PURIFICATION AND STRUCTURE ELUCIDATION OF BIOLOGICALLY ACTIVE COMPOUNDS PRODUCED BY A *Trametes* spp (JO5066) IN BIOTECHNOLOGICAL CULTURES AGAINST *Aedes aegepti* AND SELECTED MICROORGANISMS

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented for examination in any other institution.

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SM11/1963/07

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Recommendation

This thesis has been submitted for examination with our approval as the university supervisors.

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DEDICATION

To my family, especially my mom, for their moral support throughout my education.
Thanks for believing in me and God bless you all.

ACKNOWLEDGEMENT

ABSTRACT

Fungi, especially higher ones, have received a lot of attention in the recent decades due to the advances of biotechnological techniques. This is because secondary metabolism in fungal biotechnological cultures is manipulable and produces an array of compounds with diverse as well as novel chemical and biological properties. The activities can be taped into to address the myriad problems afflicting local communities in Kenya especially in agrochemical and medical sectors. Disease burden in rural settings remain a major development challenge and there is need to develop appropriate technologies to provide solutions. Diseases and disease-causing agents are spreading very fast and this is complicated by the problems of resistance to drugs by the pathogens and the vectors. For example the mosquito vector is becoming increasingly resistant to both synthetic and natural insecticides. There is also increasing evidence of resistant to conventional antibiotics of pathogenic microbes that is stimulating the development of strategies that target new or unexploited fungal bio-resources. In this light a basidiomycete, *Trametes* species (JO5066) was cultured in submerged cultures and the secondary metabolites extracted using conventional chemistry techniques like solvent-solvent extraction and liquid-solid adsorption techniques. Crude extracts were found to be actually active against larvae, culture filtrate 30-50% at 50 ppm and mycelium extract 30-70% at 500 ppm. The crude extracts were then fractionated, guided by mosquito larvicidal and antimicrobial activity. Larvicidal and antimicrobial assays were also carried out for the crude extracts and the purified compounds. The chemical structures of the purified compounds were elucidated using standard spectroscopic techniques: 1D (^{1}H; ^{13}C) and 2D (HSQC, COSY, NOESY, HMBC; DEPT) NMR experiments, assignments. Based on the NMR experiments two compounds, 2,3-dihydro-3,3-dimethyl-1H-indene-1,2,6-triol (compound **29**) and *p*-tolylmethanol (compound **30**) were elucidated. The larvicidal assay for 2,3-dihydro-3,3-dimethyl-1H-indene-1,2,6-triol was done and LC_{50} and LC_{90} values were calculated and found to be 235 ppm and 674 ppm respectively. The larvicidal assay for *p*-tolylmethanol was not done due to low yield. The study has demonstrated that secondary metabolites from the basidiomycete (J05066) can be used as mosquito larvicidal compounds and consequently help in controlling mosquito borne diseases.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS ABBREVIATIONS

^{13}C	Carbon 13 isotope
^{1}H	Hydrogen 1 isotope
CC	Column chromatography
COSY	Correlated Spectroscopy
DDT	Dichlorodiphenyltrichloroethane
DEPT	Distortion Enhancement by Polarization Transfer
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Correlation
Kex	Crude extracts from culture filtrate (Extra-cellular secondary metabolites)
LC_{50}	Lethal concentration that kills 50% of mosquito larvae
LC_{90}	Lethal concentration that kills 90% of mosquito larvae
Mex	Crude extracts from mycelium (Intra-cellular secondary metabolites)
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
TMS	Tetramethylsilane

CHAPTER ONE
INTRODUCTION

1.1 Background information

1.1.1 History of antibiotics

Microorganisms have been traditionally used to produce a variety of important substances for the pharmaceutical and food industries. Hence, primary and secondary metabolites, such as peptides, enzymes, organic acids and antibiotics produced by filamentous fungi are used for these purposes (Bennett, 1998; Demain, 2000). The discovery and development of antibiotics was one of the most significant advances in medicine in the 20th century. Unfortunately, most of them, particularly those for treating several of human infectious diseases are now ineffective. Therefore, to ensure effective drugs availability in the future, it is necessary to improve the antimicrobial use patterns and devise strategies to identify new antibiotics from previously unexplored sources (Smith and Jarvis, 1999).

During the past 50 years, several major advancements in medicine have come from lower organisms such as molds, yeast, and mushrooms (fungi). The first antibiotics: tetracycline and aureomycin were extracted from molds. These were hailed as wonder drugs for infections and communicable diseases. Back in 1928, Alexander Fleming began the microbial drug era when he discovered in a Petri dish seeded with *Staphylococcus aureus* that a compound produced by a mold killed the bacteria. The mold, identified as *Penicillium notatum*, produced an active agent that was named penicillin (Fleming, 1929; Demain and Sanchez, 2009). Penicillin has an interesting mode of action: it prevents the cross-linking of small peptide chains in peptidoglycan, the main wall polymer of bacteria. Pre-existing cells are unaffected, but all newly produced cells grow abnormally, unable to maintain their wall rigidity, and they are susceptible to osmotic lysis. A discovery was made that from natural penicillin **(1)**, semi-synthetic penicillins such as carbenicillin **(2)** and ampicillin **(3)** with various properties could be manufactured. The semi synthetic penicillins have various specific properties: resistance to stomach acids so that they can be taken orally, a degree of resistance to penicillinase (a penicillin-destroying enzyme produced by some bacteria) and extended range of activity against some Gram-negative bacteria. It is still a "front line" antibiotic, in common use for some bacterial infections although the development of penicillin-resistance in several pathogenic bacteria now limits its effectiveness.

Carbenicillin, R=
(2)

Ampicillin, R=
(3)
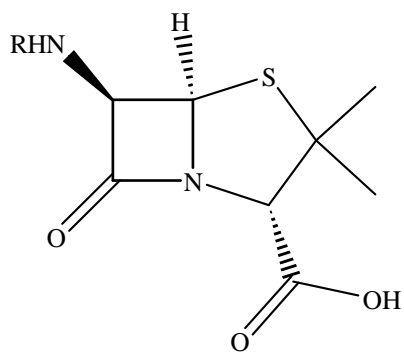
Basic penicillin structure
(1)
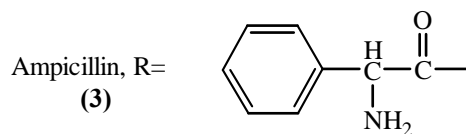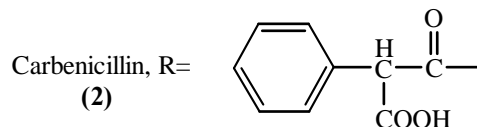
6-Aminopenicillanic acid, R=H
(4)

After the success of penicillin, drug companies and research groups progressively assembled large microorganism culture collections to try and discover additional antibiotics. The result was the discovery of antibiotics such as chloramphenicol and chlortetracycline (Mann *et al.*, 1999). All of these compounds, or derivatives thereof, are still in use as drugs today.
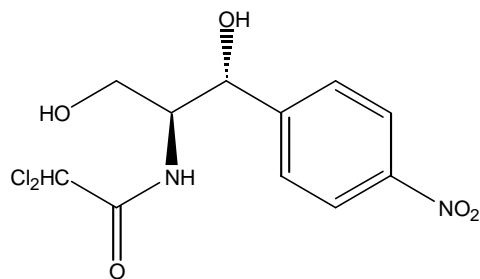
Chloramphenicol
(5)
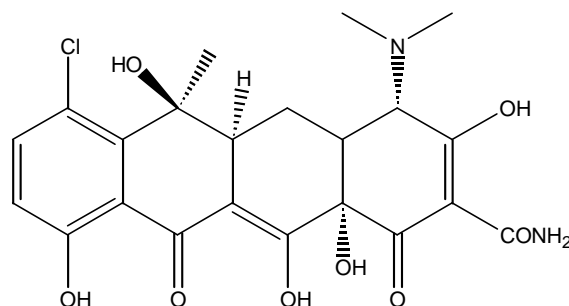
Chlortetracycline
(6)

Following the antibiotics era, in the latter part of 20th century, scientists isolated from fungi many more products important in agriculture, industry and medicine. However, emphasis started slowly shifting towards other groups of microbes, such as bacteria including actinomycetes. In the 21st century, there are reasons to believe that fungi can again occupy the center stage in the search for novel chemicals useful in industry. Fungi are known to produce a vast array of secondary metabolites that have varied biological activity. In the last decades, the wide range of pharmaceutically interesting metabolites from basidiomycetes, a large group of terrestrial fungi of the phylum basidiomycota, has been one of the most attractive groups of natural products studied (Boh *et al.*, 2003).

There has been a rapid pace of advancement in organ transplant due to cyclosporin, a drug derived from a fungus that uses insects as its host. Cyclosporin suppresses the immune system of transplant patients hence lowering tissue rejection rates. Lower organisms are used to commercially produce bread, beer, wine, cheese, organic acids, and vitamins ó including vitamin C. Basidiomycetes produce a series of biologically active compounds when grown in pure culture. They provide a rich and varied source of terpenoids that have antibiotic and antifungal properties (Shittu *et al.,* 2006).

Fungi present a universal characteristic which is its inability of doing photosynthesis; hence they are completely heterotrophic and must acquire their nutrients from the environment, from living, dying, or dead organisms. They can attack many different hosts so virulently that they kill the hosts and then absorb the released nutrients (Mauseth, 2003). In addition, some fungi are able to develop a symbiotic relationship with their hosts varying from mutualism to parasitism (Schardl *et al*., 2004).

Fungi and animals are more closely related to one another than either is to plants, diverging from plants more than 460 million years ago (Redecker *et al*., 2000). Diseases of plants typically do not afflict humans whereas diseases of fungi do (Martin, 2001). Since humans (animals) and fungi share common microbial antagonists such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa,* humans can benefit from the natural defensive strategies of fungi that produce antibiotics to fight infection from microorganisms. Hence, it is not surprising that most significant anti-bacterial antibiotics have been derived from fungi (Chihara, 1992).

The genus *Trametes* is among the approximated 100 genera of the family Polyporaceae, sub-division Polyporales in the division Basidiomycetes of the Fungal Kingdom. All members of the genera have pores of some sort on their underside. These pores can be very small, 10 per millimeter, or much larger, up to 2 mm per pore. In all cases the pores serve to increase the surface area for bearing the spores. The polypores are a fascinating group of fungi, although they are usually ignored by most mycophiles because of their typical inedibility, commonly small size, unfamiliar habitat and general obscurity. However, these fungi are very interesting from an ecological, microscopic, and biotechnological standpoint, and their microscopic features, are well worth observing. Unlike fleshy mushrooms, most of these fungi can be found even during dry weather or in the winter, since many are tough or perennial and many other produce

basidiocarps only beneath the surface of logs lying on the forest floor, where it remains wet most of the year (Hafiz *et al*., 2007).

The polypores (and corticioid fungi - those flat crusty fungi found on the underside of logs and sticks) are important in natural ecosystems as decomposers of wood, recycling the nutrients and minerals in the wood and releasing them over a long period of time - sometimes several hundred years from a single large down tree - where they can be used by other forest organisms (Zjawiony, 2004). In addition, some of these fungi are highly valued by biotechnologists because of their wood-degrading (and especially lignin degrading) abilities. *Trametes versicolor*, along with *Phanerochaete chrysosporium* is one of the fungi that have been investigated for possible use in biopulping (Levin *et al.,* 2008). All these species cause a white rot of wood. That is, the fungus decays the lignin and not the cellulose. Also some fungi cause a brown rot, which digest the cellulose and leaving the lignin.

1.1.2   The mosquito menace

Mosquitoes are responsible for the spread of more diseases than any other group of arthropods. They are known vectors of several disease-causing pathogens, which affect many millions of people all over the world. *Aedes aegypti* is known to carry dengue, yellow fever and *Chikungunya*; malaria is carried by *Anopheles sp*; and filarial disease by *Culex quinquefasciatus*. To prevent mosquito-borne diseases and improve public health, it is necessary to control them. But in the past, mosquito control programmes have been suffering from failures because of the ever-increasing insecticide resistance (Georghiou and Lagunes-tejeda, 1991; WHO, 1992).

Mosquito-borne diseases have an economic impact, including loss in commercial and labor outputs, particularly in countries with tropical and subtropical climates. However, no part of the world (both the tropics and non-tropics) is free from vector-borne diseases (Fradin and Day, 2002). One main method for the control of mosquito-borne diseases is the use of insecticides, and many synthetic agents have been developed and employed in the field with considerable success. However, most of the insecticides used to control mosquitoes are synthetic which affect the non-target population and also the mosquitoes are constantly developing resistance. Because of the acquired enhanced resistance of mosquito populations to conventional insecticides, the demand for the development of new products has emerged (Phillips**,** 2001). Synthetic insecticides have created a number of ecological problems, such as the development of

resistant insect strains, ecological imbalance, and harm to mammals. Hence, there is a constant need for developing biologically active natural materials as larvicides, which are expected to reduce the hazards to human and other organisms by minimizing the accumulation of harmful residues in the environment. Natural products are generally preferred because of their less harmful nature to non-target organisms and due to their innate biodegradability (Rahuman *et al*., 2008). In this regard, the development of techniques that would provide more efficient insect control and not have any ill effects on the non-target population, and are easily degradable are always sought (Redwane *et al*., 2002).

The discovery of DDT's insecticidal properties in 1939 and the subsequent development of organochlorine and organophosphate insecticides suppressed natural product research since the answers to insect control were thought to have been found. However, since 1947, when the first incidence of DDT resistance was encountered, more than 100 mosquito species have been reported as resistant to one or more insecticides, which resulting in the withdrawal of numerous older insecticides and making the discovery of new ones more difficult and expensive. Also animals do not metabolize DDT very rapidly; instead it is deposited and stored in the fatty tissues. The biological half life of DDT is about eight years. If ingestion continues at a steady rate, DDT builds up within the animal overtime (Ndungu *et* al., 2003). Moreover, other factors, such as the increasing environmental concern, limited much more the number of insecticides that can be used in mosquito control programs (Hemingway and Ranson 2000; Hemingway *et al*., 2002). Thus, there is a pressing need of discovering and/or developing new and effective but environmentally safer insecticides.

Polypores are a major component of the basidiomycete fungi in forest ecosystems and as wood decayers and tree pathogens, they play important ecological roles. Polypore fungi are heterogeneous, showing a great variation in their macromorphological characteristics. Besides anatomical characteristics, biochemical and molecular phylogenetic studies have been used to characterize the families of polypores (Hibbett and Donoghue 1995). Studies have shown that only a small number of the most common species of polypores such as *Ganoderma lucidum* have been evaluated thoroughly for biological activity (Wagner *et al*., 2003). Consequently polypores have not been fully exploited as sources of bioactive compounds. A wood rotting basidiomycete that colonized a piece of wood was collected from Mt. Kenya forest in July 2005 and immediately brought into pure culture. The fungus was serialized JO5066 and has been

preserved in Integrated Biotechnology Research Laboratory (IBRL) at Egerton University as a herbarium material and pure culture. The morphological and discernable microscopic features confirmed that the fungus had clamp connections ó the characteristic distinction of the basidiomycetes. The basidiomycete has been further studied using 18S RNA (ITS technique) and has been found to belong to *Trametes* species. (JO5066) was cultivated in nutrient liquid media and was found to produce mosquito larvicidal compounds at 50-100 ppm by using the crude extracts. In a previous study carried out, it was noted that the organic compounds of middle polarity are responsible for the activity. The compounds required further purification and further spectroscopic experiments to determine their chemical structures. It was interesting to find the compounds that are responsible for mosquito larvicidal and antimicrobial activity from cultures of this basidiomycete since free living insect larvae have been reported to feed on ageing fruiting bodies of *Trametes* species (Härkönen *et al*., 2003).

1.2 Statement of the problem

Antimicrobial resistance is currently the greatest challenge to the effective treatment of infections globally. There is also the resistance of mosquitoes to the synthetic and non-synthetic insecticides in the market, which poses a challenge to the control of mosquito borne diseases. Because of the emergence of resistance to synthetic and non-synthetic insecticides and antibiotics, the research and development of natural larvicidal products and antibiotics is worthwhile pursuing. Therefore, there is need to search for new natural and unexplored sources of larvicides and antibiotics.

1.3 Objectives

1.3.1 Main objective:

To culture, purify and structure elucidate biologically active compounds produced by a *Trametes* species (JO5066) in biotechnological cultures.

1.3.2 Specific objectives:

1. To culture the *Trametes* species JO5066 in submerged cultures for production of bioactive compounds.
2. To prepare crude extracts from the cultures of the *Trametes* species (JO5066).
3. To screen the crude extracts for larvicidal and antimicrobial activity.

4. To perform bioassay guided larvicidal activity and antimicrobial activity guided purification of the responsible compound(s) using chromatographic techniques.

5. To elucidate the structure(s) of the purified compound(s) using modern spectroscopic techniques.

1.4   Justification

Microbes have developed antibiotic resistance mechanisms and therefore, it is not surprising that they have become resistant to most of the natural antimicrobial agents that have been developed over the past 50 years. This resistance increasingly limits the effectiveness of current antimicrobial drugs and hence the need to search for new unexplored natural sources like tropical fungi. In spite of the continuous use of synthetic pesticides, mosquito populations have not been reduced significantly. The chemicals that have been traditionally used to eradicate mosquitoes are faced with sanctions, at times withdrawal and ultimate ban. In addition the chemicals are very expensive and given the bulk required, are not within reach of the weak economics like that of Kenya let alone the majority resource poor households. In the light of these and other attendant factors, there is a pressing need to re-evaluate the role of natural mosquito larvicidal from fungi using biotechnological techniques. It is in this regard that this research is justified to come up with new and novel compounds having antimicrobial and mosquito larvicidal compounds that can address the dearth of antibiotics and mosquito vector control agents.

CHAPTER TWO
LITERATURE REVIEW

2.1   Secondary metabolites

Plants, insects, microorganisms and marine organisms exhibit complex interactions with the environment and produce active small molecules, secondary metabolites, useful for their survival. As a consequence of their biological role, these metabolites might exhibit a broad range of biological activities. In fact, secondary metabolites also called natural products, have played a fundamental role in drug discovery and development processes for a long time (Pupo *et al.*, 2006). In addition, the structural complexity of the natural products have inspired synthetic organic chemists and also provided useful research tools for the understanding of several biochemical pathways. However, one crucial aspect to be considered for a successful discovery of useful novel natural products is the selection of the source of the compounds to be investigated. It is important to take into account that untapped sources of biological diversity are often related to new chemical diversity (Clardy and Walsh, 2004).

Drugs of natural origin have been classified as (i) original natural products, (ii) products derived or chemically synthesized from natural products or (iii) synthetic products based on natural product structures. Evidence of the importance of natural products in the discovery of leads for the development of drugs for the treatment of human diseases is provided by the fact that close to half of the best selling pharmaceuticals in 1991 were either natural products or their derivatives (Cragg *et al*., 1997). In this regard, of the 25 top-selling drugs reported in 1997, 42% were natural products or their derivatives and of these, 67% were antibiotics. The structures of around 140 000 secondary metabolites have been elucidated. Of all the reported natural products, approximately 20⸱25% show biological activity, and of these approximately 10% have been obtained from microbes. Furthermore, from the 22, 500 biologically active compounds that have been obtained so far from microbes, 45% are produced by actinomycetes, 38% by fungi and 17% by unicellular bacteria (Berdy, 2005).

It has been reported that the species of basidomycetes on earth is estimated at 140,000 yet only 10% are known. In essence, pharmacological potentials of about 90% of mushrooms on earth are yet to be exploited. A large number of the unknown species of mushrooms whose health promoting properties are unknown reside in Africa. This is because there are little or no data about them. Most available data on these mushrooms are on their nutritional compositions

(Aletor, 1993; Alofe *et al.,* 1996*;* Ola and Oboh, 2001). Edible mushrooms are widely used as an exquisite type of food due to their pleasant taste and, most specially, due to their nutritional and medicinal properties. They contain very important proteins, carbohydrates and mineral salts, besides being a very good source of fibers. They are able to synthesize a great amount of secondary metabolites that present antitumoral, antiviral, anti-inflammatory, antithrombotic, cytostatic and hypoglycemic activities (Brizuela *et al.*, 1998; Wasser and Weiss*,* 1999; Suay *et al.*, 2000).

Among the sources of bioactive metabolites, less intensively investigated organisms like the higher fungi seem highly promising in terms of new structures with interesting biological activities. In recent decades, interesting compounds of different biogenetic origins have been isolated from basidiomycetes and were found to have antibacterial, antifungal, phytotoxic, nematicidal, cytostatic, antiviral, and other pharmacological activities. Basidiomycetes inhabit most climate zones, from arctic to tropical rain forest. Fruiting bodies can be collected from substrates like leaves, wood, dung, or soil (Aqueveque *et al*., 2006). Medicinal higher fungi such as *Cordyceps sinensis* and *Ganoderma lucidum* have been used as an alternative medicine remedy to promote longevity for people in China and other regions of the world since ancient times. There is an increasing public interest in the secondary metabolites of those higher fungi for discovering new drugs or lead compounds. Research in drug discovery from medicinal higher fungi involves a multifaceted approach combining mycological, biochemical, pharmacological, metabolic, biosynthetic and molecular techniques. Many new secondary metabolites from higher fungi have been isolated and are more likely to provide lead compounds for new drug discovery, which may include chemopreventive agents possessing the bioactivity of immunomodulatory, anticancer, etc. However, numerous challenges of secondary metabolites from higher fungi are encountered including bioseparation, identification, biosynthetic metabolism and screening model issues, etc. Commercial production of secondary metabolites from medicinal mushrooms is still limited mainly due to less information about secondary metabolism and its regulation (Zhong and Xiao, 2009).

2.2 *Trametes* species as producers of enzymes

Basidiomycetes inhabit most climate zones, from arctic to tropical rain forest. Fruiting bodies can be collected from substrates like leaves, wood, dung, or soil. They comprise very different ecological groups of white rot, brown rot, and leaf litter fungi. Some of them are edible

9

and/or medicinal fungi; some have important biotechnological and environmental applications (Songulashvili *et al.,* 2007). Lignocellulolytic basidiomycetous fungi are able to degrade a series of recalcitrant organic compounds, such as lignin and diverse classes of pollutants with little or no structural homology to lignin. The degradation of lignin and other recalcitrant compounds by basidiomycetes is a co-metabolic process and is mediated by the coordinated action of an enzymatic system and various low molecular mass metabolites (Renata *et al.*, 2008).

The white-rot fungi are responsible for the most extensive biodegradation of lignin (Wu *et al.*, 2005). They have a powerful extracellular enzymatic complex, able to depolymerize this aromatic polymer into lower molecular weight compounds (Bajpai, 2004). Metabolic activity and cell growth depend on environmental conditions and specific mechanisms of enzymatic regulation, which are very important for enzymatic production. Higher fungi are heterotrophic and sessile organisms that need to excrete digestive enzymes for absorption of organic materials as a way of surviving to environmental variations. These variations constitute one or more of a multitude of environmental signals that induce specific changes in growth and development patterns as a fungus response (Read, 1994). The ligninolytic enzyme complexes of white-rot fungi significantly differ in their composition. These fungi secrete one or more of three extracellular enzymes that are essential for lignin degradation. The potential application of ligninolytic enzymes in biotechnology has stimulated the investigation of their production with the purpose of selecting promising enzyme producers and increasing of their yield (Elisashvili *et al.*, 2001; Galhaup *et al.*, 2002; Moldes *et al.*, 2004; Mikiashvili *et al.*, 2004; Vikineswary *et al.*, 2006). In addition, the understanding of physiological mechanisms regulating enzyme synthesis in lignocelluloses bioconversion could be useful for improving the technological process of edible and medicinal mushroom production (Songulashvili *et al.*, 2007). *Trametes villosa* is a strain with a proven biotechnological potential, which are able to mineralize pentachlorophenol and hexachlorobenzene in soil and to degrade reactive synthetic dyes (Machado *et al.*, 2005a; Machado *et al.*, 2005b; Machado *et al.*, 2006; Matheus *et al.*, 2000). Lignin biodegradation is fundamental to potential applications of biotechnology in the pulp and paper industry. Indeed, the rapid increase during the past decade in understanding how lignin is degraded has provided strong items to the study of possible applications of biotechnology in pulp and paper manufacturer (Hossain and Anantharaman, 2006).

2.3 Bioactive fungal secondary metabolites

Fungi especially basidiomycetes are known to produce a vast array of secondary metabolites that are gaining importance for their biotechnological applications. They produce a series of biologically active compounds when grown in pure culture. Identification of microorganisms that produce bioactive compounds is of great interest in the development of new molecules to fight against many pathogens (Dreyfuss and Chapela, 1994). Fungal secondary metabolites have high therapeutic value as antibiotics, cytotoxic substances, insecticides, compounds that promote or inhibit growth, attractor, repellent etc. (Demain, 1999). These metabolites vary in production, function and specificity to a particular fungus. Fungi are also known to show larvicidal, molluscicidal, antioxidant and free-radical scavenging activities (Keller *et al.,* 2002).

According to reported biotechnological evaluation of over 200 mushroom species in Brazil, more than 75% of screened polypores showed strong antimicrobial activity (Suay *et al.*, 2000). These activities are associated not only with small molecule secondary metabolites but also with high molecular weight cell wall polysaccharides. The major philosophy of the search for antimicrobial compounds from basidiomycetes is that humans (and animals) share common microbial pathogens with fungi, such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas areuginosa*, so that man can benefit from defensive strategies used by fungi against microorganisms (Zjawiony, 2004).

Basidiomycetes, especially polypores, have a long history of medicinal use. For instance, the tinder polypore, *Fomes fomentarius*, was used in the eighteenth and nineteenth centuries in hemostatic dressings and bandages (Roussel *et al*., 2002). Screening of crude extracts of *Ganoderma basidiocarps*, such as *Ganoderma lucidum* (Reishi mushrooms), *G. pfeifferi*, and *G. resinaceum*, revealed selective activity against *Bacillus subtilis*. Two secondary metabolites, ganomycin A (**7**) and ganomycin B (**8**), isolated from *G. pfeifferi* showed moderate growth inhibition of several bacterial strains, particularly Gram-positive strains such as *B. subtilis*, *S. aureus*, and *Micrococcus flavus* (Mothana *et al.*, 2000).
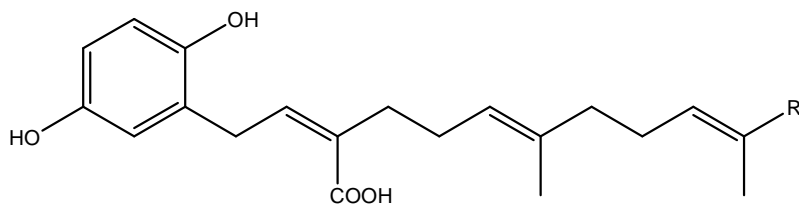
Ganomycin A (**7**) R = OH
Ganomycin B (**8**) R = H

Merulinic acids A, B, and C (**9-11**) were isolated from the fruiting bodies of the polypores *Merulius tremellosus* and *Phlebia radiata*. The merulinic acids showed antimicrobial activity particularly against *Arthrobacter citreus*, *B. subtilis*, *Corynobacterium insidiosum*, *Micrococcus roseus*, and *Sarcina lutea*. *Mycobacterium phlei* was selectively inhibited by **10** and **11**, while **9** was inactive. Similarly, *S. aureus* and *Proteus vulgaris* were inhibited only by merulinic acid B (**10**) (Wasser and Weiss, 1999).
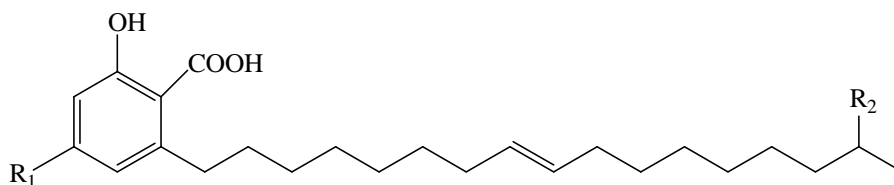
Merulinic acid A (**9**) R$_1$=OH, R$_2$=H
Merulinic acid B (**10**) R$_1$=H, R$_2$=OH
Merulinic acid C (**11**) R$_1$=H, R$_2$=H

It is interesting that mycelia cultures of *M. tremellosus* do not produce merulinic acids, but instead a highly antifungal sesquiterpenoid, merulidial (**12**). This occurrence serves as an example of the influence of different life cycle stages on the production of fungal secondary metabolites. Biological activities of merulidial (**12**) are associated with the presence of two aldehyde functions in the molecule (Wasser and Weiss, 1999).
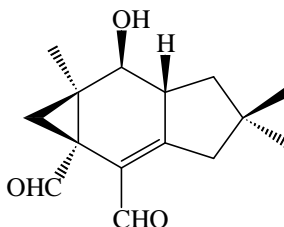
Merulidial (**12**)

Antimicrobial sesquiterpenes, desoxyhypnophilin (**13**), hypnophilin (**14**), 6,7-epoxy-4-hirsutene-5-ol (**15**), and 6,7-epoxy-4-hirsutene-1,5-diol (**16**), with a hirsutane skeleton were isolated from the wood-decaying polypore *Lentinus crinitus* (Abate and Abraham, 1994).
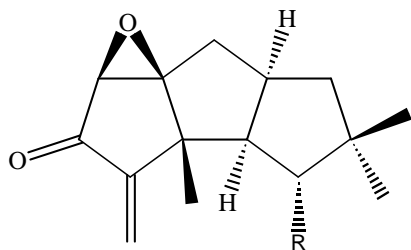
Desoxyhypnophilin **(13)** R = H
Hypnophilin **(14)** R = OH
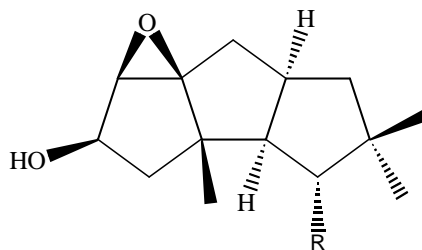
6,7-Epoxy-4-hirsutene-5-ol **(15)** R = H
6,7-epoxy-4-hirsutene-1,5-diol **(16)** R = OH

Desoxyhypnophilin (**13**) and hypnophilin (**14**) are active against the Gram-positive bacterium *Bacillus cereus*, and spores of *Aspergillus niger*, *A. flavus*, and *Mucor rouxii.* The ,-unsaturated exomethylene ketone system, present in these compounds, is responsible for antimicrobial activity (Abate and Abraham, 1994).

Sorbicillactone A (**17)**, the first member of the novel class of sorbicillin-derived alkaloids, isolated from a strain of *Penicillium chrysogenum* from a specimen of the Mediterranean sponge *Ircinia fasciculata*. It exhibits promising activities in several mammalian, viral, and neuronal test systems, in particular a highly selective cytostatic activity against murine leukemic lymphoblasts (L5178), the ability to protect human T cells against the cytopathic effects of HIV-1, and it is capable to act as a neuroprotective agent in primary neurons (Bringmann *et al.*, 2003; Bringmann *et al.*, 2005).

Sorbicillactone A

(17)
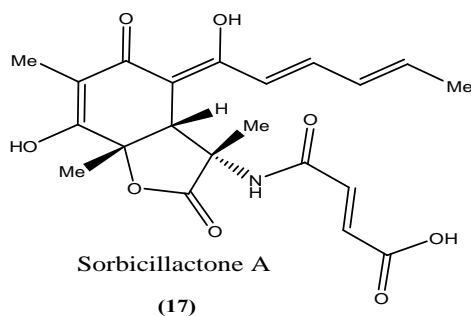
Heptemerones C, D and G (**18**, **19**, **20**), were isolated from the broth of submerged cultures of *Coprinus heptemerus*, a basidiomycete which previously had not been known to produce secondary metabolites. The compounds were bacteriostatic towards *Pseudomonas fluorescens*, *Micrococcus luteus*, *Corynebacterium insidiosum*, *Bacillus brevis* and *B. subtilis*. Phytotoxic activities were only recorded for heptemerones towards *Oryza sativa*. The most active compound was heptemerones G because of the presence of two reactive groups, an , -unsaturated aldehyde function and an , -unsaturated ketone group (Kettering *et al.,* 2005).
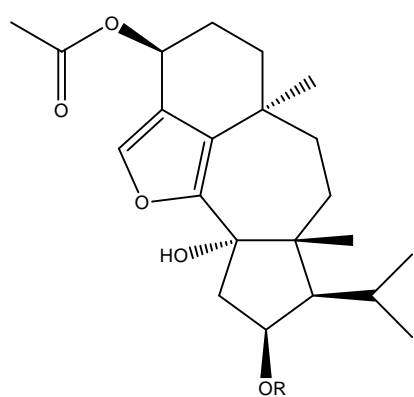
(18) Heptemerones C, R=CH₃
(19) Heptemerones D, R=Ac
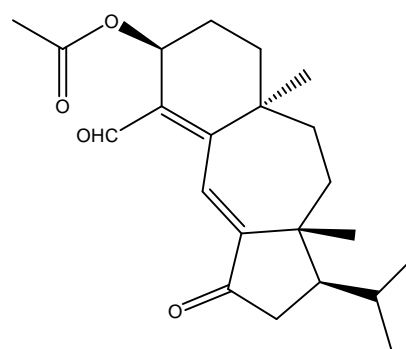
Heptemerones G
(20)

Two polyacetylenes have been isolated from *Gymnopilus spectabilis*: hepta-4,6-diyn-3-ol (**21**) and 7-chloro-hepta-4,6-diyn-3-ol (**22**). They showed strong biological activity which is believed to be due to the presence of the triple bonds (Aqueveque *et al*., 2006).
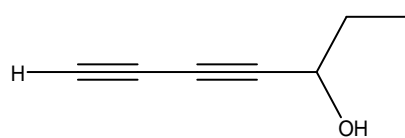
Hepta-4,6-diyn-3-ol
(21)
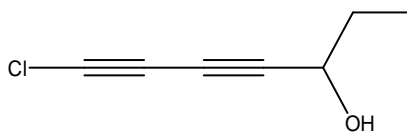
Hepta-4,6-diyn-3-ol
(22)

2.4   Natural products from endophytic fungi

Endophytes are microorganisms that live inside living host plant tissues without causing symptoms of disease. Endophytes might be defined as microorganisms that can be detected at a

given moment within the tissues of an apparently healthy plant host (Schulz and Boyle, 2005), and they have been found to produce a significant number of interesting natural products (Tan and Zou, 2001, Gunatilaka, 2006 and Zhang *et al.*, 2006). They have proven to be promising sources of new and biologically active natural products which are of interest for specific medicinal or agrochemical applications (Strobel, 2002). Considering certain plants have been known to have therapeutic principles is of great interest in the field of resourceful use of biodiversity by employing biotechnological techniques.

Studies have shown that fungal endophytes are ubiquitous in plant species (Faeth and Hammon, 1997; Huang *et al.,* 2007) and are mutualistic to their host. At least some of them are thought to be receiving nutrition from the plant in exchange for producing special substances such as secondary metabolites to protect the host from successful attack by fungi, pests and mammals. An array of natural products has been characterized from endophytes, which includes anti-cancerous, anti-oxidants, anti-fungal, anti-bacterial, anti-viral, anti-insecticidal and immunosuppressants (Tejesvi *et al.*, 2007). Endophytes have the ability to produce a huge chemical diversity, including alkaloids, peptides, steroids, terpenoids, isocoumarins, quinones, phenylpropanoids and lignans, phenols and phenolic acids, aliphatic compounds, lactones, and others. Among these compounds, several have interesting biological activity. Chaetominine **(23)**, an alkaloid with a new framework, was extracted from *Chaetomium sp*. IFB-E015, an endophytic fungus from *Adenophora axiliflora*. Chaetominine showed cytotoxicity against the human leukemia K562 and colon cancer SW1116 cell lines higher than the drug 5- fluorouracil (Jiao *et al*., 2006).
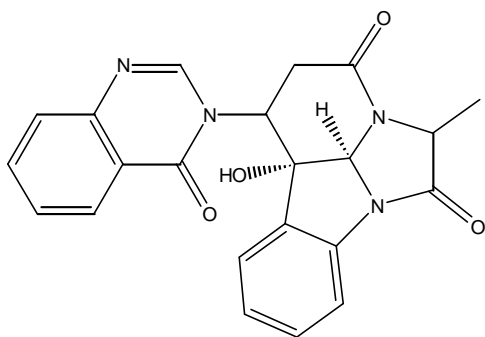
Chaetominie **(23)**
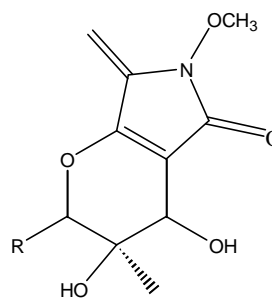
Phaeosphoramide **A (24)** R = β C_5H_{11}
B (25) R = α C_5H_{11}

Phaeosphoramides A **(24)** and B **(25)**, two new carbon skeleton derivatives, were isolated from the endophytic fungus *Phaeosphaeria avenaria*. Phaeosphoramide A was found to be an

inhibitor of the signal transducer and activator of transcription (STAT)-3, which plays a vital role in regulating cell growth and survival, constituting a target for anticancer therapy (Maloney *et al.*, 2006).

2.5 *Trametes* basidiomycetes in enzyme therapy

Higher basidiomycetes have been known to possess medicinal and nutritional properties for thousands of years. Mushrooms for example have been used in clinical nutrition because they exhibit anti-tumour, immune modulating, cardiovascular and antimicrobial properties (Wasser and Weiss, 1999).

Mushroom biomass contains many complex substances of therapeutic interest such as proteinbound polysacharide complexes and lentinan; secondary metabolites such as: terpenes, alkaloids and lactones and enzymes such as: laccase, superoxide dismutase, glucose oxidase and peroxidase (Ng, 1998; Karmali and Oliveira, 1999). It has been known that enzyme therapy plays an important role in several clinical conditions such as in cancer treatment, malignant lymphoma and cardiovascular disorders (Ossowski *et al.*, 1996; Gubareva, 1998).

A number of pathological damages such as carcinogenesis and cellular degeneration related to aging process are due to reactive oxygen species produced by sunlight, ultraviolet radiation, chemical reactions and metabolic processes. These reactive oxygen species (i.e superoxide radicals) are toxic to living cells since they oxidize and degrade important biological macromolecules such as lipids and proteins. Superoxide dismutase catalyses the destruction of superoxide radicals and hence protects oxygen ó metabolizing cells from the harmful effect of these free radicals. Several research workers have shown that superoxide dismutase is involved in some diseases such as Parkinsonøs disease, cancer and anemia. Several mushroom species are known to contain substances which mimic superoxide dismutase activity (Jacob *et al.,* 2001; Ichinose *et al.,* 2002).

Another important enzyme system consist of cytochrome P-450 which is located in the endoplasmic reticulum and play an important role in metabolism and detoxification of 4 endogenous substances (Ichinose *et al.,* 2002). This enzyme system has been also found in some higher basidiomycete fungi. Thrombin is an important protease of the coagulation system and therefore it is a suitable target for inhibition of blood coagulation. There are a number of secondary metabolites in mushrooms which play an important role as thrombin inhibitors (Doljak *et al.,* 2001).

2.6 Fungal secondary metabolites as potential sources of mosquito larvicides

Diseases transmitted by blood-feeding mosquitoes have constantly been spreading, despite the significant advances in medicine and public health achieved throughout the last century. Apart from the apprehensive effort to develop vaccines, the most successful means to control the mosquito-borne diseases have been focused on suppressing the host interaction by using insecticides (Justice *et al*., 2003). The global remedy for mosquito control continues to be selective application of residual synthetic insecticides. The public health benefit delivered by these, both in the tropics as well as in the temperate zones, cannot be overemphasized - they save thousands of lives each year. However, their side effects on both humans and the environment are worrisome. Therefore, the developments of resistance by insects are issues of grave concern (Scholte *et al*., 2003). Despite several methods being used for mosquito control, the spread of malaria and other parasitic diseases is still a major problem in the developing countries. For instance in the last decade there have been consistent reports from Kenya that malaria outbreaks occur in areas where the disease was previously non-existent, such as highlands around Mount Kenya (Boko *et al*., 2007).

It is important to conduct effective mosquito prevention programs by eliminating breeding habitats or applying pesticides to control the early life stages of the mosquito. Prevention programs, such as elimination of any standing water that could serve as a breeding site, help reduce the adult mosquito population and the need to apply other pesticides for adult mosquito control. Using larvicides is the best way to control mosquitoes. Larviciding involves applying pesticides to breeding habitats to kill mosquito larvae. Larviciding can reduce overall pesticide usage in a control program. Killing mosquito larvae before they emerge as adults can reduce or eliminate the need for ground or aerial application of pesticides to kill adult mosquitoes.

New innovative compounds to control mosquitoes require the availability of the large number of compounds. Chemo-diversity in nature offers valuable source e.g. secondary metabolites, previously regarded as waste products are now recognized for their resistant activity against pests and diseases. Nature has developed an enormous diversity during several billions of years of evolution. It is estimated that there are at least 250,000 different plant species, up to 30 million species of insects, 1.5 million species of fungi and similar number of algae and prokaryotes in existence (Crous *et al*., 2006). All of these species coexist in ecosystems and

interact with each other in several ways in which chemistry plays a major role for example in defense, pollination and symbiosis. In basic terms, these organisms share a similar biochemistry for a living cell, but in addition to that they produce a wide variety of secondary metabolites that are involved in the interactions between organisms. Considering the number of organisms and the almost infinite number of interactions it is not surprising that an enormous wide variety of secondary metabolites has evolved within organisms. In Kenya there is a rich biodiversity that is underutilized especially for addressing problems that are unique to the region in terms of diseases like malaria and its vector. Every year, malaria kills more than 1 million people worldwide and everyday there are people dying of other mosquito transmitted diseases.

(Oxiran-2-yl) methyl pentanoate (**26**) was isolated form a basidiomycete JO5444 and showed larvicidal activity. The activity is believed to be due to the presence of the epoxide and the ester (Njogu *et al*., 2009). In addition compound (**26**), has an ester functional group a property found in many compounds isolated from basidiomycetes for example pulvinic acids isolated from mycelia cultures of *Suillus bovines* (Besl *et al*., 2008)
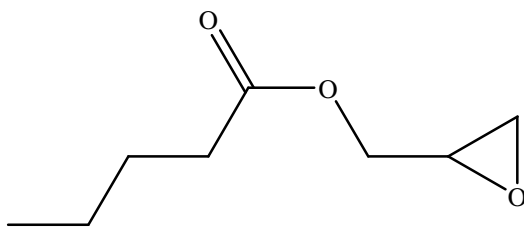
(Oxiran-2-yl)methyl pentanoate **(26)**

2.7   Bacteria resistance

Antibiotic resistance is not a recent phenomenon, on the contrary, this problem was recognized soon after the natural penicillins were introduced for disease control, and bacterial strains held in culture collections from before "the antibiotic era" have also been found to harbour antibiotic-resistance genes. In some cases the situation has now become alarming, with the emergence of pathogenic strains that show multiple resistance to a broad range of antibiotics (Cernicka *et al*., 2007). As antibiotics and other antibacterial agents such as antibacterial lotions, soaps, and sprays are used to kill bacteria, the species pool for the stronger bacteria. Since antibacterial agents do not kill 100% of bacteria, they leave a few of the bacteria that are resistant. These bacteria continue to grow, and in fact, they flourish due to an increase in nutrients that their weaker counterparts would have competed for. Besides antibacterial agent

use, over-prescription of antibiotics weeds out weak bacteria and leaves resistant strains. More commonly, bacteria that are targeted by antibiotics develop mechanisms to fight back against antibiotics. New antibiotics that are active against resistant bacteria are required. Bacteria have lived on the earth for several billion years. During this time, they encountered in nature a wide range of naturally occurring antibiotics. To survive, bacteria developed antibiotic resistance mechanisms. Therefore, it is not surprising that they have become resistant to most of the natural antimicrobial agents that have been developed over the past 50 years (Hancock, 2007). This resistance increasingly limits the effectiveness of current antimicrobial drugs (Demain and Sanchez, 2009). Consequently bacterial, viral drug resistances as well as the spread of fungal and parasitic diseases necessitate the search for additional antibiotic compounds with activity at low concentrations and with reasonably low toxicity to humans (Hoffman *et al*., 2008).

During the last five decades several pathogenic micro-organisms have developed resistance to the available antibiotics (Evans *et al*., 2006; Singh *et al.,* 2006). Infections by multi-drug resistant isolates of *Candida* sp., *Staphylococcus epidermidis*, *S. aureus*, *Streptococcus* sp., *Enterococcus* sp. and *Escherichia coli*, among others, have become more frequent thus stimulating the search for new antibiotics with novel mechanisms of action (Kotra and Mobashery*,* 1998; Morschhaeuser *et al.,* 2000; Sandven, 2000; Thomson and Moland, 2000; Singh *et al.,* 2006; Srivastava *et al.,* 2007). Bacteria become antibiotic-resistant by mutating existing genes or acquiring new ones that can encode for efflux pumps or deactivating enzymes.

2.8   Fungal Secondary metabolites as antibiotics

Since the discovery of penicillin there have been several antibiotics discovered from fungi and other sources notably the actinomycetes. As an example several secondary metabolites have been identified from *Penicillium corylophilum*, as well as their biological activities. Antimicrobial metabolites from *P. corylophilum* were extracted and an alkaloid, fumiquinozoline F **(27)**, derived from tryptophan plus anthranilic acid with antimicrobial activity was isolated by using chromatographic methods (Marley *et al.,* 2004).
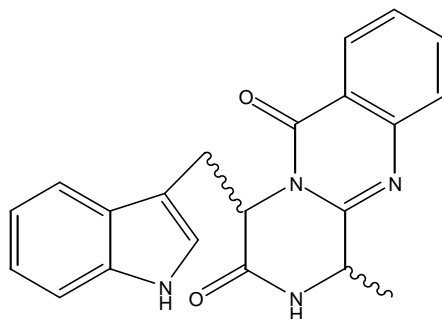
Fumiquinozoline F (**27**)

The first investigations on the potential of basidiomycetes as sources of antibiotics were performed by Anchel, Hervey and Wilkins in 1941 (Sandven 2000; Florey *et al*., 1949), when they examined extracts of fruiting bodies and mycelia culture from over 2000 species. They succeeded in the isolation and identification of pleuromutilin (Kavanagh *et al*., 1950), a diterpene that is especially useful for the treatment of mycoplasm infections in animals (Brizuela *et al*., 1998; Rosa *et al*., 2003) and served for the development of the first commercial antibiotic of basidiomycete origin.

Phellinone **(28)**, a new furanone derivative, has been isolated from the stem-cooked rice culture broth of *Phellinus linteus* KT&GPL-2. *Phellinus linteus* known as *Sangwhang* in Korea is an orange colored mushroom. Phellinone showed antimicrobial activity against *Bacillus subtilis* IAM 1069 (Yeo *et al*., 2007).
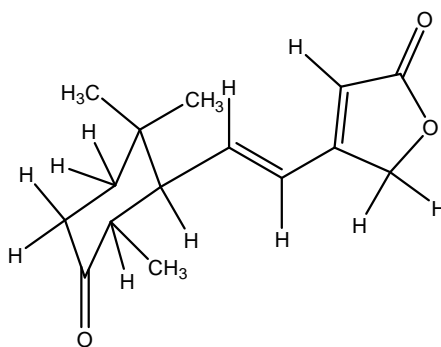
4-((E)-2-((1S,6S)-2,2,6-trimethyl-5-oxocyclohexyl
vinyl) furan-2(5H)-one ((Phellinone)

(28)

CHAPTER THREE
MATERIALS AND METHODS

3.1   Reagents and chemicals

The main chemicals and reagents used are: ethanol, methanol (99.98% purity) (Reag. Ph. Eu.) Panreac Quimica of Spain, ethyl acetate (GPR) Belami fine chemicals, cyclohexane 99.99% purity (ARG) Fisher Scientific, UK, acetone (GPR) Kobian Kenya Limited, silica gel (MN 60 Kieselgel 0.063 ó 0.2 mm/70 -230 mesh AST for column chromatography) Machery ó Nagel Germany and Mitsubishi HP21-DIAION resin.

3.2   Equipments

The equipments used are: autoclave ó Danfoss 59407-3 No. 375, analytical Balance ó Precision 310M Swiss quality, pH meters ó Fishes Acument ® model 620A, magnetic stirrer hotplate ó Gallenlamp, vacuum rotary evaporator type 349/2, biological safety cabinet (SterilGARD The Baker Company Sanford ó Class II Type A/B3 US PAT NO. 3,895,570), incubator, heating mantle, heating module ó Pierce Chemical Company Silli-ThermÎ , liebig condenser and suction pump,

3.3   Apparatus and other materials

Capillary tubes, 250 ml and 100 ml conical flask, 100 ml and 10 ml measuring cylinders, buchner filtration system, filter funnel, filter papers, petri dishes, chromatographic tanks, Chromatographic column (glass), 250 ml glass beaker, 100 ml glass beaker, 250 ml round bottomed flask, 500 ml separating funnel, inoculating blade (spear), acid washed sand, TLC plate, {Macharey-Nagel, ALUGRAM ® SIL G/UV254 0.25 mm Duren, Germany}, cotton wool, aluminium foil, glucose testing strips (Diabur-test ® 5000 (Roche), 20 µl and 200 µl Eppendorf micropipette and disposable tips, syringes, droppers with rubber suction bulbs, data recording forms, disposable cups (glass bowls), vials, paper discs *Aedes aegypti* larvae in their late third and early fourth instars. Fungal test organism: *Candida albicanus* and bacterial test organisms *Salmonela typhimurium*, *Escherilia coli*, *Bacillus subtilis* and *Staphylococcus aereus* (S140). The used glassware were cleaned with a detergent and thoroughly rinsed with clean water and dried in an oven.

3.4 Preliminary preparations

3.4.1 Re-growth of the *Trametes* species (JO5066)

From agar slants in the Integrated Biotechnology Research Laboratory, agar pieces with mycelia were cut from the slants and inoculated onto potato dextrose agar (PDA) plates under sterile conditions inside the laminar flow hood. Then the Petri dishes were sealed sterile using the Para film and left to grow under ambient laboratory conditions of 12 hour light/dark cycles for 21 days.

3.4.2 Preparation of liquid media

Malt extract 1%, yeast 0.4% and glucose 0.4% were dissolved in 250 ml of tap water to form starter cultures. Then replicates of 1 L scale in 2 L flasks with the pH of each adjusted to 5.5 by use of sodium hydroxide and hydrochloric acid. They were corked with cotton wool plugs, wrapped with aluminium foil and autoclaved at 121°C and pressure 1.5 bars for 15 minutes. The media was sterilized twice after which let to cool.

3.4.3 Inoculation of the *Trametes* species (JO5066) strain in the liquid media

A well grown pure culture of the *Trametes* species (JO5066) strain on PDA plate was cut into several agar plugs using sterile inoculating blade and in each conical flask (250 ml) four agar plugs with mycelium of the *Trametes* species (JO5066) was introduced then allowed to grow as still cultures with regular agitations at ambient conditions. The growth of the culture was then closely monitored and evaluated daily to check the biomass build up and the presence of any contamination and stopped after 7 days. A well grown 250 ml starter culture was then used to inoculate into a 1 L scale. This was allowed to grow until the glucose level in the culture was exhausted. It was done in replicates (at least fifty flasks) to ensure a high yield of the crude extracts.

3.5 Preparation of crude extracts

After growth was stopped, the culture filtrate was then separated from mycelium by filtration using a Buchner filtration system and both extracted as follows.

3.5.1 Culture filtrate crude extract (Kex)

The combined culture filtrate was passed through a Mitsubishi HP21-DIAION resin packed in a glass column (diameter of 2.5cm and height of 50cm) thrice. The column was then

eluted with 100% acetone (2 L), followed by 100% methanol (2 L) and the eluents collected. Each organic extracts was concentrated under reduced pressure using rotary evaporator to remove acetone and methanol, respectively. The aqueous test concentrate was extracted with equal volume of ethyl acetate thrice and the combine ethyl acetate extract dried with anhydrous sodium sulphate. The dried organic extract was concentrated using the rotary evaporator at temperatures not exceeding 50°C. The crude extract left was won with methanol and kept in screw capped vials at 4°C awaiting tests and analysis.

3.5.2   Mycelium crude extract (Mex)

The mycelium was soaked in 3 L acetone immediately after filtration for 4 hours under constant agitation using a magnetic stirrer to ensure all the compounds are extracted. It was then filtered using Buchner filtration system and the wet mycelium was dried. The dry extract was re-dissolved in 4 L water and extracted with 1 L of ethyl acetate ten times. The ethyl acetae solution obtained was then dried with anhydrous sodium sulphate to and concentrated using rotary evaporator. The crude extract obtained was kept in screw capped vials at 4°C awaiting tests and further analysis.

3.6   Biological Activity Testing

Both the crude extracts and the purified fractions obtained were tested for larvicidal and antimicrobial activities. All fractions were monitored by thin layer chromatography until a single spot was obtained. The pure fractions were then won using methanol and then carefully evaporated to dryness and subsequently characterized.

3.6.1   Larvicidal assay

In order to establish LC_{50} and LC_{90} values, that is, the concentrations of extracts in parts per million required to kill 50% and 90% of *Aedes aegypti* larvae (obtained from the laboratory of the Pyrethrum Board of Kenya Nakuru), within 24 hours, multiple 10-fold dilutions of the extract stock solution was prepared to provide a working concentration range. Two replicate assays were carried out for every sample concentration, each with 10 larvae. Larvae were observed at the start of the assay, 2 hours, 4 hours, and 8 hours interval and after 24 hours and considered dead when they did not respond to stimulus or when they did not rise to the surface of the solution (WHO, 2005). Negative controls accompanied each assay and involved treating larvae with water and methanol. The LC_{50} and LC_{90} were calculated only for the most active

extracts. The dead larvae counted after every 2 hours, was used to calculate the percentage mortality (**Eq 1**) reported from the average for the three replicates taken (WHO, 2005).

$$\% \ Mortality = \frac{Number \ of \ dead \ larvae}{Number \ of \ Larvae \ Introduced} \ x \ 100 \qquad \textbf{Eq1}$$

3.6.2 Antimicrobial assays

The antimicrobial activity was carried out using agar diffusion assay technique for crude extract and enriched fractions. Using adjustable (analogue) volume micropipette, known quantities of either the crude extract or the enriched fractions were put on to paper discs (6 mm). The dried impregnated paper discs were carefully placed onto the agar surface of the test plates with selected laboratory test strains of bacteria and fungi. This was followed by incubation at 37°C for 18 hours. The results were evaluated as inhibition zones around the discs for the active fractions and quantified by measuring the diameter of the inhibition zones.

3.7 Purification of the compounds

Purification was carried out with column chromatography packed with silica gel as the stationary phase. The mobile phases constituted discrete gradients of increasing solvent polarity from non-polar to polar solvents using cyclohexane-ethyl acetate-methanol mixtures.

3.8 Column chromatography

A glass chromatographic column was mounted vertically and approximately 40 g of silica gel suspended in cyclohexane, and then swirled vigorously until homogenous slurry was obtained. The slurry was degassed using a water pump to remove trapped gases and then slurry packed ensuring that no air was introduced into the column. The dry silica gel adsorbed sample was loaded onto the column uniformly above the slurry and anchored in place with acid washed sand. The column was then developed with discrete solvent gradient system with increasing polarity from cyclohexane to ethyl acetate and finally 100% methanol.

These eluents were collected from the column and pooled into several fractions based on thin layer chromatography (TLC) analysis. The fractions were concentrated by removing the organic solvents under reduced pressure using rotary evaporator. The final crude extract were won using minimal volume pure methanol and then transferred to a screw-capped vial carefully labeled and kept under refrigeration. These fractions were then screened for larvicidal and

antimicrobial activity using the same procedure as in section 3.6 and the active fractions underwent further chromatographic techniques in order to clean them.

3.9 Thin Layer Chromatography

Thin layer chromatography was used to determine whether the active fractions obtained were pure. They were spotted on silica coated aluminium thin layer chromatography plate and then developed in solvents of different polarities to give the best separation. Then viewed under a UV lamp with 365 nm and 254 nm wavelengths to determine the active spots and finally sprayed with *p*-anisaldehyde and put in the oven to dry at 110ºC for 10 minutes the spots finally obtained were viewed. If two or more spots were obtained then the fractions were concluded to be unclean and further purification had to be done. The spots with similar R_f values were pooled together. For the third set of purification isocratic solvent system was used.

3.10 Structure elucidation: Nuclear Magnetic Resonance spectroscopy

The data of the chemical structure of the purified compound from enriched intermediate fractions I (compound **29**) and II (compound **30**), in consistent with other structures given in this document (section 4.2.3) was obtained from an NMR spectrometer. The purified compounds were dissolved in deuterated chloroform $CDCl_3$). (^{1}H-NMR and ^{13}C-NMR) and two dimensional (COSY, HMBC, HSQC and NOESY) experiments were performed. All experiments were carried out in Bruker AV 300 MHz spectrometer. All the spectra were recorded at room temperature and the chemical shifts recorded/given in parts per million (ppm) relative to the TMS peak.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1   Results of cultivation, purification and structure elucidation

4.1.1   Cultivation of the basidiomycete, *Trametes* (JO5066)

The basidiomycete JO5066 was cultivated as still cultures under laboratory ambient conditions and the growth was stopped after 21days when the glucose levels were depleted. Immediately growth was stopped, crude extracts were prepared for both intra- and extra-cellular secondary metabolites as summarized in the scheme below in (**figure 1**). The crude extract from culture filtrate (Kex) was 1.2 g while the mycelium crude extract (Mex) was 1.6 g.

Figure 1: Scheme of extraction crude extracts

20 L of culture broth

16.5 L culture filtrate   140 g dry weight mycelium

1.2 g crude extract (Kex)   1.6 g crude extract (Mex)
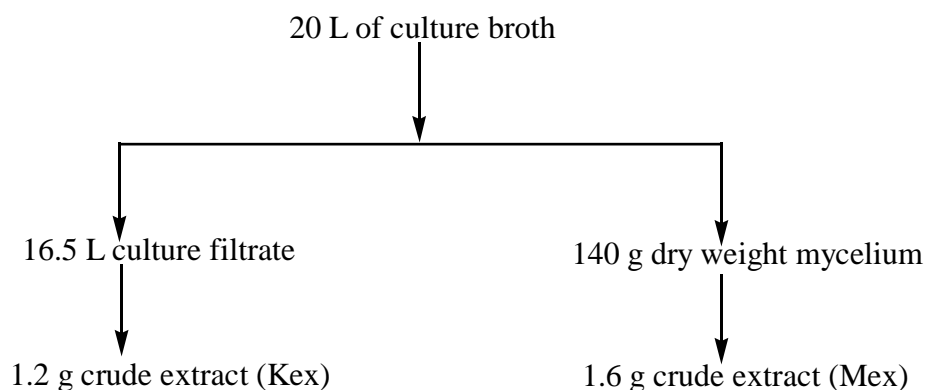
4.1.2   Larvicidal activity of the crude extracts

Both Kex and Mex crude extracts were tested for larvicidal activity as described in section 3.6.1. Each of the crude extracts was tested in the concentration ranges 50 ó 1000 ppm, in which larval mortality was scored after 2, 4, 8 and 24 hours. The results obtained for Kex and Mex are summarized in **(table 1 and 2)**, respectively. The values recorded were the average of a duplicate experiment. The control was set up using distilled water and methanol added without the sample and there was no mortality observed.

Table 1: Mosquito larvicidal activity for culture crude extract (Kex) against *A. aegypti* larvae

Concentration (ppm)	% Mortality			
	2 hours	4 hours	8 hours	24 hours
50	30	30	40	50
100	40	30	50	70
200	60	90	100	100
500	100	100	100	100
1000	100	100	100	100

In the case of Kex it was noted that there was activity for as low as 50 ppm concentration within the range 30 ó 50% mortality for the whole period of mortality (**table 1**). It was also observed that 100% mortality occurred for concentrations from 500 ppm except for 200 ppm after 8 hours.

Table 2: Mosquito larvicidal activity for Mycelium crude extract (Mex) against *A. aegypti* larvae

Concentration (ppm)	% Mortality			
	2 hours	4 hours	8 hours	24 hours
50	0	0	0	0
100	0	0	0	0
200	0	0	0	0
500	30	40	40	70
1000	100	100	100	100

For Mex, activity was observed from 500 ppm in the range 30 ó 70 % for the whole period the experiment was evaluated. It is only at 1000 ppm that 100% mortality was observed. Kex (**table 1**) was noted to have more activity as compared to Mex (**table 2**).

4.1.3   Antimicrobial activity of the crude extracts

Both the Kex and Mex crude extracts were tested for antimicrobial activity in agar diffusion assay and the results recorded as diameter of the inhibition zones as summarized in table 3 below. The test organisms were the yeast *C. alibicans* and the bacteria; *S. typhimurium*, *E.coli*, *B. subtilis* and *S. aureus*.

Table 3: Antimicrobial bioassay for crude extracts

	Diameter of inhibition zone (mm)				
Crude extracts	*C. albicans*	*S. typhimurium*	*E. coli*	*B. subtilis*	*S. aureus*
Kex	15 mm	13 mm	10 mm	12 mm	16 mm
Mex	9 mm	(-)	10 mm	(+)	10 mm

Key: (-)   negative test

(+)   positive test

The antifungal activity of both crude extracts against *C. albicans* indicated that Kex had a larger zone of inhibition than the Mex. Mex showed no activity for *S. typhimurium*, while activity for *B. subtilis* was minimal hence could not be measured. However, there was a mixed pattern of activity against the bacteria with Kex showing relatively larger zones of inhibitions than those of Mex. Therefore Kex which was noted to be more active was picked for further analysis.

4.1.4   Bioactivity activity guided purification of compounds from Kex

The biologically active compounds in culture filtrate (Kex) were targeted for purification using bioactivity guided purification. This was done using silica gel chromatography. The crude extract was purified according to the scheme summarized in (**figure 2)** below;

Figure 2: Scheme of purification of intermediate fractions

1.2 g Kex
Gradient silica gel chromatography
ethylacetate in cyclohexane

390 mg Intermediate Fraction II
Gradient silica gel chromatography
70% ethylacetate in cyclohexane

37 mg of pure compound JO5066K337

160 mg Intermediate Fraction I
Gradient silica gel chromatography
50% ethylacetate in cyclohexane

8 mg of pure compound JO5066K232
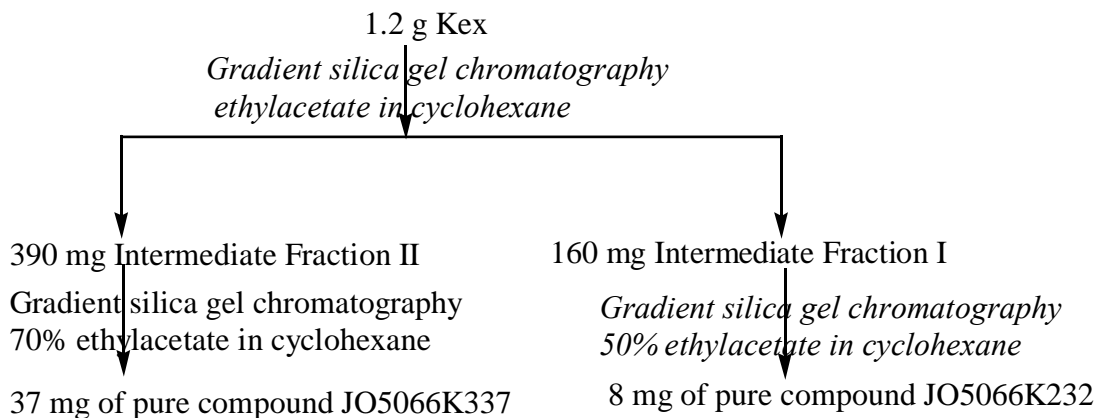
The 1.2 g yield of the crude extract (Kex) was subjected to chromatography with gradient elution with increasing polarity of ethyl acetate in cyclohexane. The active compounds eluted with gradient mobile phase (cyclohexane and ethylacetate) to afford 160 mg of an intermediate fraction 1 and 390 mg fraction labeled II respectively. These intermediate fractions were further subjected to chromatographic separation, which led to elution of 8 mg and 37 mg of pure compounds JO5066K232 with 50% ethyl acetate in cyclohexane and JO5066K337 with 70% ethyl acetate in cyclohexane respectively.

4.1.5 Mosquito larvicidal activity of the pure compounds

The purified compound, JO5066K337, was tested for larvicidal activity against *A. aegypti* and the results of the test are summarized in (**table 4)** below;

Table 4: Larvicidal bioassay for 2,3-dihydro-3,3-dimethyl-1H-indene-1,2,6-triol against *A. aegypti* larvae

Concentration (ppm)	% Mortality			
	2 Hours	4 Hours	8 Hours	24 Hours
50	0	0	0	10
100	0	0	0	50
200	0	0	20	80
500	0	10	70	100
1000	10	60	100	100

The results given in table 4 indicate that there was no observable activity up to 8 hours since the start of the experiment. From the results it is the mortality data at 24 hours that was correlated to obtain $LC_{50} = 235$ ppm   and $LC_{90} = 674$ ppm.

4.1.6 Results of NMR experiments

The purified compound was subjected to one- and two-dimensional NMR experiments. The ^{1}H-NMR, ^{13}C-NMR and distortionless enhances polarization transfer (DEPT) experiments were carried out and the corresponding spectra are attached in appendices 1-30.

4.2 Discussion of the results

4.2.1 The growth of the basidiomycete JO5066

The morphological and discernable microscopic features confirmed that the fungus JO5066 had clamp connections, which are the characteristic distinguishing features of a basidiomycete. This established that the fungus (JO5066) used for this study was a basidiomycete. The basidiomycete was further identified using molecular markers 18S RNA (ITS) technique (Baldwin, 1992; Gardes and Bruns, 1993; Chen *et al.*, 2001) and was found to

belong to *Trametes* genus. The species could not be determined unequivocally. Cultivation of the basidiomycete in the malt liquid medium, took 21 days for glucose levels to be exhausted and the residual glucose left had stagnated indicating that it was not supporting growth. In submerged cultures, normally the growth kinetics and the fungal morphology in broth fermentation are highly dependent on the culture conditions, such as carbon sources, C/N relation, initial pH and temperature, agitation intensity and aeration rate (Shu *et al*., 2004). In the present study the malt extract was the carbon and nitrogen source, glucose ó simple carbon source and yeast ó nitrogen source. Glucose was the starter source of carbon, before the basidiomycete employs its efficient enzymatic systems to immobilize glucose from the sugar macromolecules in the malt extract to support further growth development (Lopez *et al*., 2003). The growth was allowed under ambient conditions as still cultures with occasional agitations to enhance aeration. The pH was initially pre-set at 5.5, which is an established optimum for growth of the basidiomycete (Shu *et al*., 2004).

4.2.2 Bioassay tests

Once growth of the basidiomycete was stopped, the mycelium was separated from the culture filtrate, from which respective crude extracts were prepared. The crude extract (Kex) when tested for larvicidal activity showed 100% mortality at 50 ppm after 8 hours while the Mex extracts showed 100% mortality at 1000 ppm after 2 hrours for the larvicidal assays (**Table 1 and 2**), indicating that the Kex extract was more active than the Mex extract. In the antimicrobial assays (**Table 3**) the mycelial crude extract (Mex) showed no activity against bacterial test organisms; *S. typhimurium* and had minimal activity against; *B. subtilis*. The inhibition zone for Kex against *S. aureus* (S140) was 16 mm while for the Mex the inhibition was 8 mm, indicating that the Kex extract was more active compared to Mex. However, there was antifungal activity against *C. albicans* with an inhibition zone of 15 mm for Kex.

4.2.3 Structure elucidation of the purified compounds
Structural elucidation of compound JO5066K337

The pure compound JO5066K337 was obtained as a yellow liquid weighing 37 mg. The chemical structure of the compound was determined based on 1D and 2D NMR experiments (**appendices 1-22**). The spectral data from the experiments are summarized in table 5 for the compound. From this the structure of compound JO5066K337 was discerned and proposed to be
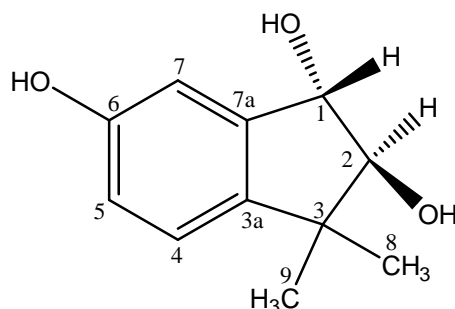
2,3-dihydro-3,3-dimethyl-1H-indene-1,2,6-triol (**29**). This maybe a new compound being reported for the first time from cultures of a basidiomycete. From ^{13}C-NMR spectrum there were 11 discernable carbon signals, which when analyzed together with ^{1}H-NMR and DEPT spectra indicated that there were 4 quaternary carbons, 5 methine carbons and 2 methyl carbons. It was evident that the compound had no methylene carbons. The information was found to be consistent with the molecular formula, $C_{11}H_{14}O_3$, with unsaturation index (UI) of 5.

$$\text{Unsaturation index} = \frac{(n - r + 2)}{2} \quad \textbf{Eq 2}$$

$$C_nH_{n+r}\ (C_{11}H_{14}O_3)$$

Where: $n = 11, r = 3$

$$UI = (11-3+2)/2 = 5$$

2, 3-dihydro-3, 3-dimethyl-1H-indene-1, 2, 6-triol **(29)**

From the 11 signals there were 6 typical aromatic sp^2 hybridized carbons; 111.8, 124.3, 129.3, 132.8, 135.7 and 168.3 ppm, with the last one (C-6) being oxygenated typified by the chemical shift in the deshielded range and consistent with a phenol moiety. From these carbons C-3a (124.3 ppm) and C-7a (132.8 ppm) are quaternary and C-4 (135.7 ppm), C-5 (129.3 ppm) & C-7 (111.8 ppm) are bearing a proton each.

The remaining 5 carbons were accounted for by 2 methyl carbons, 11.4 ppm (C-8) and 16.8 ppm (C-9), with both attached to a quaternary carbon 22.1 ppm (C-3). This leaves 2 carbons that are accounted for as oxygenated sp^3 hybridized that are part of a rigid ring system, C-1 (83.3 ppm) and C-2 (78.9 ppm). Carbons C-1, C-2 and C-3 are part of five-membered ring that is fused to the aromatic system as deciphered from the 2-D NMR experiments COSY, NOESY and

HMBC. The two protons (4.72 ppm & 3.72 ppm) attached to C-1 and C-2, respectively are in a *trans*- orientation supported by a ^3J-value of 2.1 Hz characteristic of such a stereochemistry common in sugars (Friebolin, 2005). It is on this basis that the relative configuration on C-1 and C-2 were proposed.
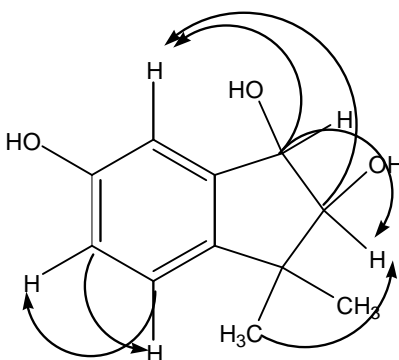
Observed $^1H – ^{13}C$ HMBC couplings for the proposed structure

The HMBC (**appendices 12**-**14**) spectrum revealed that the methine carbons (C-4:135.7ppm and C-5: 129.3) are correlating with protons with chemical shifts 5.86 ppm and 6.47 ppm (**table 5)**. Also the methine carbons (C-1: 83.3ppm and C-2: 78.9 ppm) are correlating with protons with chemical shifts 3.72 ppm and 4.72 ppm respectively.
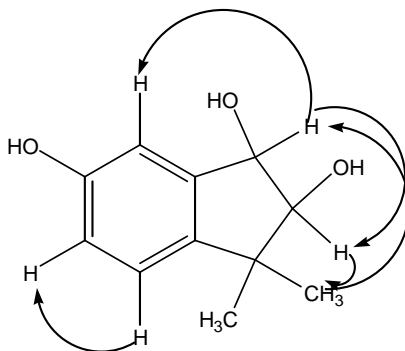
Observed $^1H – ^1H$ NOESY couplings for the proposed structure

From NOESY (**appendices 19-21**) protons with chemical shifts 6.47 ppm and 5.86 ppm, 4.72 ppm and 3.72 ppm, 3.72 ppm and 1.09 ppm were seen to correlate with each other.-From the spectral information and the above arguments, the structure of compound JO5066K337 was proposed to be 2,3-dihydro-3,3-dimethyl-1H-indene-1,2,6-triol (**29**).

Table 5: Summarized data from 1-D and 2-D NMR experiments for compound JO5066K337

Position	^{13}C	^{1}H	DEPT	HMBC	COSY	NOESY
1	83.3	4.72	CH	H-7/ H-2/ H-4/ H-5	H-1/H-2/H-5	H-5/H-4/H-6/H-7/H-2
2	78.9	3.72	CH	H-7	H-1/H-2/H-9	H-8/H-5/H-7
3	22.1	-	-	-	-	-
3a	124.3	-	-	-	-	-
4	135.7	6.47	CH	H-5/H-1	H-4/H-5	-
5	129.3	5.86	CH	H-4	H-4/H-5/H-1	H-4/H-5
6	168.3	-	-	-	-	-
7	111.8	5.27	CH	H-2	-	H-2/H-5
7a	132.8	-	-	-	-	-
8	11.4	1.09	CH_3	-	-	H-7/H-2/H-5/H-6
9	16.8	2.02	CH_3	H-7/ H-2	-	-

Structural elucidation for compound JO5066K232

The other pure compound coded JO5066K232 was obtained as an orange liquid weighing 8 mg. The chemical structure of the compound was determined based on 1D and 2D NMR experiments (**appendices 22-30**). The spectral data from the experiments are summarized in table 6 for the compound. From the spectral information the structure of compound JO5066K232 was discerned and proposed to be *p*-tolylmethanol (**30**). From ^{13}C-NMR spectrum there were 8 discernable carbon signals, which when analyzed together with ^{1}H-NMR and DEPT spectra indicated that there were 2 quaternary carbons, 4 methine carbons, 1 methylene carbon and 1 methyl carbon. The information was found to be consistent with the molecular formula, $C_8H_{10}O$, with unsaturation index of 4.

$$C_8H_{10}O \qquad n = 8, r = 2$$

$$UI = (8-2+2)/2 = 4$$
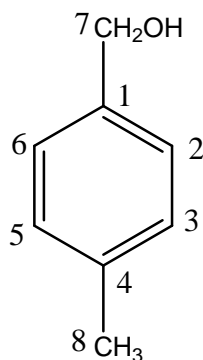
p-Tolylmethanol (**30**)

From the 8 signals there were 6 typical aromatic sp^2 hybridized carbons; 147.1, 113.1, 130.3, 154.5, 130.3 and 113.1 ppm. From these carbons C-1 (154.5 ppm) and C-4 (147.1 ppm) are quaternary and C-2 (113.1 ppm), C-3 (130.3 ppm), C-5 (130.3 ppm) and C-6 (113.1 ppm) are bearing a proton each. The remaining 2 carbons were accounted for by a methylene carbon C-7 (64.0 ppm) and methyl carbon, C-8 (14.5 ppm). Carbons C-1, C-2, C-3 C-4, C-5 and C-6 are part of the aromatic system as deciphered from the 2-D NMR experiments COSY, NOESY and HMBC (**appendices 26-30**). It can be seen that (**table 6**) from COSY spectrum that protons with

chemical shifts: 6.44 ppm, 7.26 ppm and 7.26 ppm; 6.44 ppm, 7.26 ppm and 6.44 ppm are correlating. This information was used to propose the structure of the compound.

Table 6: Summarized data from 1-D and 2-D NMR experiments for compound JO5066K232

Position	^{13}C	^{1}H	DEPT	HMBC	COSY
1	154.5	-	-	H-7	-
2	113.1	6.44	CH	-	H-3/H-5
3	130.3	7.26	CH	-	H-6//H-2
4	147.1	-	-	H-8	-
5	130.3	7.26	CH	-	H-6//H-2
6	113.1	6.44	CH	-	H-3/H-5
7	64.0	3.92	CH_2	-	-
8	14.5	2.43	-	-	H-8

CHAPTER FIVE
CONCLUSION AND RECOMMENDATIONS

5.1   Conclusion

The research findings in this work revealed that the basidiomycete, *Trametes* sp (JO5066) produced biologically active compounds when grown in liquid submerged cultures for 21 days. The crude extract from culture filtrate showed both larvicidal activity against *A. aegyptii* and antibacterial and antifungal activities. The crude extract showed 100% mortality of the mosquito larvae for a concentration of 50 ppm within 24 hours. The extract had significant activity against both Gram negative and Gram positive bacteria with inhibition zones of 13~15 mm in agar diffusion assay. For the only fungal target organism used, *C. albicans* the inhibition zone of 15 mm was measured. These observations clearly demonstrated that the *Trametes* sp (JO5066) was producing biologically active compounds.

The crude extracts when fractionated and purified for the active compounds responsible for the observed biological activities afforded two compounds 2,3-dihydro-3,3-dimethyl-1H-indene-1,2,6-triol (**29**) and *p*-tolylmethanol (**30**) whose structures were elucidated based on NMR experiments. It is only the former that was obtained in sufficient amounts to allow further testing of activities and performance of spectroscopic experiments. The latter was obtained in low amounts that allowed only for spectroscopic studies. 2,3-Dihydro-3,3-dimethyl-1H-indene-1,2,6-triol (**29**) showed clear larvicidal activity. The compound **29** had a considerable activity with LC_{50} of 235 ppm and LC_{90} of 674 ppm against the third instar larvae of *A. aegyptii*. The larvicidal activity of the pure compound was noted to be lower than for the crude extract. This may be due to synergism present in crude extract This is a major finding for this study given that compound **29** maybe a new compound reported for the first time from cultures of a fungal source. In addition the larvicidal activity for the compound **29** is reported in this thesis for the first time. With these findings all the objectives of the study were met as were prepared at the beginning.

5.2   Recommendations

This study addressed the set objectives but there were limitations. If these are addressed the quality of the results would be greatly enhanced. However, for further work I would recommend;

- To complete the spectroscopic analysis, mass spectrometric analysis need to be carried out.

- The proposed chemical structure for compound **29** should be confirmed to establish the stereochemical aspects using organic synthesis techniques.

- Further larvicidal assay in addition to critical activities like toxicity studies needs to be done to validate the potential application of compound 29 as a commercial larvicidal agent.

- The producing organism, *Trametes* sp (5066) should be taxonomically identified to species level and cultivated in much larger scales to produce more of the active compound **29**.

- Use of modern equipment and techniques to isolate the active compounds that might not have been accomplished in this study.

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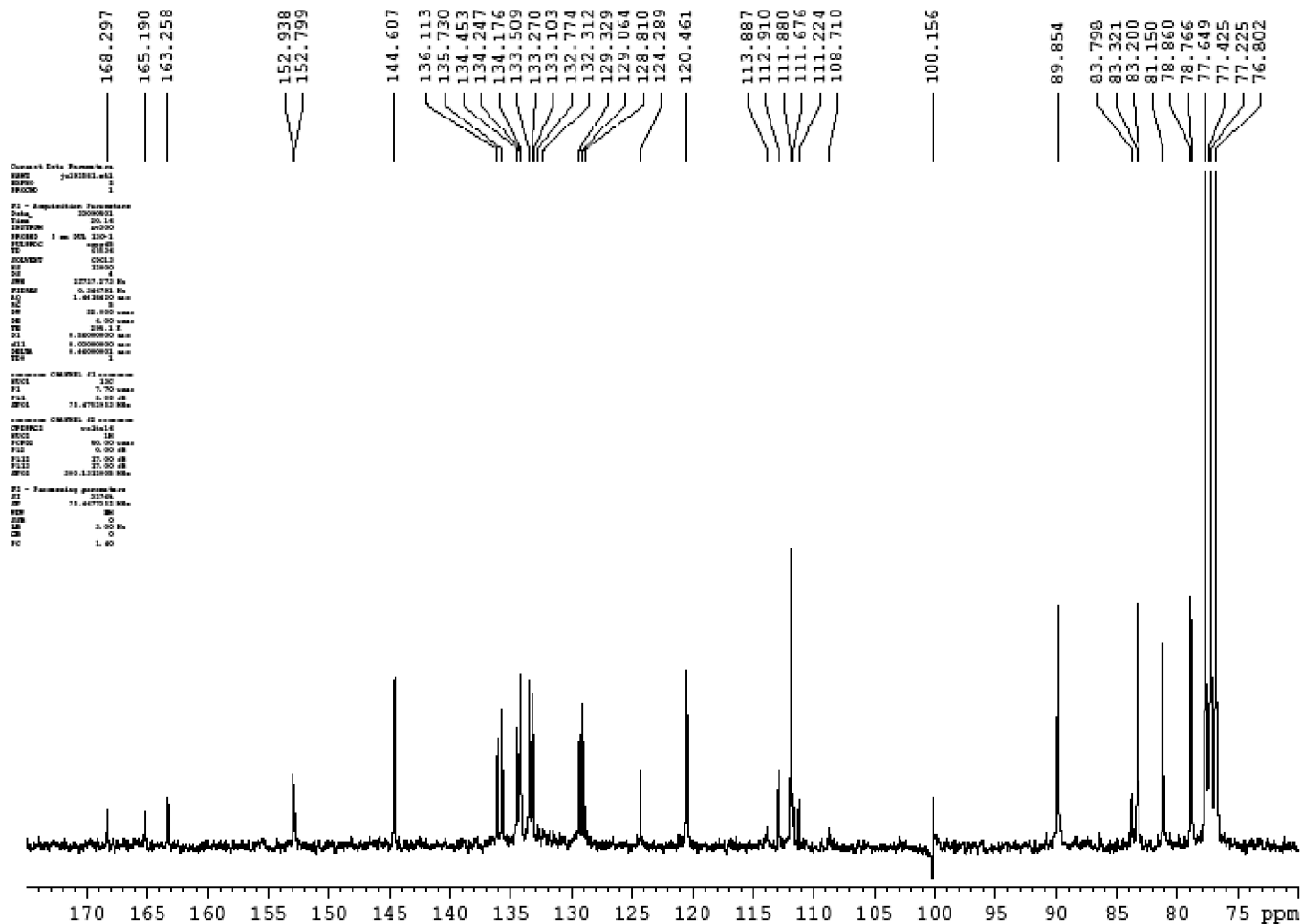
APPENDICES

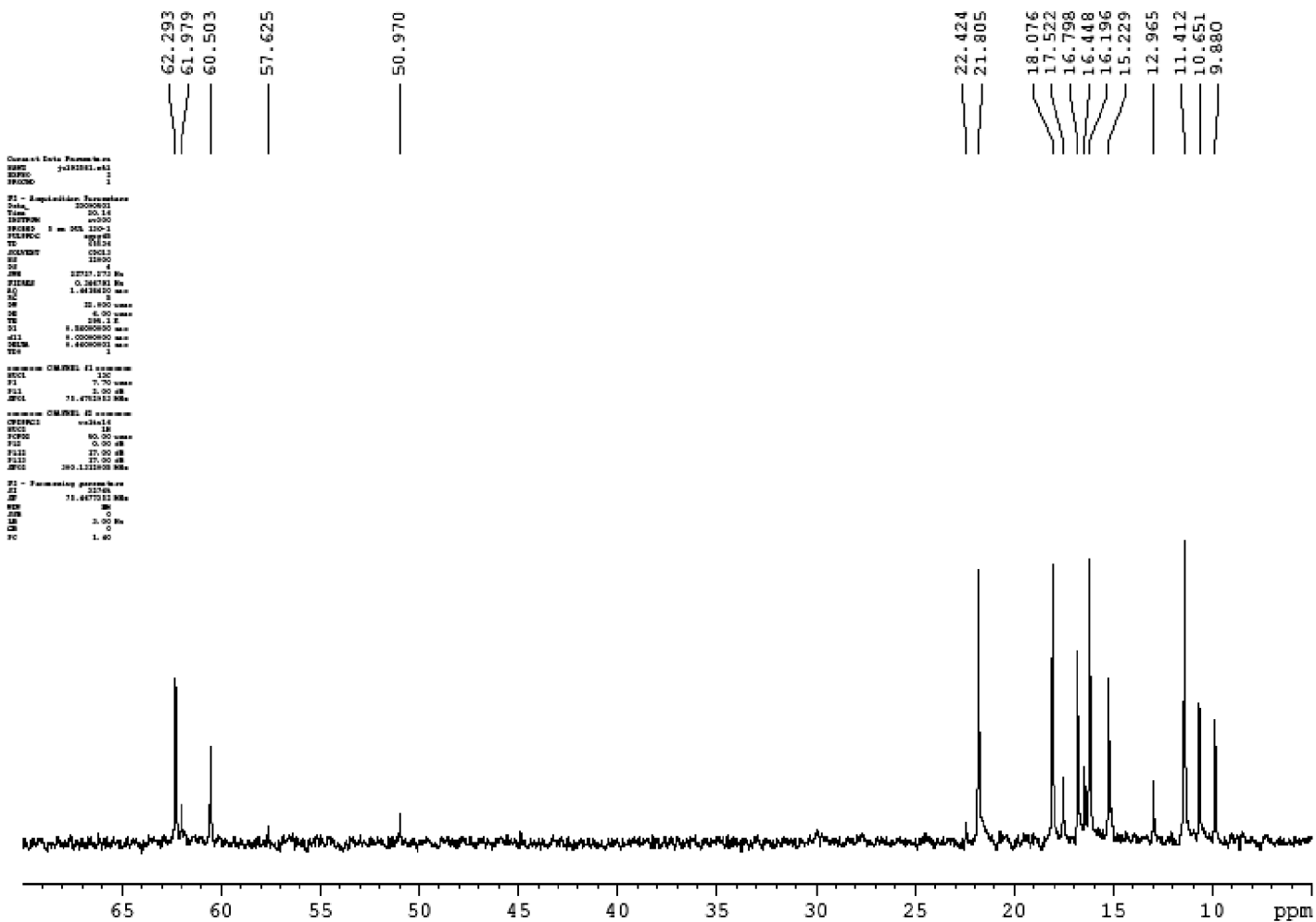**Appendix 1: ^{1}H NMR spectrum for compound 29**

1H 337 in CDCl3 300MHz proton spectrum
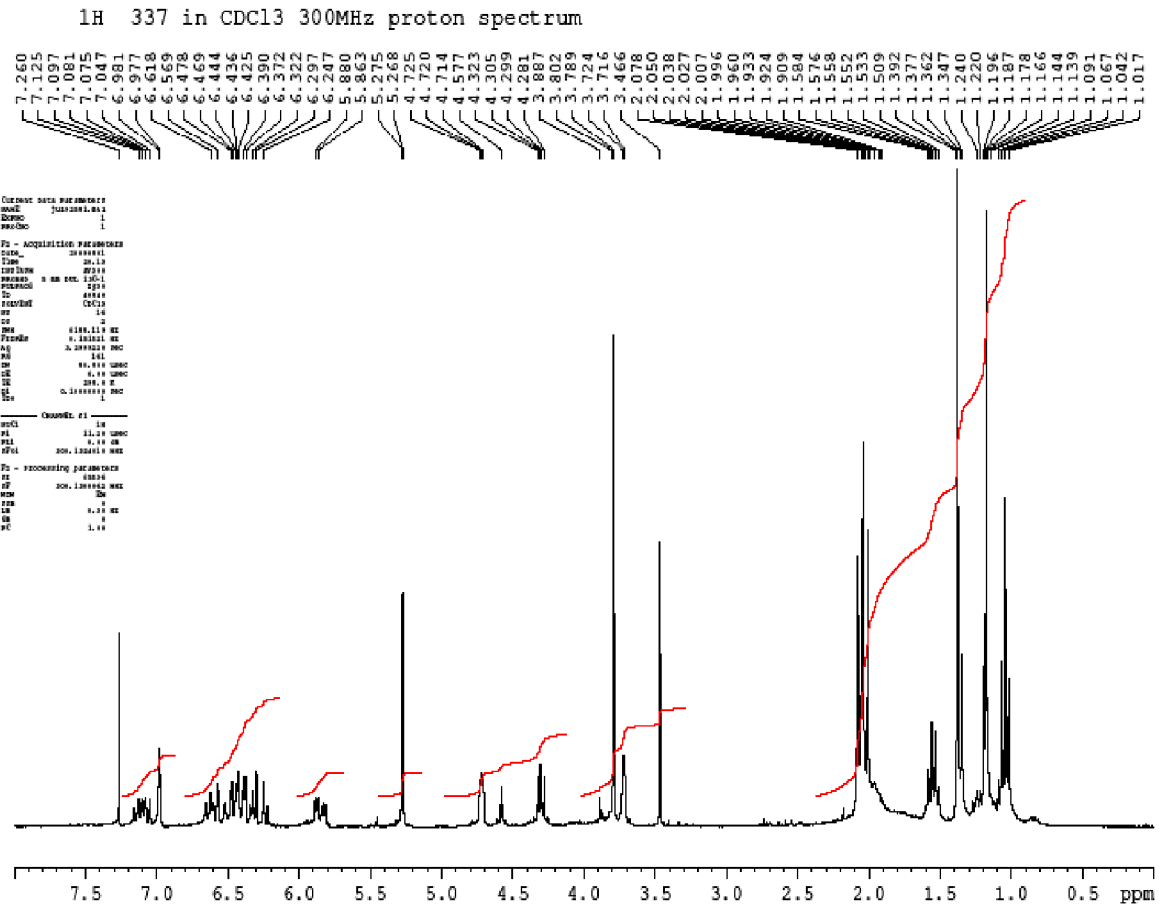
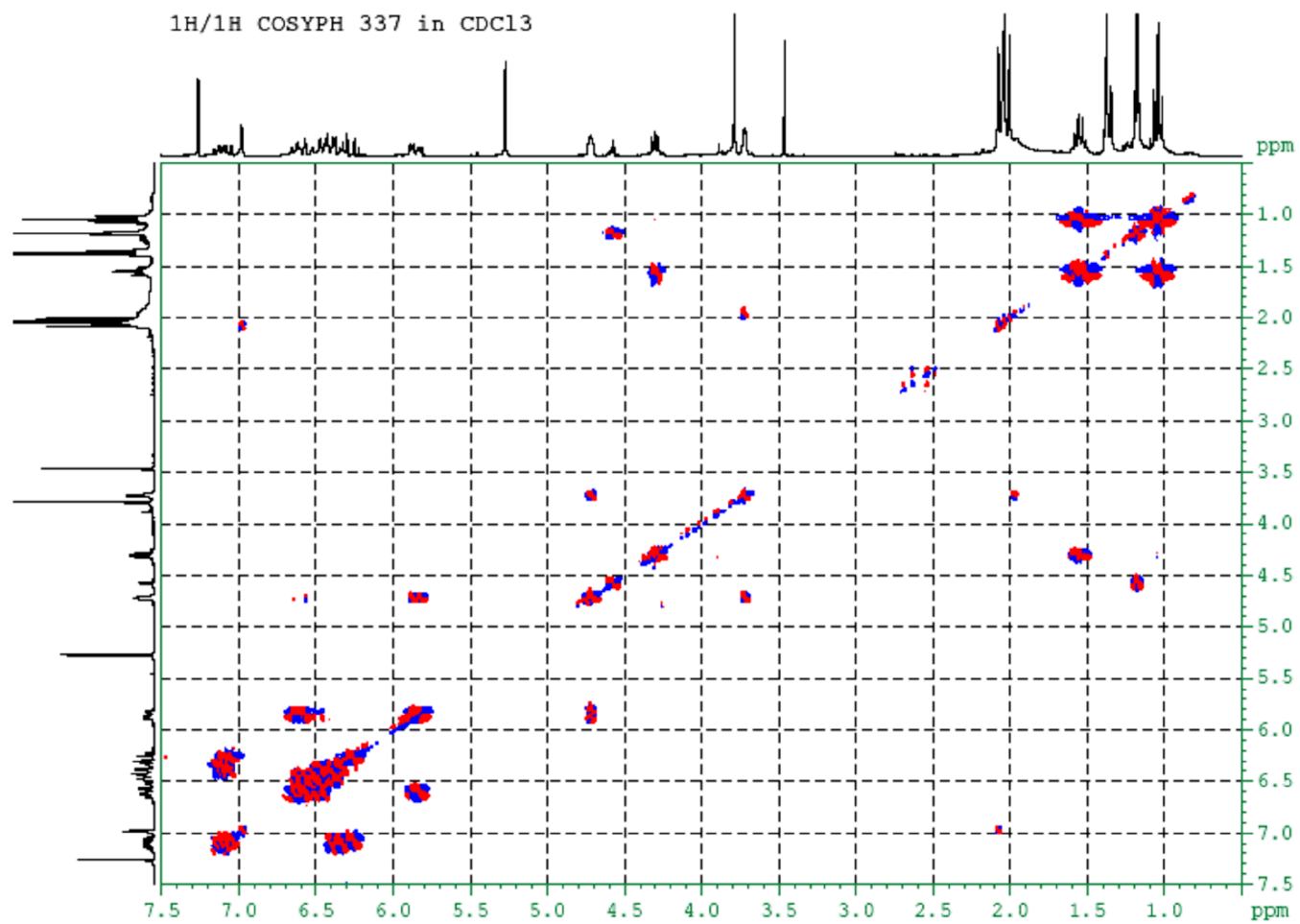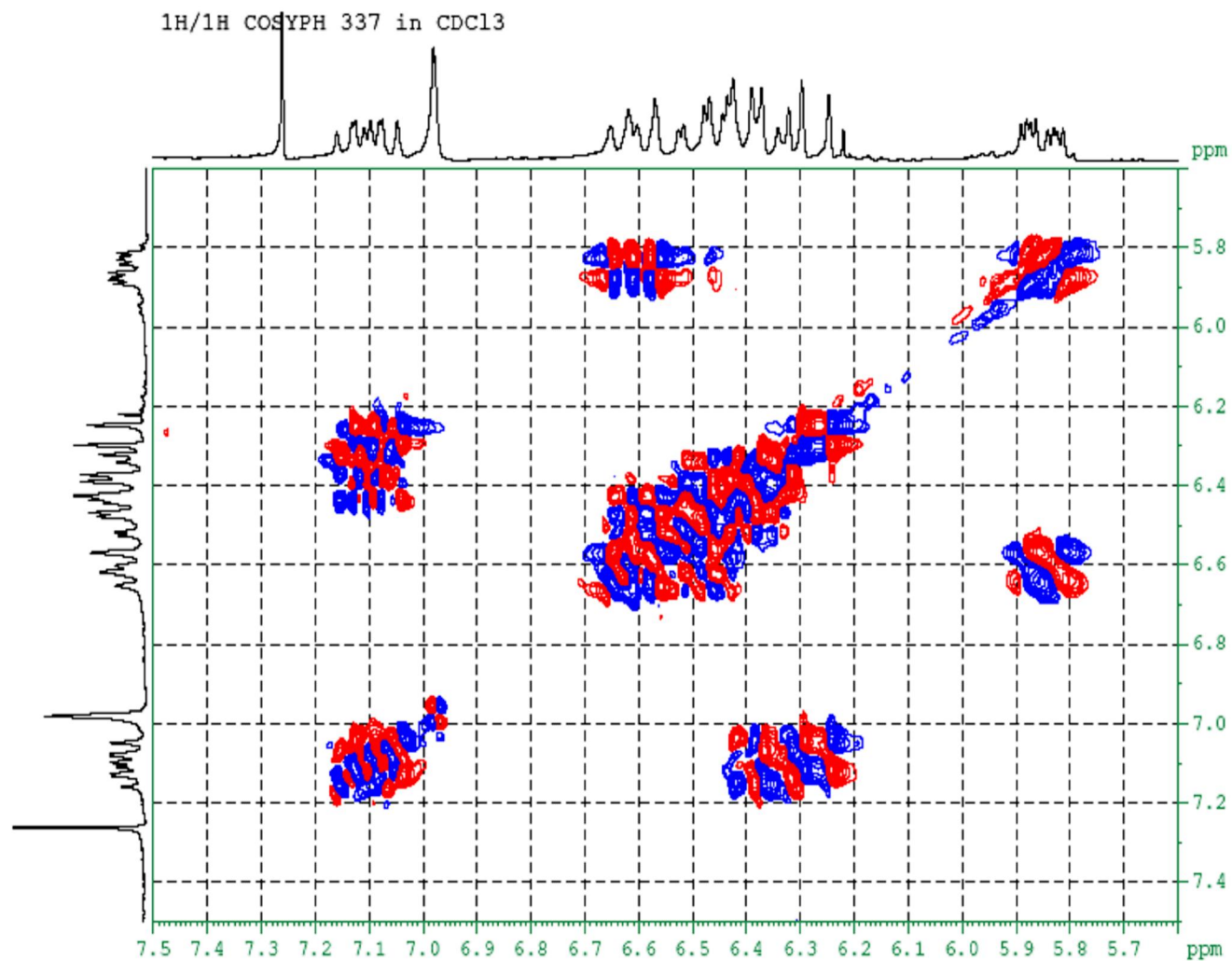**Appendix 2: ¹H NMR spectrum for compound 29**
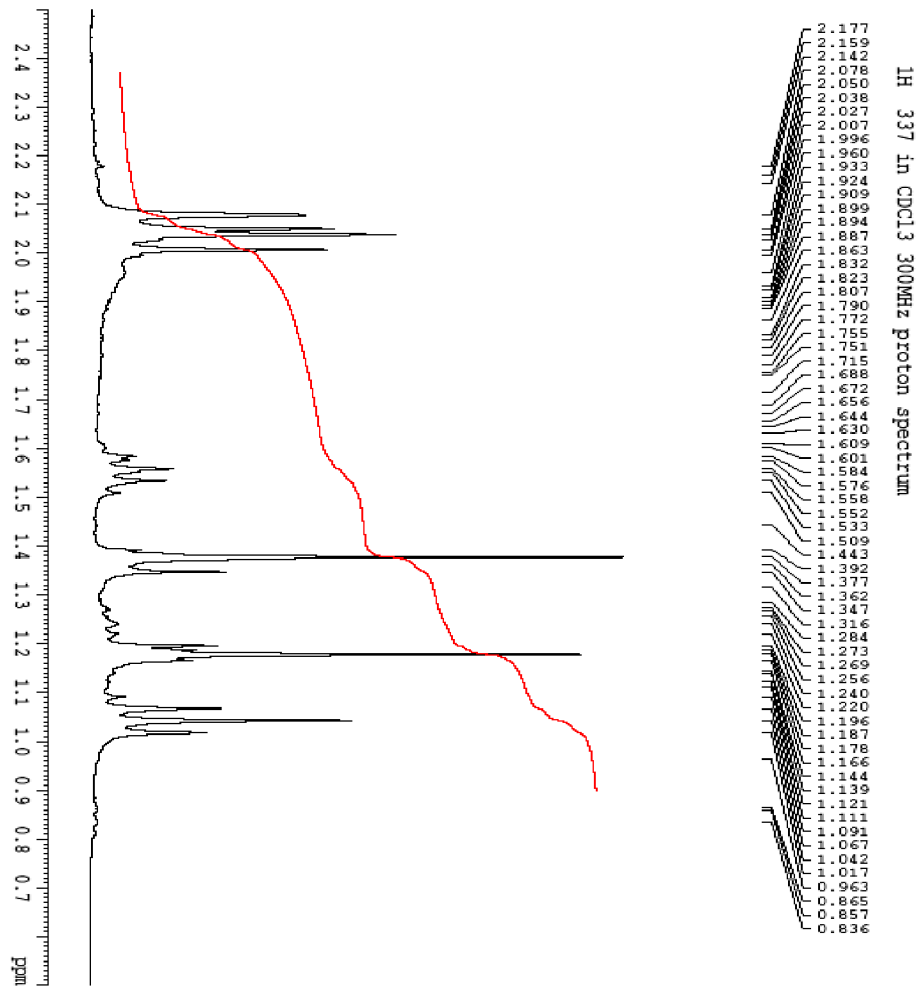
Appendix 3: ^{13}C NMR spectrum for compound 29

Appendix 4: ^{13}C NMR spectrum for compound 29
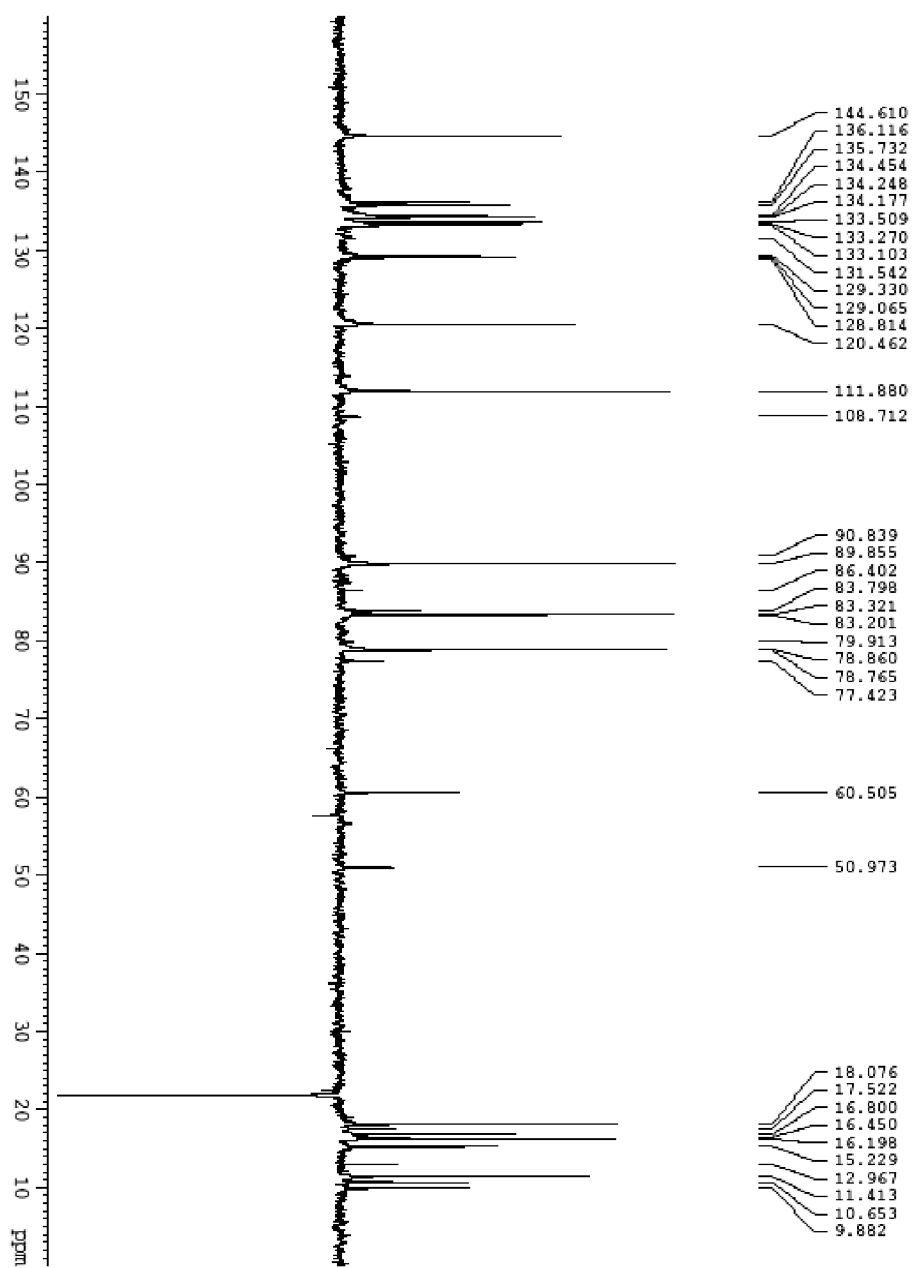
Appendix 5: COSY NMR spectrum for the compound 29

1H/1H COSYPH 337 in CDCl3

Appendix 6: COSY NMR spectrum for the compound 29

1H/1H COSYPH 337 in CDCl3

Appendix 7: COSY NMR spectrum for the compound 29

1H/1H COSYPH 337 in CDCl3
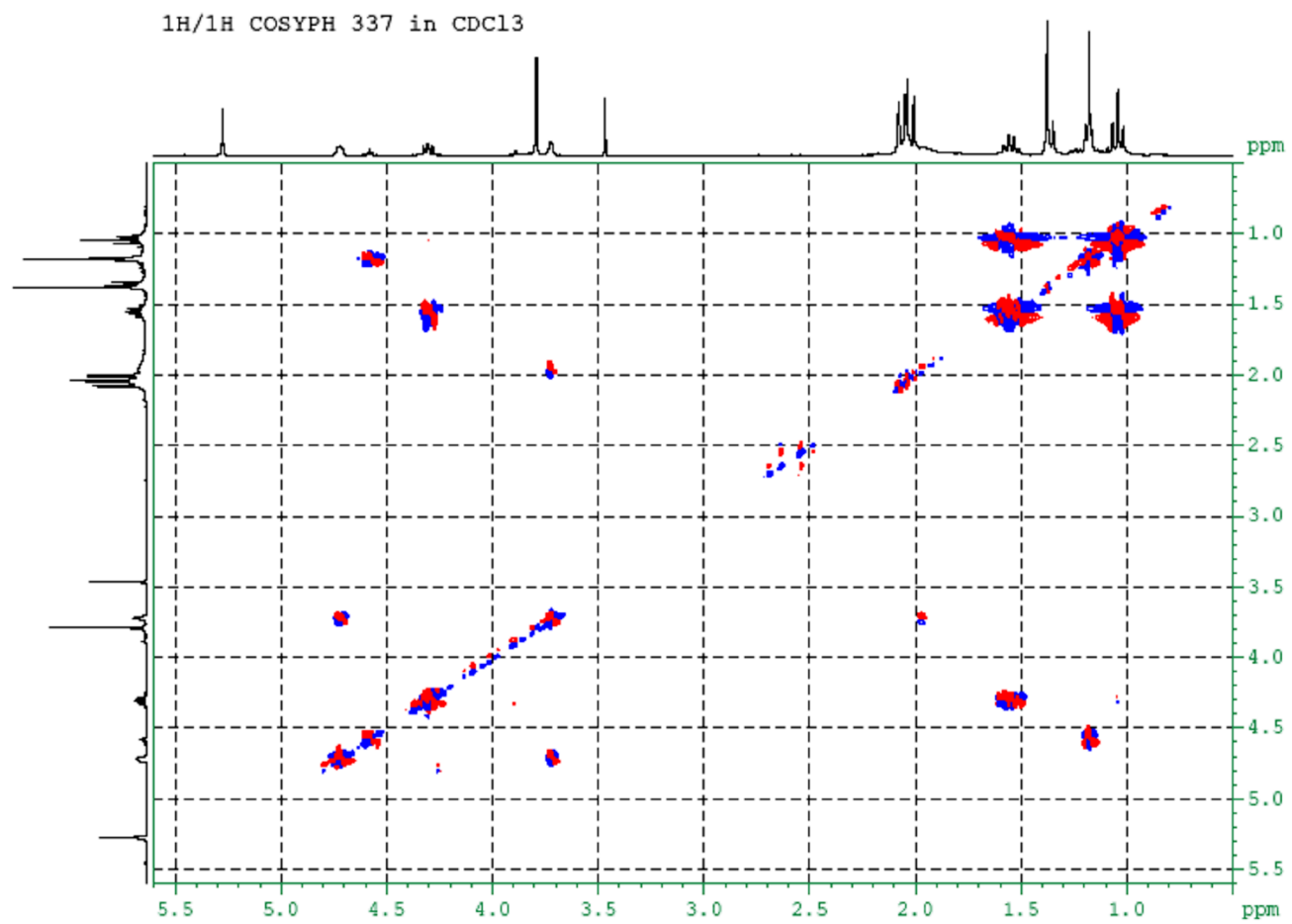
Appendix 8: DEPT spectrum for compound 29

144.610
136.116
135.732
134.454
134.248
134.177
133.509
133.270
133.103
131.542
129.330
129.065
128.814
120.462

111.880
108.712

90.839
89.855
86.402
83.798
83.321
83.201
79.913
78.860
78.765
77.423

60.505

50.973

18.076
17.522
16.800
16.450
16.198
15.229
12.967
11.413
10.653
9.882

Appendix 9: DEPT spectrum for compound 29

144.610

136.116
135.732
134.454
134.248
134.177
133.509
133.270
133.103
131.542
129.330
129.065
128.814

120.462

111.880

108.712

90.839
89.855

86.402
83.798
83.321
83.201
79.913
78.860
78.765
77.423

60.505

50.973
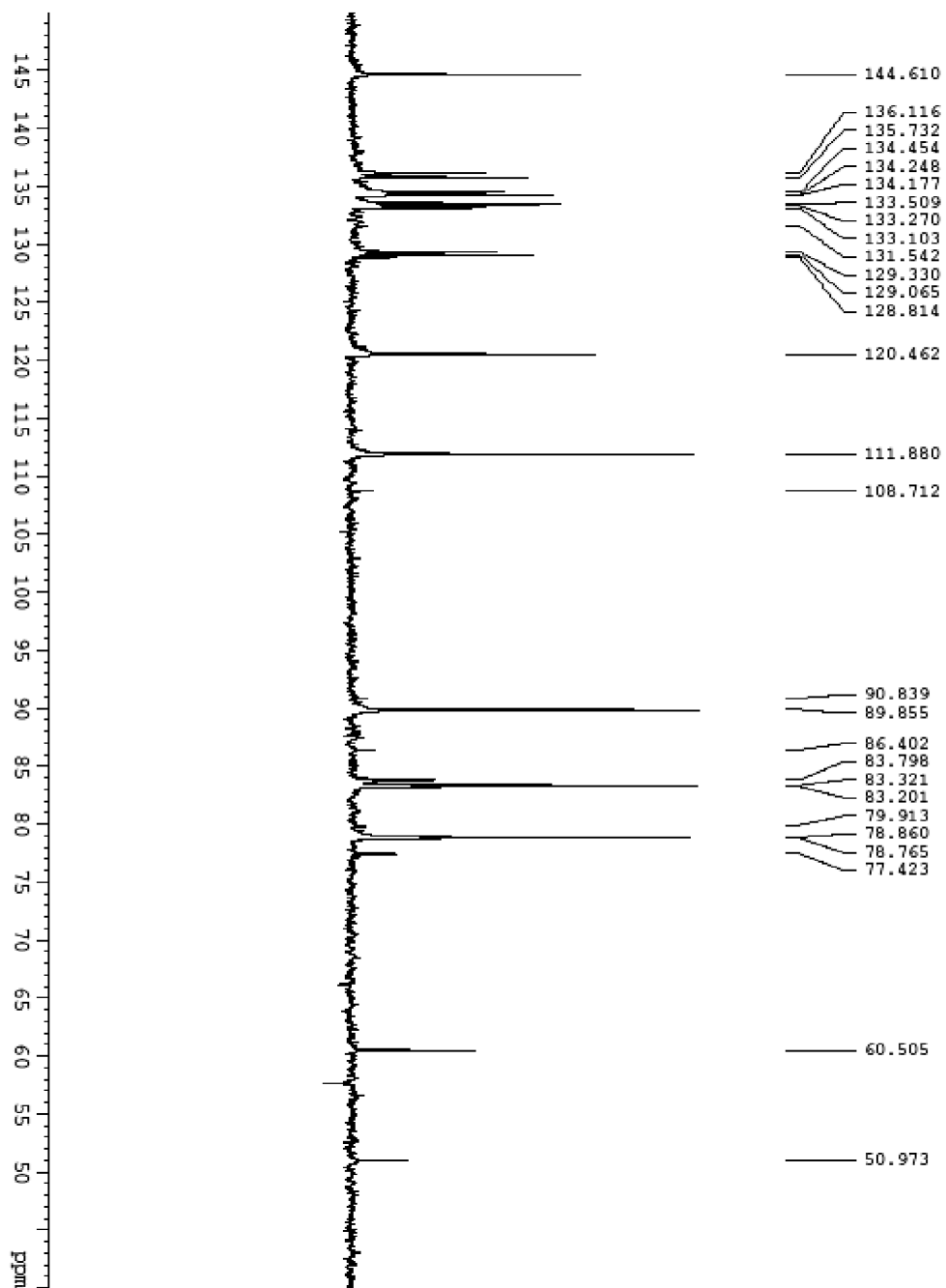
Appendix 10: DEPT spectrum for compound 29
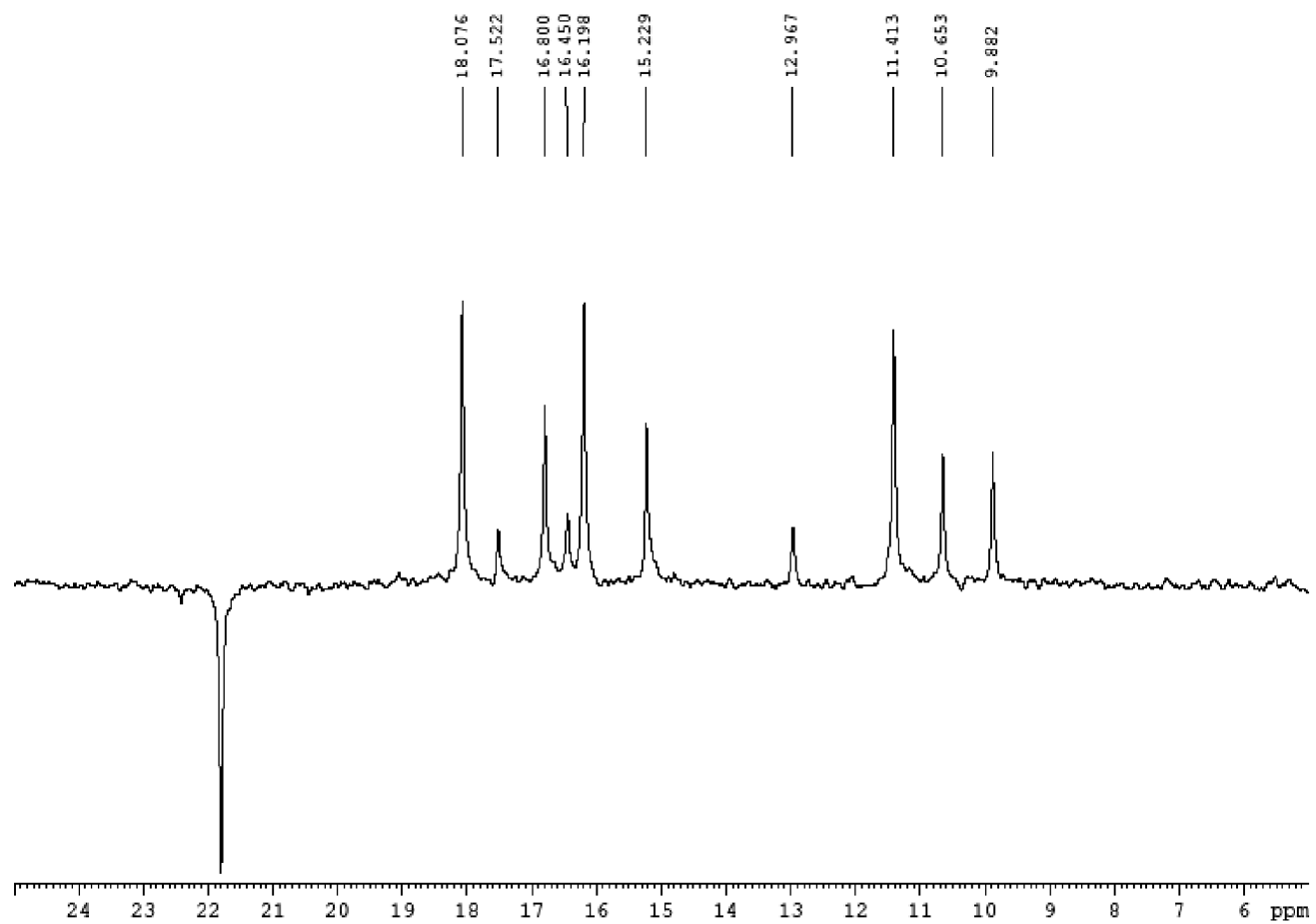
Appendix 11: DEPT spectrum for compound 29

136.116
135.732
134.454
134.248
134.177
133.509
133.270
133.103

131.542

129.330
129.065
128.814

120.462

111.880

108.712

59
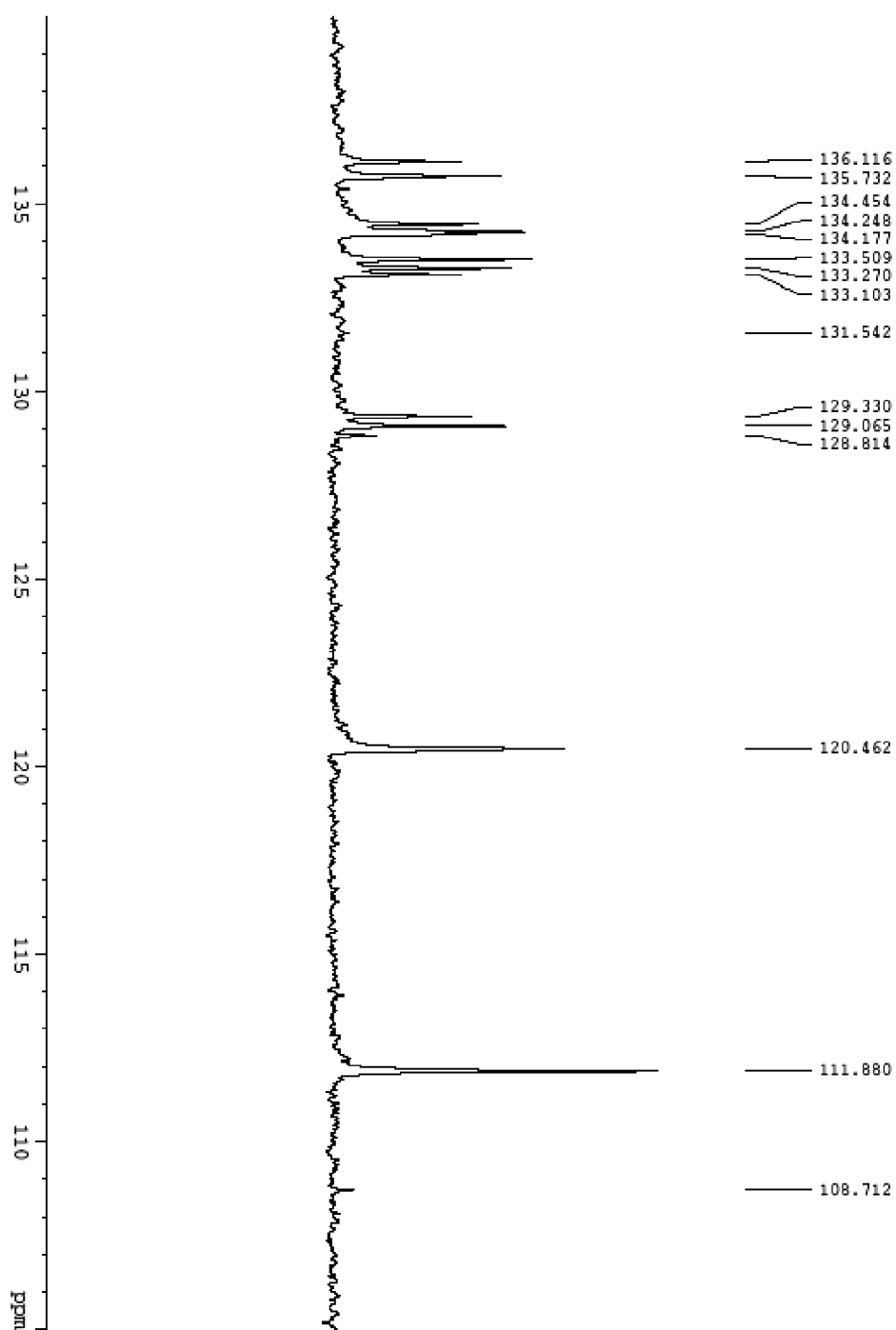
Appendix 12: HMBC spectrum for compound 29

1H/13C HMBC LP, 300/75 MHz 2D spectrum, 337 in CDCl3
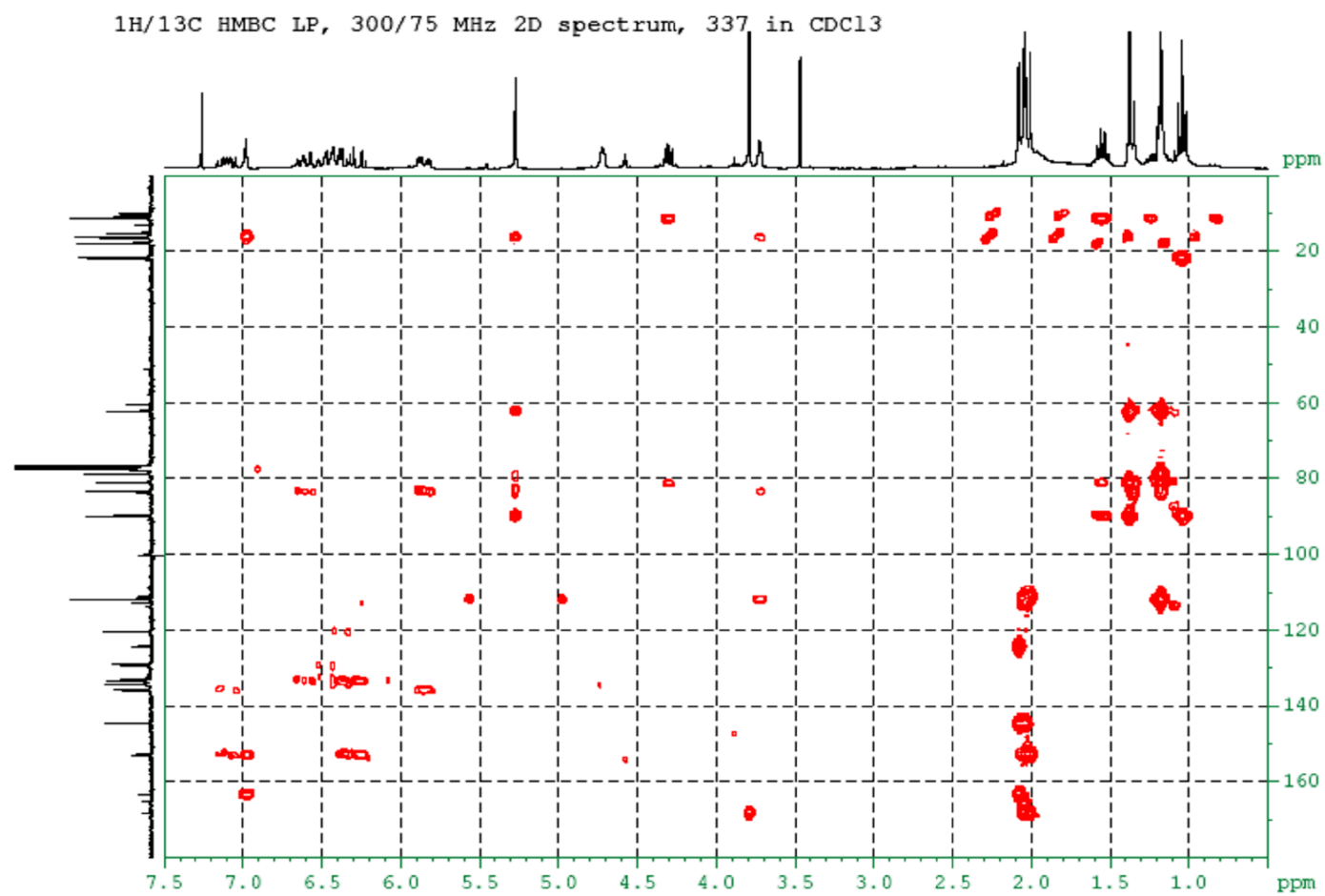
Appendix 13: HMBC spectrum for compound 29

1H/13C HMBC LP, 300/75 MHz 2D spectrum, 337 in CDCl3
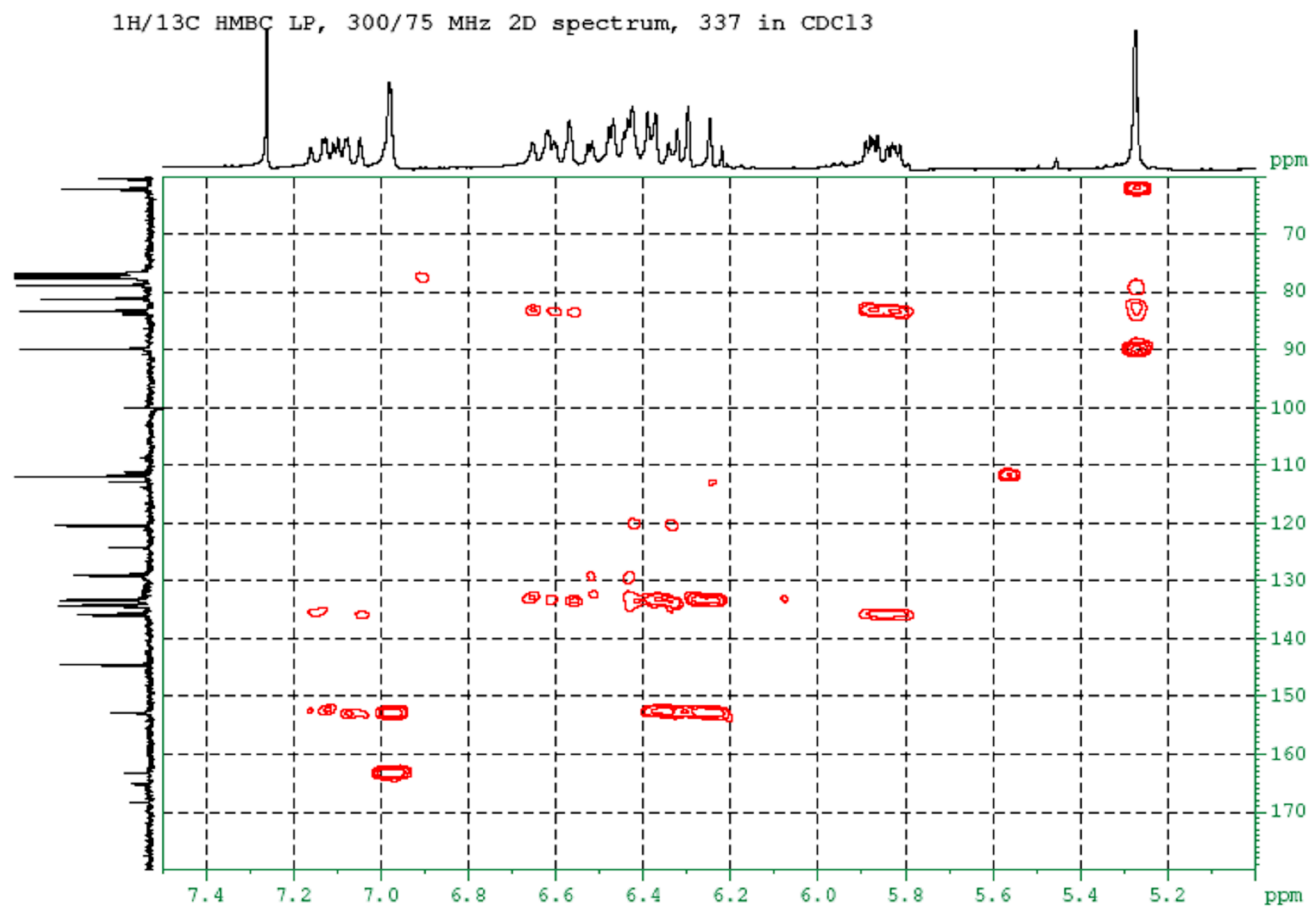
Appendix 14: HMBC spectrum for compound 29

1H/13C HMBC LP, 300/75 MHz 2D spectrum, 337 in CDCl3
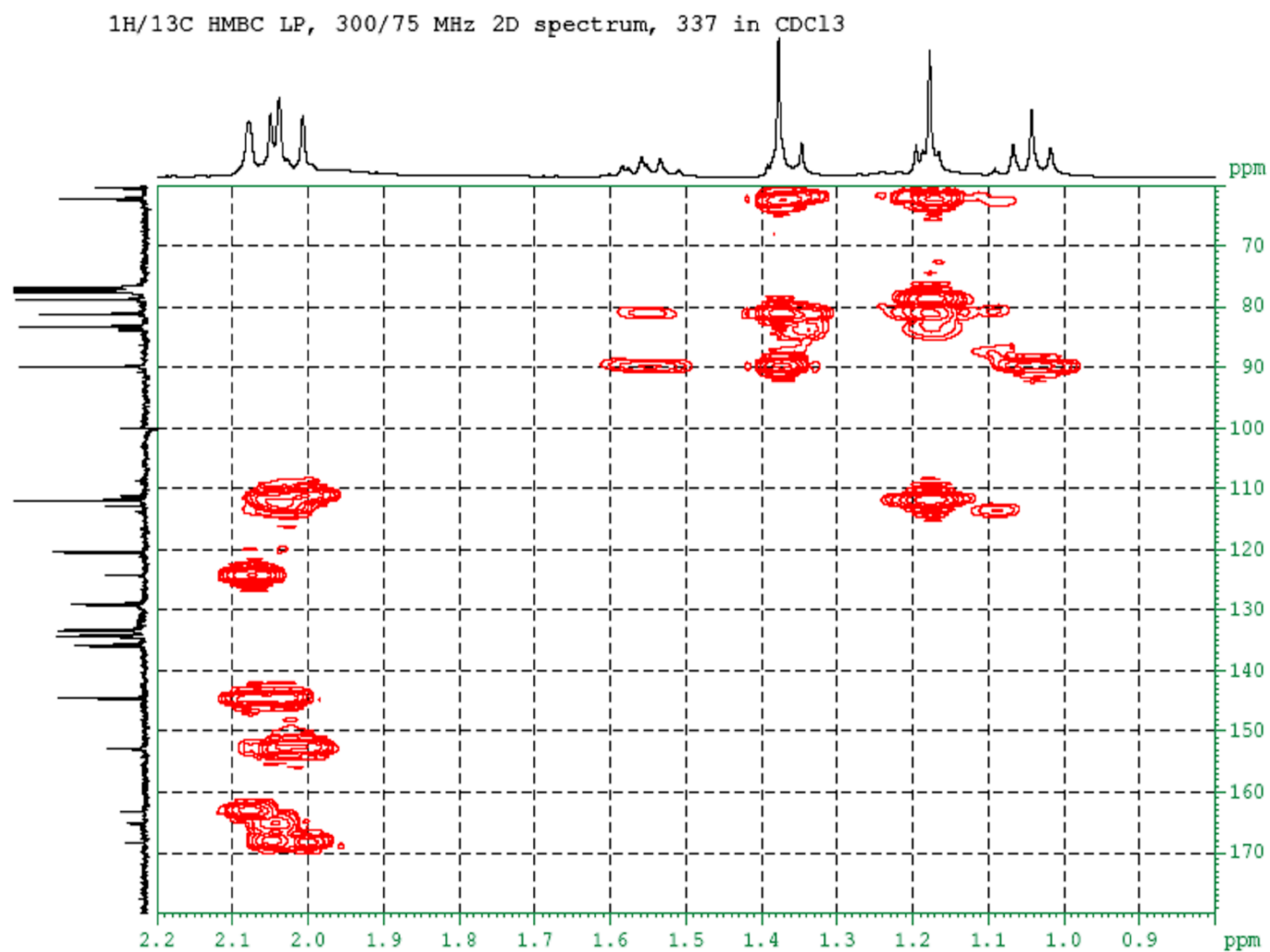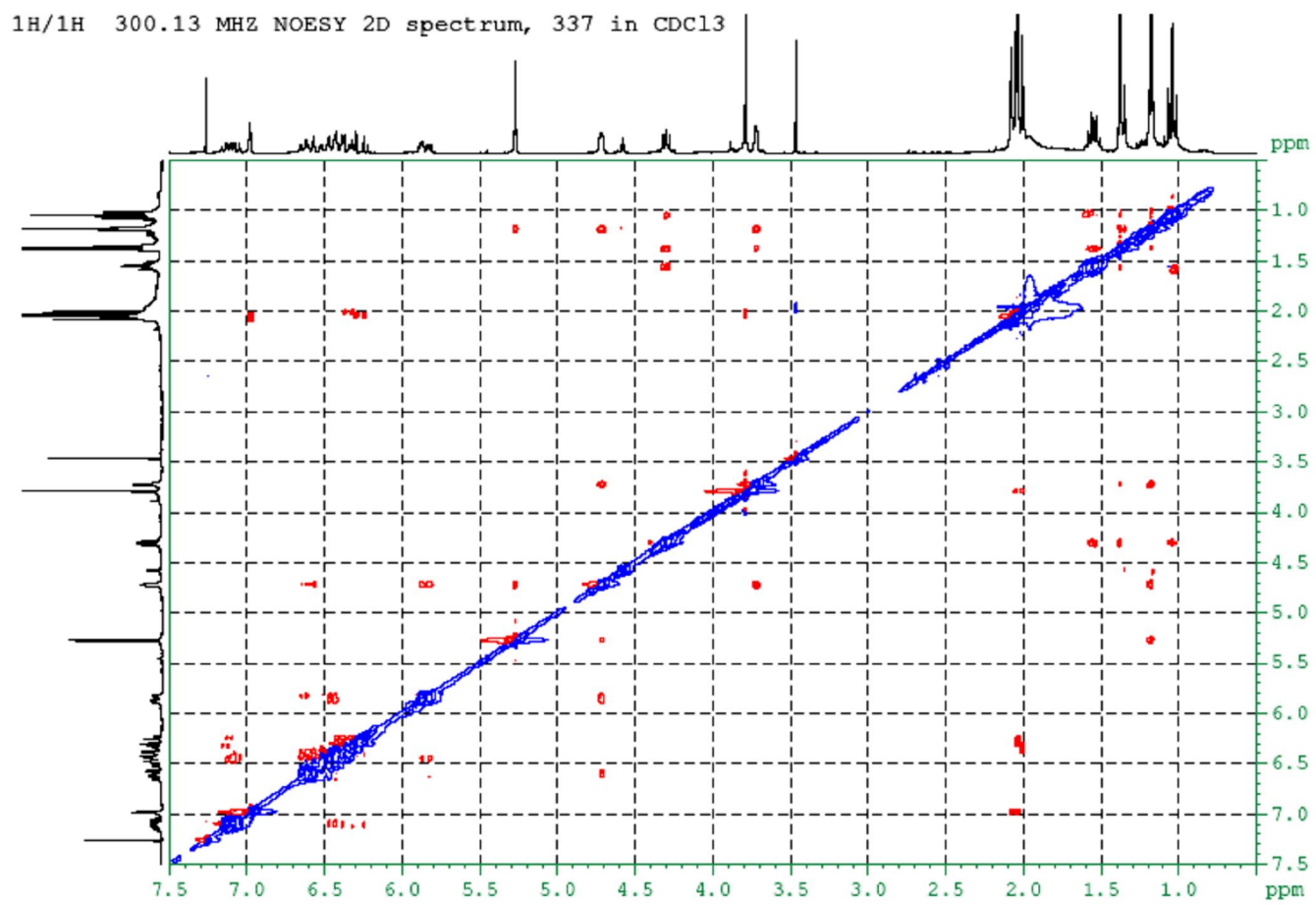
Appendix 15: HSQC-DEPT spectrum for compound 29

1H/13C HSQC-DEPT, 337 in CDCl3 300/75 MHz Phase sens. spectrum.
MAY NEED MANUAL PHASE CORRECTION.
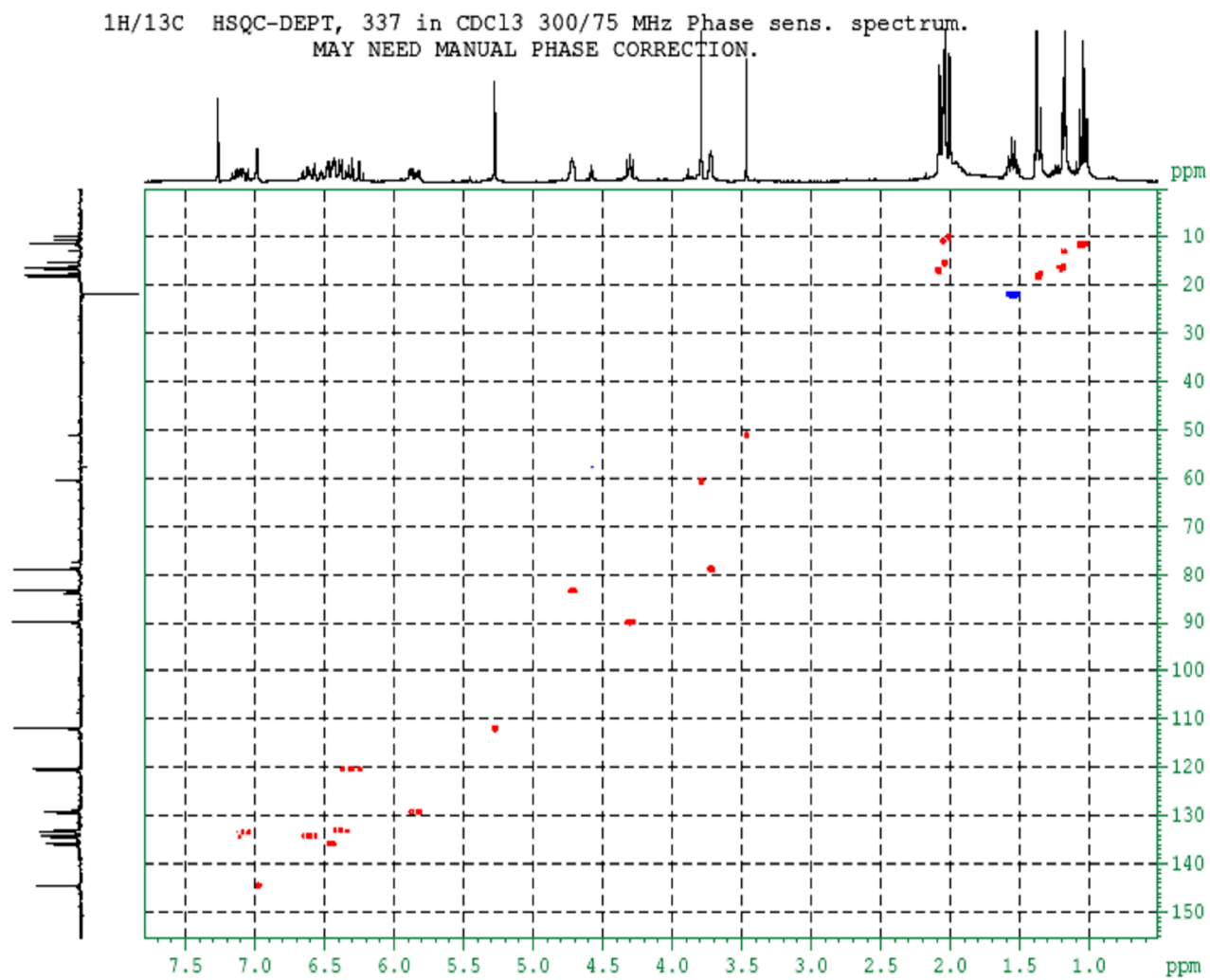
Appendix 16: HSQC-DEPT spectrum for compound 29

1H/13C HSQC-DEPT, 337 in CDCl3 300/75 MHz Phase sens. spectrum.
MAY NEED MANUAL PHASE CORRECTION.
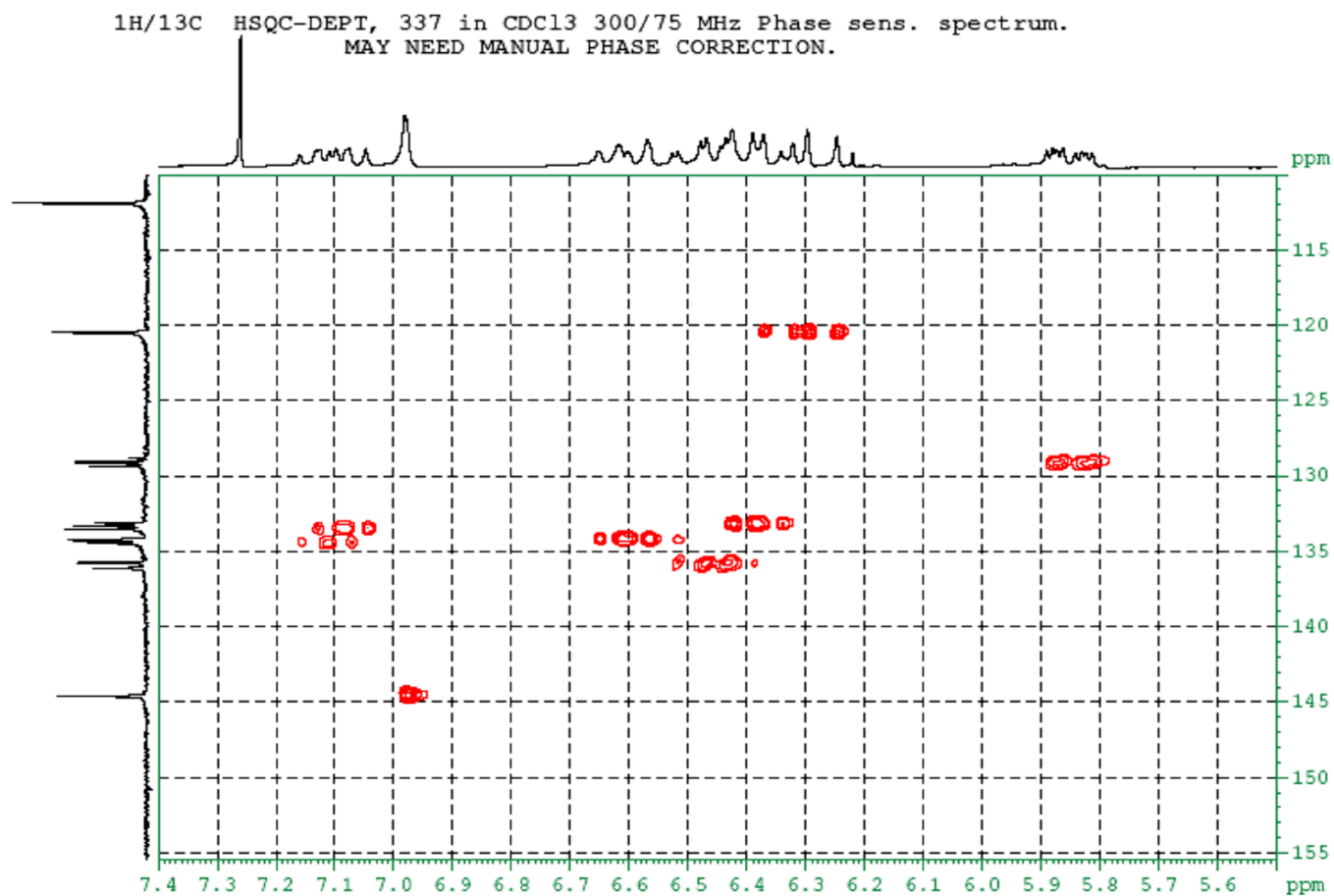
Appendix 17: HSQC-DEPT spectrum for compound 29

1H/13C HSQC-DEPT, 337 in CDCl3 300/75 MHz Phase sens. spectrum.
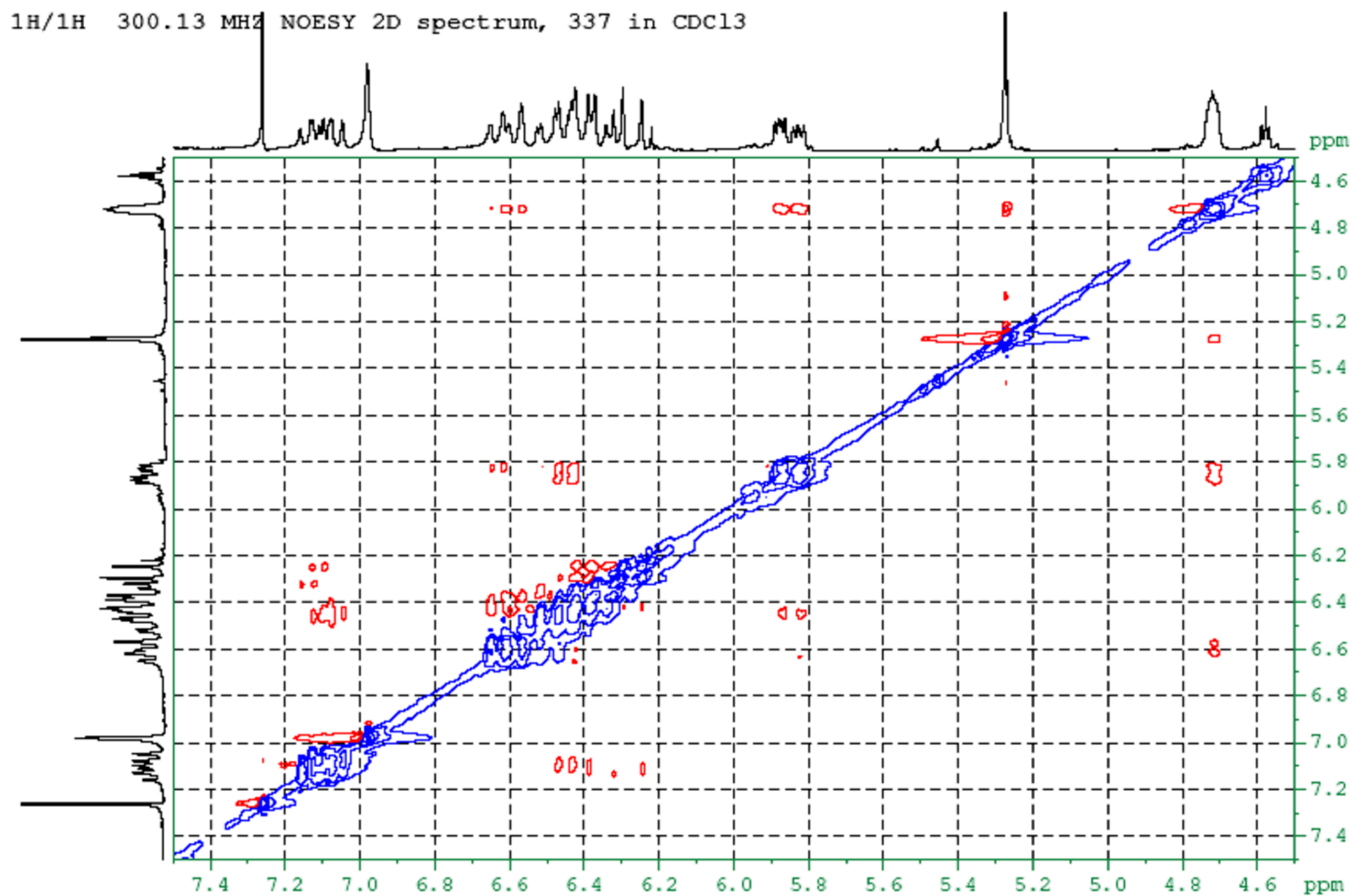MAY NEED MANUAL PHASE CORRECTION.
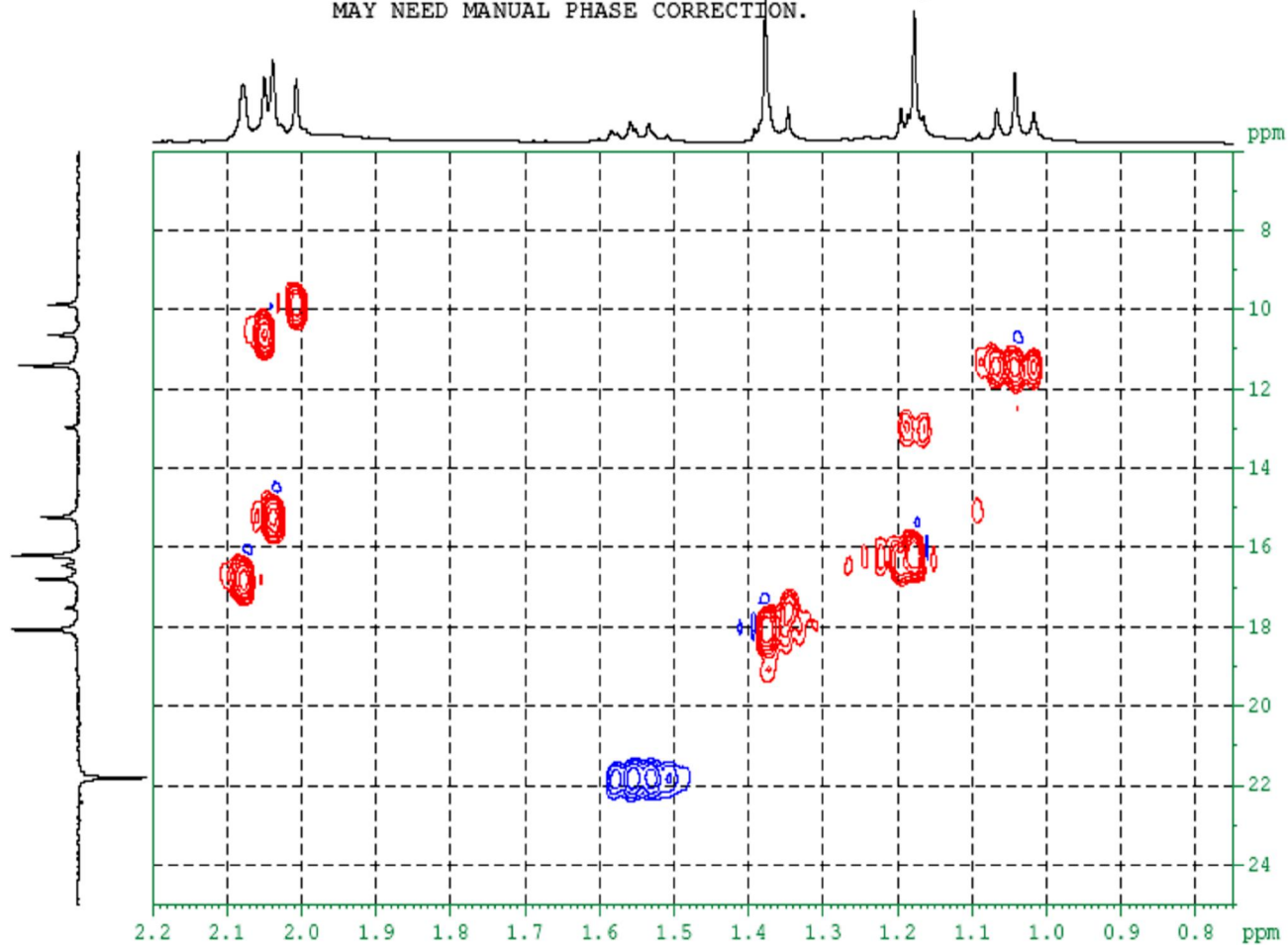
Appendix 18: HSQC-DEPT spectrum for compound 29

1H/13C HSQC-DEPT, 337 in CDCl3 300/75 MHz Phase sens. spectrum.
MAY NEED MANUAL PHASE CORRECTION.
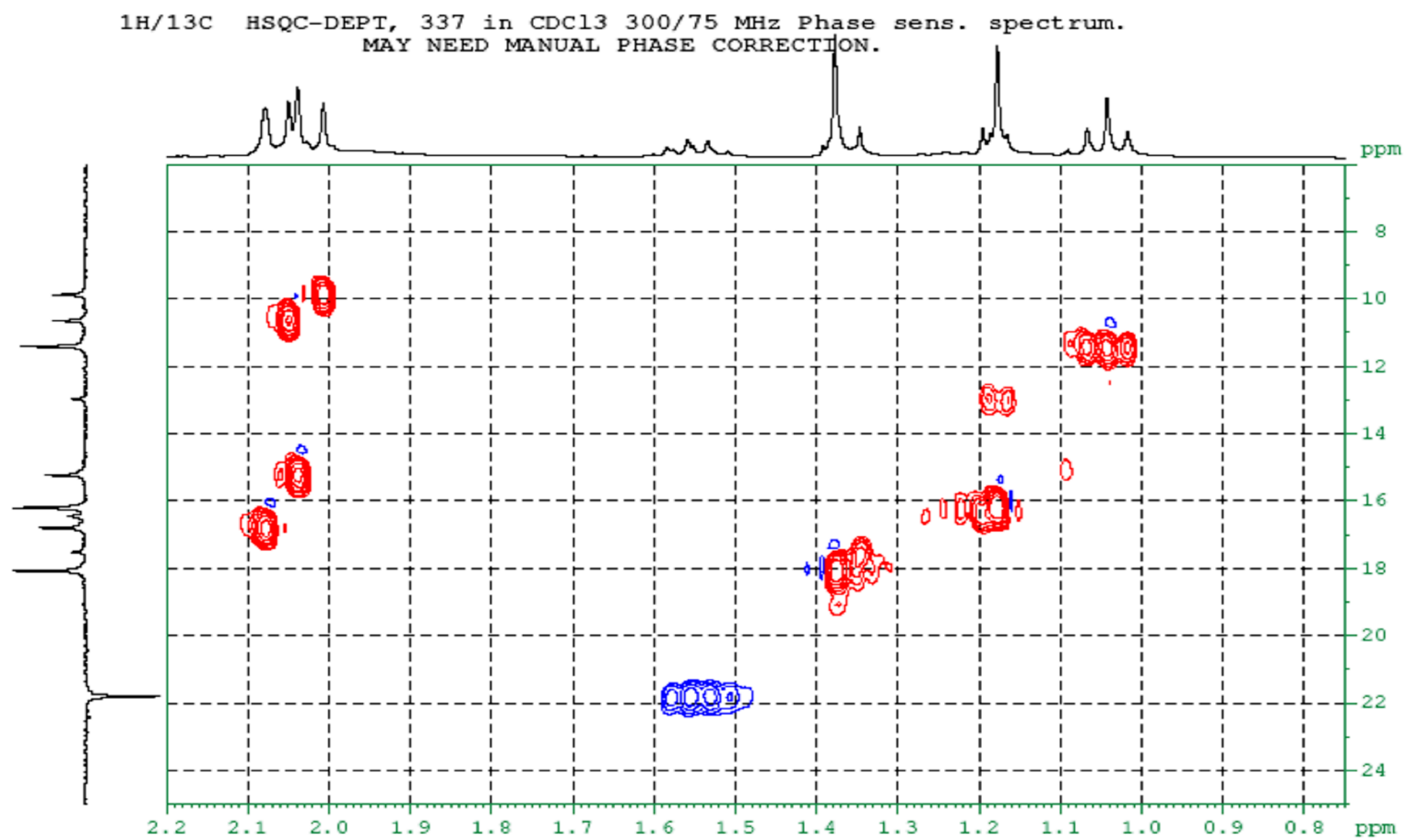
Appendix 19: NOESY spectrum for compound 29

1H/1H 300.13 MHZ NOESY 2D spectrum, 337 in CDCl3

Appendix 20: NOESY spectrum for compound 29

1H/1H 300.13 MHz NOESY 2D spectrum, 337 in CDCl3

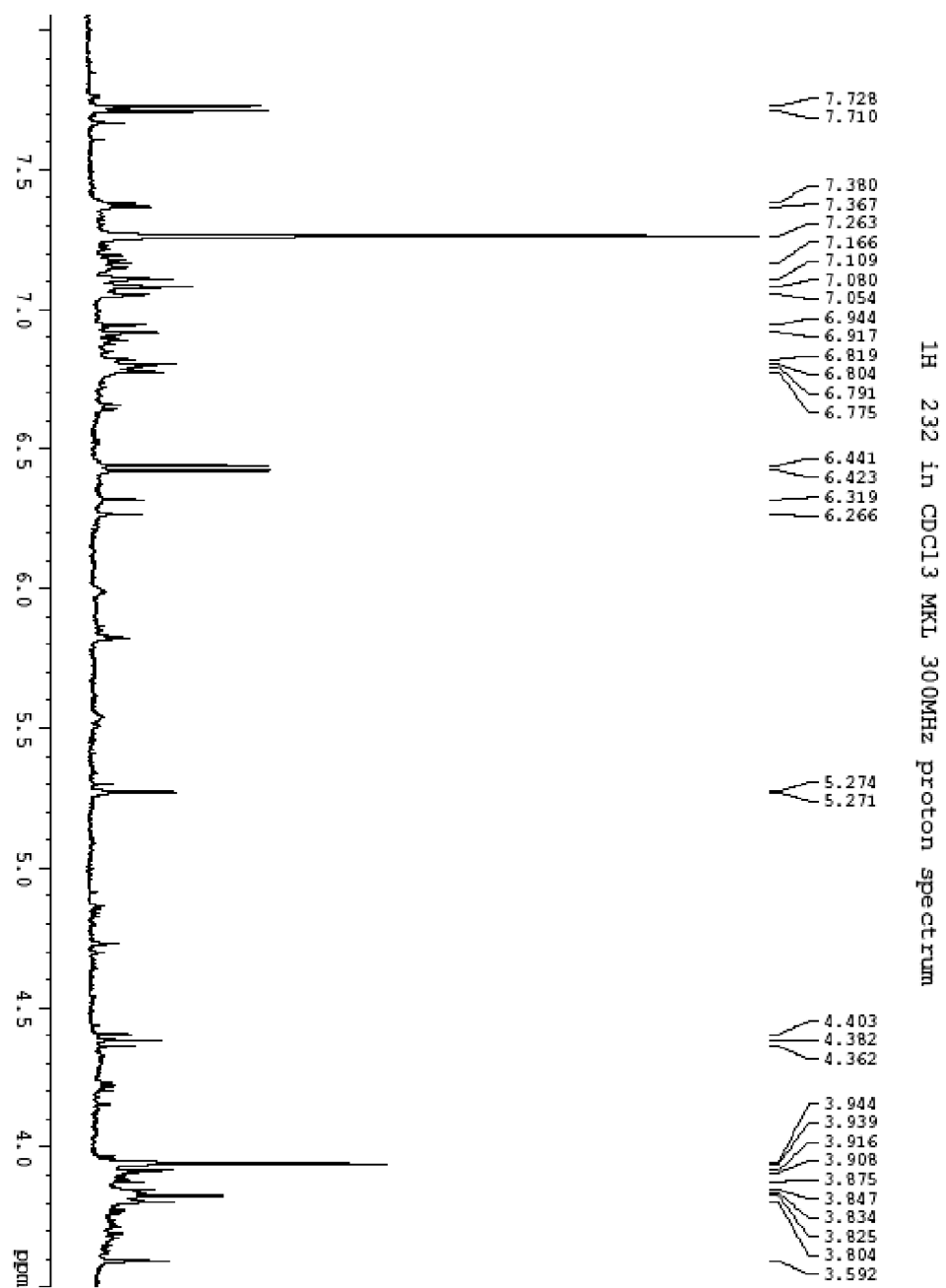**Appendix 21: NOESY spectrum for compound 29**

1H/1H 300.13 MHZ NOESY 2D spectrum, 337 in CDCl3
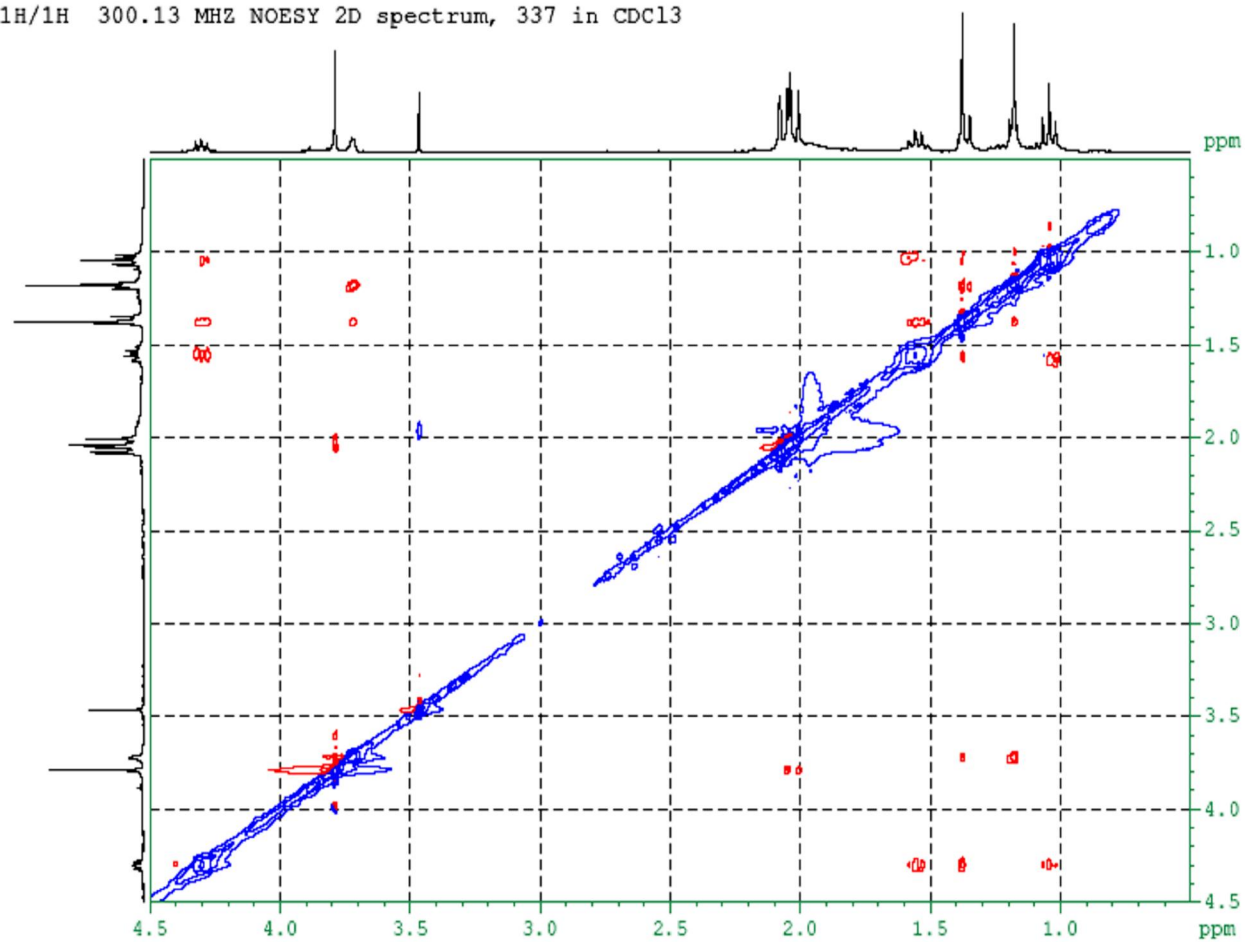
Appendix 22: ¹H spectrum for compound 30

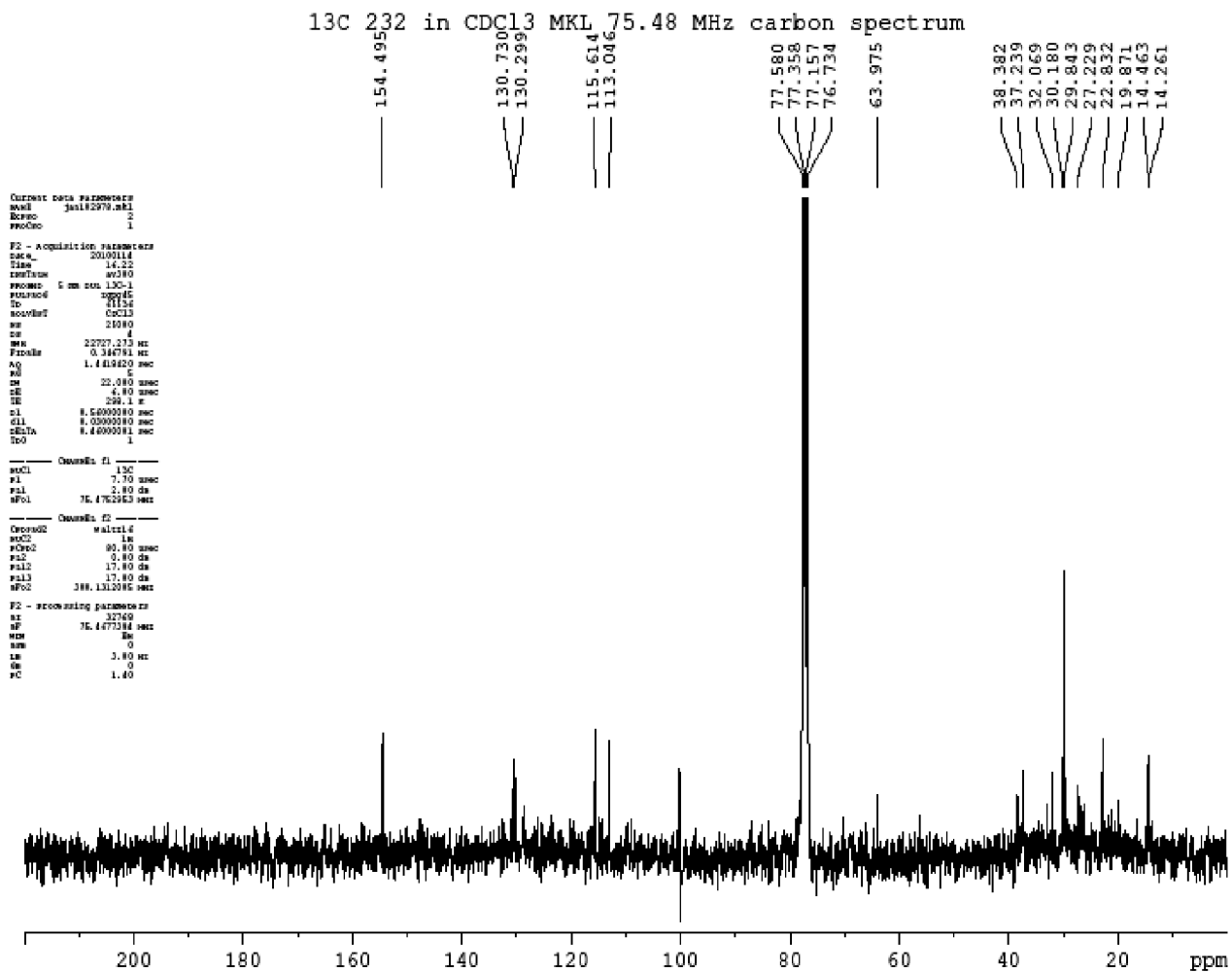1H 232 in CDCl3 MKL 300MHz proton spectrum

7.728
7.710

7.380
7.367
7.263
7.166
7.109
7.080
7.054
6.944
6.917
6.819
6.804
6.791
6.775

6.441
6.423
6.319
6.266

5.274
5.271

4.403
4.382
4.362

3.944
3.939
3.916
3.908
3.875
3.847
3.834
3.825
3.804
3.592

Appendix 23: ¹H spectrum for compound 30

1H 232 in CDCl3 MKL 300MHz proton spectrum
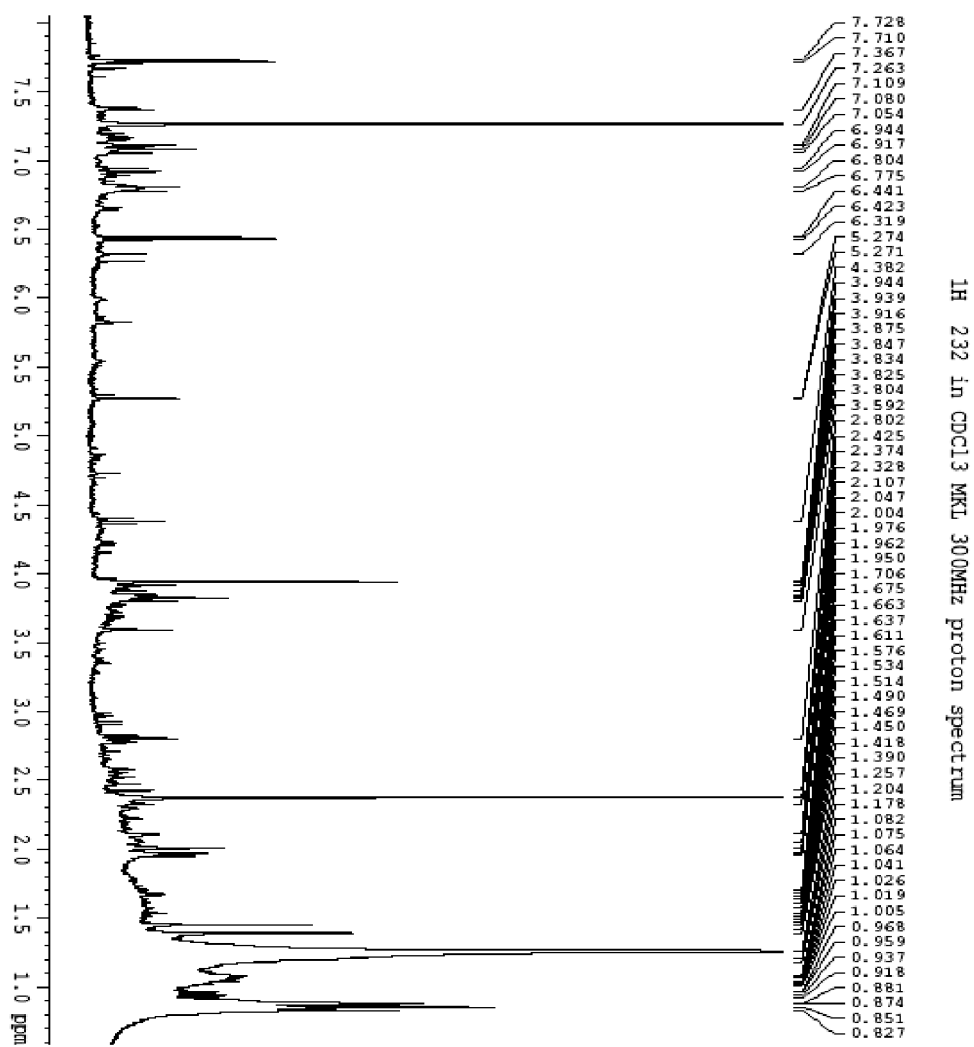
Appendix 24: ^{1}H spectrum for compound 30

1H 232 in CDCl3 MKL 300MHz proton spectrum

2.824
2.802
2.781
2.574
2.526
2.473
2.425
2.374
2.328
2.304
2.291
2.221
2.107
2.047
2.028
2.004
1.976
1.962
1.950
1.784
1.730
1.706
1.675
1.663
1.637
1.611
1.576
1.534
1.514
1.490
1.469
1.450
1.418
1.390
1.257
1.204
1.178
1.082
1.075
1.064
1.041
1.026
1.019
1.005
0.968
0.959
0.937
0.918
0.881
0.874
0.851
0.827

0.070
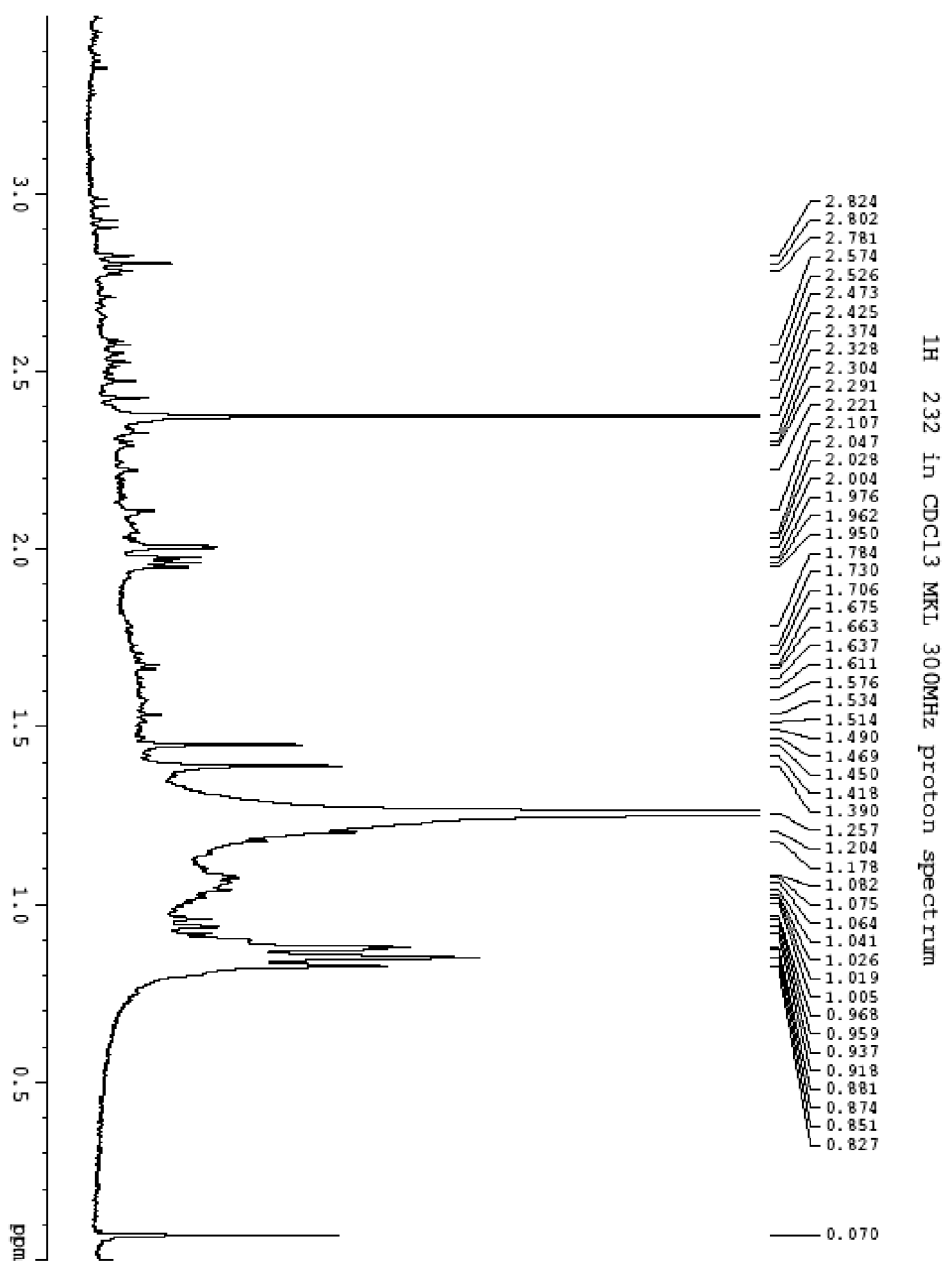
Appendix 25: ^{13}C spectrum for compound 30

13C 232 in CDCl3 MKL 75.48 MHz carbon spectrum

Appendix 26: COSY spectrum for compound 30

COSYPH 232 in CDCl3 MKL
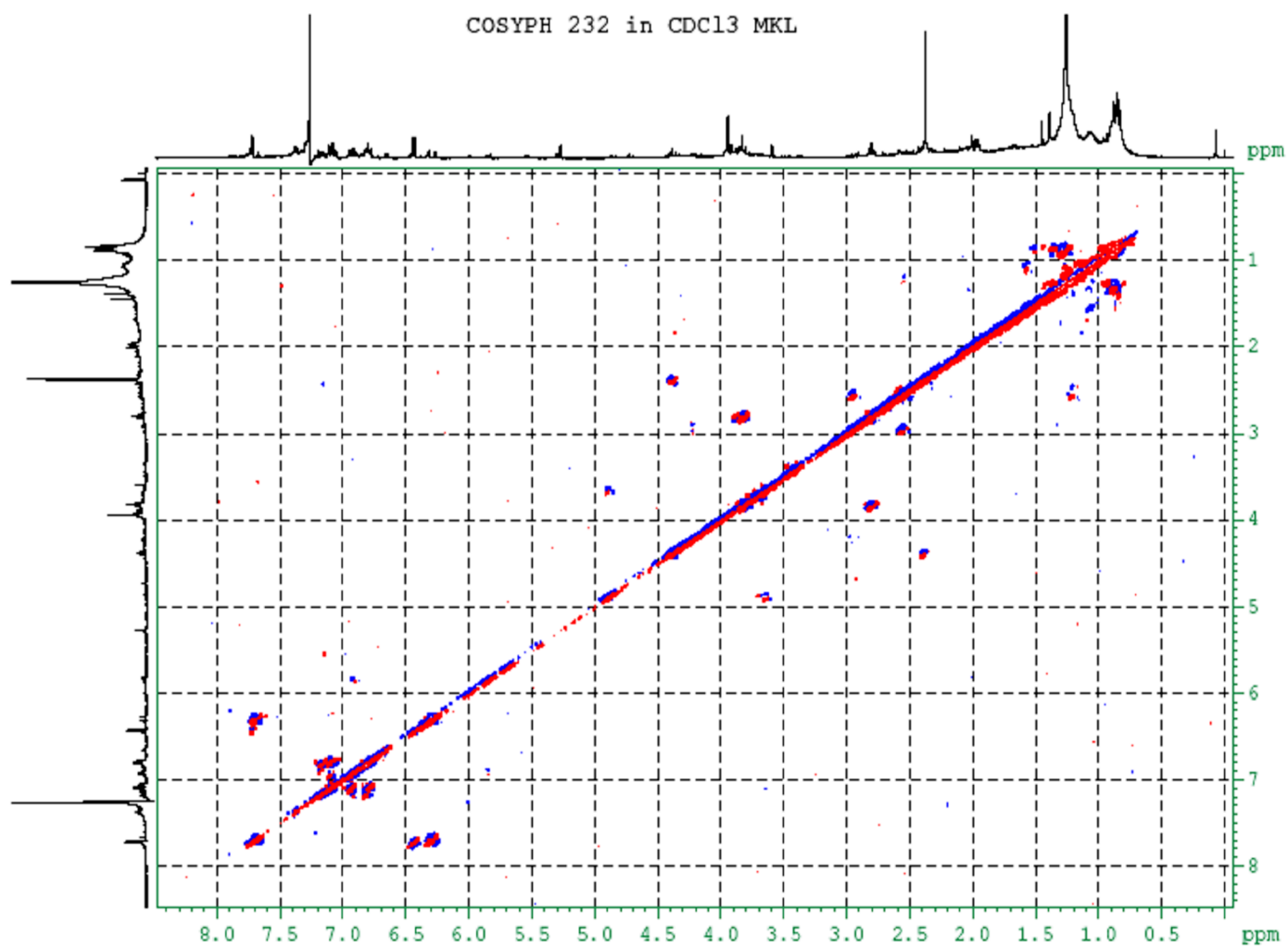
Appendix 27: DEPT spectrum for compound 30

154.496
147.092
13C DEPT135 232 in CDCl3 MKL 75.48 MHz carbon spectrum
130.731
130.301
128.710
126.216
123.676
115.615
114.916
114.442
113.052
109.608
77.360
74.009
56.119
32.896
21.200
19.880
14.467
14.266
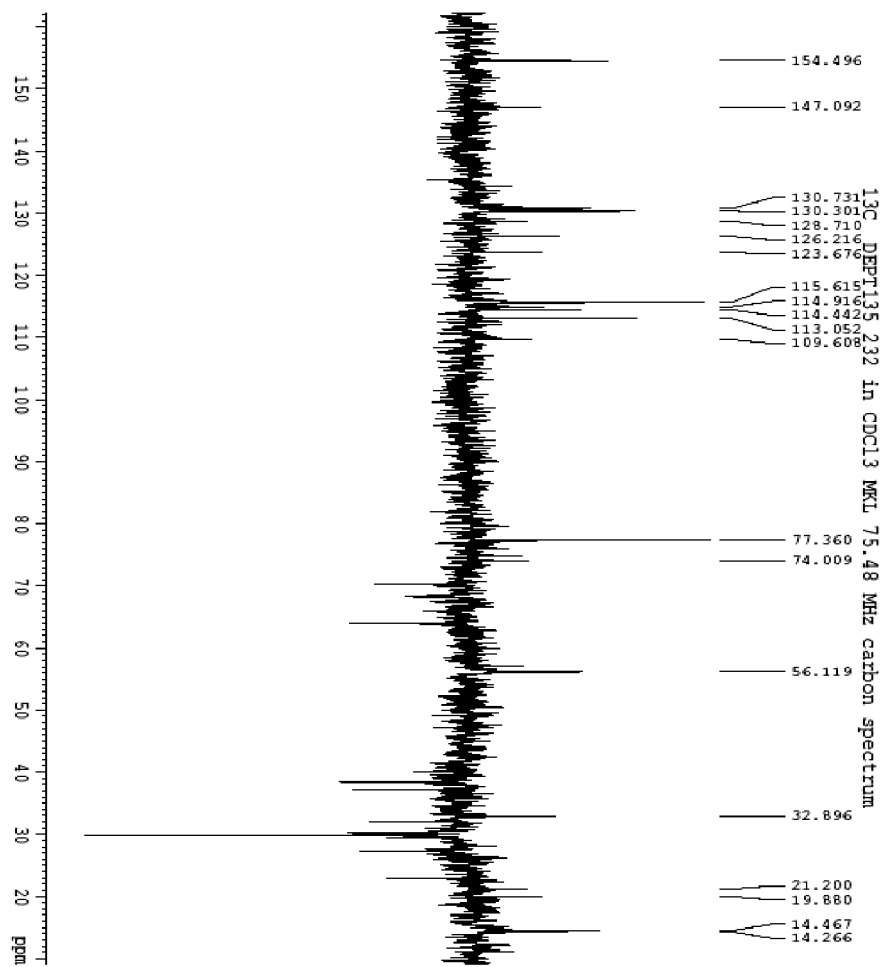
Appendix 28: HMBC spectrum for compound 30

H/13C HMBC LP, 300/75 MHz 2D spectrum,232 in CDCl3 MK
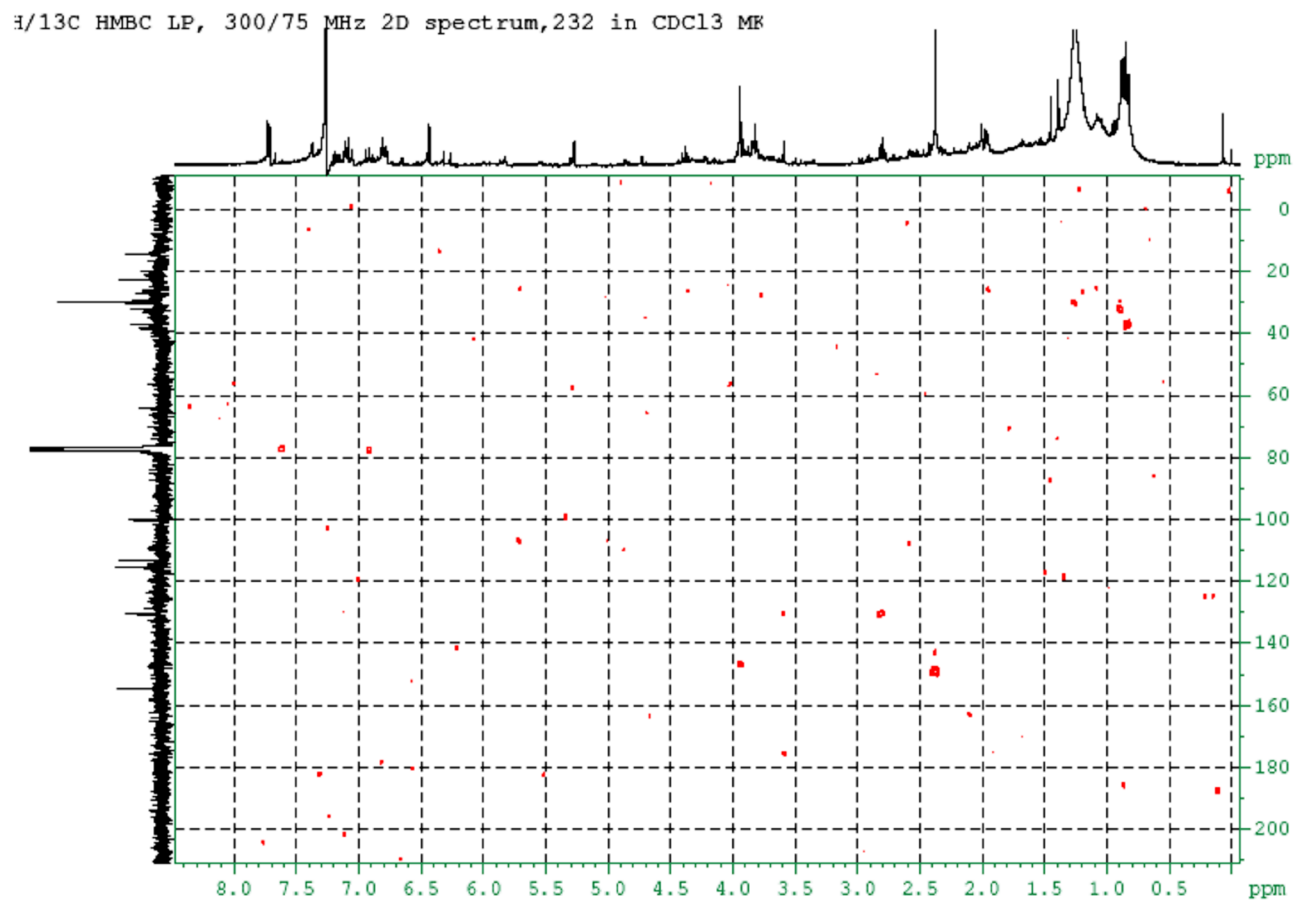
Appendix 29: HSQC-DEPT spectrum for compound 30

HSQC-DEPT, 232 in CDCl3 MKL 300/75 MHz Phase sens. s]
MAY NEED MANUAL PHASE CORRECTION.
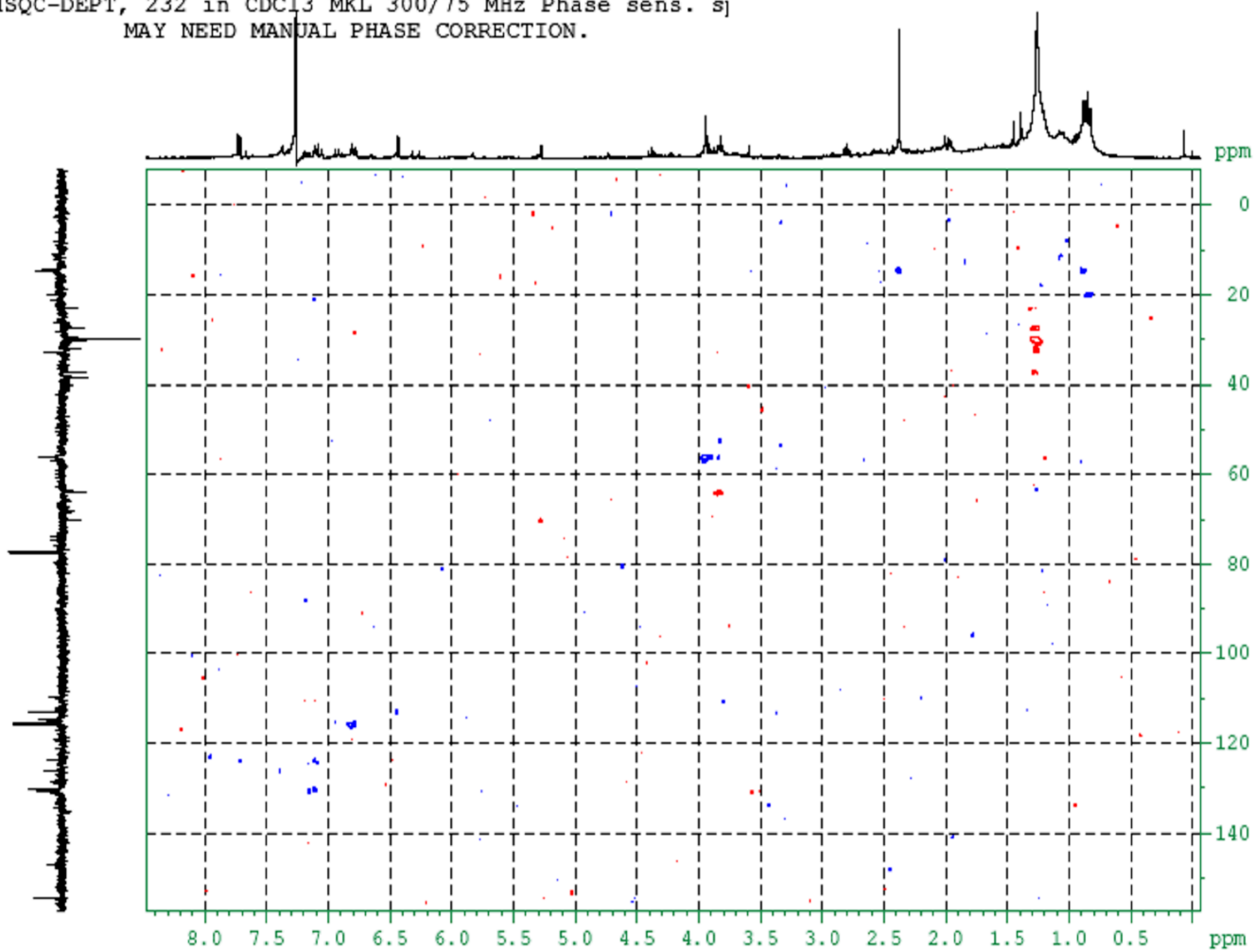
Appendix 30: NOESY spectrum for compound 30

1H/1H 300.13 MHZ NOESY 2D spectrum, 232 in CDCl3 MKL