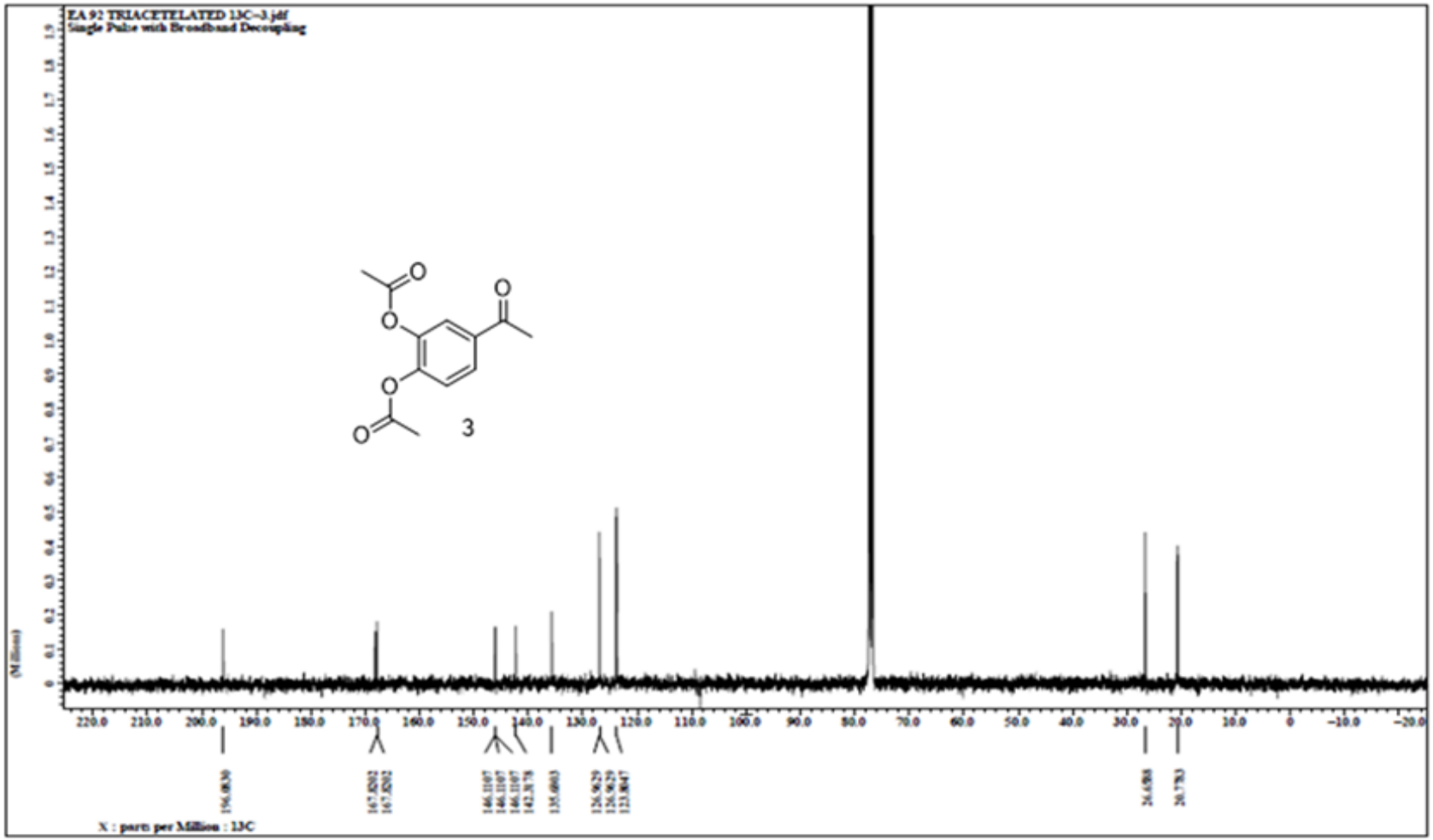
Appendix A2: ^{13}C NMR Spectrum for Compound **2** in $CDCl_3$



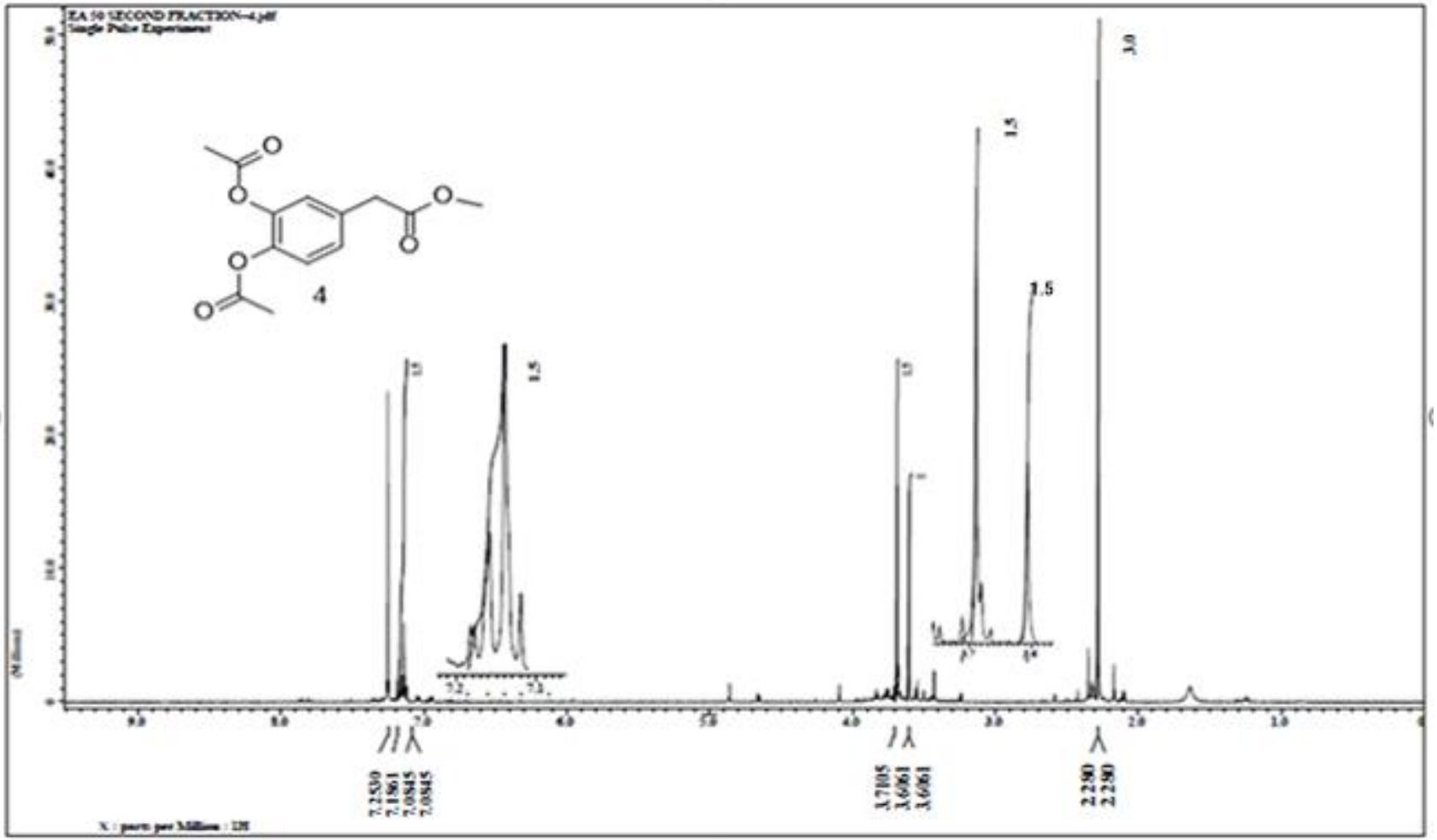
Appendix B1: ^{1}H NMR Spectrum for Compound **3** in $CDCl_3$



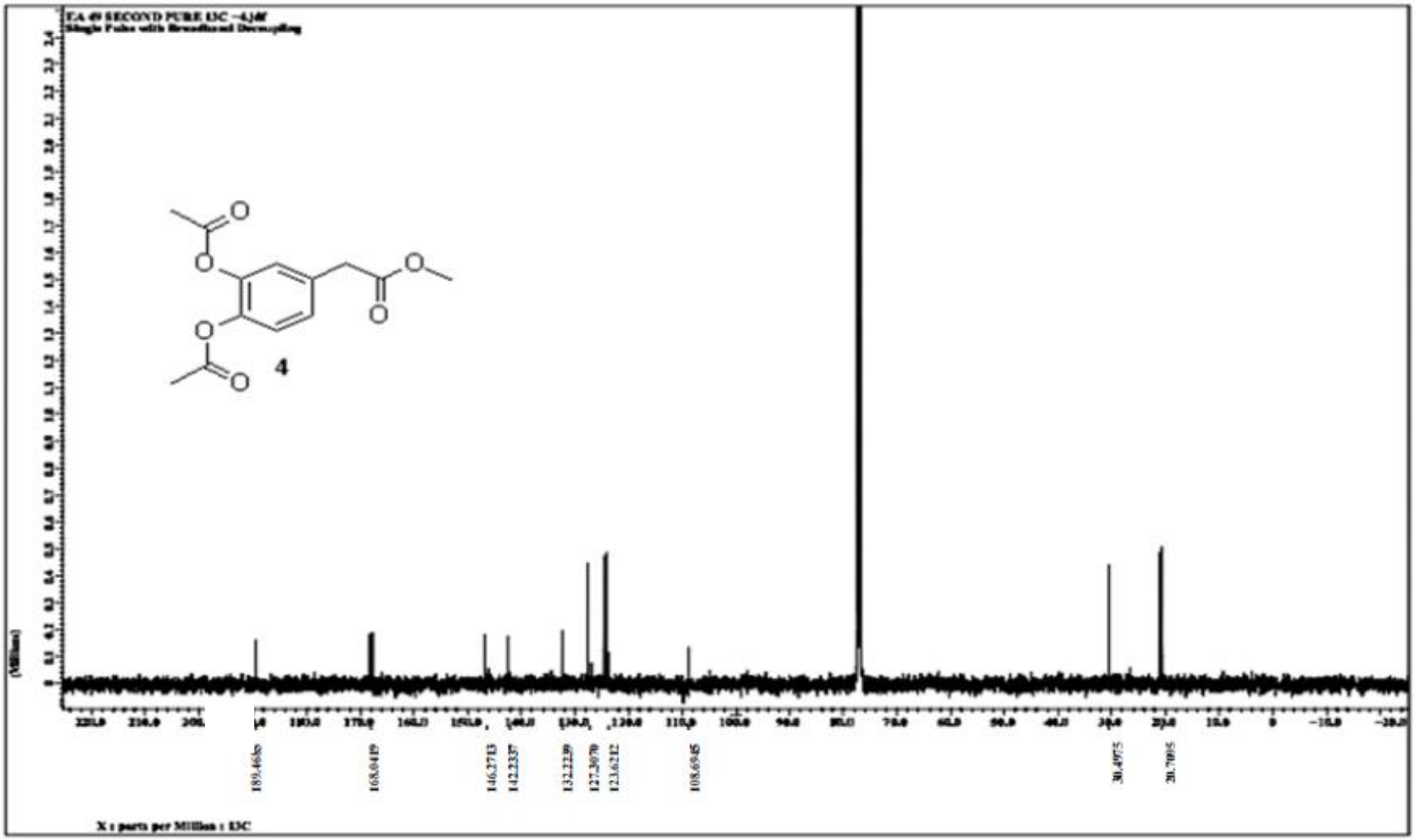
Appendix B2: ^{13}C NMR Spectrum for Compound **3** in $CDCl_3$



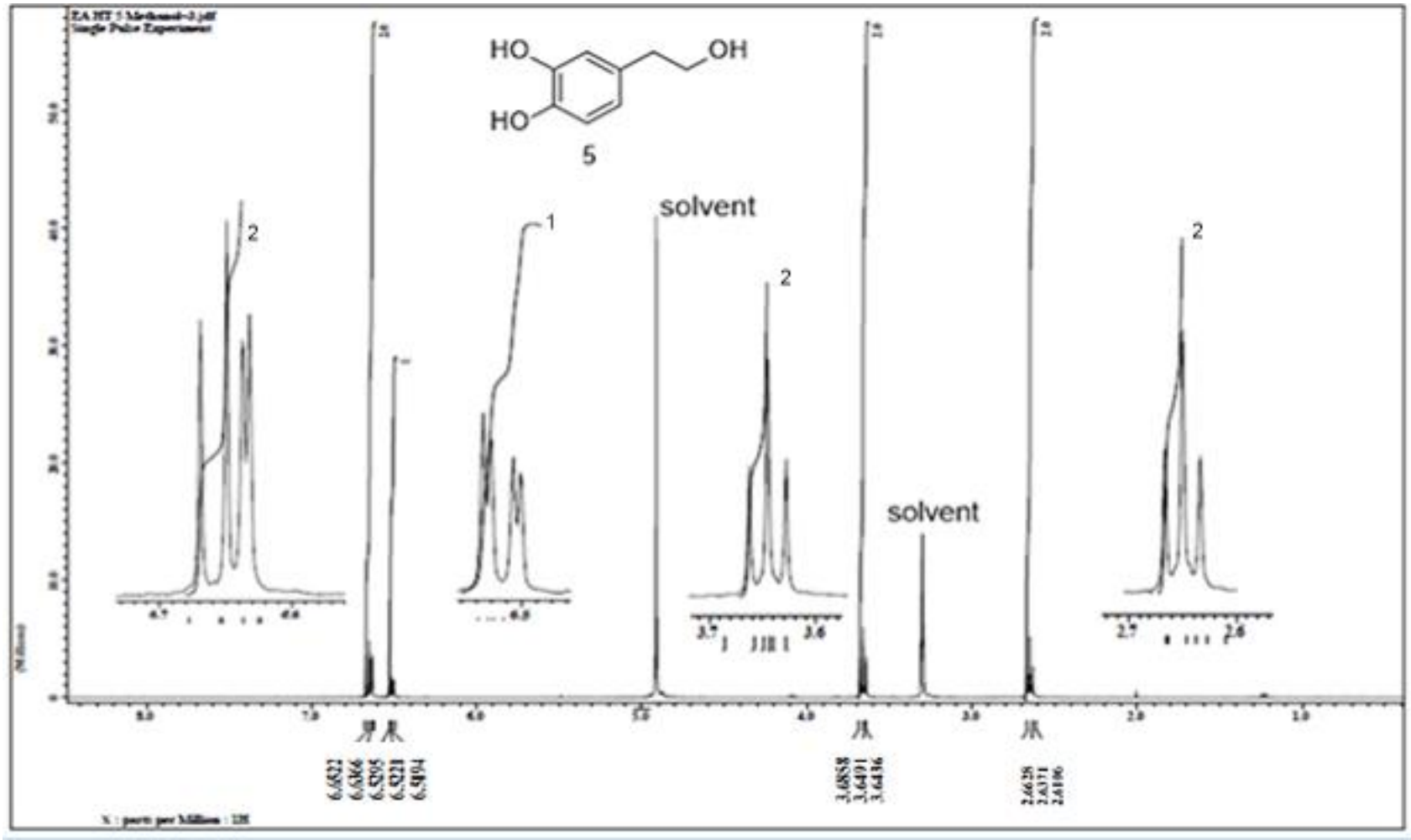
Appendix C1: ^{1}H NMR Spectrum for Compound **4** in $CDCl_3$



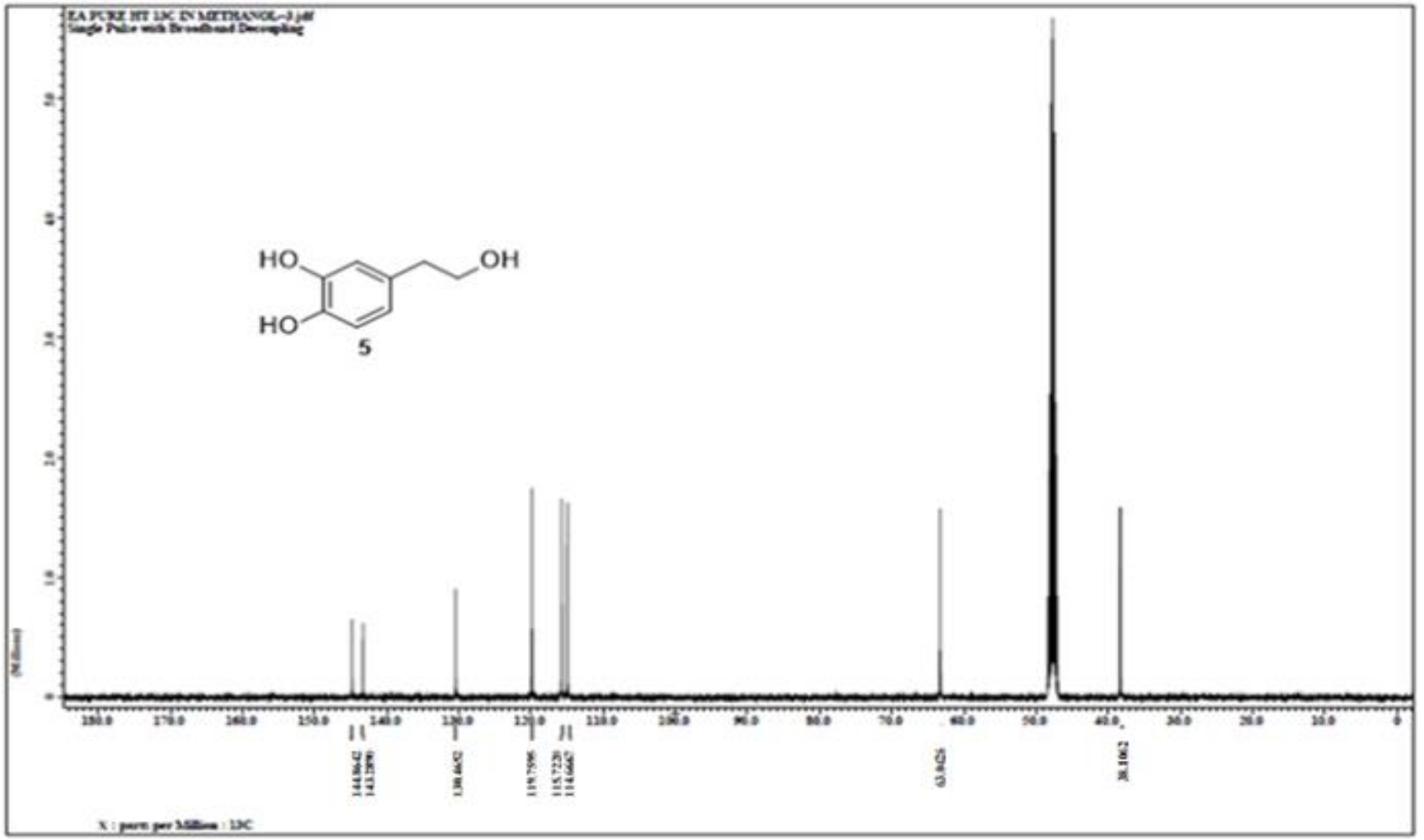
Appendix C2: ^{13}C NMR Spectrum for Compound **4** in $CDCl_3$



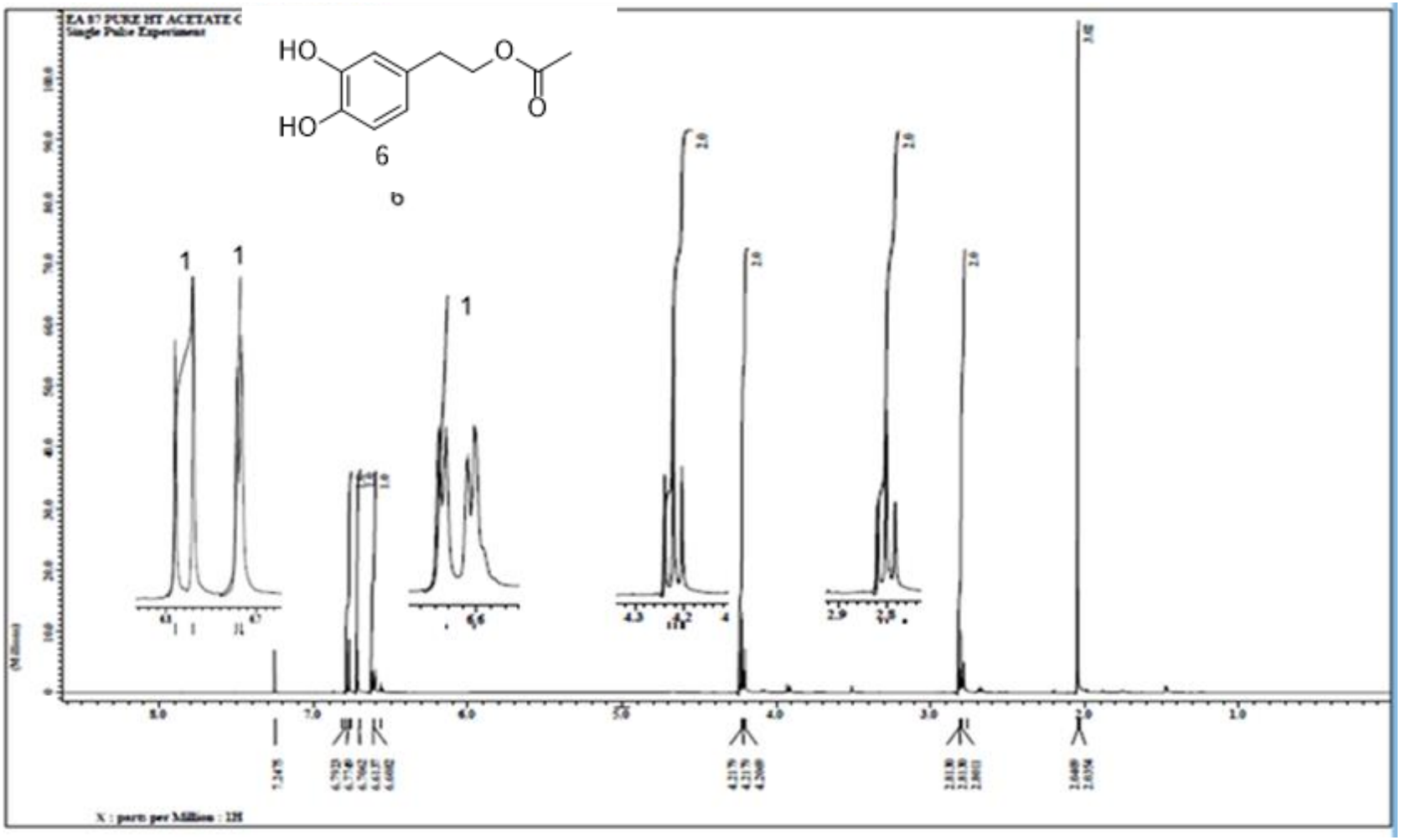
Appendix D1: ^{1}H NMR Spectrum for Compound **5** in (CD_3OD)



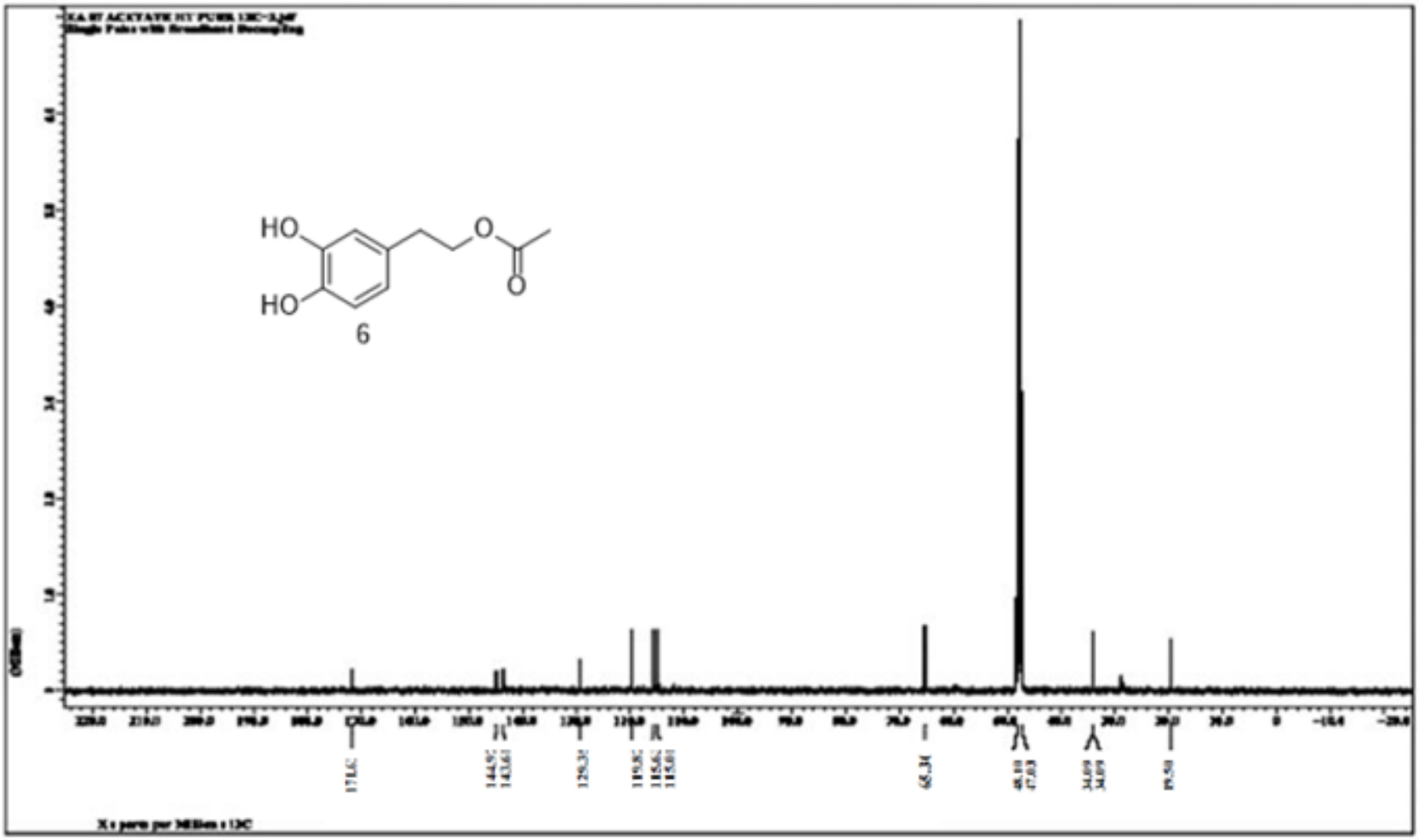
Appendix D2: ^{13}C NMR Spectrum for Compound **5** in (CD_3OD)



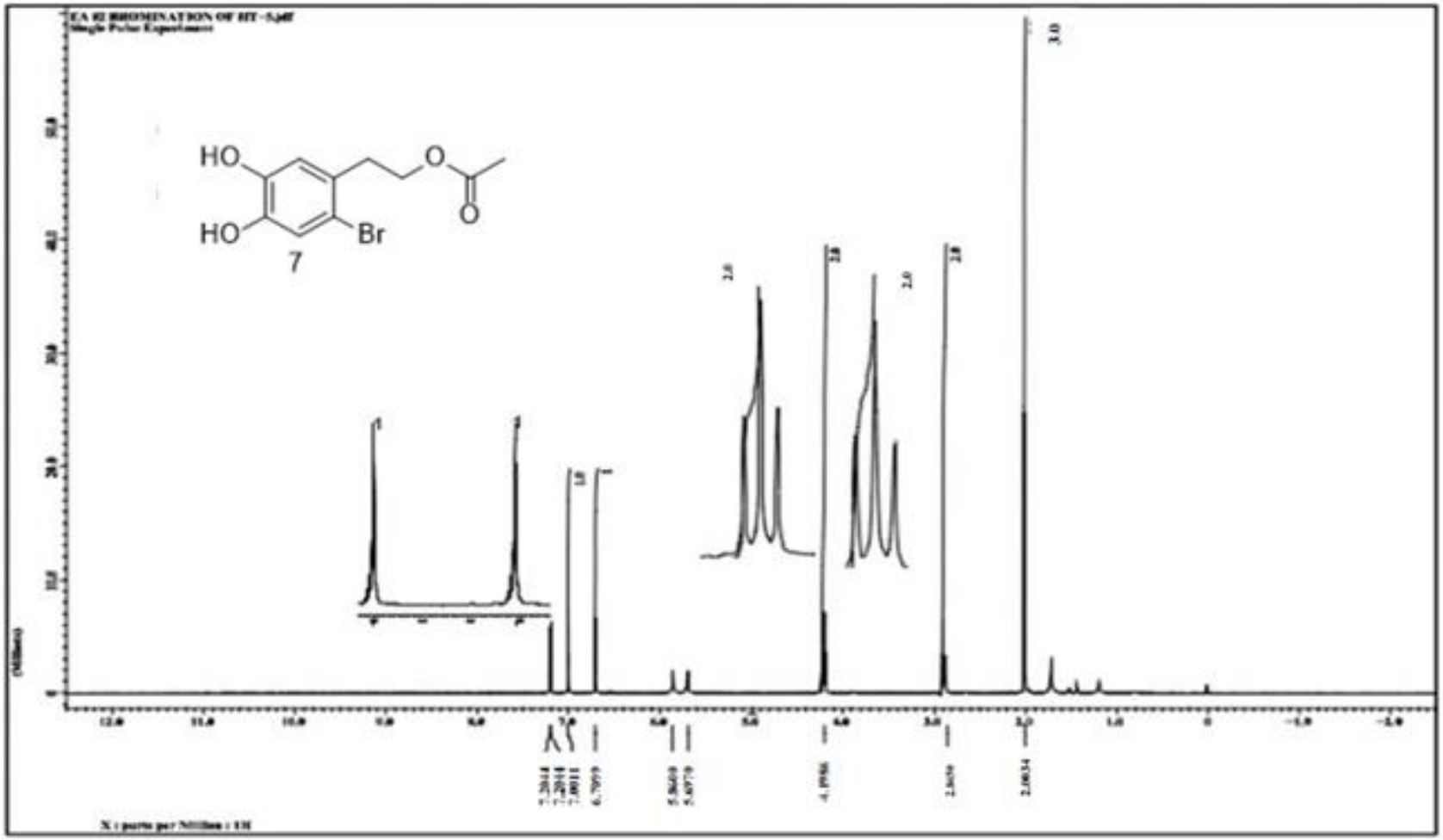
Appendix E1: ^{1}H NMR Spectrum for Compound **6** in $CDCl_3$



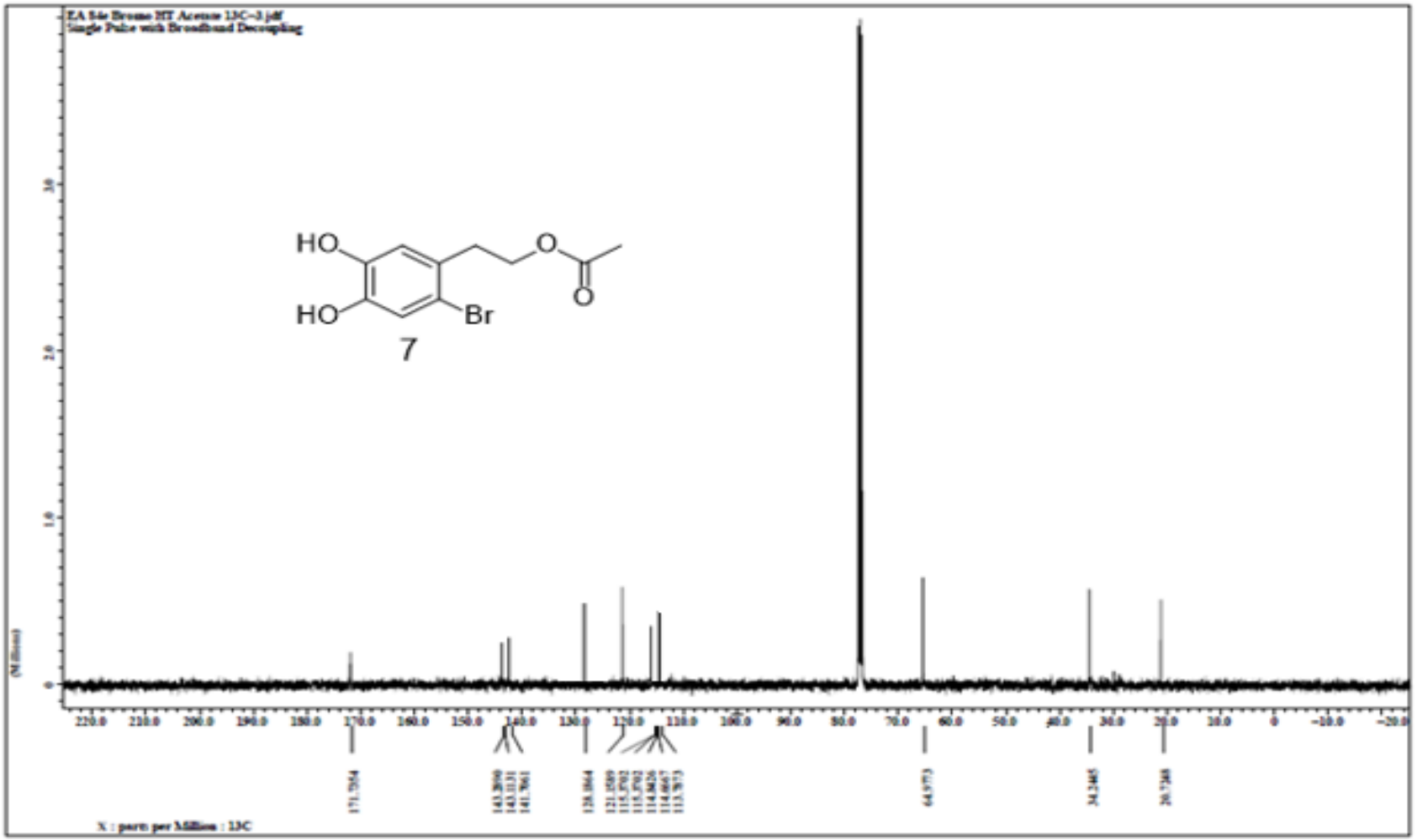
Appendix E2: ^{13}C NMR Spectrum for Compound **6** in $CDCl_3$



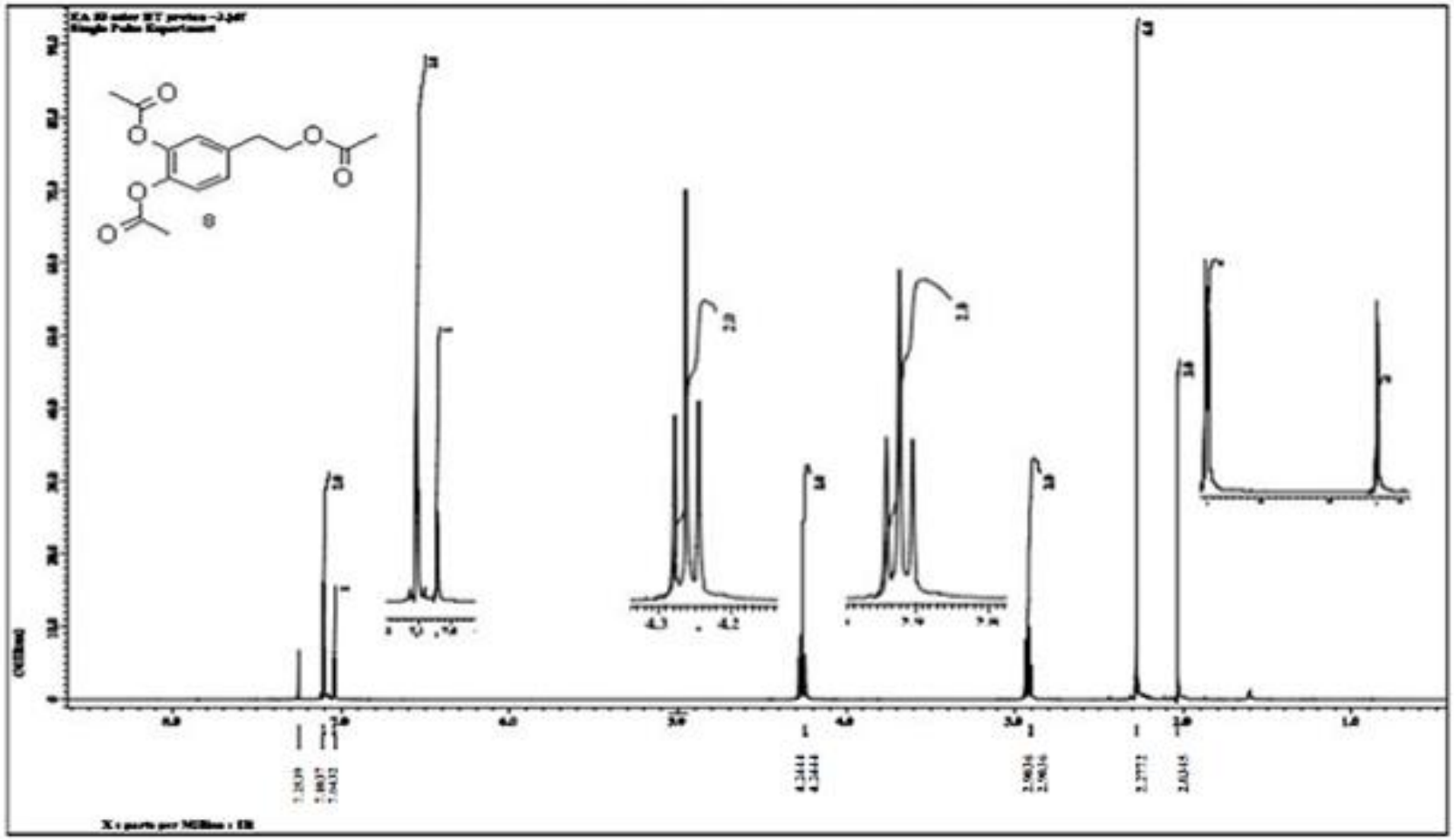
Appendix F1: ^{1}H NMR Spectrum for Compound **7** in $CDCl_3$



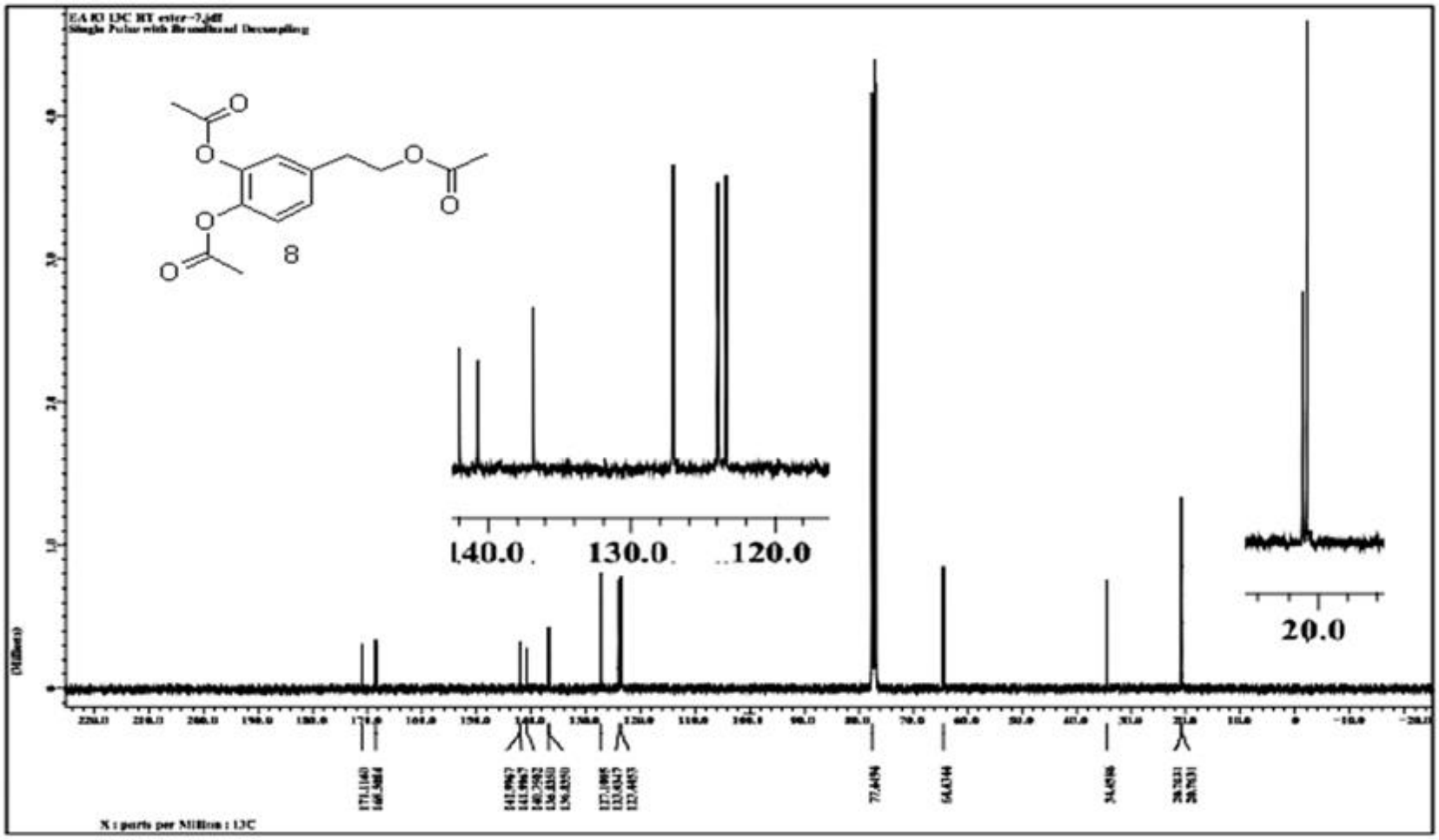
Appendix F2: ^{13}C NMR Spectrum for Compound **7** in $CDCl_3$



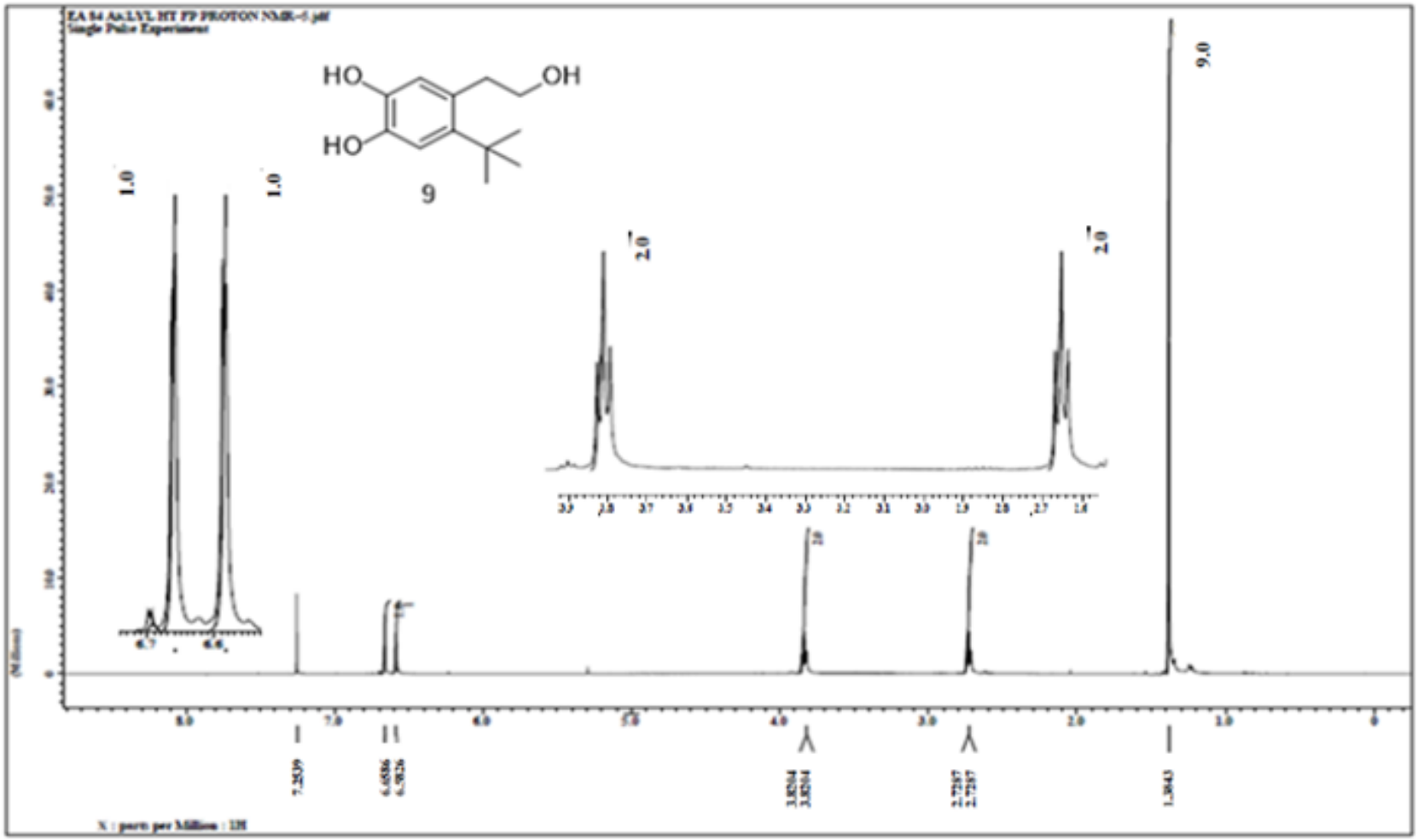
Appendix G1: ^{1}H NMR Spectrum for Compound **8** in $CDCl_3$



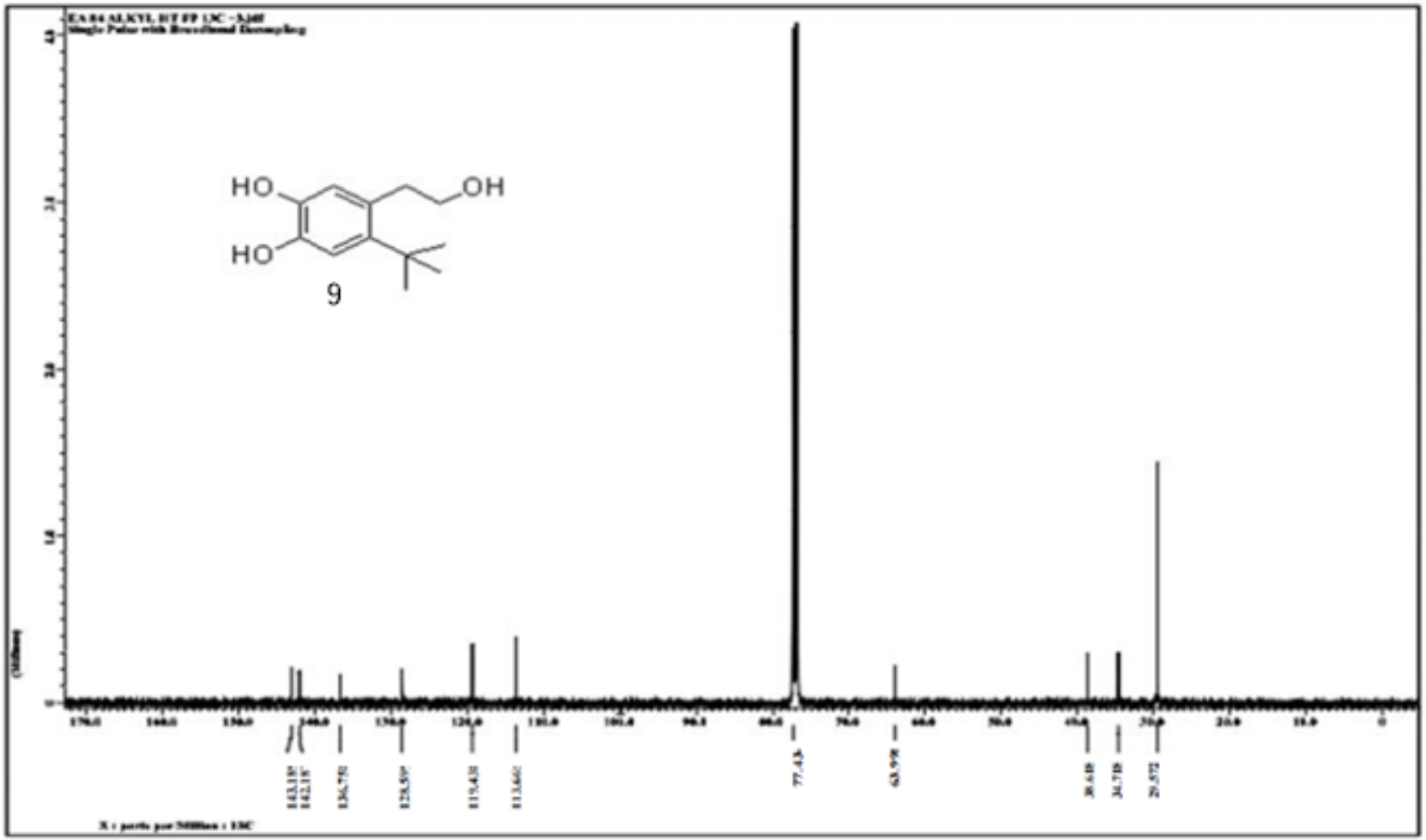
Appendix G2: ^{13}C NMR Spectrum for Compound **8** in $CDCl_3$



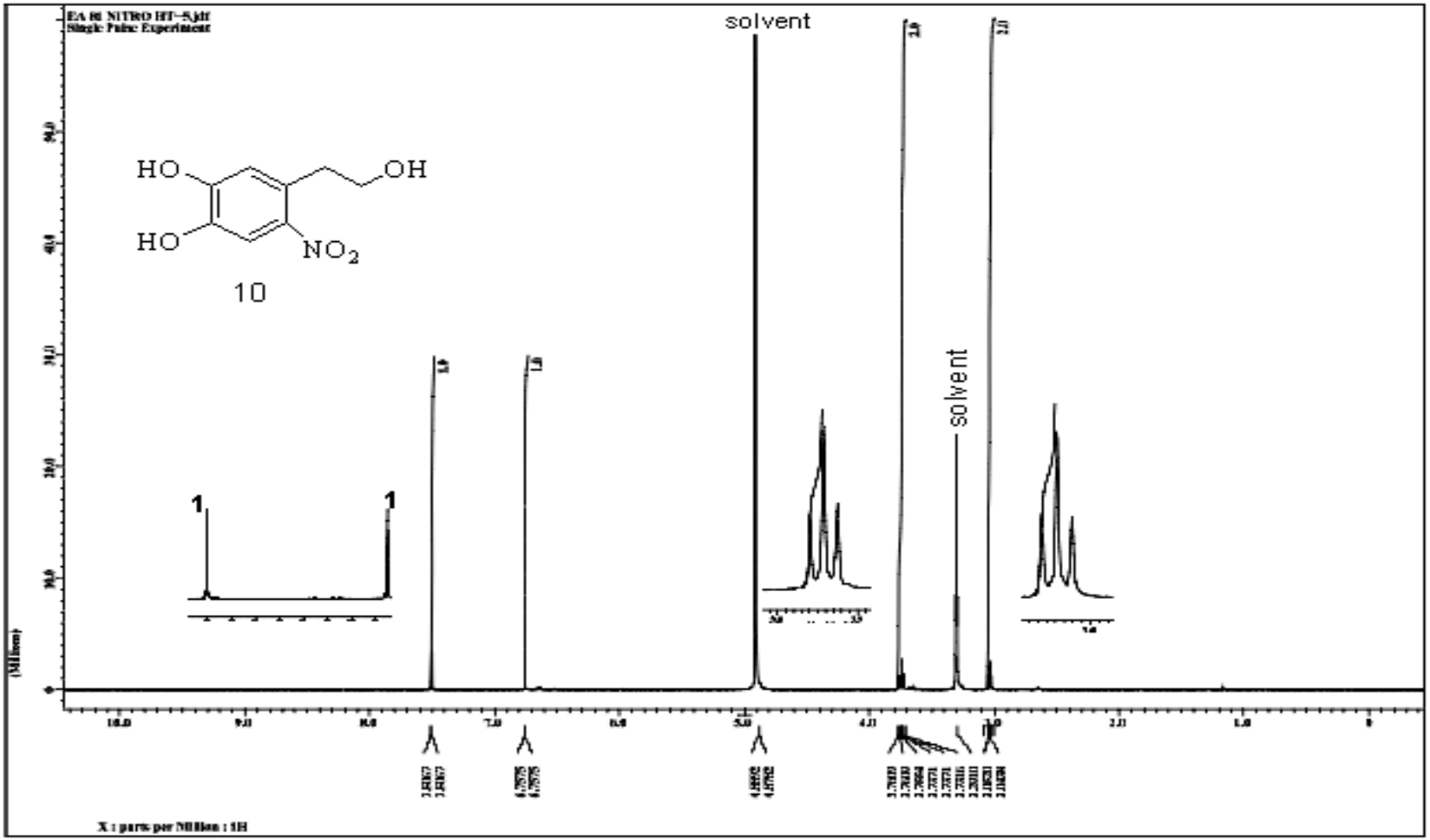
Appendix H1: ^{1}H NMR Spectrum for Compound **9** in $CDCl_3$



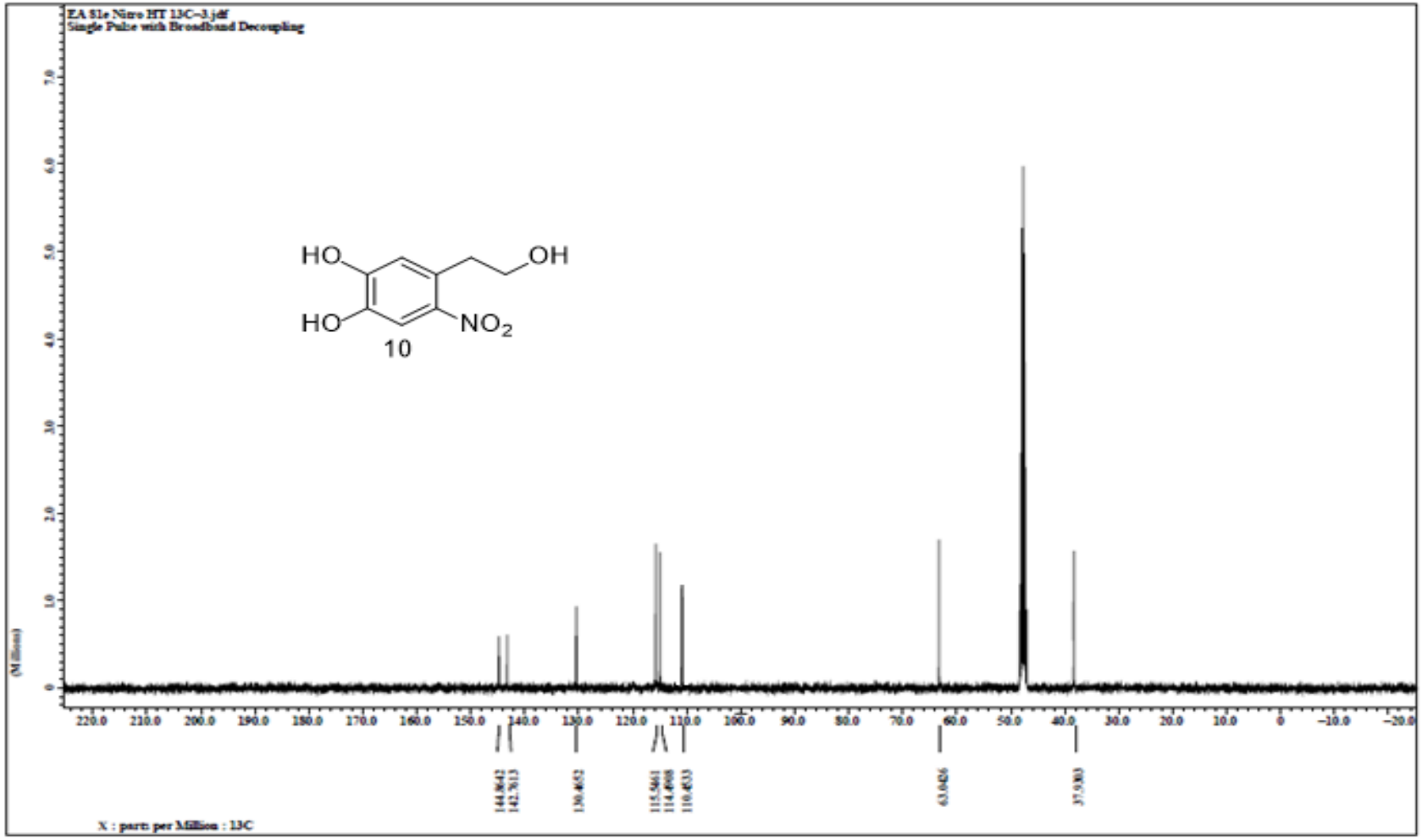
Appendix H2: ^{13}C NMR Spectrum for Compound **9** in $CDCl_3$



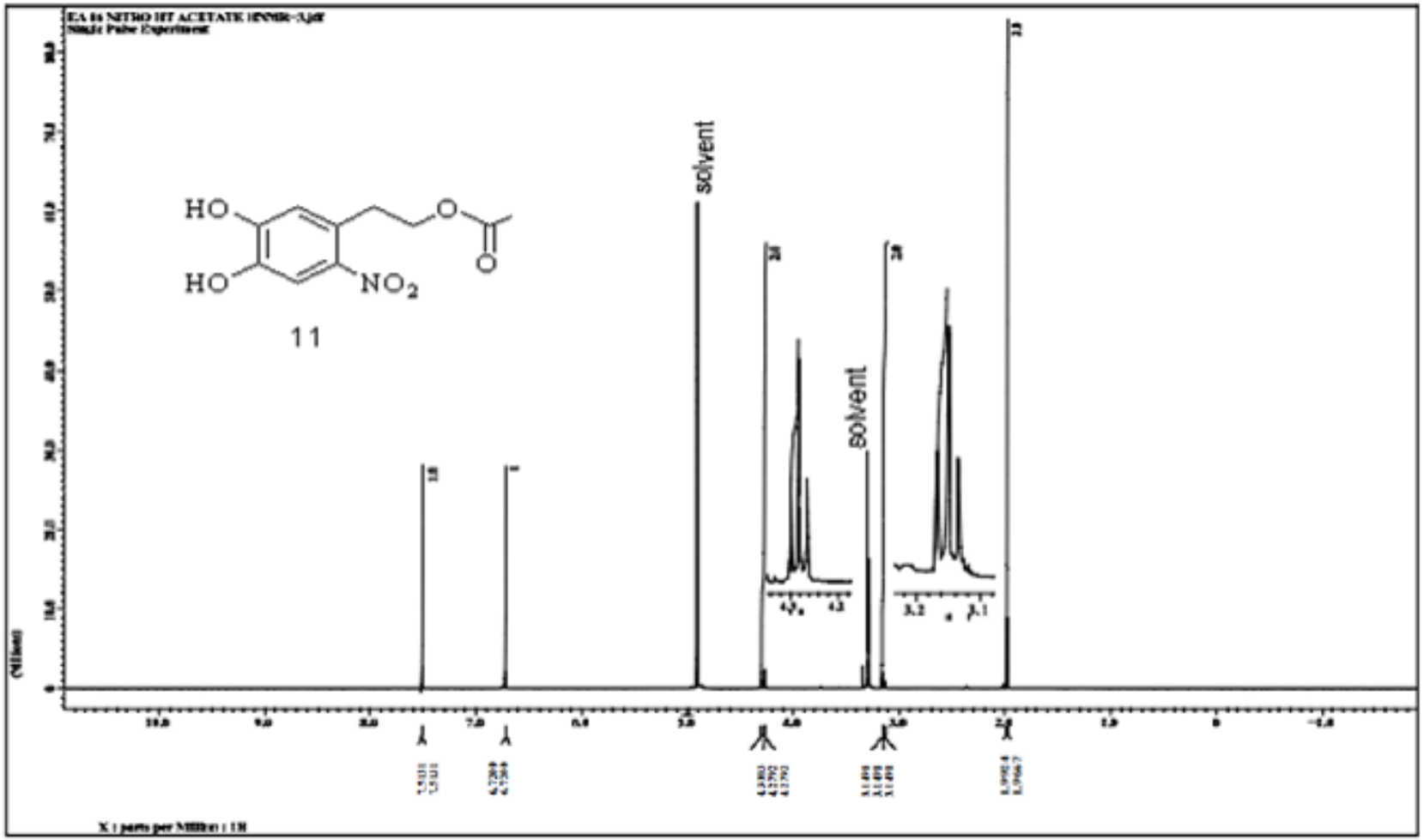
Appendix I1: ^{1}H NMR Spectrum for Compound **10** in (CD_3O_D)



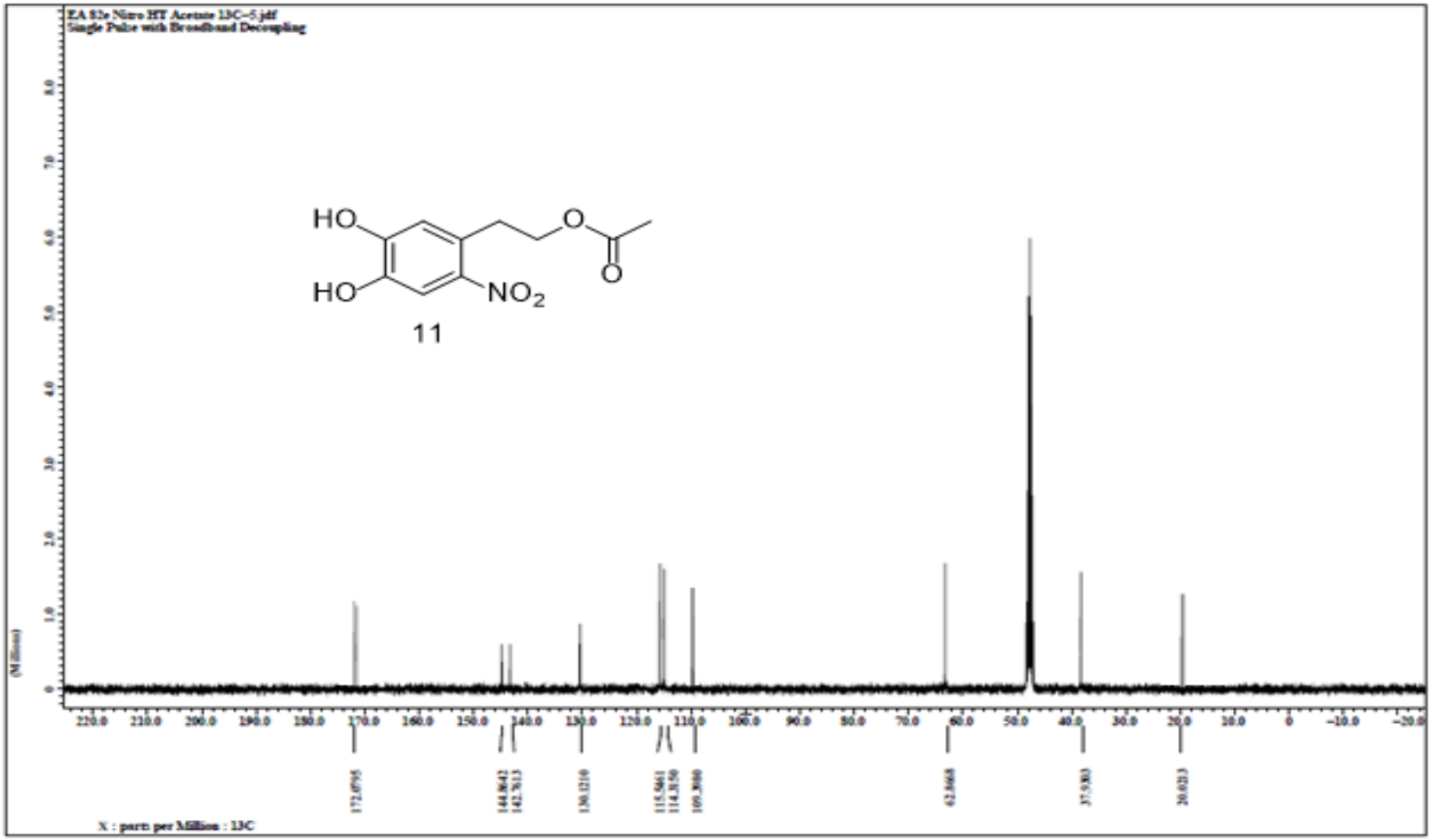
Appendix I2: ^{13}C NMR Spectrum for Compound **10** in (CD_3O_D).



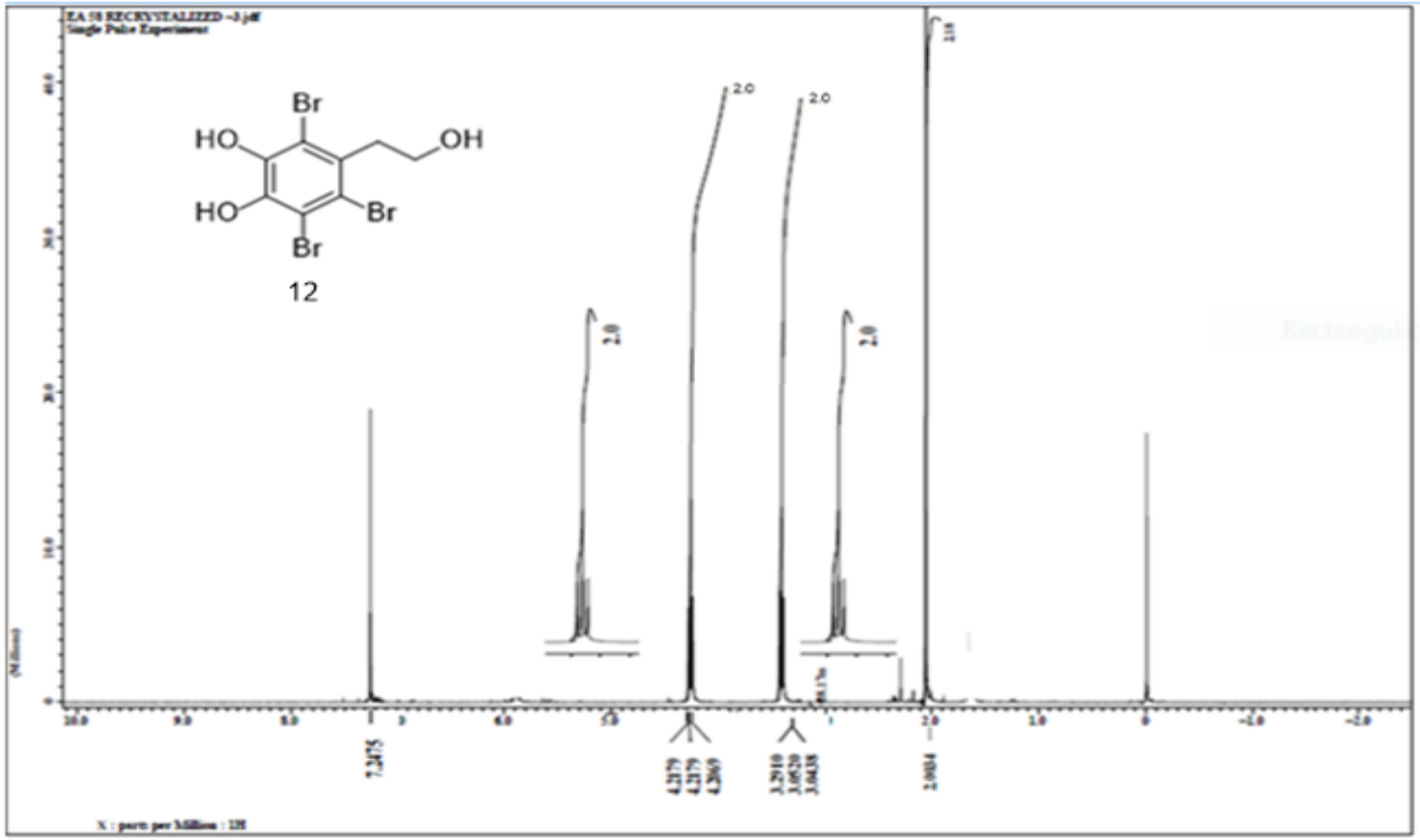
Appendix J1: ^{1}H NMR Spectrum for Compound **11** in (CD_3O_D)



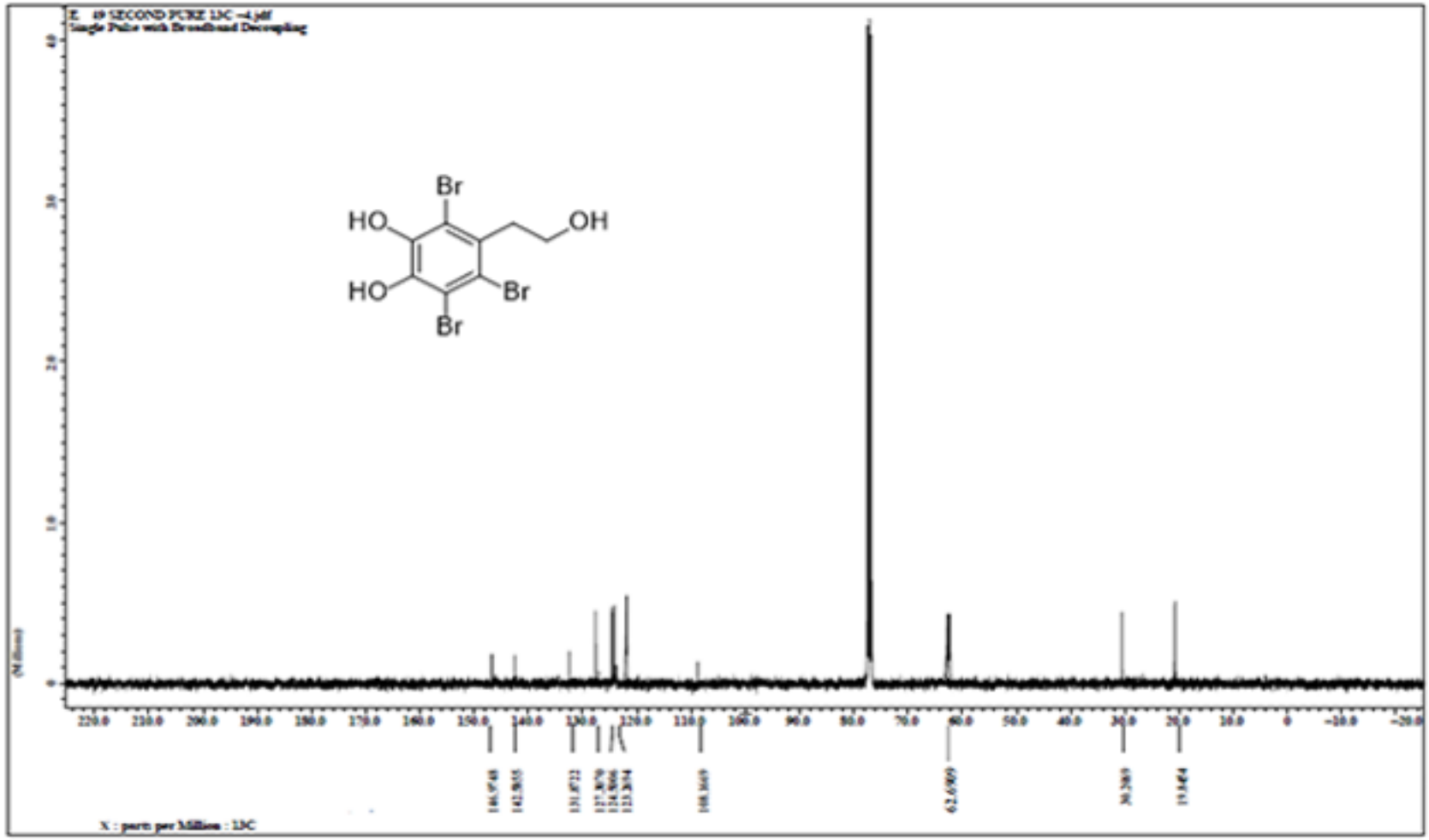
Appendix J2: ^{13}C NMR Spectrum for Compound **11** in (CD_3OD)



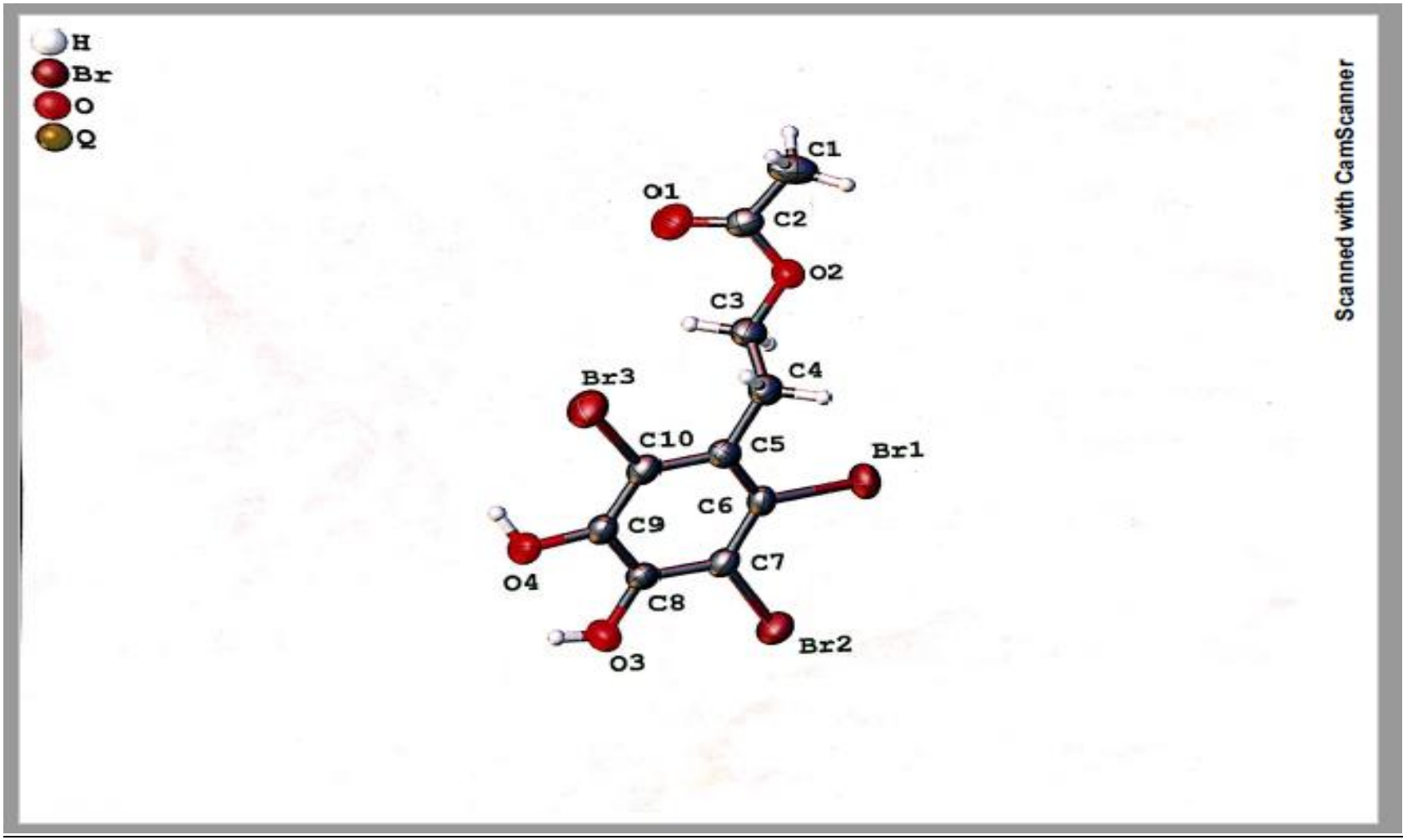
Appendix K1: ^{1}H NMR Spectrum for Compound **12** in $CDCl_3$



Appendix K2: ^{13}C NMR Spectrum for Compound **12** in $CDCl_3$



Appendix L: X-ray Crystallography for Compound **12**



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