ISOLATION AND PURIFICATION OF MOSQUITO LARVICIDAL COMPOUNDS FROM EXTRACTS OF A BASIDIOMYCETE JO5289

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A thesis submitted to the Graduate School in partial fulfilment for the requirements of Master of Science Degree in Chemistry of Egerton University

EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

Declaration:

I, Chirchir D. K., hereby declare that this is my original work and has not been submitted wholly or in part for any award in any other institution of learning to the best of my knowledge.

Signed:……. Date:…….

SM11/1802/06

Recommendation:

We wish to confirm that this thesis was done under our supervision and has our approval to be presented for examination as per the Egerton University regulations.

Dr. J. O. Omolo

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Signed:……. Date:……

Dr. P K. Cheplogoi

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DEDICATION

This thesis is dedicated to my beloved parents, whose vision and energy, moral, financial and emotional support has positively shaped my destiny.

ACKNOWLEDGEMENT

My gratitude goes to Egerton University graduate school and Chemistry department for the opportunity offered to me to pursue Master of Science degree in Chemistry. Many thanks go to Pyrethrum Board of Kenya for provision of mosquito larvae used in the bioassay in this project. I am also grateful to Africa Institute of Capacity Development (AICAD) and International Foundation of Science (IFS) for supporting this project. I wish to acknowledge all staff members of the Department of Chemistry, Egerton University, for their moral support that gave me ample time and peace of mind during my studies. I am greatly indebted to my supervisors - Dr. Omolo and Dr. Cheplogoi for their tireless guidance, valuable advice, encouragement, and their useful ideas, which came from their wide experiences. My heartfelt gratitude goes to Dr. Rotich, the Dean Faculty of Science, for his constant advice that has nurtured my maturity as a young scientist. I also express my sincere gratitude to Prof. D.A Mulholland and Dr. Moses Langat of Surrey University – UK for NMR analysis. Lastly I salute my colleagues, for their moral support. May the blessing of Almighty God be upon us.

ABSTRACT

Mosquitoes present a worldwide, public health and nuisance challenge since they transmit human diseases including west Nile virus, malaria, dengue, Japanese encephalitis, filiarisis and other viral diseases throughout the globe. An obvious method for preventing the spread of these diseases is to control mosquito vector population by insecticides and synthetic agents, which have been developed and employed in the field with considerable success. As a result of insects' resistance and adverse non-target effects, the current trend is to reduce or eliminate many of these older insecticides and develop safer, biodegradable, low costs larvicides for vector control. The secondary metabolites from higher fungi were thus investigated for possible mosquito larvicidal activity to control mosquitoes. A basidiomycete serialized JO5289 was collected from undisturbed habitat in Londiani forest in Rift Valley province. The strain was preserved as agar slant and the corresponding herbarium material kept in a fungal culture collection in the Integrated Biotechnology Research Laboratory (IBRL) at Egerton University. On initial screening it was found to produce active compounds in liquid culture against *Aedes aegypti* larvae. It was cultured in sterile submerged nutrient liquid malt media and growth conditions were manipulated to trigger the generation of secondary metabolites. From the culture two sets of crude extracts were prepared with intracellular secondary metabolites prepared from mycelium (Mex) while extracellular secondary metabolites prepared from the cultured filtrate (Kex). The crude extracts were tested for larvicidal activities against late third instar larvae and early fourth instar larvae of *Ae. aegypti* before activity guided purification of the active compounds was carried out. These afforded eleven purified compounds which were subjected to NMR experiments (both 1D and 2D), that were used to determine the chemical structures of the compounds. Two mosquito larvicidal compounds were purified; 4-(2-hydroxyethyl)phenol and 3-methoxy-5-methyl-1,2-benzenediol with LC_{50} values of 231 and 237ppm, respectively against *Ae. aegypti* larvae after 24 hours. The third compound that was purified 2-hydroxy-4-(4-hydroxychroman-7-yl)but-3-enal whose larvicidal activity was not determined due to low yield. These compounds (4-(2-hydroxyethyl)phenol and 3-methoxy-5-methyl-1,2-benzenediol) have been produced from cultures of a basidiomycete and reported to have mosquito larvicidal activity for the first time. Despite the limitations of necessary research equipment, the study has demonstrated the potential of a basidiomycete JO5289 as a source of mosquito larvicidal compounds. It is the recommendation of this work that these compounds can be evaluated further for product development.

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LIST OF ABBREVIATIONS

CC	Column Chromatography
COSY	Correlated spectroscopy
DDT	Dichloro Diphenyl Trichloroethane
DEPT	Distortionless Enhancement by Polarisation Transfer
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single-Quantum Correlation
IBRL	Integrated Biotechnology Research Laboratory
IR	Infra Red
JO5289	This is the serial number of the basidiomycete strain.
LC_{50}	Lethal concentration that kills 50% of mosquito larvae
LC_{90}	Lethal concentration that kills 90% of mosquito larvae
MS	Mass Spectroscopy
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Spectroscopy
TLC	Thin Layer Chromatography
UV/Vis	Ultra violet –visible spectroscopy

CHAPTER ONE
INTRODUCTION

1.1 Background information

Mosquitoes are flying insects in the family Culicidae that are found around the world and there are about 3500 species. The females of most mosquito species suck blood (hematophagy) from other animals. This has made them the most deadly disease vector known, killing millions of people over thousands of years and continuing to kill more by the spread of the infectious diseases (Cepleanu, 1993). Several mosquito species belonging to genera *Anopheles*, *Culex* and *Aedes* are vectors of pathogens of various diseases like malaria, filiarisis, Japanese encephalitis (JE), dengue and dengue hemorrhagic fever, yellow fever and others (Becker *et al.*, 2003). Indeed, the present recrudescence of these diseases is due to high number of breeding places and the increasing resistance of mosquitoes to the current commercial insecticides (Chowdhury and Chandra, 2008).

In addition to personal protection and educating the public, the most successful method of minimizing the incidence of mosquito-borne diseases is to eradicate and control the mosquito vectors. This is performed principally by systematic treatment of the breeding places through a combination of environmental management and applications (Rozendaal, 1997). For most mosquito borne diseases, there are no effective methods by which to control the advance of epidemic in tropical regions (ICMR, 2003).

Despite the immense resource presented by natural flora, control of mosquitoes still depends basically on the use of synthetic pesticides. They have become an essential component of insect pest management and in the past they have included mostly small molecular weight compounds, for example, organochlorines, organophosphates, carbamates and others (Wood, 2003).

The discovery of Dichloro Diphenyl Trichloroethane's (DDT) insecticidal properties in 1939 and the subsequent development of organochlorine and organophosphate insecticides suppressed natural product research since the answers to insect growth regulation were thought to have been found (Harzsch and Hafner, 2006). However, DDT ceased to be used due to insect resistance and environmental impact. Animals do not metabolize DDT very rapidly; instead it is deposited and stored in the fatty tissues and its biological half-life is about 8 years and if

ingested continues to build up at steady rate (Ndungu *et al*., 2003). In addition, the high cost of synthetic pyrethroids, environmental and food safety concerns, the unacceptability and toxicity of many organophosphate and organochlorine and the increasing insecticide resistance on a global scale have ruled out the miracle of chemical technology as a solution when it comes to containing the upsurge in mosquito population (ICMR, 2003).

The synthetic larvicides are no doubt effective but have now been discarded due to deleterious effects on the ecosystem and non-target organisms. More concerted efforts have to go into studies to make environmentally friendly compounds viable for field use in vector control operations (Singh *et al*., 2006). Therefore the search for an alternative vector control methods including biological control has attracted the attention of many researchers (Neetu *et al*., 2007). An alternative for conventional chemical control is the utilization of natural products from fungi in the search for environmentally safe, biodegradable, low cost larvicides for vector control (Killeen *et al*., 2002).

Despite its enormous historical successes, mosquito larval control remains a largely forgotten tool for mosquito control. With increasing interest in integrated vector management the potential of antilarval measures in Africa needs to be re-evaluated (WHO, 2005). The advantage of targeting the larval stage is that mosquitoes are killed before they disperse to human habitations and that mosquito larvae, unlike adults, cannot change their behaviour to avoid control activities targeted at larval habitat (Chapman, 1974).

A large number of bioactive and structurally diverse fungal metabolites have been isolated and characterized over the years and some of these have been used for the development of valuable pharmaceuticals and pesticides (Hawksworth, 2000). Ever since the discovery of penicillin, fungi have been recognized as sources of novel compounds important in human health. In addition to providing new pharmacologically active compounds, tropical fungi acts as sources of new antifungal agents, insecticidal compounds (both larvicidal and adulticidal) and enzymes for industrial use including bio conversions (Ndungu *et al*., 2004). Unlike other mosquito larvicidal biocontrol agents such as bacteria, microsporidia, viruses; fungi can infect and kill insects without being ingested (Scholte *et al*., 2005).

Mosquito larvae of different species display different susceptibility to the same insecticides. In general, *Aedes* larvae are more robust and less susceptible to insecticides than *Culex* larvae. The susceptibility of *Anopheles* larvae can vary since they can be more or less

susceptible than *Culex* and *Aedes* larvae to insecticides. *Ae. aegypti* is commonly used in insecticide screening trials because it is usually less susceptible and is easy to colonize in the laboratory (Essam *et al*., 2005). Thus *Ae. aegypti* was used in this project as a serotome laboratory specimen.

1.2 Statement of the problem

There is currently a scarcity of viable insecticides (both larvicides and adulticides) to control mosquitoes. This is a consequence of emergence of resistance to synthetic and non-synthetic chemical insecticides which has led to an increase in mosquito population and hence an increase in the spread of mosquito borne diseases. In addition there are inadequate tools targeting the larval stage of mosquito; an effective way of tackling the vector. These have prompted the search and development of natural larvicidal products. However, the current research on natural larvicides exclusively utilizes plants which are limited due to the time required for maturation in enough quantities to meet the ever rising demand for larvicides as opposed to fungi (basidiomycetes).

1.3 Objectives

1.3.1 General objective

To isolate and characterize larvicidal compounds from a basidiomycete JO5289 against larvae of *Ae. aegypti*.

1.3.2 Specific objectives

(i) To screen the crude extracts (Mex and Kex) for larvicidal activity against *Ae. aegypti.*

(ii) To purify larvicidal compounds from mycelium and culture filtrate crude extracts (Mex and Kex) using chromatographic techniques.

(iii) To elucidate the structure of purified compounds using standard spectroscopic techniques.

(iv) To determine the larvicidal activity of purified and characterised compounds.

1.4 Hypotheses

(i) The crude extracts from the submerged cultures of basidiomycetes JO5289 have larvicidal activity against *Ae. aegypti*.

(ii) The larvicidal compounds from the crude extracts can be purified using chromatographic techniques.

(iii) Chemical structures of pure compounds can be elucidated using spectroscopic techniques.

(iv) The purified compounds have significant larvicidal activity against *Ae. aegypti*.

1.5 Justification

Fungal genetic resources have been shown to display great diversity between and within species and more so the production of biologically active molecules. Most of these have been exploited for the benefit of mankind especially in pharmaceutical industry. However, little if any research has been reported on possibility of utilizing the biologically and chemically diverse secondary metabolites from fungi for mosquito control. The preliminary bioassay of JO5289 showed good larvicidal activity against *Ae. Aegypti*.

CHAPTER TWO
LITERATURE REVIEW

2.1 Mosquitoes

The medical importance of mosquitoes as vectors of serious diseases that cause morbidity, mortality, economic loss, and social disruption such as malaria, lymphatic filariasis, and viral diseases is well-documented (Becker *et al.*, 2003). Mosquitoes not only cause nuisance by their bites but also, contribute significantly to poverty and social debility in tropical countries (Jang *et al.*, 2002).

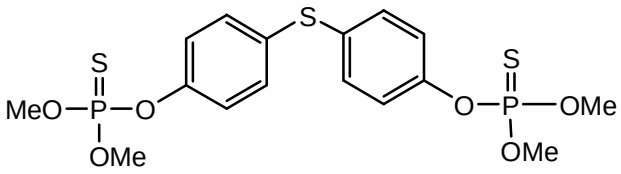
Aedes aegypti is a mosquito associated to human, and the vector for infection with dengue and yellow fever, endemic diseases covering tropical and subtropical regions. Although yellow fever has been reasonably brought under control, no vaccine is available against most mosquito borne diseases, their control being restricted to combating the vector by attacking the larval breeding places (Geris *et al.*, 2008). An obvious method for preventing the spread of these diseases is to control the vector population by insecticides and synthetic agents which have been developed and employed in the field with considerable success (Neetu *et al*., 2007).

Effective management of mosquitoes entails getting rid of the situations that are attracting them to breed and kill the larvae before they become adults (Chowdhury and Chandra, 2008). The main aim of mosquito management operations is to reduce the number of pests or disease vector species to a level where the impact on the adjacent human population is kept to an acceptable level. The long term goal is to maintain the mosquito populations below this threshold (Jang *et al.,* 2002).The most effective mosquito control strategies integrate well established and proven mosquito management techniques which incorporate physical, chemical and biological control methods. Integrated control aims at giving the best combination of methods to give maximum long-term control at most efficient overall cost (Neetu *et al*., 2007).

There are many methods used for mosquito control. Some target the larval stage while others are used to kill or repel adults. The most efficient approach to control the vector is to target the immature stages of their life cycle. If mosquitoes do breed, larviciding allows control measures to be used in targeted areas while the mosquito larvae are still concentrated in breeding pools and before adult mosquitoes spread throughout the community (Rahuman *et al.*, 2009).

2.2 Application of synthetic chemicals to control mosquitoes

Historically chemical insecticides from synthetic sources have been used in mosquito control (Tia *et al*., 2006). Control of adult mosquitoes is the most familiar aspect of mosquito control and is accomplished by ground-based applications or *via* aerial application of chemical pesticides. Generally modern mosquito management programs in developed countries use low-volume applications of pesticides, although some programs may still use thermal fogging. The non-systemic organophosphorus insecticide temephos (**1**) is most often used for control of *Ae. aegypti* but the inefficiency of this drug and growing resistance in the dengue vector has been reported (Geris *et al.,* 2008).



1

The role of DDT in combating mosquitoes has been the subject of considerable controversy. While some argue that DDT deeply damages biodiversity, others argue that DDT is the most effective weapon in combating mosquitoes and hence mosquito borne diseases (Chowdhury and Chandra, 2008). While some of the disagreement is based on differences in the extent to which disease control is valued as opposed to the value of biodiversity, there is also genuine disagreement amongst experts about the costs and benefits of using DDT (Curtis, 2000). Moreover, DDT-resistant mosquitoes have started to increase in numbers, especially in tropics due to mutations, reducing the effectiveness of this chemical; these mutations can rapidly spread over vast areas if pesticides are applied indiscriminately (Chevillon *et al.*, 1999).

Repeated use of synthetic insecticides for mosquito control has disrupted natural biological control systems and led to resurgences in mosquito populations (Tia *et al.,* 2006). Most of the adulticiding programs suffer shortcomings since it involves spraying pesticides indiscriminately with little being done to restrict breeding (Geris *et al*., 2008). Mosquitoes tend to develop resistance to chemical pesticides over time, which render the chemicals less effective. Adulticides generally present considerable risks to all living organisms and kill beneficial insects and natural mosquito predators such as dragonflies and beetles (Nakamura *et al*., 2004). It has also resulted in the development of resistance, undesirable effects on non-

target organisms and fostered environmental and human health concern which initiated a search for alternative control measures (Hayes, 1991).

Synthetic chemical larvicides continue to be applied for controlling mosquitoes in most parts of the world (Rahuman *et al.,* 2009). Many of these chemicals are toxic to human, plant and animal life and resistance can be problematic in maintaining control especially with organophosphate and pyrethroid larvicides (Amer and Mehlhorn, 2006). Pesticides exposure among humans has also been linked to immune dysfunction, various forms of cancer and birth defects. It is therefore, necessary to identify a safe, eco-friendly alternative source of larvicides in order to reduce mosquito menace (Chowdhury and Chandra, 2008). It is clear therefore that two major drawbacks with the use of chemical insecticides are that most are non-selective and are facing a problem of resistance.

Resistance to insecticides is increasing, yet few insecticides and tools are available for resistance management. In this regard there is an urgent need for the development of techniques that would provide more efficient insect control incur ill effect on non-target population and are easily degradable (Redwane *et al*., 2002).

2.3 Mosquito larvicides from plants

Humans have used plant parts, products and metabolites in the pest control since historical times. Plants are the chemical factories of nature, producing many chemicals, some of which have medicinal and pesticidal properties. Members of the plant families – Asteraceae, Cladophoraceae, Meliaceae, Oocystaceae and Rutaceae possess various types of activity against many species of mosquitoes (Vasudevan, 2009). Wood (2003) lists some important phytochemical products such as pyrethrum, derris, quassia, nicotine, hellebore, anabasine, azadiractin, d-limonene, campor and terpenes that have been used in the developed countries before the advent of synthetic organic insecticides.

Plants are considered as a rich source of bioactive chemicals (Wink, 1993) and they may be an alternative source of mosquito control agents. Botanicals offer an advantage over synthetic pesticides because they are less toxic, less prone to the development of resistance and easily get biodegraded (William, 2007). Natural products of plant origin with insecticidal properties have been tried in the past for control of variety of insect pests and vectors (Desai, 2002). For instance, essential oils of leaf and bark of *Cryptomeria japonica* demonstrated high larvicidal activity against *Ae. aegypti* (Diptera: Culicidae) larvae (Cheng *et al.,* 2003). It has

also been reported that it shows great biological activities including antifungal (Cheng *et al.*, 2005, 2006), antibacterial, termiticidal and antimite (Gu *et al.*, 2007).

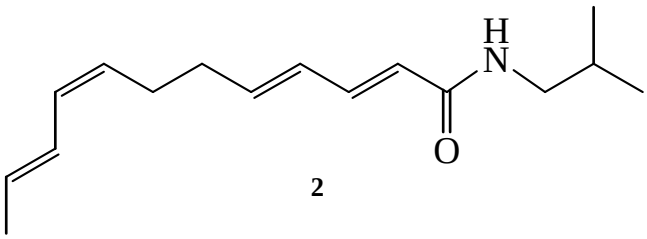
Mullai *et al*. (2008) reported that the crude extract of *Citrullus vulgaris* was found to be effective against *Ae. aegypti* in 24 hours with LC_{50} value of 42.76ppm and LC_{90} value of 85.76ppm. One of the most studied botanical species with good pesticidal attributes is the neem tree (*Azadirachta indica*) whose extracts have shown considerable activity and multiple mode of action against agricultural pests, forest insects and insects of public health (Vasudevan *et al.*, 2009).

The plant extracts of *Corian drumsativam, Ferula asafetida* and *Trigonella foenumgraceum* were found to be effective and showed encouraging results against *Ae. aegypti* and *Culex* (Diptera: Culicidae) mosquito larvae (Harve and Kamath, 2004). Long before the advent of synthetic insecticides, plants and their derivatives were used to kill pest of agriculture, veterinary and public health. Sosan *et al.* (2001) reported larvicidal activities of essential oils of *Ocimum gratissium*, *Cymbopogon citrus* and *Ageratum conyzoides* against *Ae. aegypti* which had achieved 100% mortality at 120, 200 and 300ppm concentrations, respectively.

In addition, it was reported that the essential oil of *Ipomoea cairica* possesses remarkable larvicidal properties as it could produce 100% mortality in the larvae of *Cx. tritaeniorhynchus, Cx. Quinquefasciatus, Ae. aegypti, An. stephensi* mosquitoes at concentrations ranging from 100 to 170ppm (Rahuman *et al.*, 2009). Dwivedi & Kawasara (2003) found acetone extract of *Lantana camara* to be most effective against *Cx. quinquefasciatus* larvae at the dose of 1 ml/100ml.

Larvicidal activities of the plant extracts vary according to the plant species, the parts of the plant, the geographical location where the plants were grown and the application method. Use of these botanical derivatives in mosquito control instead of synthetic insecticides could reduce the cost and environmental pollution (Sosan *et al.*, 2001).

The use of plants as repellant is widespread in different communities in Africa and the performance of many has been evaluated experimentally (Snow *et al.*, 1999). N- isobutyl-2E,4E,8E,10Z-dodeca-2,4,8,10–tetraenamide (**2**) was isolated from *Spilanthes mauritiana* and gave 100% mortality against third instar larvae of the *Ae. aegypti* at 10^{-5} mg/mol (Jondiko, 1986).



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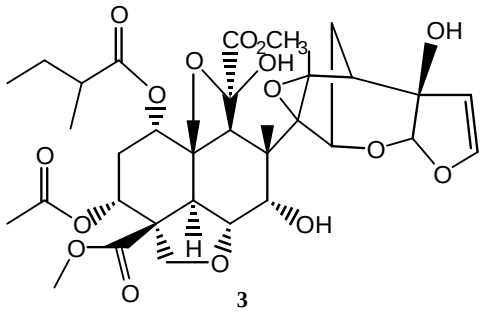
Numerous plants have been shown to produce pesticide compounds, as a chemical defense mechanism against predators or infection. *A. indica* (Meliaceae) commonly known as Neem, was investigated for antimalarial compound and was found to have mild activity (Vasudevan *et al.,* 2009). Azadirachtin (**3**) is one of the insecticidal compounds found from the seeds of *A. indica.*



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Though Neem products show a high larvicidal activity, which is attributed to the epoxide ring function, they do not show adulticidal action It has been suggested that azadirachtin acts as an anti-ecdysteroid and thus kills the larvae by growth inhibition effect (ICMR, 2003).

Natural larvicides from plants though potent in laboratory settings are limited in terms of large scale production and commercialization to meet the ever rising demand for larvicides as opposed to fungi. Tropical higher fungi are a rich yet quite less exhausted resource for the search of natural larvicides. This has called for the search of more larvicides from fungi.

2.4 Fungal metabolites with insecticidal properties

Microbial natural products remain the most promising source of novel secondary metabolites. The impact of microbial biodiversity favours the chance of isolating new antibiotics and development of new molecules to help in fighting many pathogens (Busi *et al.,* 2009). Fungi produce a wide range of secondary metabolites with high therapeutic value as

antibiotics, cytotoxic substance, insecticides, compounds that promote or inhibit growth, attractors, repellent and others (Demain, 1999). These metabolites are being exploited in different fields of medicine and industries (Huisman and Gray, 2002).

Fungi are extremely diverse group of heterotrophic organisms that are exploited by human for various biotechnology applications. They are used in the production of foods, beverages, organic acids, enzymes, polysaccharides and antibiotics and other pharmaceuticals; as agents of biological control of insect pest, pathogenic fungi and weeds and in biomass conversion (Getha *et al.,* 2009). Among fungi classes, ascomycetes are reported to be active producers of antimicrobial compounds which have high therapeutic values (Busi *et al.,* 2009).

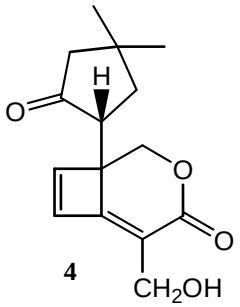
Intensive screening of microorganisms for substances of value in medicine or agriculture has revealed a wide range of biologically active secondary metabolites. The reason for secretion of these compounds by microorganisms is not exactly understood. In fact, competition for the habitat and nutrients causes many species of fungi to excrete substances that inhibit growth, or may even cause the death of organisms in their vicinity, such as bacteria, fungi, and insects (Geris *et al*., 2008). Moreover, certain fungi have entomopathogenic activity, infecting and killing insects *via* production of secondary metabolites. One such compound is bassianolide, a cyclodepsidipeptide produced by the fungus *Beauveria bassiana*, which elicits atonic symptoms in silkworm larvae (Nakamura *et al*., 2004).

Pythopathogenic fungi are prolific producers of structurally diverse biologically active secondary metabolites (Guzman-Lopez *et al.,* 2007). High level of environmental stress together with intense and frequent interaction with other organism promote production of metabolically diverse compounds (Wagenaar *et al*., 2002).

Polypores are a large group of terrestrial fungi of the phylum basidiomycota (basidiomycetes) and along with certain ascomycota are major sources of pharmacologically active substances (Getha *et al.,* 2009). There are about 25000 species of basidiomycetes of which about 500 are members of the aphyllophorales; apolyphyletic group that contains polypores. The secondary metabolites of polypores exhibit a wide range of biological activities such as antimicrobial, antiviral, antifungal, anticancer, cardiovascular, anti-inflammatory, antioxidant, immunostimulating, nematocidal and other activities (Zjawiony, 2004). Studies have shown that only a small number of the most common species of polypores such as

Ganoderma lucidium have been evaluated thoroughly for biological activity (Getha *et al.*, 2009).

Several secondary metabolites from polypores show phytotoxic activity. A parasite of conifers, the polypore *Heterobasidium annosum* produces phytotoxic bicyclic sesquiterpenes, fomanosin (**4)** and fomannoxin (**5**). Both compounds are toxic to the green algae *Chlorella pyrenoidosa* (Zjawiony, 2004).



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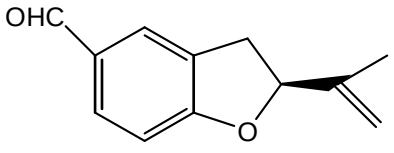

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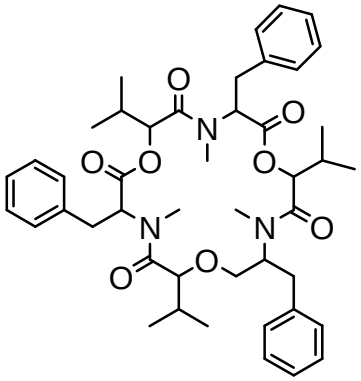
Some secondary metabolites isolated from polypores have been reported to have insecticidal activity. An example is beauvericin (**6**) from *Laetiloporus sulphureus* as well as several ascomycetes that exhibit significant insecticidal activities. Beauvericin is produced by the bright yellow polypore commonly known as 'chicken of the wood'. Beauvericin also is considered as a mycotoxin produced by hypocrealean ascomycetes in grain (Zjawiony, 2004).



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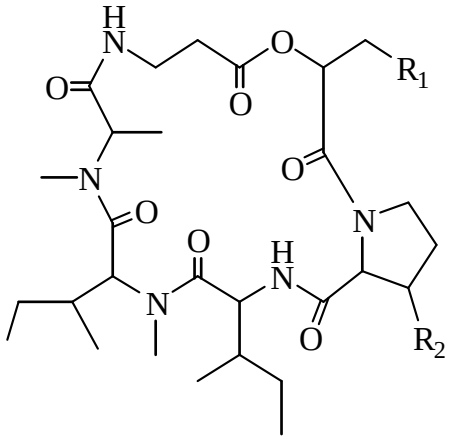
Bio-assay guided fractionation of the organic extracts from liquid cultures of an undescribed species of entomopathogenic fungus from the genus *Aschersonia* by silica gel column chromatography and reversed phase HPLC afforded two cyclic depsipeptides i.e. destruxin A4 (**7**) and destruxin A5 (**8**). Another compound previously indentified homodestruxin B (**9**) was isolated as a product of the fungus. The three compounds showed

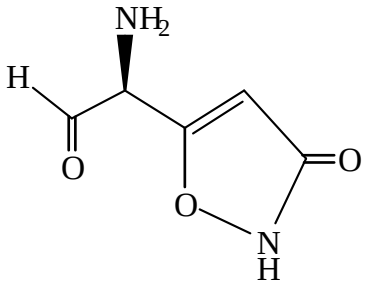
insecticidal activity against *Drosophila Melanogastus.* The general structure of the three compounds is given below (Stuart and Gibson, 1996).



R_1;-CH=CH_2, R_2=H **(7)**

R_1;-CH=CH_2, R_2=CH_3 **(8)**

R_1;-CHMe_2, R_2=H **(9)**



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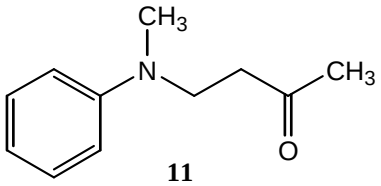
Insecticidal activity against houseflies was reported and has been known for some time for ibotenic acid (**10**) isolated from fruiting bodies of *Amanita muscaria, A. stabiliformis* and *A. pauturina*. It was also isolated from basidiomycetes *Polyporus sulphurous* (Deol *et al.,* 1978) and from entomopathogenic fungi, *Beauveria bassiana.* Insecticidal activity was also exhibited against mosquito larvae, brine shrimps, houseflies and cockroaches, cardiac cells *in vitro*.

Screening of *Aspergillus goraklpurensis* for the productions of bioactive secondary metabolites resulted in 4-(N-methyl N-phenylamino)butan-2-one (**11**) and itaconic acid (**12**). Whereas itaconic acid showed antibacterial and antifungal activity at 150ppm, 4-(N-methyl-N-phynylamino)butan-2-one showed potent lethality against *Spodoptera litura* 4th instar larvae with LC_{50} value of 330.69ppm (Busi *et al*., 2009).



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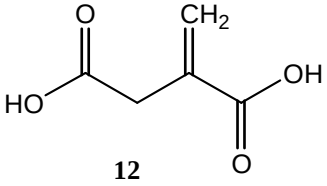


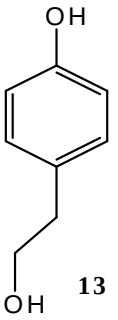
12

The wide range of pharmaceutical metabolites from basidiomycetes has been one of the most attractive groups of natural products studied (Getha *et al.,* 2009). The first investigation on the potential of basidiomycetes as antibiotics were carried out in 1941 when the extracts of fruiting bodies and mycelia cultures from over 2000 species were investigated (Nakamura *et al.,*

2004). Pleuromutilin, a diterpene that is especially useful for the treatment of mycoplasm infections in animals was one of the first commercial antibiotics developed from basidiomycete. Despite their potentials and enormous diversity, studies aiming at the discovery of bioactive compounds from basidiomycetes were hampered by difficulties such as slow growth rate and low product yields (Getha *et al.,* 2009).

The current trend in research is the utilizations of the submerged cultures since they have the potential advantages for higher mycelia production in a compact space and for a shorter incubation time with a lesser chance of contamination. Further optimization of the culture medium composition and physiochemical conditions of growth allows regulation of fungal metabolism in order to obtain standardized nutraceutical substance in higher yield. Mycelia formed by growing pure cultures in submerged culture are best technique for obtaining consistent and safe mushroom products (Shibata *et al.,* 2004).

Extracellular tyrosol (**13**) and tryptophol (**14**) were produced simultaneously from a *Ceratocystis adiposa* culture. Tyrosol is a compound with pharmaceutical interest that present antioxidant activity a property used in atheroscelorosis treatment and tryptophol is reported to have antibiotic and phytotoxic activities. Tryptophol production has also been reported in *Aspergillus niger*, agrobacterium *tumefaciens* and *Rhizobium* species (Guzman – Lopez, 2007).



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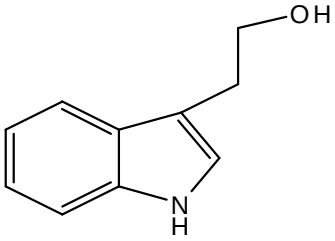

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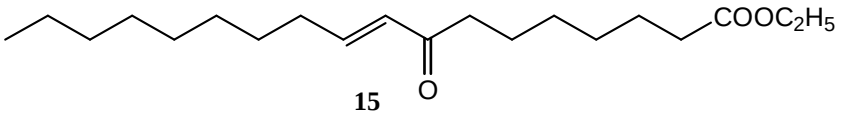
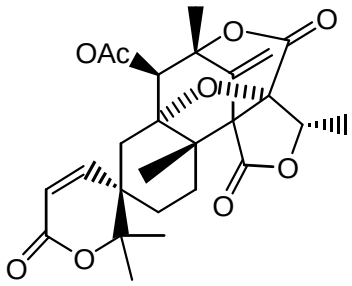
Two compounds possessing estrogenic activity were isolated from the mycelium of medicinal fungus *Cordiceps ophioglossoides*. These compounds were identified as ethyl (E)-8-oxo-9-octadecenoate (**15**) and tyrosol (**13**)



15

The above compound (**15**) had also been isolated as an aldehyde dehydrogenase inhibitor from higher basidiomycete mushroom *Clitocybe clavipes* (Tricholomatacea) (Kawagishi *et al.,* 2002). The cordyceps species has been regarded as an elixir of life as well as a valuable tonic from ancient times (Mizuno, 1999).

The fungus *Penicilium sp* from *Melia azidarach* roots produced a series of meroterpenoids. Of particular interest are dehydroaustin (**16**) and acetoxydehydroaustin (**17**). Based on chemical structure comparison, these compounds could be precursors of the meroterpenoid austin (**18**). Meroterpenoids are natural products of mixed biosynthetic origin that are partially derived from terpenoids and exhibit important biological activities.



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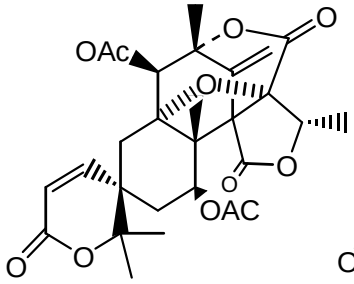

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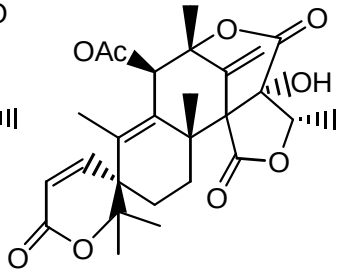
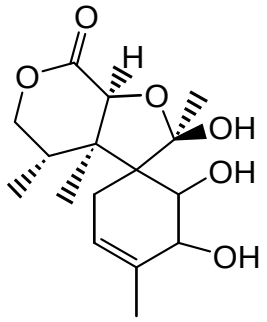


18

Dehydroaustin and acetoxydehydroaustin exhibited larvicidal activity against third instar larvae of *Ae. aegypti* with LC_{50} values of 2.9 and 7.3ppm, respectively, while austin displayed a very low mortality (Geris *et al*., 2008). The larvicidal activity displayed by the meroterpenoid **16** and **17** is probably related to the δ-spirolactone system. Moreover, the additional OAc group in **17** seems to significantly reduce the larvicidal activity. Further, the very low activity of **18** compared to **16** and **17** suggest that the additional bridging furan ring in the latter two compounds also significantly influences activity. This could indicate a hydrophobic binding reactivity site in this part of the molecule.

A novel sesquiterpenoid cyclopinol (**19**) has been isolated from the basidiomycete *Boletus calopus* (Liu *et al.,* 2008). Though its biological activity was not determined, it portrayed the great potentials of basidiomycetes as a rich source of bioactive metabolites.



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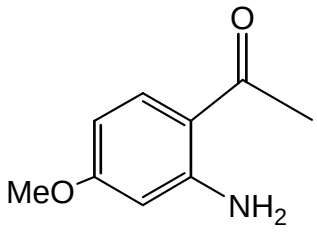

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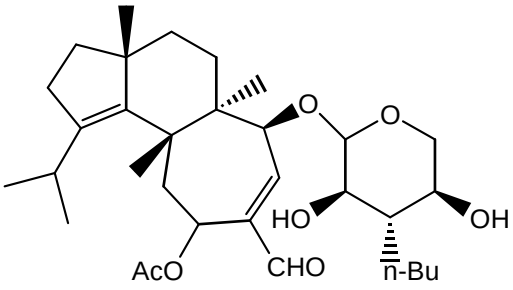
The basidiomycete *Cortinarious umidicola* that grows under the trees in the mountainous region of Kunming in China is associated with edibility and toxicity property. The fruiting bodies of this fungus produced a novel natural pyridine derivative 3-aldyhyde -2-amino-6-methoxypyridine (**20**) (Lin and Ji-Ku, 2003).

A novel cyathane–xyloside derivative, erinacine R (**21**), was isolated from the mycelia of basidiomycete *Hericium erinacium*. Erinacines and their xylosides are currently attracting much attention because of their unique biological activities. Erinacines are known to have a potent stimulating activity for nerve growth factor synthesis. *Hericium erinacium* is a mushroom belonging to the family Hericiaceae and has been known as a Chinese medicine or food in China and Japan (Ma *et al*., 2008).



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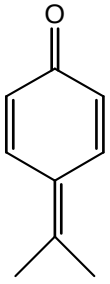

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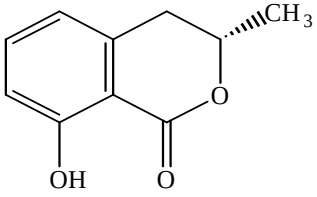
The basidiomycete *Thelephora aurantiotincta* (Cornor) belongs to the family Thelephoraceae. It grows in symbiosis with pine trees and a *p*-terpenyl, aurantiotinin (**22**) was isolated from the fruiting bodies of the basidiomycete *T. aurantiotincta* Cornor (Lin and Ji-Ku, 2003).

2.5 Bioactive fungal metabolites recently isolated from Kenyan basidiomycetes

The Kenyan basidiomycetes have been studied and have proved to be rich in bioactive metabolites. Some larvicidal active compounds were isolated from the basidiomycete serialized

JO5182. Three compounds; methyl-2-hydroxy-4-methoxybenzoate (**23**), 2,4-dimethoxyphenol (**24**) and (R)-(-)-8-hydroxy-3-methyl-3,4-dihydro-1H-2-benzopyran-1-one (**25**) were isolated (Kendagor, 2008).



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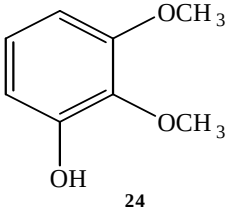

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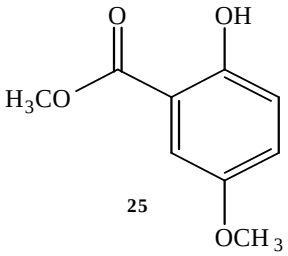

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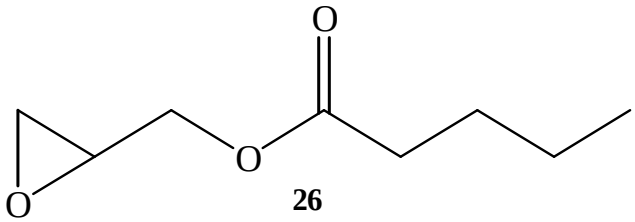
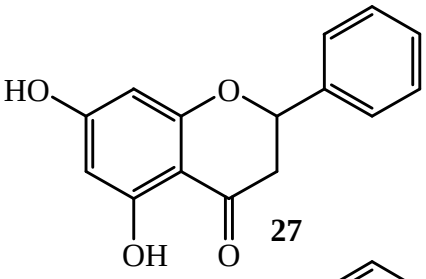
The basidiomycete JO5444 was also studied and a novel compound identified as (oxiran-2-yl) methyl pentanoate (**26**) was isolated. The LC_{50} and LC_{90} in 24 hours of the compound against larvae of *Ae. Aegypti* in their late third and early fourth instars were found to be 1.50 and 1.90ppm. This was quite a high activity as compared to the compounds isolated from other fungi (Njogu *et al*., 2009).



26

Some antibacterial compounds were isolated from basidiomycete JO5191, *Collybia* species (**27, 28, 29, 30**) and their minimum inhibitory concentration (MIC) were found to be in the range of 3ug/ml–12ug/ml, agreeing considerably well with those reported from previous literature (Mutambi, 2007).



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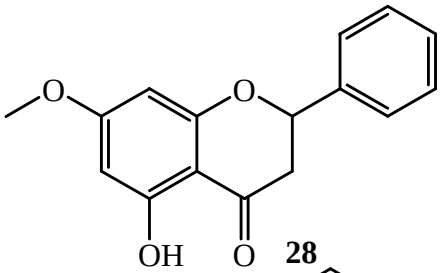

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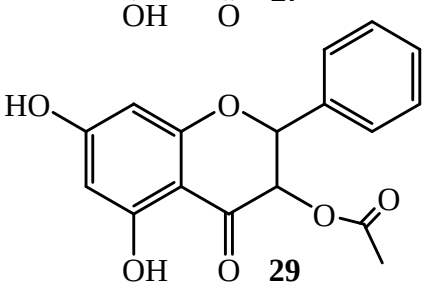

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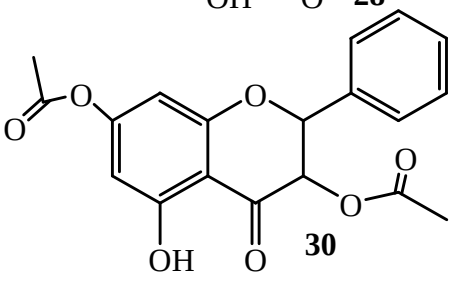

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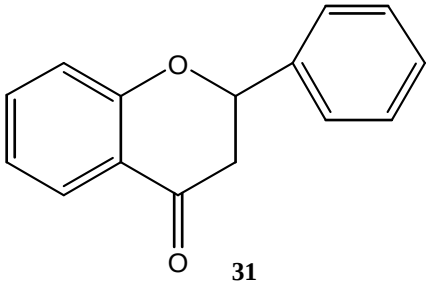
2.6 Flavonoids

Flavanoids are polyphenolic compounds primarily of 15 carbon atoms, two benzene rings joined by a linear three carbon chain (**31**). They are found majorly in the plant kingdom and are products of secondary metabolism *via* the shikimate and acetate pathways (Bravo, 1998). Many flavonoids are easily recognized as flower pigments, however their occurrence is not restricted to flowers but include all parts of the plants. Over 5000 flavonoids have been isolated from plants, making it the largest and most widely distributed group of polyphenols (Cook and Samman, 1996).

Flavonoids have common physiochemical properties and biological activities among them. They provide pigmentation to the plants which is important for organism reproduction purposes. They have also been postulated to participate in energy transfer pollen germination and resistance against infection (Ross and Kasum, 2002).



31

Flavonoid is a product from cinnamoyl-CoA starter unit with chain extension using three molecules of malonyl – CoA. This initially gives a polyketide, which according to the nature of the enzyme responsible, can be folded in two different ways. This allows aldol or Claisen-like reactions to occur, generating aromatic rings (Harborne and Williams, 2000).The biosynthesis of Flavonoids is outlined in the scheme below.

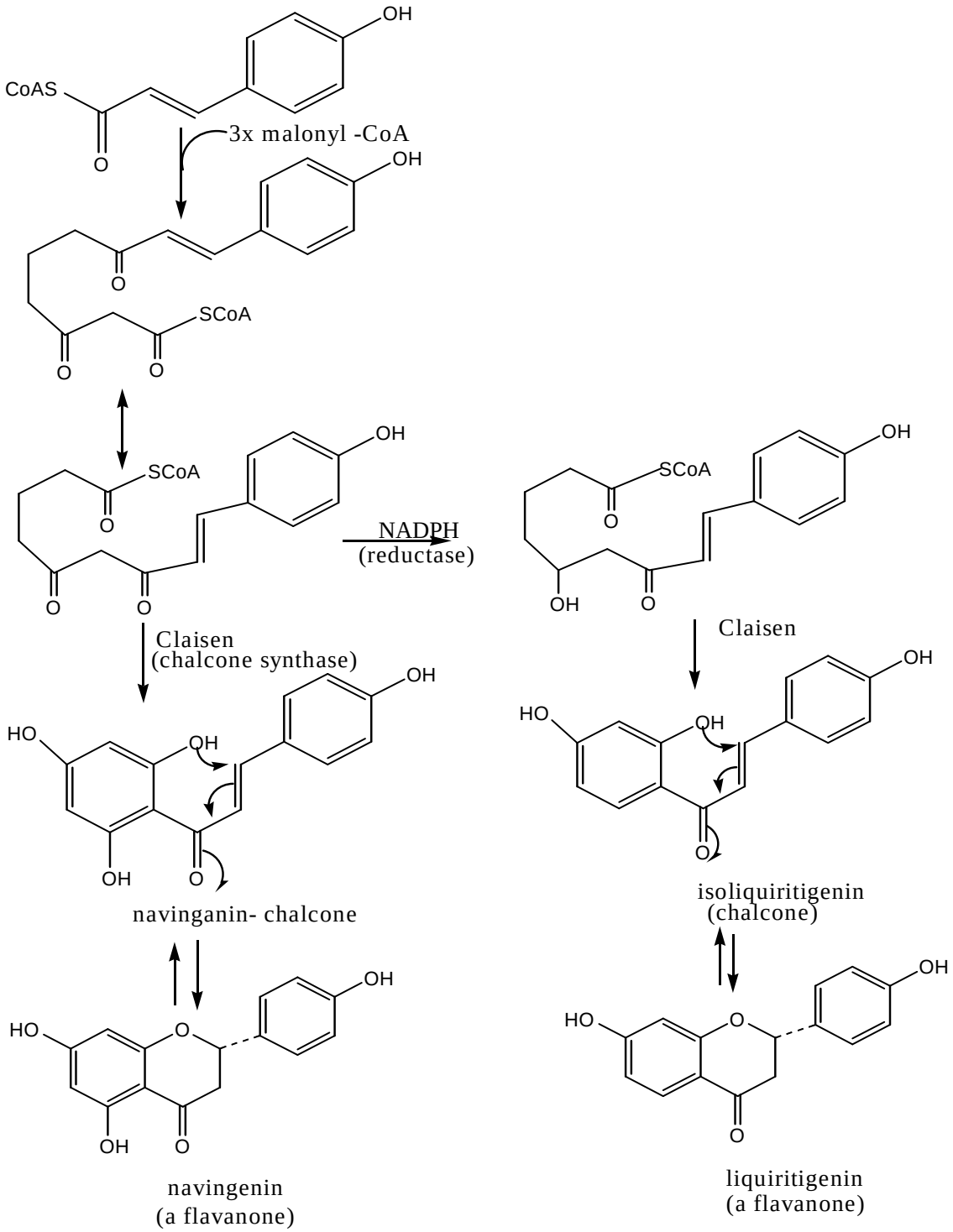


Figure 1: Biosynthesis of flavonoids

2.7 Carbon source and growth of basidiomycetes

The growth kinetics and fungal morphology in broth fermentation are highly dependent on culture conditions such as carbon sources, carbon/nitrogen relation, initial pH and temperature, agitation intensity and aeration rate (Shu *et al*., 2004). A large amount of work has reported the effects of environmental parameters on biomass concentration and on the yield of bioactive compounds by mushroom, but those researches are limited to few kinds of mushrooms (Tang and Zhong, 2002).

Many kinds of mushroom frequently require starch, glucose, sucrose for their submerged cultures. Carbon source as a component of culture medium plays an important role in the growth and in the production of bioactive compounds by basidiomycetes and other fungi (Vahidi *et al.,* 2006). Carbon compounds range from simple molecules like proteins, proteins, polysaccharides and lipids (Barnett, 1981). Number of waste materials have been considered for this purpose, for example a waste product obtained from distillation of fermented cane juice (Falanghe, 1964).

Basidiomycete utilizes a wide range of carbon sources for growth and production of active compounds. The sources include simple sugars such as glucose, sucrose, maltose, lactose and complex sugars such as molasses (Vahidi *et al.,* 2006). Garraway and Evans (1984) explained that fungal species may have the ability to utilize a particular carbon source for vegetative growth but may be unable to use it for production of specialized structures.

Vahidi *et al.* (2006) investigated the effects of different carbon sources on growth and production of antifungal agents by *Gymnopillus spectabillis*. They used different carbon sources including glucose, fructose, lactose, maltose, manitol and sucrose at concentration of 10g/l and slow carbon releasing sources including malt extract and soluble starch at the concentration of 2g/l. The results showed that the highest activity (90% inhibition of spore germination) and biomass concentration (2.4 g/l) were obtained when glucose was used as carbon source. The results are tabulated in table 1.

Table 1: Total biomass concentration and antifungal activity of *G. spectabilis* growth in simple sugars **(**Vahidi *et al.*, 2006)

Carbon source	Biomass (g/l)	Inhibition (%)
Glucose	2.5	90
Maltose	1.7	55
Lactose	1.2	47
Sucrose	1.5	26
Fructose	2.3	52
Manitol	1.4	32
Xylose	1.1	35

Combination of glucose with malt extract as slow releasing carbon source also increased activity to 100% inhibition. The results obtained from use of slow releasing carbon source and combination of glucose plus malt extract and starch is shown in table 2.

Table 2: Total biomass concentration and antifungal activity of *G. spectabillis* growth in complex carbon source **(**Vahidi *et al.*, 2006)

Carbon source	Biomass (g/l)	Inhibition (%)
Malt extract	2	84
Soluble starch	1.6	37
Glucose + malt extract	2.3	100
Starch + glucose	2.1	84

Increased antifungal activity of fungus in media containing a simple sugar like glucose plus slow releasing carbon source like malt extract can be explained by high production of secondary metabolites when their producing organisms grow in complex media (Martin and Demain, 1980). It is evident that in media containing glucose plus a more slowly utilized carbon source, glucose is usually used first and after glucose depletion, the second carbon source is used in secondary metabolites biosynthesis (Vahidi *et al.*, 2006).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample collection and preservation

The fruiting body of a basidiomycete serialized JO5289 was collected from a natural habitat in Londiani Forest in July 2005. The basidiomycete was brought into pure culture by trapping spores from a sterile piece from the underside (the hymenium) of the fruiting body. The mushroom had certain distinct macroscopic features like the morphology of the stipe, gills and pilleus. The colour of the lamella and the spores were also distinct features that were observed. The basidiomycete grew in the PDA plates as moist brown mycelium. The hyphal strands were found to be septate and had clamp connection which was the basis of its classification as a basidiomycete. The basidiomycete was preserved as agar slants with the corresponding herbarium material in the Integrated Biotechnology Research Laboratory (IBRL) at Egerton University.

3.2 Preliminary preparation

Gloves, working benches, blades and generally the working environment were sterilized using 70% ethanol while the liquid media was heat sterilized using an autoclave (Danfoss 59407-3 No. 375). The liquid media was constituted by dissolving 10g malt media, 4g of glucose (Kobian Kenya limited, Nairobi) and 4g of yeast extract in 1L of tap water. The pH was adjusted to 5.5 using 1.0M NaOH and 1.0M HCl, dispensed in 250ml Erlenmeyer flasks, corked with cotton wool plugs, wrapped with aluminium foil. The setup was sterilized at a temperature of 121^{o}C and pressure of 1.5 bars for about 15 minutes. Potato Dextrose Agar (PDA) plates were prepared by autoclaving 9.75g PDA suspended in 250ml distilled water, cooling to ambient temperature and then dispensed as 15 ml per sterile Petri dishes in sterile lamina flow hood. The basidiomycete JO5289 was grown on the PDA plates from the preserved agar slants. The plates were incubated at ambient temperature for three weeks in IBRL.

3.3 Cultivation of the basidiomycete JO5289 in liquid media

The sterilized liquid media was used to culture the basidiomycete JO5289. Agar plugs (1cm x 1cm) from a well grown PDA plate were cut and then introduced asceptically into 250ml liquid media, which was constituted as described in section 3.2 above. These were

allowed to grow at ambient temperature and upon assuming steady growth rate were used to inoculate lL scale of culture. A total of twenty 1L scale replicates were prepared and allowed to grow under ambient temperature conditions. The replicate cultures were regularly agitated and monitored closely for good growth and any contamination. The growth was also closely monitored and evaluated daily to check for glucose depletion using glucose testing stripes. Immediately glucose levels in the culture were depleted, the growth was stopped and the mycelia were separated from culture filtrate by vacuum filtration, from which crude extracts for intracellular and extracellular secondary metabolites were separately prepared.

3.4 Preparation of crude extracts

3.4.1 Crude extracts from mycelium targeting intracellular secondary metabolites

Mycelium that was obtained from the filtration was freeze dried to afford a dry weight mycelium. The dried mycelium was weighed and dissolved in 1.5L of acetone under constant agitation for 2 hours. The mixture was set-up in a covered Erlenmeyer flask to reduce the evaporation of the solvent. The acetone extract was obtained by filtration using Buchner filtration system and concentrated to dryness at a reduced pressure using a rotary evaporator. The dried crude extract was weighed, labelled as Mex, tested for larvicidal activity (according to section 3.6) and stored in a screw-capped vials kept at 4°C awaiting bioassay testing, chromatographic fractionation and purification.

3.4.2 Crude extracts from culture filtrate targeting extracellular secondary metabolites

The combined volume of the culture filtrate, obtained by filtration in section 3.3, was passed three times through a resin (Mitsubishi HP21 DIAION resin) packed in a glass column. The resin was packed in a vertically mounted column of glass (diameter 2.5cm and height 60cm) using distilled-deionised water. Once all the culture filtrate passed through the resin, the trapped secondary metabolites were eluted with 1500ml of acetone followed by 1000ml of methanol. The eluents were collected and concentrated to a residual aqueous remain, which was extracted five times with ethyl acetate. The combined ethyl acetate extract was dried using anhydrous sodium sulphate and concentrated using rotary evaporator under reduced pressure. The dried crude extracts were transferred into screw capped vials, weighed, labelled as Kex and kept at 4°C awaiting chromatographic fractionation and purification.

3.5 Purification of crude extracts

The mycelia crude extract (Mex) and culture filtrate crude extract (Kex) from sections 3.4.1 and 3.4.2 were separately fractionated and purified using repeated silica chromatography. Column chromatography work was guided by thin layer chromatography (TLC) to determine the solvent system and the level of purity at different stages of the fractionation and purification. Suitable solvent system was determined by spotting the respective crude extracts (Mex and Kex) on aluminium coated silica TLC plates, which were developed in various TLC chambers saturated with various solvents in different ratios. At this stage the solvents used were dichloromethane, cyclohexane, ethyl acetate and methanol. The developed plates were visualized using UV lamp and then sprayed with anisaldehyde spraying reagent before drying the plate in the oven to aid further visualization. From the TLC results, the best solvent system i.e. the one that gave better separation was used in the column chromatography.

A vertically mounted glass column was slurry packed with silica gel suspended in the less polar organic solvent. This slurry was constituted by suspending about 40g of silica gel in the first solvent. It was degassed by use of a suction pump and poured into the column. This was anchored in place by use of acid washed sand. The silica adsorbed sample was introduced slowly into the column with the aid of pipette and the sample was also anchored in place by use of acid washed sand so as to avoid turbulence during elution. Various fractions were collected and every eluent collections were spotted on a TLC plate. TLC analysis was used to determine the purity of the eluents which were later pooled into various fractions based on TLC results and then concentrated under reduced pressure. Repeated column chromatography and TLC were done until fractions were deemed clean. The weight of the purified fractions was determined and then transferred to screw-capped vials and stored in fridge at 4°C awaiting larvicidal tests and structure elucidation experiments.

3.6 Mosquito larvicidal assay

3.6.1 Source of mosquito larvae

The mosquito larvae used in this project were obtained from Pyrethrum Board of Kenya, Nakuru where they are reared under standard conditions. Both the crude extracts and the purified samples were subjected to mosquito larvicidal activity test against late 3^{rd} and early 4^{th} in star larvae of *Ae. aegypti*. The late third and early fourth in star larvae were used since the

response of freshly hatched larvae is not fully developed and they do not migrate satisfactorily during the first few hours (Essam *et al*., 2005). The larvicidal tests were carried out in the IBRL, Egerton University.

3.6.2 Mosquito Larvicidal assay

Standard methods for assaying larvicidal activity in scientific research laboratories as recommended by the WHO were followed in all experiments (WHO, 2005). Both the crude extracts and the purified samples were subjected to mosquito larvicidal activity. Bioassays were carried out in duplicate using 10 larvae for each replicate assay. Several fractions of varying concentration (20, 50, 100, 200, 500 and 1000ppm) were made and tested against the larvae of *Ae. aegypti*. Aqueous solutions of methanol were employed as the control experiments. The larvae were placed in test plastic pots containing 4 ml of the test solution. Larvae were considered dead when they were unable to reach the surface of the solution when the test plates were shaken. The number of dead larvae was determined at the start of the experiment and after 2, 4, 8, and 24 hours to monitor the larval mortality.

3.7 Structure elucidation (NMR spectroscopy)

Structures of purified larvicidal active compounds were elucidated using Nuclear Magnetic Resonance (NMR) spectroscopic technique. NMR spectroscopic experiments were performed on a 300 MHz Bruker AVANCE NMR spectrometers at the Division of Chemical Sciences, Faculty of Health and Medical Sciences, University of Surrey, Guildford, UK. The spectra were recorded in deuterated chloroform ($CDCl_3$) and the chemical shifts were recorded in ppm (parts per million) relative to the solvents. The deuterated chloroform was referenced according to the central line at δ 7.26 in the ^{1}H NMR spectrum and at δ 77.23 in the ^{13}C NMR. The purified compounds were dissolved in deuterated chloroform ($CDCl_3$) in a clean vial. The solution was then transferred to an NMR tube and was placed in the probe for analysis. The same sample was used to obtain the spectra (^{1}H, ^{13}C, COSY, DEPT, NOESY and HMBC) data for the compound. Data was acquired from the NMR machine as computer print out. From the spectroscopy experiment, structures were proposed based on the interpretation of the combined spectra for each compound.

3.8 Statistical analysis

The percentage mortality were recorded and the average taken for the replicate experiments for each time interval. The result of bioassay was subjected for analysis using regression line analysis. The values of various concentrations were plotted against percentage mortality and regression equation was obtained The LC_{50} and LC_{90} values which were the concentration values for killing 50% and 90%, respectively of the mosquito larvae in 24 hours were obtained from the regression equation.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Cultivation, purification, larvicidal and structure elucidation

4.1.1 Cultivation of the basidiomycete in solid and liquid media

The basidiomycete JO5289 was grown on PDA, which was initially white cottony mycelium that turned moist brown with age. The hyphal strands were found to be septate and had clamp connection which was the basis of its classification as a basidiomycete. The hyphal strands in a well grown mycelium indicated the presence of clamp connection which is characteristic feature of basidiomycetes. The basidiomycete JO5289 took 21 days to grow in liquid malt media at ambient temperature. Glucose served as the initial carbon source before the basidiomycetes can immobilise carbon from more complex carbon matrix in molasses. After 21 days the culture was separated into mycelium and culture filtrate from which crude extracts, Mex and Kex were prepared, respectively (figure 2).

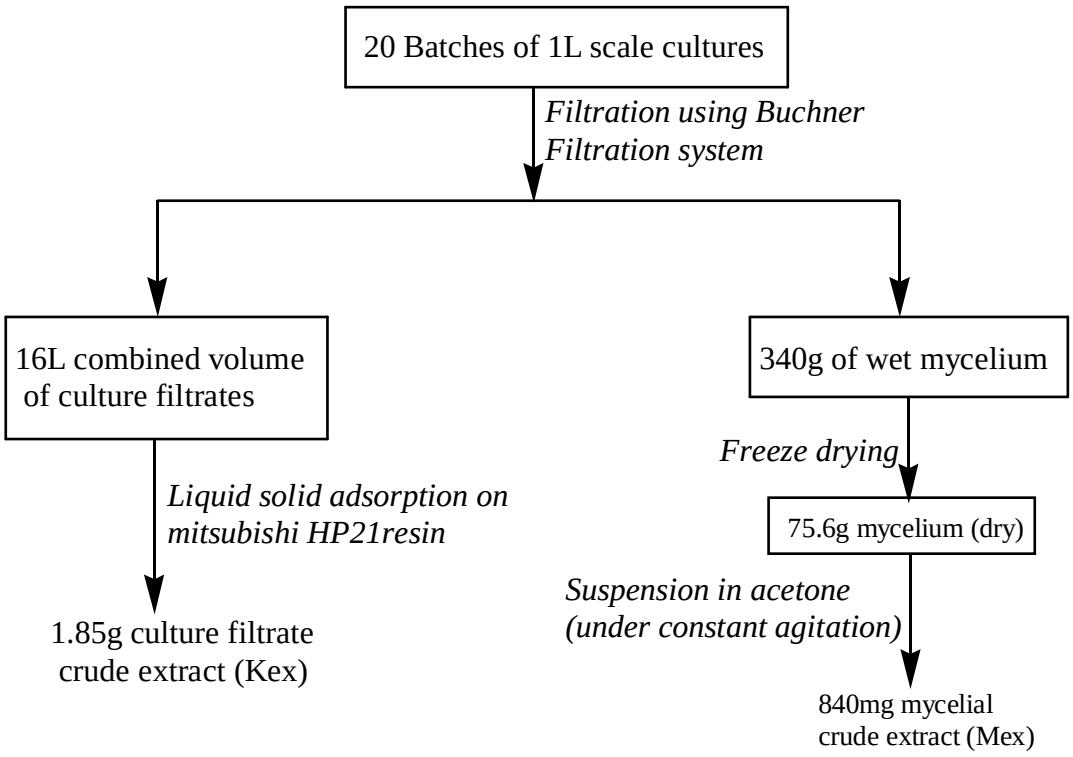


Figure 2: Flow scheme illustrating the preparation of mycelial (Mex) and culture filtrate (Kex) crude extracts

4.1.2 Larvicidal tests of the mycelia and culture filtrate crude extracts (Mex and Kex)

The mycelial crude extract was prepared in defined concentrations in the interval 0 to 1000ppm. They were then tested for larvicidal activity against *Ae. aegypti*. The crude extracts prepared at different concentrations were tested in set-ups that were evaluated for percent mortality after 2, 4, 8 and 24 hours. All the tests were done in duplicate and the mean value is reported in the table 3.

Table 3: Larvicidal test for the crude extracts (Mex)

	Percentage mortality of the crude extracts			
Concentration (ppm)	2 hours	4 hours	8 hours	24 hours
0	0	0	0	0
50	0	0	0	0
100	0	0	0	0
200	0	20	40	60
500	0	30	40	80
1000	20	50	80	100

From the table 3, it was noted that higher concentration of 1000ppm was more efficacious especially after 24 hours, which had 100% mortality. After 24 hours the 500ppm had 80% mortality and 200ppm had 60% mortality. For concentrations less than 200ppm, there was no percent mortality observed at all even after 24 hours. However, the observed activity at 1000ppm after 24 hours of 100% mortality was still significant given that it was observed for crude extract. Hence the crude extract was further investigated for the compounds responsible for activity using activity guided separation and purification.

Culture filtrate crude extract (Kex) was similarly processed and tested in duplicate for larvicidal activity against *Ae. aegypti* like the mycelia crude extract (Mex). The results are summarised in the Table 4 below.

Table 4: Larvicidal test for crude extracts (Kex)

	Percentage mortality of the crude extracts			
Concentration (ppm)	2 hours	4 hours	8 hours	24 hours
50	0	0	0	0
100	0	0	0	0
200	0	0	0	20
500	0	20	20	80
1000	0	40	40	100

Like in the case of the mycelia crude extract above, only 1000ppm showed larvicidal activity after 24 hours. In contrast to Mex, the Kex had either no or very weak activity at concentrations below 500ppm. It was also noted that the Kex had relatively weaker activity compared to the Mex, but this can only be approximate and not conclusive since these are crude extracts.

4.1.3 Purification of culture filtrate crude extract (Kex) of the basidiomycete JO5289

The larvicidal activity observed in the culture filtrate extract was investigated to identify the compounds responsible for activity. This was carried out by use of thin layer chromatography and column chromatography. The culture filtrate crude extract (1.85g) obtained from the batch cultivation (Figure 2) was chromatographed by using isocratic solvent system of 90% cyclohexane and 10% ethyl acetate. A total of 40 fractions were collected, each having a volume of approximately 10ml. Each of the fractions was spotted on TLC plate. The developed TLC plate was allowed to dry and was viewed under UV radiation (λ = 254 and 365nm). The plate was further sprayed with *p*-anisaldehyde solutions and dried for 15 minutes at 110°C. These two procedures enable the fractions to be pooled together into 3 main fractions based on the pattern and R_f values of the spots on the TLC plate. These main fractions are referred to as intermediate product I, II and III as outlined in figure 3.

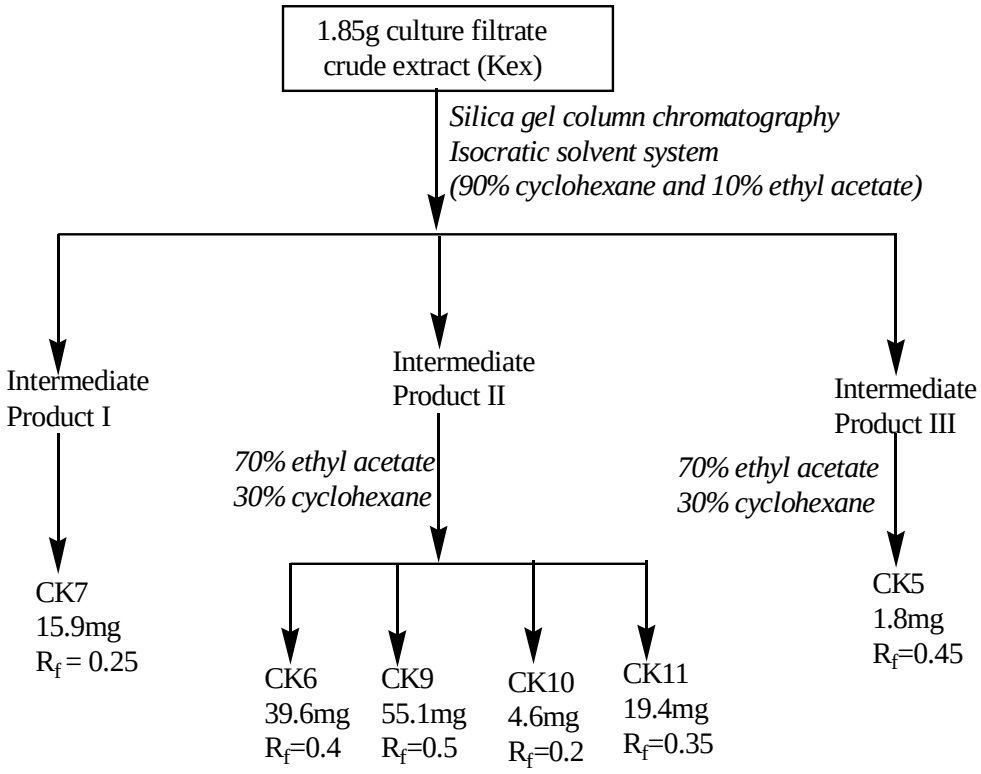


Figure 3: Summary of the purification scheme of Culture filtrate extracts (Kex)

Intermediate product I was appearing as a single spot on TLC plate when visualised by the UV radiations and after spraying with *p*-anisaldehyde. This was handled as a pure compound and was labelled CK7 before storing it at 4°C awaiting further analysis. The intermediate product II and III was subjected to further isocratic silica gel chromatography using a solvent system of 70% ethyl acetate and 30% cyclohexane. From the eluents collected, 4 pure compounds were obtained. These were labelled CK6, CK9, CK10 and CK11 and kept at 4°C awaiting further analysis.

The intermediate product III was similarly subjected to further purification and afforded one pure compounds labelled as CK5 as outlined in the purification scheme above (figure 3). It should be noted that for every compound purified, the corresponding yield and the R_f value are also given in the purification scheme. All the six pure compounds from Kex were tested for larvicidal activity and also subjected to NMR experiments to determine the chemical structures.

4.1.4 Purification of mycelium (Mex) of the basidiomycete JO5289

The mycelium extract (Mex) was subjected to silica gel chromatography using isocratic solvent system of 70% cyclohexane and 30% ethyl acetate. Two intermediate products IV and V were obtained (Figure 4). Intermediate product IV had several spots on TLC analysis and it was established that the compounds were separating with isocratic solvent system of 90% cyclohexane and 10% ethyl acetate. Consequently intermediate product IV led to purification of four compounds that were labelled CM1, CM2, CM5 and CM7 as shown in the figure below.

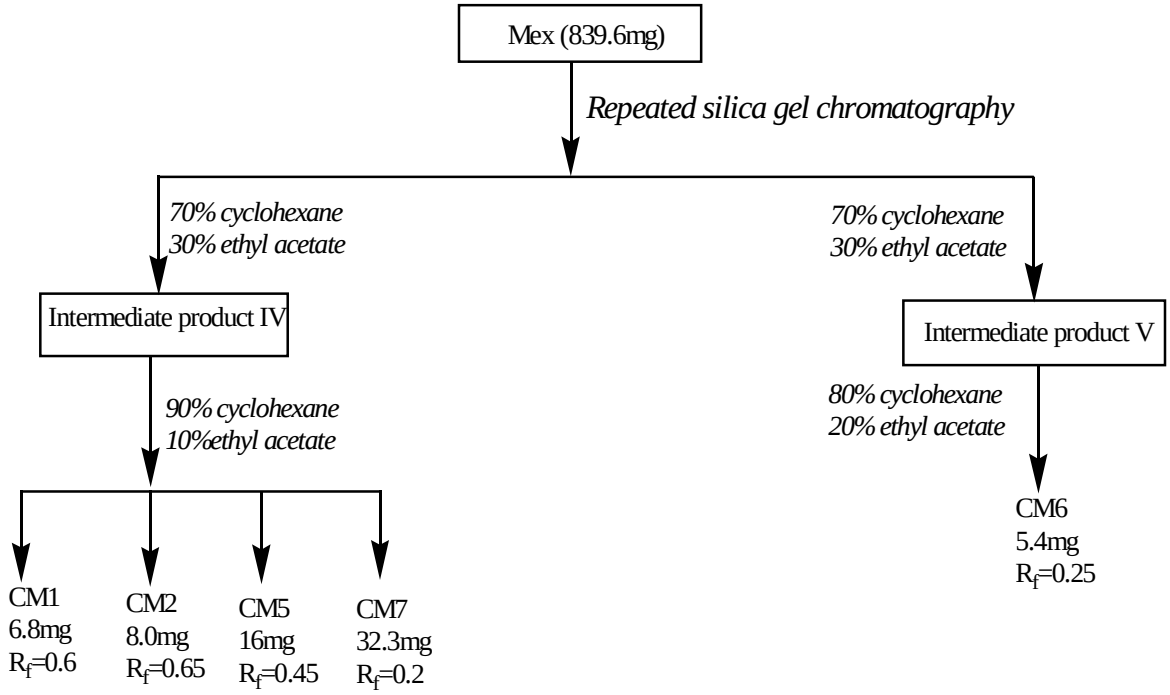


Figure 4: Summary of the purification scheme of mycelium extract (Mex)

The intermediate product V was further purified with isocratic solvent system of 80% cyclohexane and 20% ethyl acetate. The purification afforded one pure compound that was labelled CM6. It was noted that it gave a brown spot when sprayed with *p*-anisaldehyde. Each of the compounds purified for mycelium crude extract, the yield and the R_f values are also given in the purification scheme in figure 4 above. The pure compounds were tested for larvicidal activity and also subject to NMR experiments to help in elucidating the structures.

4.1.5 Larvicidal tests of compound CK6

The results of larvicidal tests for compound CK6 are summarised in table 5. It was clear that sensitivity of *Ae. aegypti* larvae was concentration dependent correlated at 24 hours. At other evaluation intervals (after 2, 4, and 8 hours) there was no significant activity observed and correlation to be determined. It was also noted that there were not much activity below concentration of 100ppm. But after 24 hours, it was statistically significant to conclude correlate the tested concentrations and the percentage mortality.

Table 5: Percentage mortality for pure compound CK6

Concentration (ppm)	2 hours	4 hours	8 hours	24 hours
100	0	0	0	20
150	0	0	0	40
200	0	0	0	50
300	0	0	20	60
400	0	10	30	80
LC_{50}	-	-	-	231ppm
LC_{90}	-	-	-	453ppm

The control had all the larvae active after 24 hours. From the table 5 results, the concentration at 100ppm showed 20% mortality after 24 hours, 150ppm had 40% mortality, 200ppm had 50% mortality, 300ppm had 60% and 400ppm had 80% mortality after 24 hours. Regression analysis was used to determine the LC_{50} and LC_{90} values (Appendix XXVIII). The regression equation is $Y= 0.180X + 8.362$ and the $R^2 = 0.950$. From the regression equation the values of LC_{50} and LC_{90} were obtained as 231ppm and 453ppm respectively. The R^2 value shows a significant correlation between the results.

Larvicidal tests for fraction CK11 was also done and the results were tabulated in table 6. The LC_{50} and LC_{90} values at 2 hours and 4 hours were not done since mortality was only observed at higher concentration. From the table 6 it is clear that in the tested concentration range, it was possible to correlate to the observed percentage mortality after 8 and 24 hours. The LC_{50} values at 8 hours and 24 hours were moderate thus was considered significant. The control had all the larvae active after 24 hours. Moreover the absence of larval mortality in negative

control in 24 hours is a clear indication that the real cause of mortality is the active compounds in the extracts.

Table 6: Percentage mortality for compound CK11

Concentration(ppm)	2 hours	4 hours	8 hours	24 hours
150	0	0	0	20
200	0	0	10	30
250	0	0	20	50
300	0	20	50	90
400	10	40	60	100
LC_{50}	-	-	345	237
LC_{90}	-	-	499	350

4.2 Structure elucidation

All the 11 compounds purified from both the mycelium (Mex) and culture filtrate (Kex) crude extracts were sent for NMR analysis in University of Surrey, United Kingdom. These were labelled CK5, CK6, CK7, CK9, CK10, CK11 from Kex. and CM1, CM2, CM5, CM6 and CM7 from Mex. However, only 3 (CK6, CK5 and CK11) gave complete spectra necessary for structure elucidation and their structures are discussed below. The rest were either not pure enough or not sufficient to carry out detailed NMR experiment. It is note worthy that all compounds purified from Mex fell under the group of compounds whose structures were not elucidate.

4.2.1 Compound CK6 (Appendix I to XIII)

The NMR spectra (both 1D and 2D) were used in interpretation of structure of CK6; the major compound in the spectra. From the ^{13}C NMR spectrum (appendix IV), six signals are evident (δ = 154.7, 115.7 130.3, 130.8, 64.1 and 38.4) corresponding to the 8 carbons present in the molecule. From the Distortionless Enhanced by Polarization Transfer (DEPT) (Appendix VIII), three different types of carbon atoms were evident; two pairs of methine carbon atoms (δ = 115.7 and 130.3ppm), two methylenic carbon atom (δ = 38.4 and 64.1) and two quaternary

carbons (δ = 130.8 and 154.7ppm). The signals at δ = 115.7 and 130.3ppm were isochronous, each representing 2 pairs of carbon atoms in a benzene ring that was para disubstituted, indicating unequivocally that the ring was having some symmetry. The compound contained an aromatic ring as was evident from the six characteristic carbon chemical shifts. The carbon signal at 154.6ppm was represented oxygenated aromatic carbon attached to a hydroxyl (OH^-) functional group. Heteronuclear Single-Quantum Correlation (HSQC) experiment confirmed that sp^2-hybridsed methine proton signals at δ = 6.78 and 7.08 were connected directly to the isochronous carbon atoms with chemical shifts δ = 115.7 and 130.3, respectively. The carbon signal at δ = 64.1 is typical of aliphatic oxygenated sp^3-hybridised carbon attached to an oxygenated centre (OH).

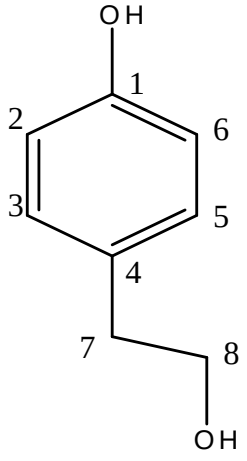
Further information from HSQC experiment indicated that the proton signals at δ = 2.79 and 3.81 were respectively on aliphatic sp3-hybridised carbons at 38.4 and 64.1 respectively. The latter aliphatic carbons are adjacent to each other and this is confirmed by an isolated spin system they display in the COSY experiment. In addition, COSY spectra further indicate clearly presence of a mirrored spin system for the aromatic protons (δ = 6.78 and 7.08ppm). The HMBC (Appendix IX) spectrum clearly reveals that the respective aromatic methine proton at δ = 6.78 and 7.08 are correlating with aromatic carbon atoms at δ = 154.7, 130.8 and 115.7 but not the carbon at δ = 130.3ppm for 6.78 and not δ = 115.7ppm for the proton δ = 7.08ppm. The latter observation pointed to the fact that the protons are positioned meta- with respect to the carbons that they are not correlating with. All these arguments are summarised in table 7 below.

Table 7: NMR data for CK6

Entry	1H (δ)	1H (δ)*	^{13}C(δ)	^{13}C(δ) *	COSY	DEPT	HMBC (H→C)
1	-	-	154.7	154.1	-	-	-
2	6.78	6.77	115.7	115.4	H3	-CH-	C1,C2,C4
3	7.08	7.09	130.3	130.0	H2	-CH-	C1,C2,C4
4	-	-	130.8	130.2	-	-	-
5	7.08	7.09	130.3	130.0	H6	-CH-	C6,C4,C1
6	6.78	6.77	115.7	115.4	H5	-CH-	C1,C5,C4
7	2.79	2.82	38.4	38.3	H8	$-CH_2-$	C4,C5,C3,C8
8	3.81	3.83	64.1	63.8	H7	$-CH_2-$	C7,C4

* Guzman-Lopez *et al.* (2007)

All the above information is summarized in the table 8 and the foregoing arguments led to the structure of the compound to be proposed as 4-(2-hydroxyethyl)phenol (**13**) drawn below. This compound has the synonyms as 2-(4-hydroxyphenyl) ethanol, 4-hydroxyphene ethyl alcohol and is commonly known as tyrosol.



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The structure of the compound was further confirmed by comparison of ^{1}H NMR and ^{13}C NMR data with those in the literature as shown in table 7. Tyrosol (**13**) had been obtained from mycelia of an ascomycetes fungus *Cordyceps ophioglossoides* as an estrogenic substance (Kawagishi *et al.*, 2004). It has also been obtained from phytopathogenic fungus *Ceratocystis adipose* as an antioxidant (Guzman-Lopez *et al.*, 2007). Its production had been reported in other *Ceratocystis* species before including *Ceratocystis fimbriatacoffea* (Gremand and Tabacchi, 1996), *C. clarigera*, *C. ips* and *C. huntii* strains (Ayer *et al.*, 1986). Furthermore literature survey confirms that tyrosol is produced from tyrosine and is commonly produced in fungal cultures. The biosynthetic pathway shows that there is an initial oxidation, deamination reaction, followed by decarboxylation and reduction of the resulting aldehyde (Turner and Aldridge, 1983).

Tyrosol generally has pharmacological interests and shows an antioxidant activity. It has also been used in atheroscelrosis treatment, protecting low density lipoproteins (LDL) from oxidation which play a role in the initiation and progress of cardiovascular diseases (Guzman-Lopez *et al.*, 2007). Phytotoxic activity of tyrosol has been observed in lettuce leaves and certain toxicities in mice (Ayer *et al.*, 1986). However, to our knowledge this was the first time the larvicidal activity was reported for tyrosol.

4.2.2 Compound CK11 appendix XIV to XIX

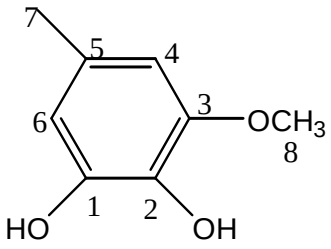
^{1}H-NMR and ^{13}C-NMR as well as other NMR experiments are summarised in table 8. The ^{13}C NMR (appendix XV) had 6 discernable carbon signals but accountable for 8 carbon atoms. Two signals at (δ = 154.5 and 113.2) were isochronous, each representing a pair of carbon atoms. Six of the 8 carbon atoms were characteristic aromatic carbons (δ = 113.2, 113.2, 128.9, 129.6, 154.5 and 154.5ppm). The carbon signals at δ=154.5 is typical an oxygenated aromatic carbon that is often associated with phenolic (¯OH) group and methoxy oxygen. From DEPT experiment (appendix XVII) only the isochronous carbon signals at δ = 113.2ppm were indicated to be connected to methine hydrogens (δ = 6.43 and 7.71ppm). From the COSY spectra (appendix XVI) these methine protons are locked in a kind of a spin system suggesting that they are in the neighbourhood of each other. The COSY spectrum also shows the interactions of protons at carbon δ = 113.7. These protons (δ = 6.43 and 7.71ppm) are clearly splitting one another with a coupling constant of J = 5.4Hz. The observed coupling constant is

midway between those reported for *meta*-coupling (1~3Hz) and *ortho*-coupling (7~9Hz) (Friebolin, 2005). Since typical *ortho*-coupling in benzene ring systems are enhanced, the observed *J*-value was interpreted as *meta*-coupling. In addition it was clear from ^{13}C-NMR that the carbon chemical shifts at δ = 51.7ppm was characteristic of a methoxy carbon and the carbon signal at δ = 14.3 is a methyl carbon.

Table 8: NMR Data for CK11

ENTRY	^{13}C (δ)	^{1}H(δ)	DEPT
1	154.5	7.72-	-
2	129.6	-	-
3	154.5	7.72	-
4	113.2	6.43	-
5	128.9	-	-
6	113.2	6.42	-CH-
7	14.3	0.88	$-CH_3$
8	51.7	3.67	$-OCH_3$

The DEPT experiment (appendix XVII) further confirmed the presence of a methoxy carbon at δ = 51.7ppm, a methyl carbon at δ = 14.3ppm. From the same experiment and ^{13}C NMR data, it was also evident that the carbons at δ = 128.9, 129.6, and 154.5ppm were quaternary carbons. There was no much information discerned from the HMBC experiment except an authentic correlation between the methoxy protons (on carbon at δ = 51.7ppm) and one of the aromatic carbon δ = 154.5ppm. The NOESY spectrum of the compound was not run due to low yield. Based on the spectral data (Table 8) for CK11 and the foregoing arguments, the structure of the compound CK11 was proposed as 3-methoxy-5-methyl-1,2-benzenediol (**33**).



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4.2.3 Compound CK5 appendix (XX to XXVII)

^{1}H-NMR, ^{13}C-NMR, DEPT, HMBC, NOESY, COSY and HSQC-DEPT experiments were performed on compound CK5 and the corresponding spectra are attached as appendices (XX to XXVII). From all the experiments the data are summarised in table 9 below.

Table 9: NMR Data for CK5

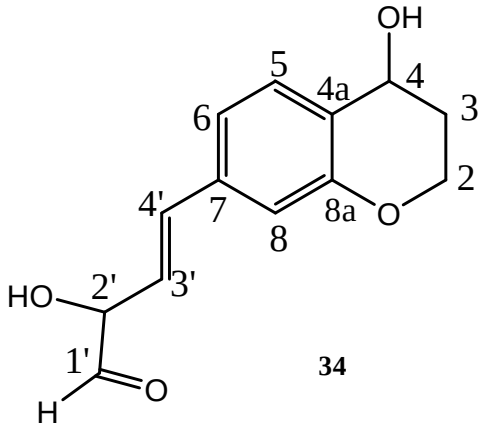
ENTRY	13C(δ)	HSQC-DEPT(δ)	DEPT	COSY	HMBC(H→C)	NOESY
1’	179.3	9.62	-CH-			
2’	59.4	6.05	-CH-	H3’		
3’	125.8	7.06	-CH-	H2’, H8		
4’	128.3	7.19	-CH-			
2	63.6	4.87/3.47	$-CH_2$	H3		H3
3	40.6	3.47	$-CH_2-$	H2		H2
4	77.4	7.19	-CH-			
4a	129.8	-	-C			
5	129.1	7.22	-CH-	H6	C6	H6
6	129.8	6.78	-CH-	H5	C5	H5
7	129.8	-	-C			
8	105.7	6.02	-CH-	H3’		
8a	129.1	-	-C			

From the ^{13}C spectrum (appendix XXII) there are 10 signals accounting for 13 carbon atoms, indicating that two signals are isochronous. The carbon signal at δ = 129.1 possibly accounts for one methinic carbon (C5) and one quaternary carbon (C8a). Similarly the carbon signal at δ = 129.8 was intepred as triply isochronous and was attributed to one methinic carbon (C6) and two quaternary carbon (C4a & C7). Whereas this is a rare occurrence, it was the best deduction arrived at for this molecule. Equally there was a typical carbonyl carbon chemical shift at δ = 179.3, which clearly showed presence of an aldehydic carbon. In the spectrum there was also evidence of aromatic carbons signals at δ = 129.8, 129.1, and 105.7ppm with the first two signals being respectively doubly and triply isochronous accounting for the five carbon atoms. In the same region of the spectrum there was evidence of two sp^2 hybridised carbons at δ = 125.8, which were isochronous and were assigned as olefinic carbons. This information was corroborated by a clear isolated spin system involving the respective two methine proton in the

COSY spectra (appendix XXIII). This information unequivocally helped to distinguish these signals from the aromatic sp^2 hybridised carbons that also characteristically observed in this region. Further upfield, there were three oxygenated sp^3 hybridised carbon signals at δ = 59.4, 63.6 and 77.4. DEPT spectra (appendix XXIV) revealed the presence of eight methinic carbons including aldehydic one and two methylenic carbons. This indicated that there were three quaternary carbons in the molecule.

The COSY spectrum further revealed a coupling and splitting pattern between protons at 59.4ppm and 125.8ppm; a possible indication that they are in the neighbourhood of one another. A similar splitting is evident between protons at 125.8ppm and 105.7pm, 63.6ppm and 40.6ppm, & 129.1ppm and 129.8ppm. The NOESY spectrum (appendix XXVII) shows the interaction of protons at 63.6ppm and 40.6ppm in space. A similar interaction is seen between protons at 129.1ppm and 129.8ppm. The HSQC-DEPT (appendix XXVI) indicated clearly that the methinic protons on oxygenated carbon (63.6ppm) were non- degenerated protons resonating at 3.47ppm and 4.87ppm. This was clear evidence that the oxygenated carbon was part of a rigid ring system typical of unsaturated ring fused to an aromatic ring system. The HMBC spectrum (appendix XXV) indicates the interaction of protons at 129.1ppm and 129.8ppm. It should noted that HMBC experiment for this compound did not give much information.

However, from the other experiments tabulated in table 9 and the foregoing arguments as well as literature information, the structure of compound CK5 was proposed and identified as 2-hydroxy-4-(4-hydroxy-chroman-7yl) but-3-enal (**34**).



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Compound **34** is a chromanone derivative and such compounds have been isolated from fungi and plants (Lee *et al.*, 2007). Among the known naturally occurring chromanones, nearly

all have alkyl substituent at the C2 (40.6ppm) or C3 (60.6ppm) position. They have also been synthesised by chemical methods for their extensive bio-activities such as antifungal, antibacterial, antitumour and antiviral activities (Li *et al.*, 2007). From this study three compounds had their complete structures proposed based on NMR experiment. The three compounds belong to two classes of compounds; the phenols and chromans (a derivative of flavonoids).

From the purification scheme (figures 3 and 4) and the foregoing discussion, a brief summary on the purification, bioassay and structure elucidation of compounds isolated from Basidiomycete JO5289 is presented in table 10 below.

Table 10: Summary on the purification, bioassay and structure elucidation of purified compounds

Sample	Compounds purified	Compounds tested	Compounds with structure
Kex	CK5, CK6, CK7, CK9, CK10 and CK11	CK6 and CK11	CK5, CK6 and CK11
Mex	CM1, CM2, CM5, CM6 and CM7	None	None

4.2.4 Biological activities of compounds

Of the three compounds, it is only tyrosol (**13**) and 3-methoxy-5-methyl-1,2-benzenediol (**33**) had their respective LC_{50} and LC_{90} determined. Tyrosol (**13**) had LC_{50} and LC_{90} of 231ppm and 453ppm, respectively. 3-methoxy-5-methyl-1,2-benzenediol (**33**) had LC_{50} and LC_{90} of 237ppm and 350 ppm, respectively. The observed LC_{50} and LC_{90} values were significant though they were not within the range of those previously isolated larvicidal compounds reported from the literature. Sesquiterpenoids such as (E)–nerolidol and farnesol showed LC_{50} values of 17ppm and 13ppm, respectively. Ocimenone, a monoterpenoid isolated from *Tagetes minuta* oil exhibited LC_{50} value of 40ppm and a triterpene from *A. indica* showed an LC_{50} value of 21ppm (Geris *et al.,* 2008). However, the study has revealed the potential of this basidiomycete as source of larvicidal compounds.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The basidiomycete JO5289 was grown in defined liquid nutrient media (1% malt extract, 0.4% yeast extract and 0.4% glucose) and produced secondary metabolites that were harvested as crude extracts for both mycelium (Mex) and culture filtrate (Kex). Both Mex and Kex were tested for mosquito larvicidal activity against *Ae. aegypti* with respective LC_{50} of 408 and 458ppm at 24 hours. Whereas both showed similar larvicidal activity at concentration of 1000ppm after 24 hours, Kex was relatively weaker. Both crude extracts were closely analysed using TLC and further purified using column chromatography. From Mex, five compounds were purified while Kex gave six pure compounds and these are (CM1, CM2, CM5, CM6, CM7, CK5, CK6, CK7, CK, CK10, and CK11).

All the purified compounds were subjected to both 1-D and 2-D NMR experiments for structure elucidation. From these only compounds CK5, CK6 and CK11 had all the requisite experiments for structure elucidation, hence chemical structures were proposed for three compounds. The structures of the three pure compounds were determined based on NMR spectral data. Two of them; 4-(2-hydroxyethyl)phenol, and 3-methoxy-5-methyl-1, 2-benzenediol were active against *Ae. aegypti* larvae with LC_{50} values of 231 and 237ppm at 24 hours, respectively. However, larvicidal activity of 2-hydroxy-4-(4-hydroxy-chroman-7yl)but-3-enal was not determined due to low yield.

The larvicidal activity of the pure compounds was higher than the crude extracts. This depicts that purification enhanced the activity of the compounds and thus confirms the fourth hypothesis to hold. Generally the isolated compounds showed moderate larvicidal activities compared with those reported from literature.

The study has revealed the potential of Kenya basidiomycete JO5289 as a source of mosquito larvicidal compounds despite the low LC_{50} values reported. Full identifications of the components present in the active fractions that might be responsible for the larvicidal activity will be important in realizing full exploitation of the fungus for biotechnological applications.

5.2 Recommendations

- The taxonomic identification of the basidiomycete JO5289 should be done so as to determine its exact classification.
- The basidiomycete JO5289 should be scaled up during cultivation to increase the yield in order to fully exploit the basidiomycete. This will enable the structure elucidation of the active compounds whose structures were not elucidated due to low yield.
- Repeated bioassay coupled with analytical fractionation is needed to confirm the actual active compounds responsible for larvicidal activity of this basidiomycete. More advanced purification techniques such as HPLC should be applied to maximize the yield.
- The mode of action of the active compound as well as possible effects on non target organism should be studied before it can be practically used as natural mosquito control agent.
- The products of this research should be synthesized to confirm their structure and be recommended as a candidate for formulations as larvicide

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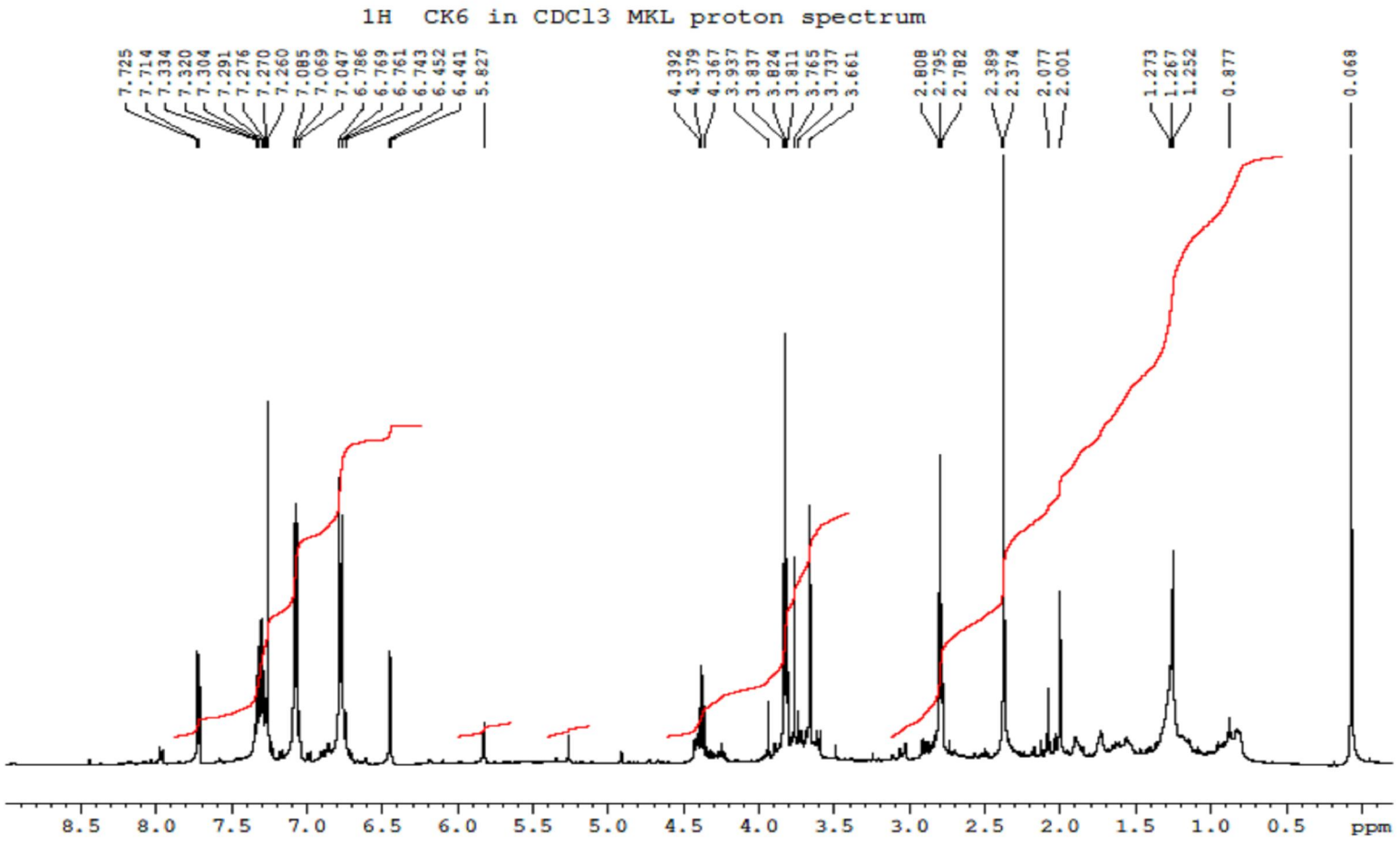
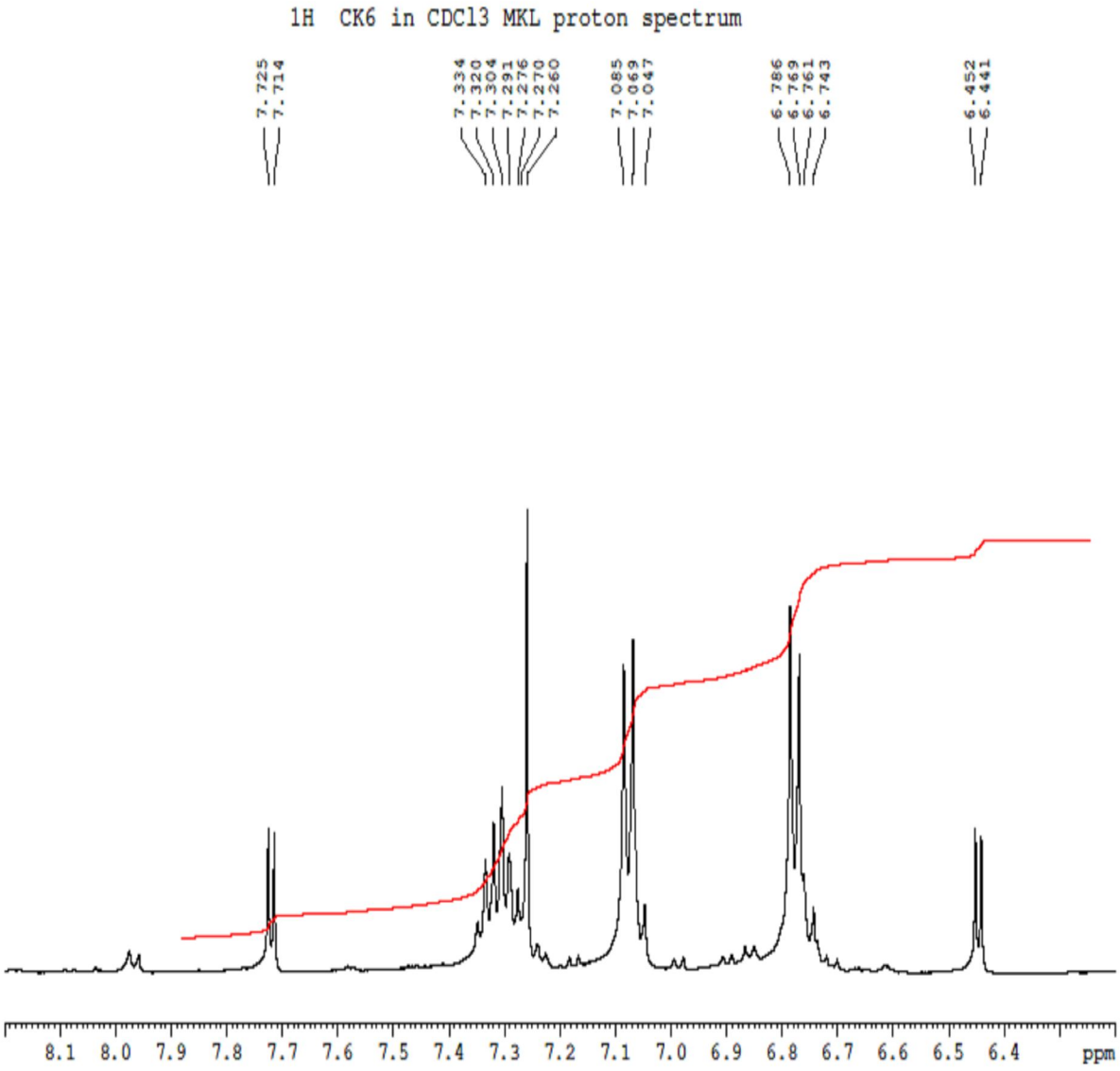
APPENDICES

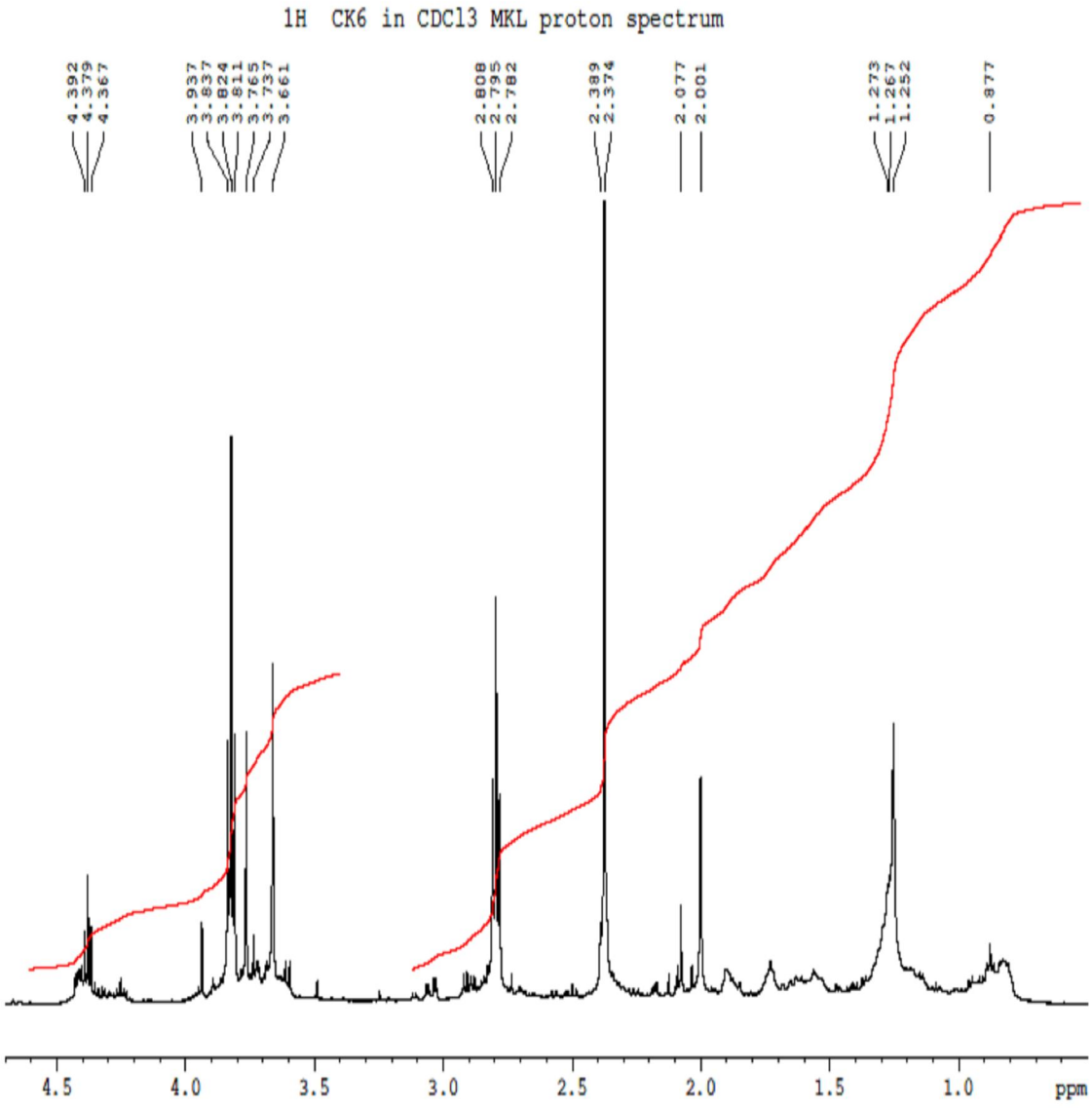
Appendix I: ^{1}H - NMR spectrum for CK6



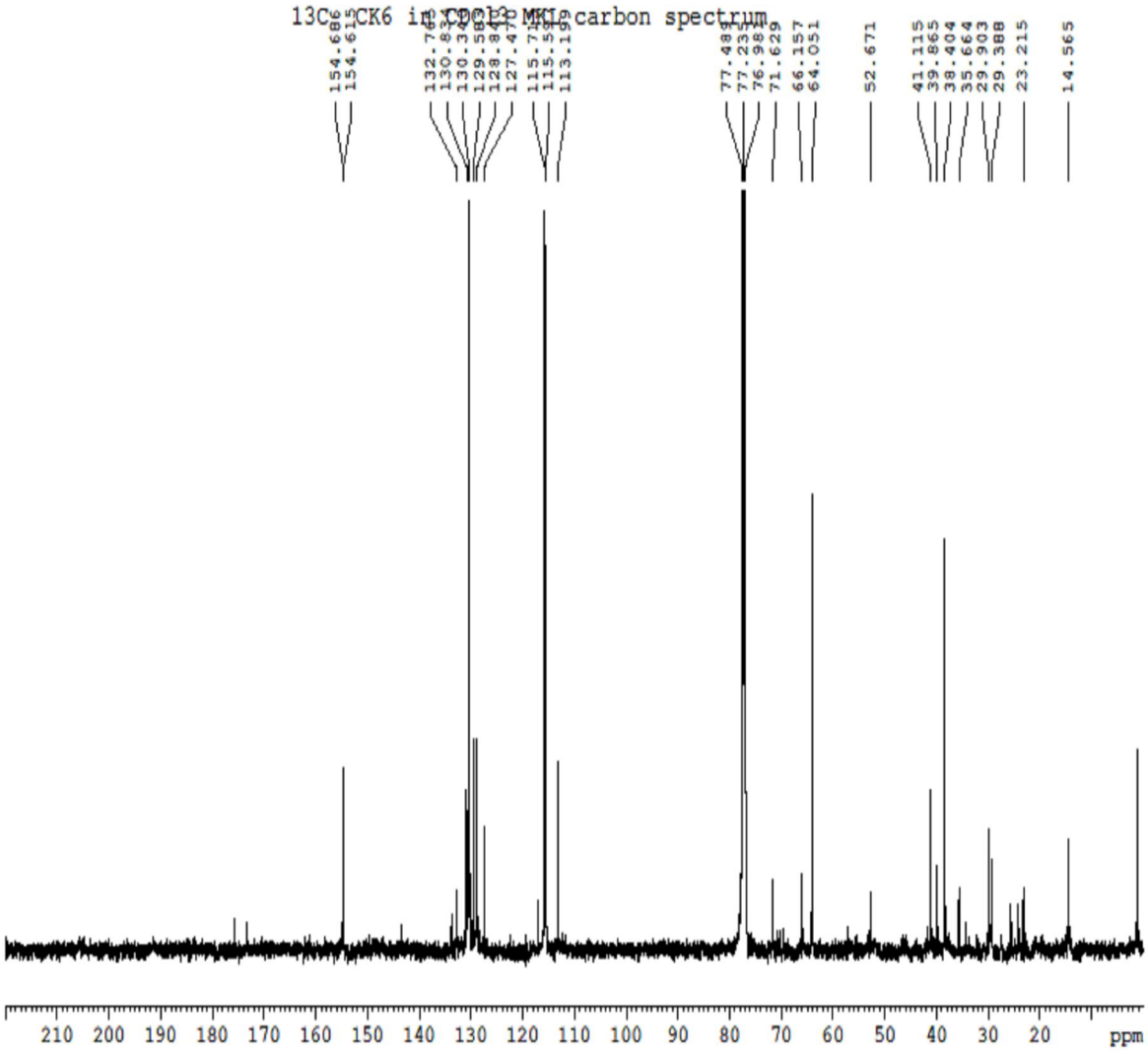
Appendix II: ^{1}H NMR Spectrum for CK6



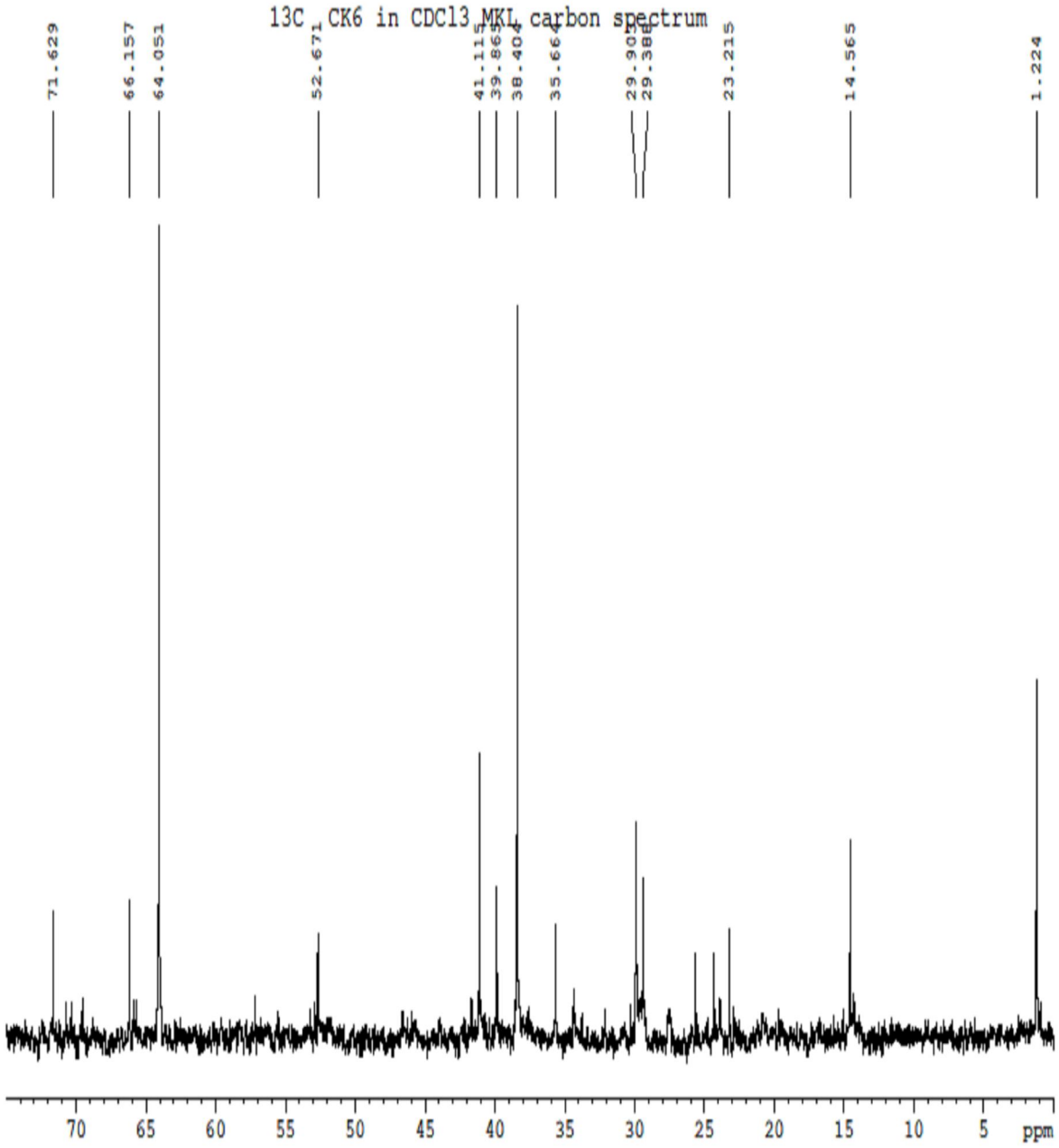
Appendix III: ^{1}H NMR Spectrum for CK6



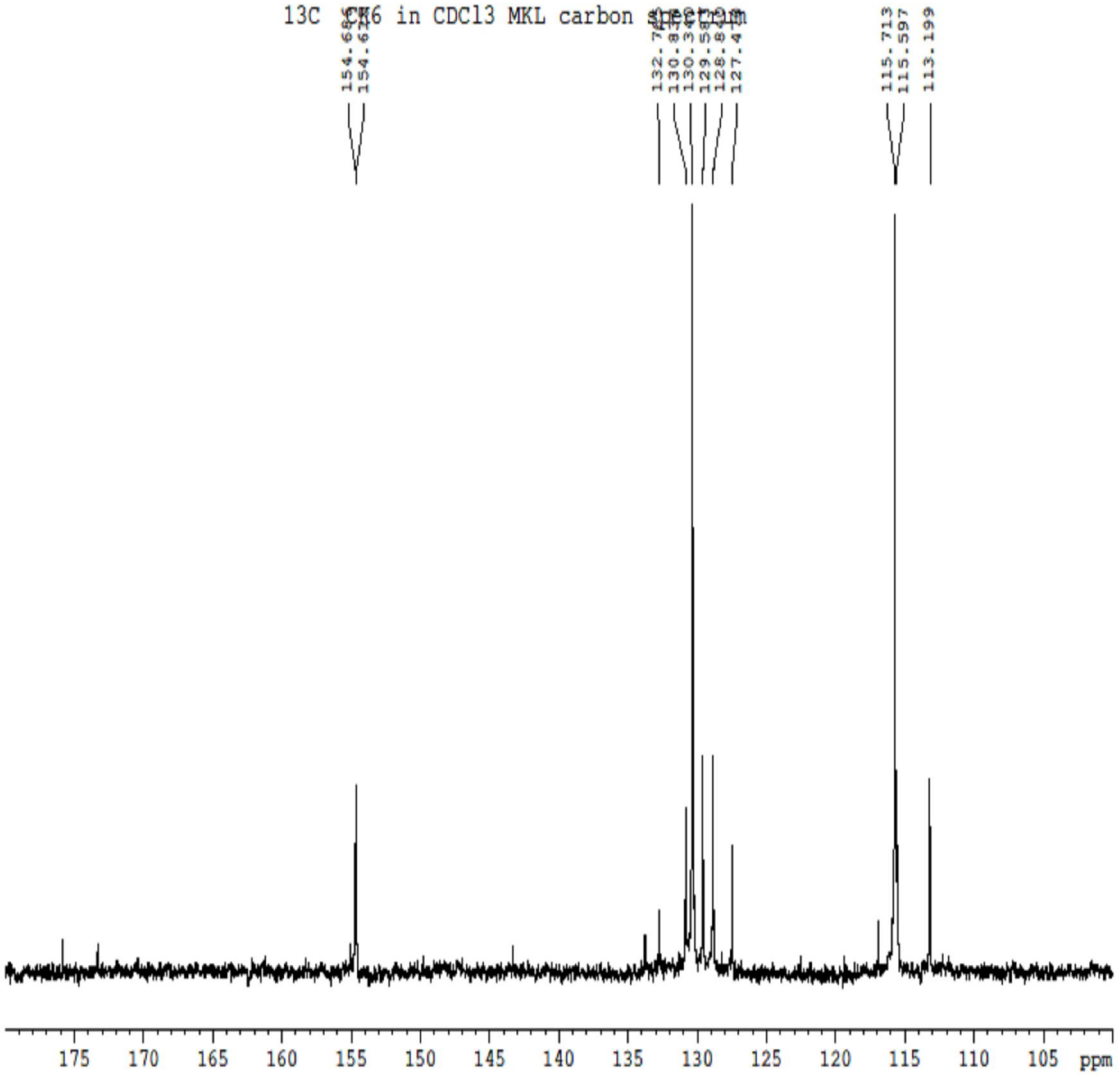
Appendix IV: ^{13}C NMR spectrum for CK6



Appendix V: ^{13}C NMR Spectrum expanded for CK6

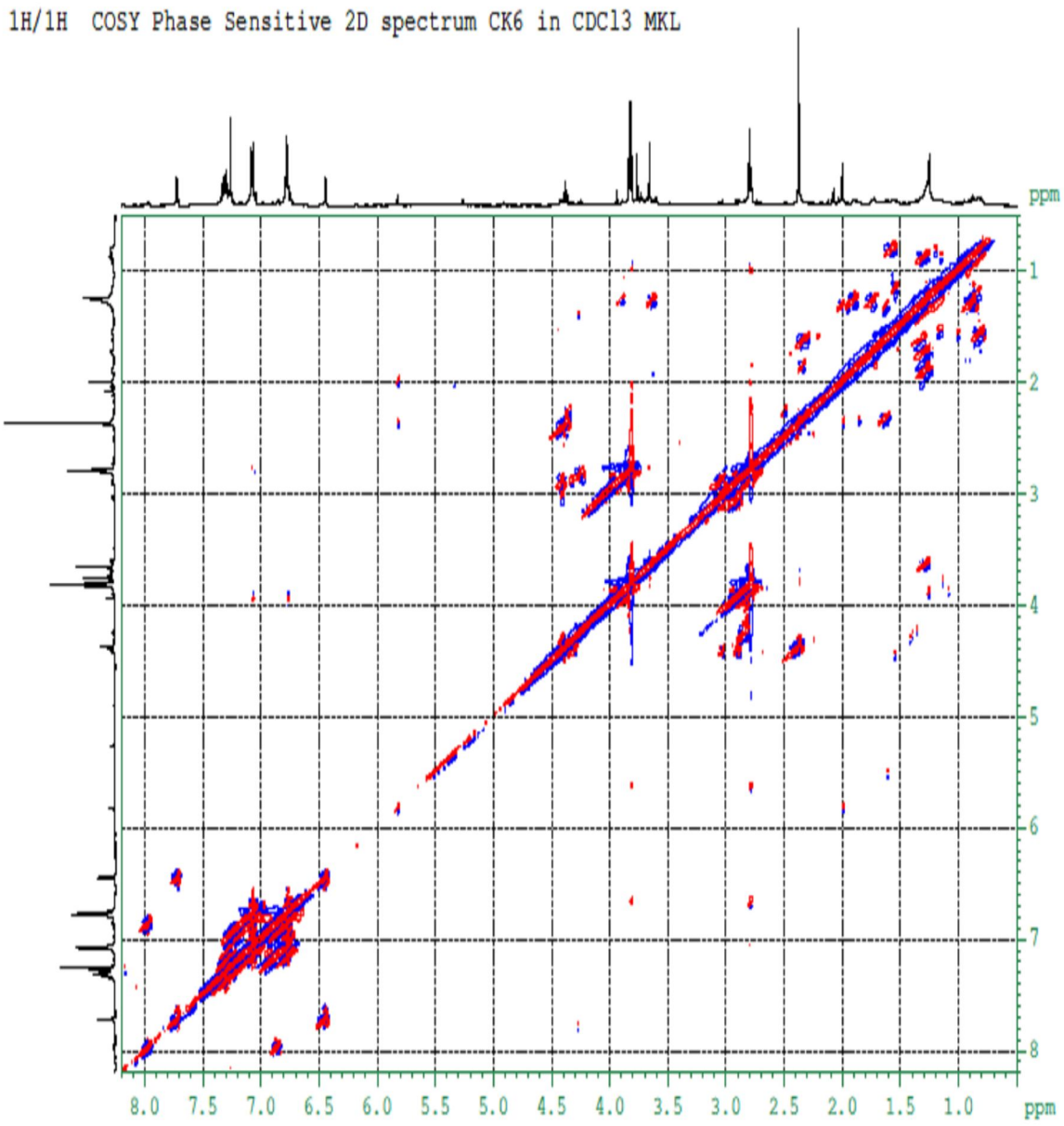


Appendix VI: ^{13}C NMR Spectrum expanded for CK6

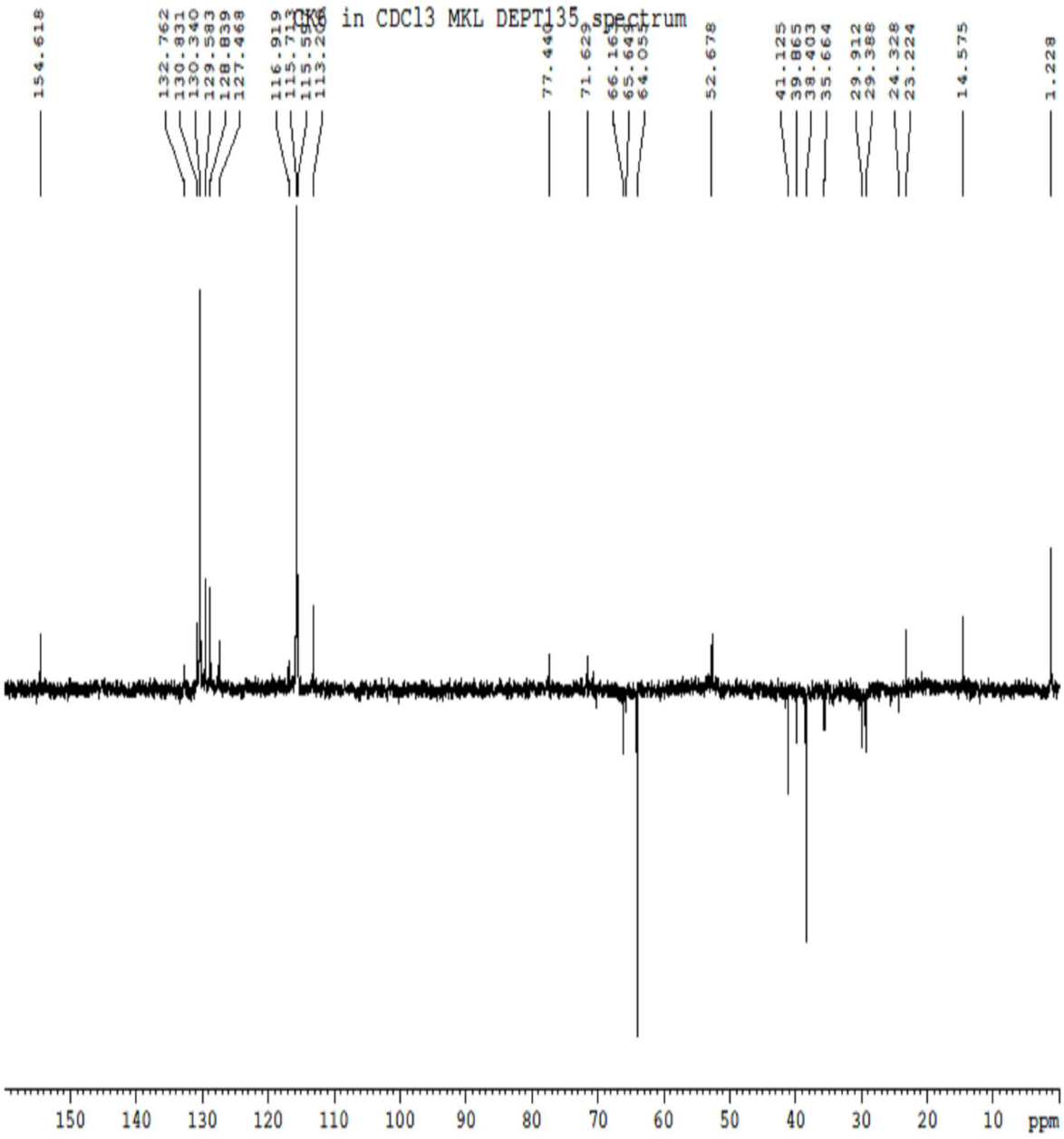


1

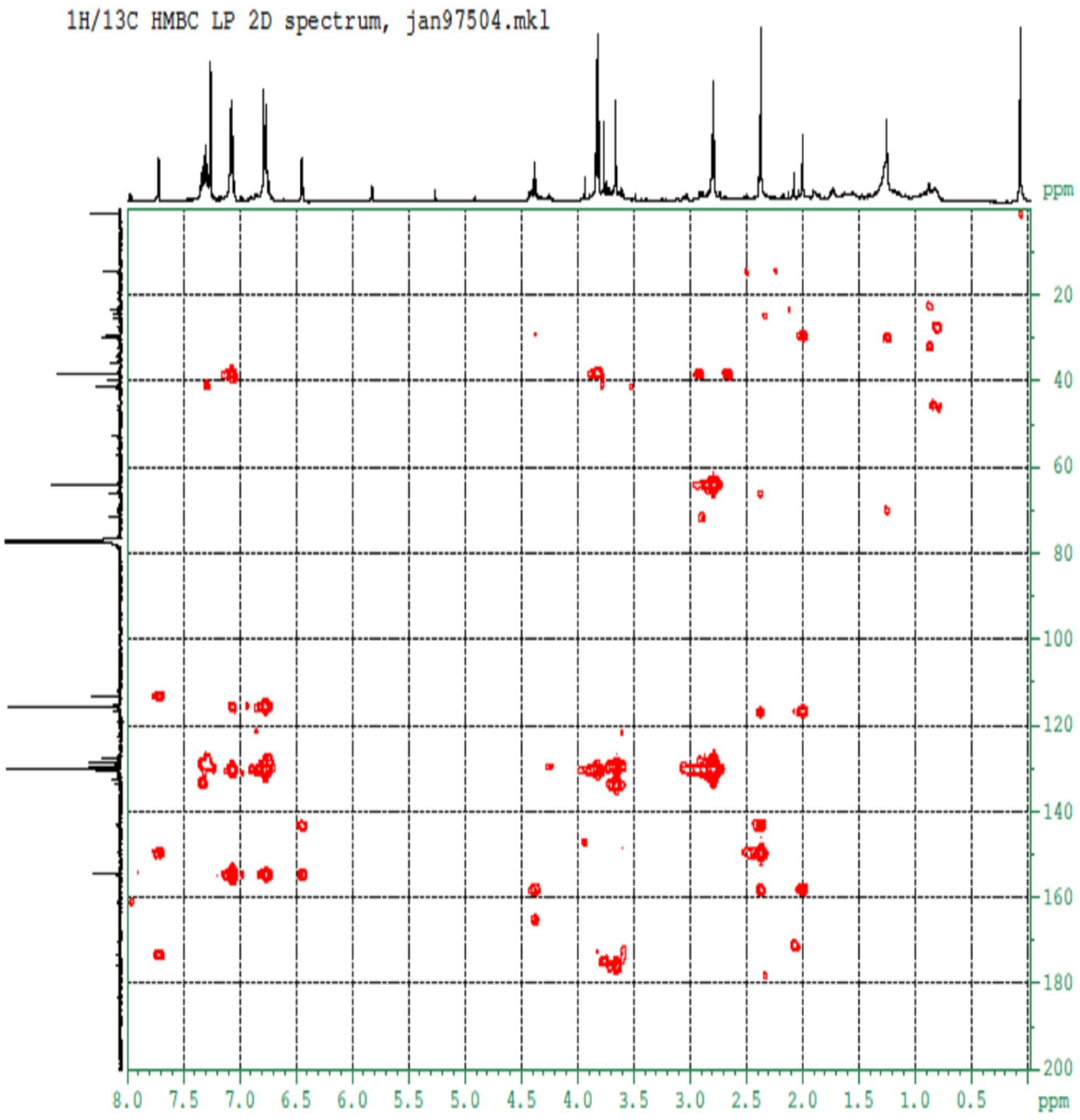
Appendix VII: COSY NMR Spectrum for CK6



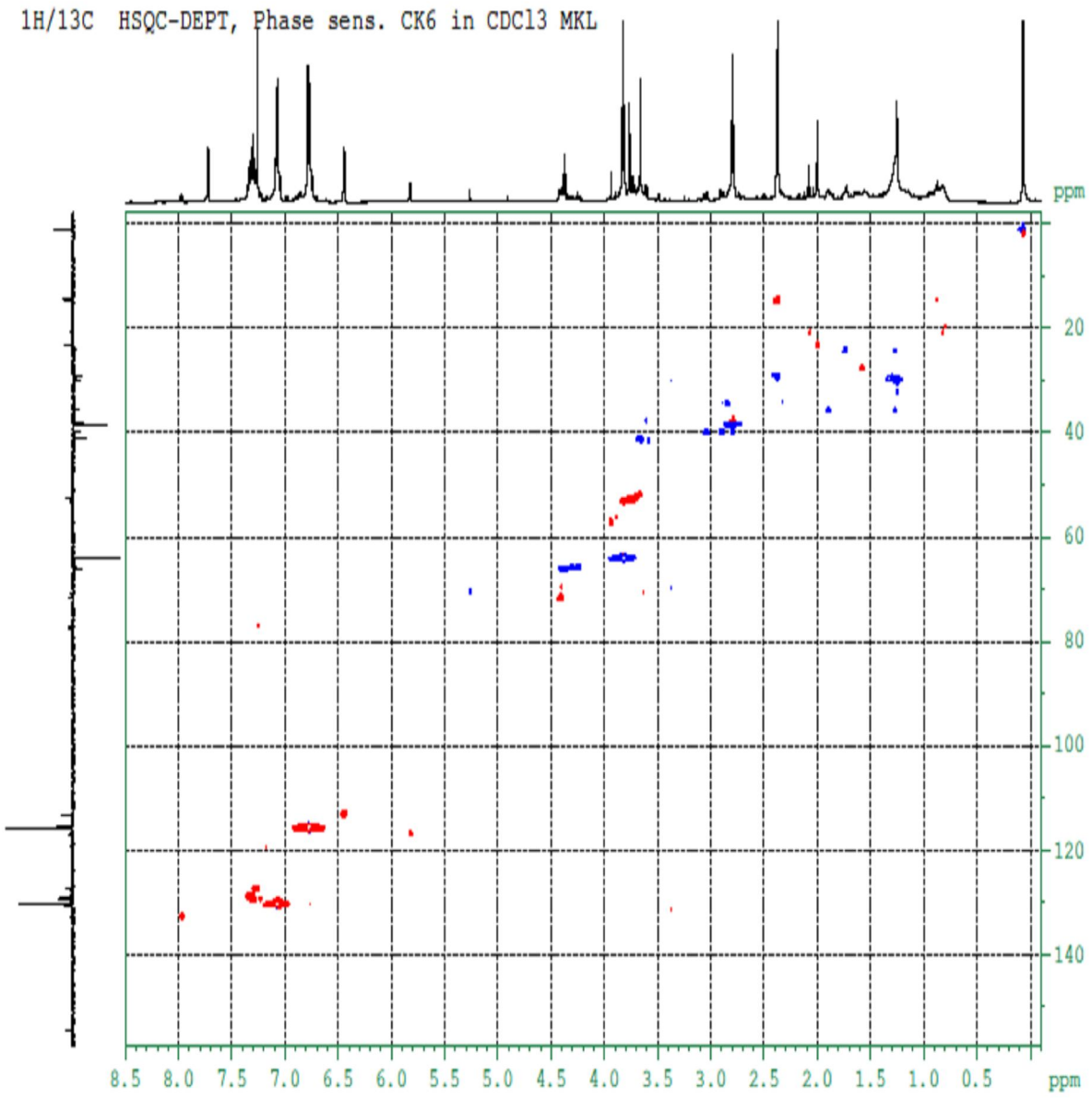
Appendix VIII: DEPT NMR Spectrum CK6



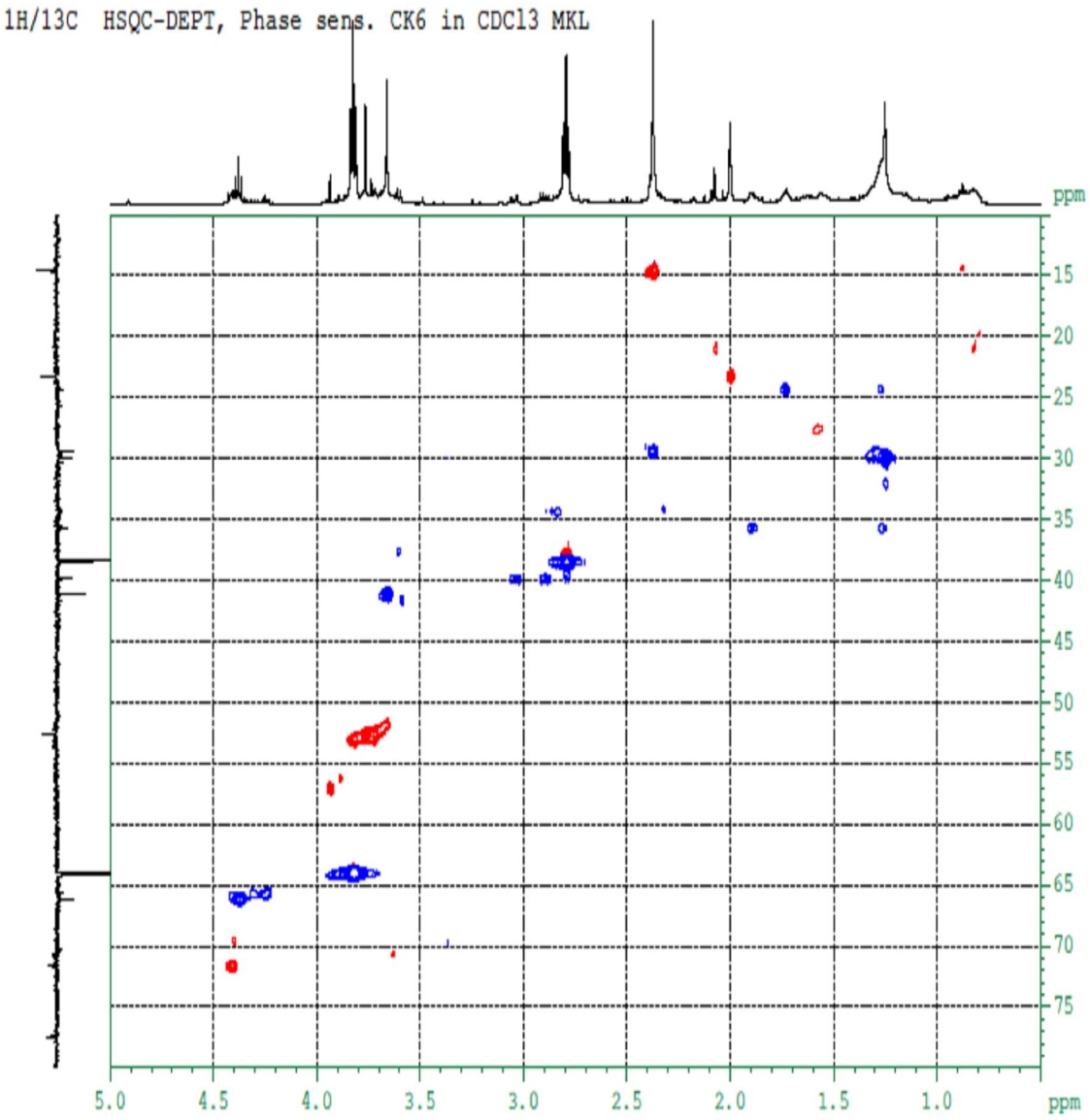
Appendix IX: HMBC NMR Spectrum CK6



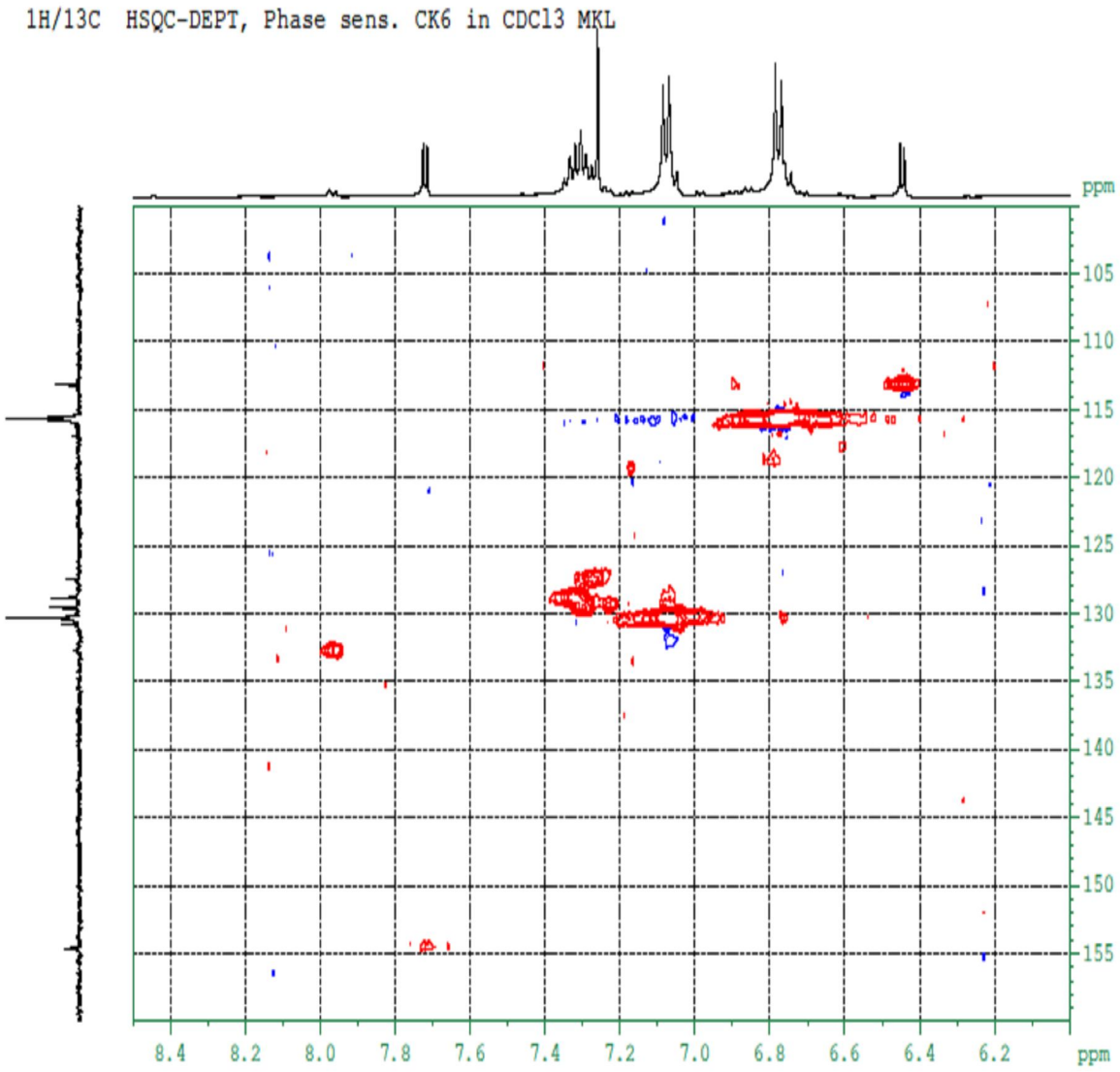
Appendix X: HSQC _ DEPT NMR Spectrum CK6



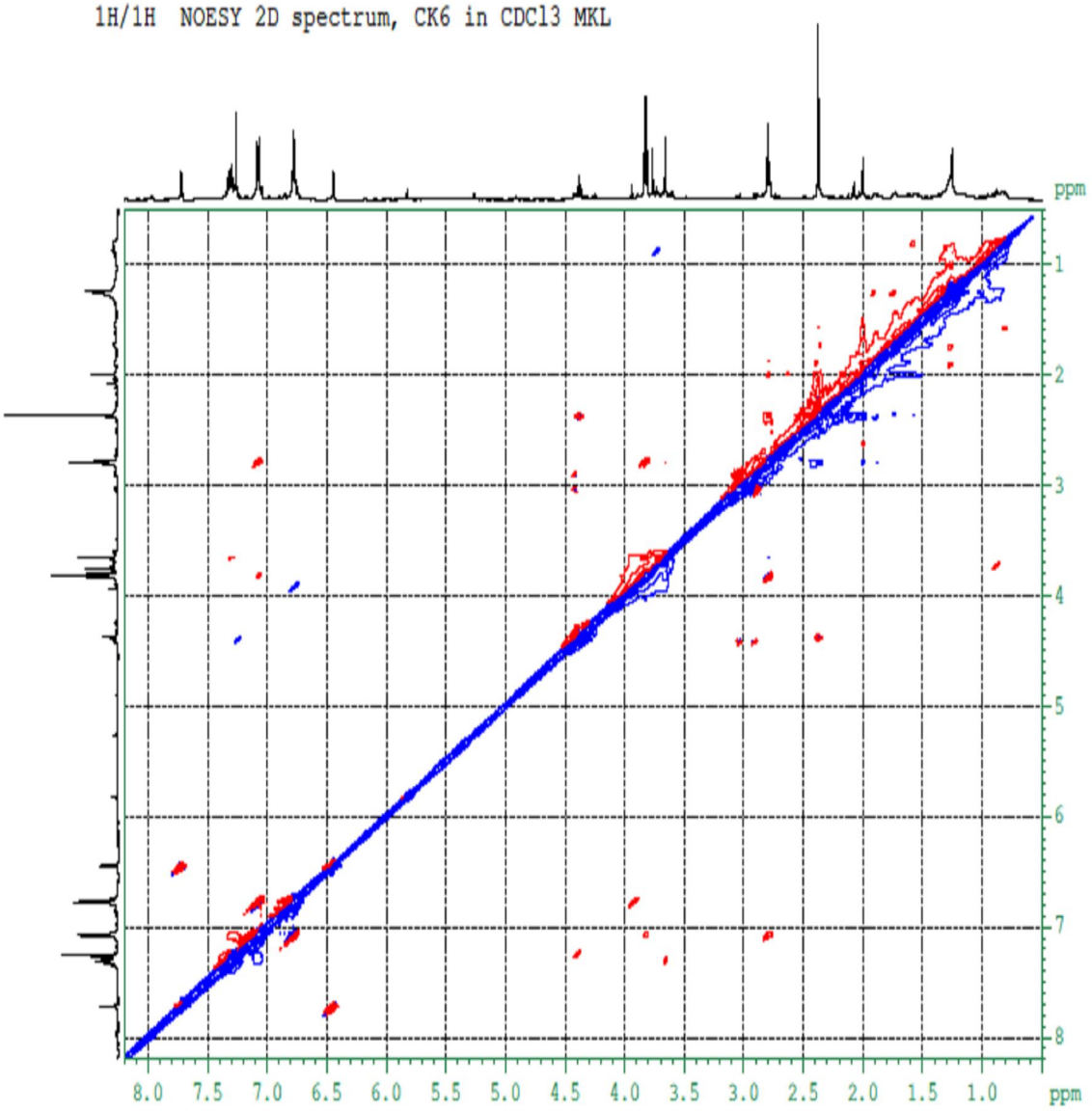
Appendix XI: HSQC_DEPT NMR Spectrum expanded CK6



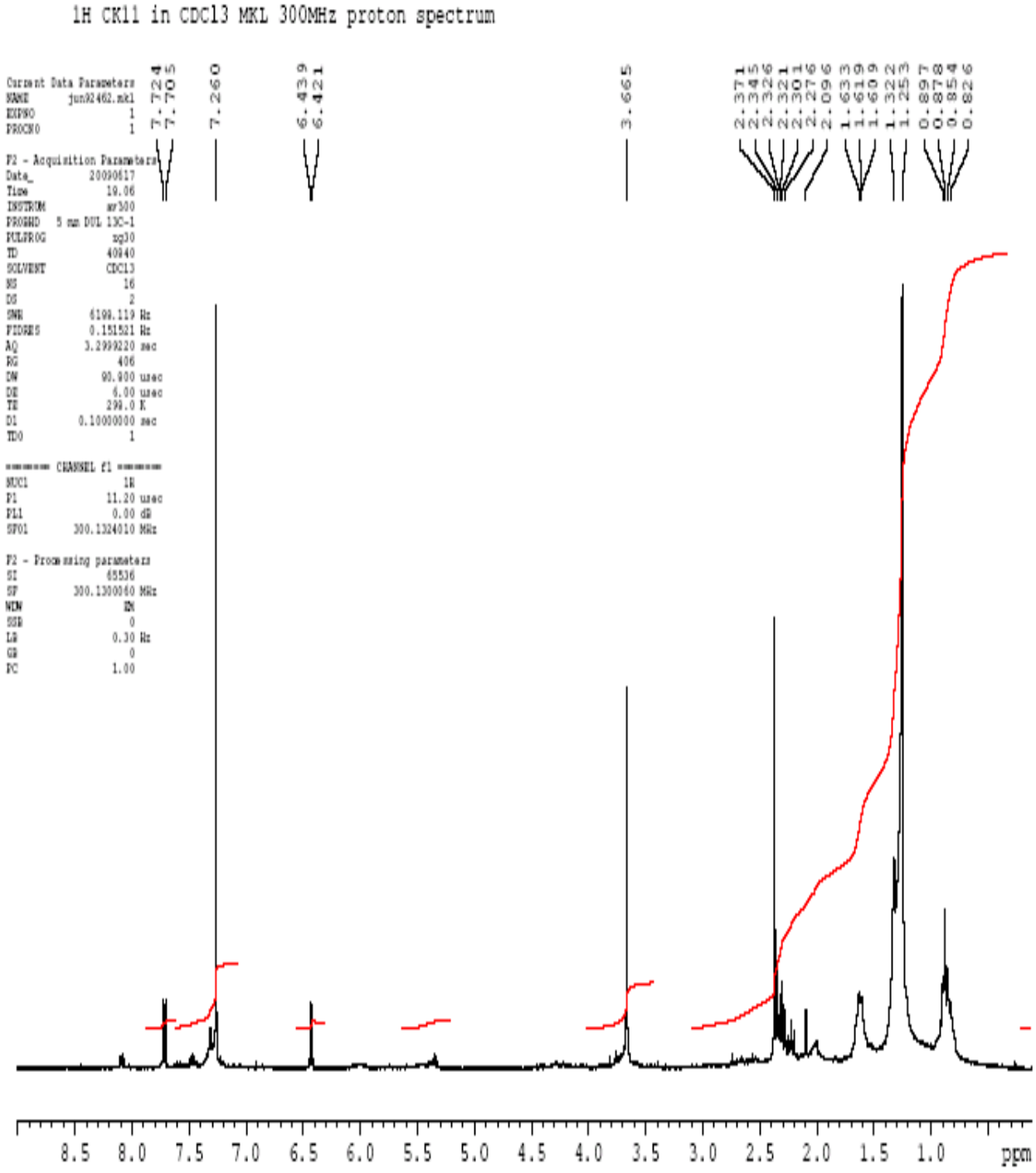
Appendix XII: HSQC_DEPT NMR Spectrum expanded CK6



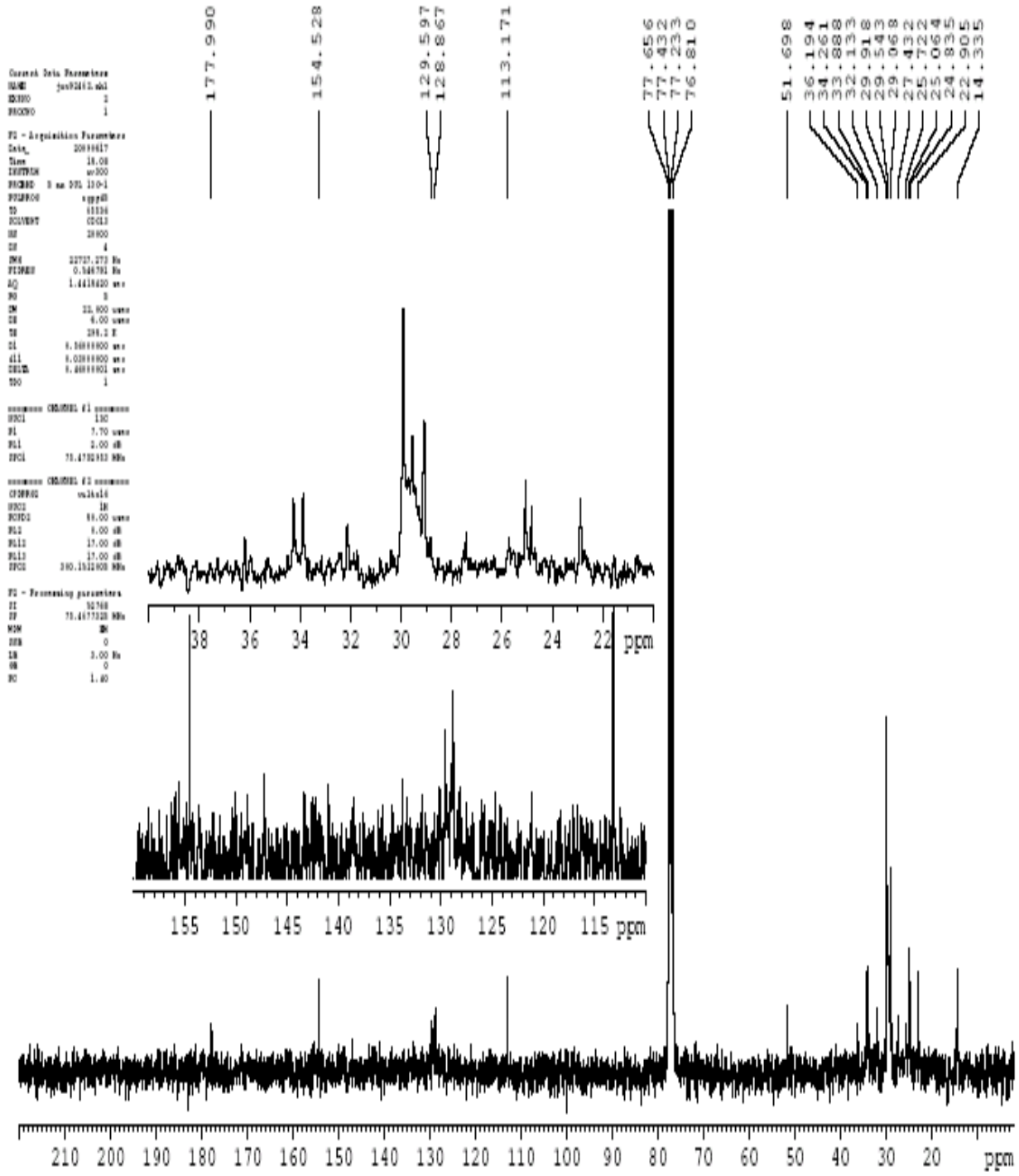
Appendix XIII: NOESY NMR Spectrum CK6



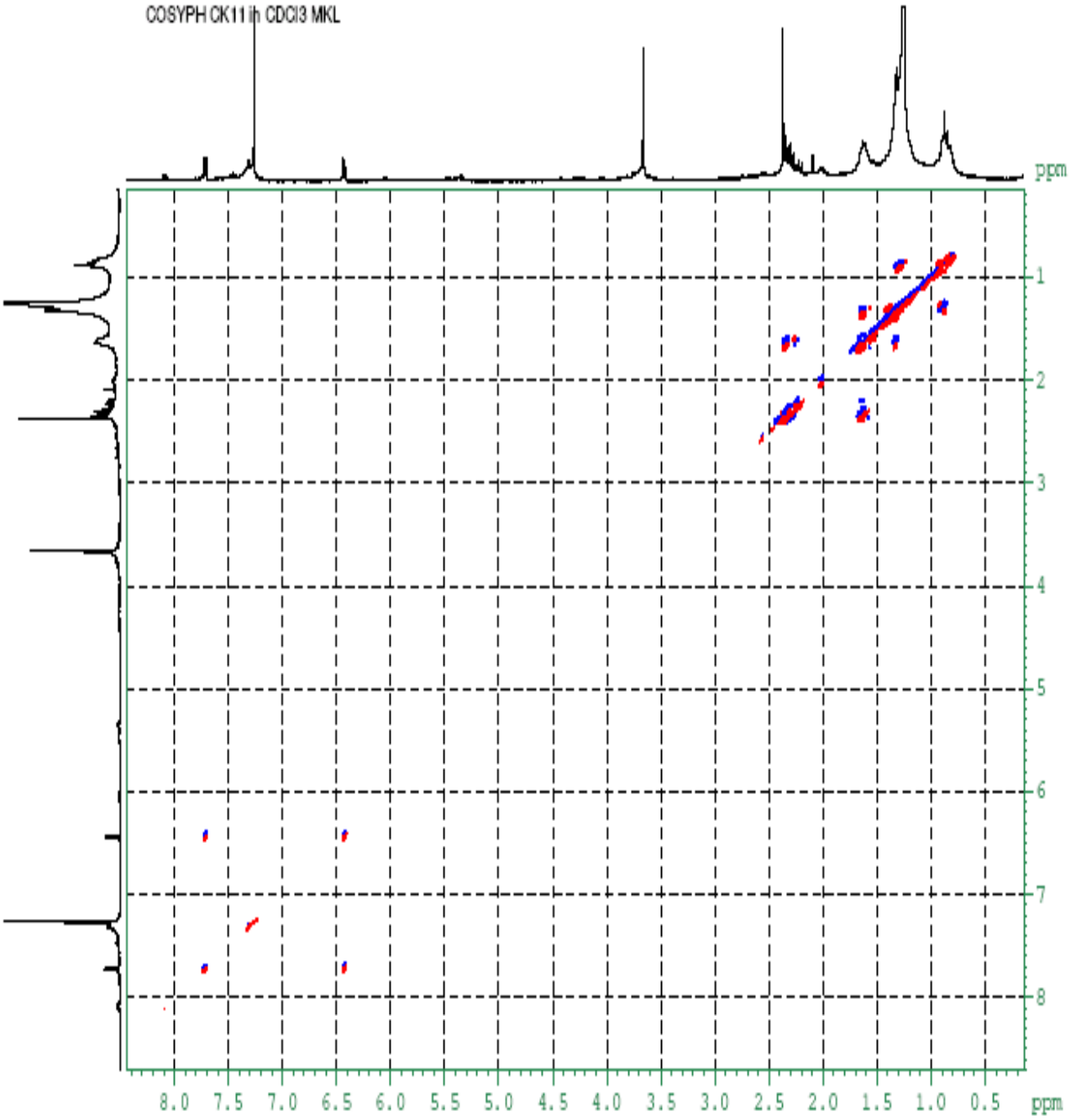
Appendix XIV: ^{1}H NMR Spectrum CK11



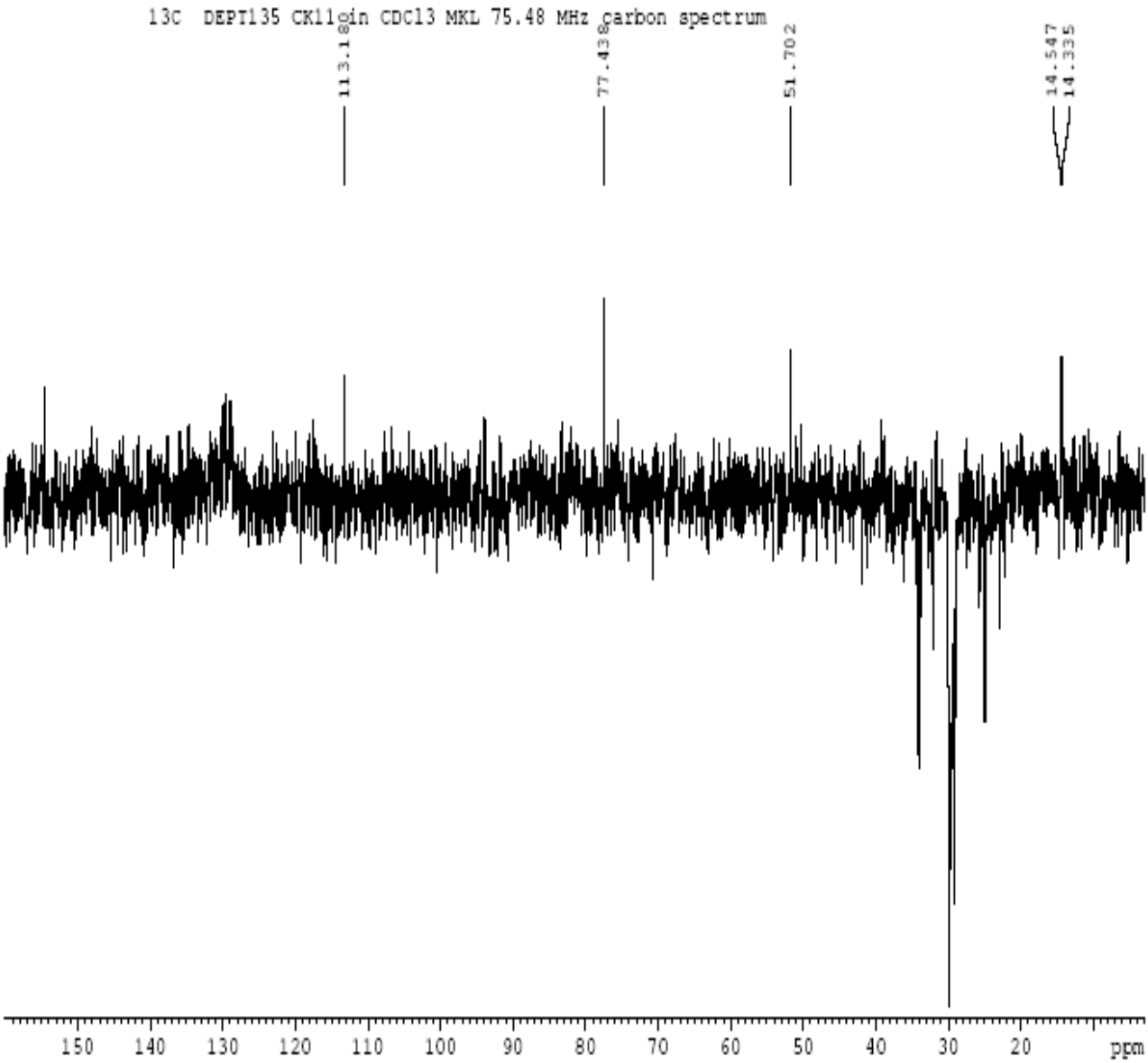
Appendix XV: ^{13}C NMR Spectrum CK11



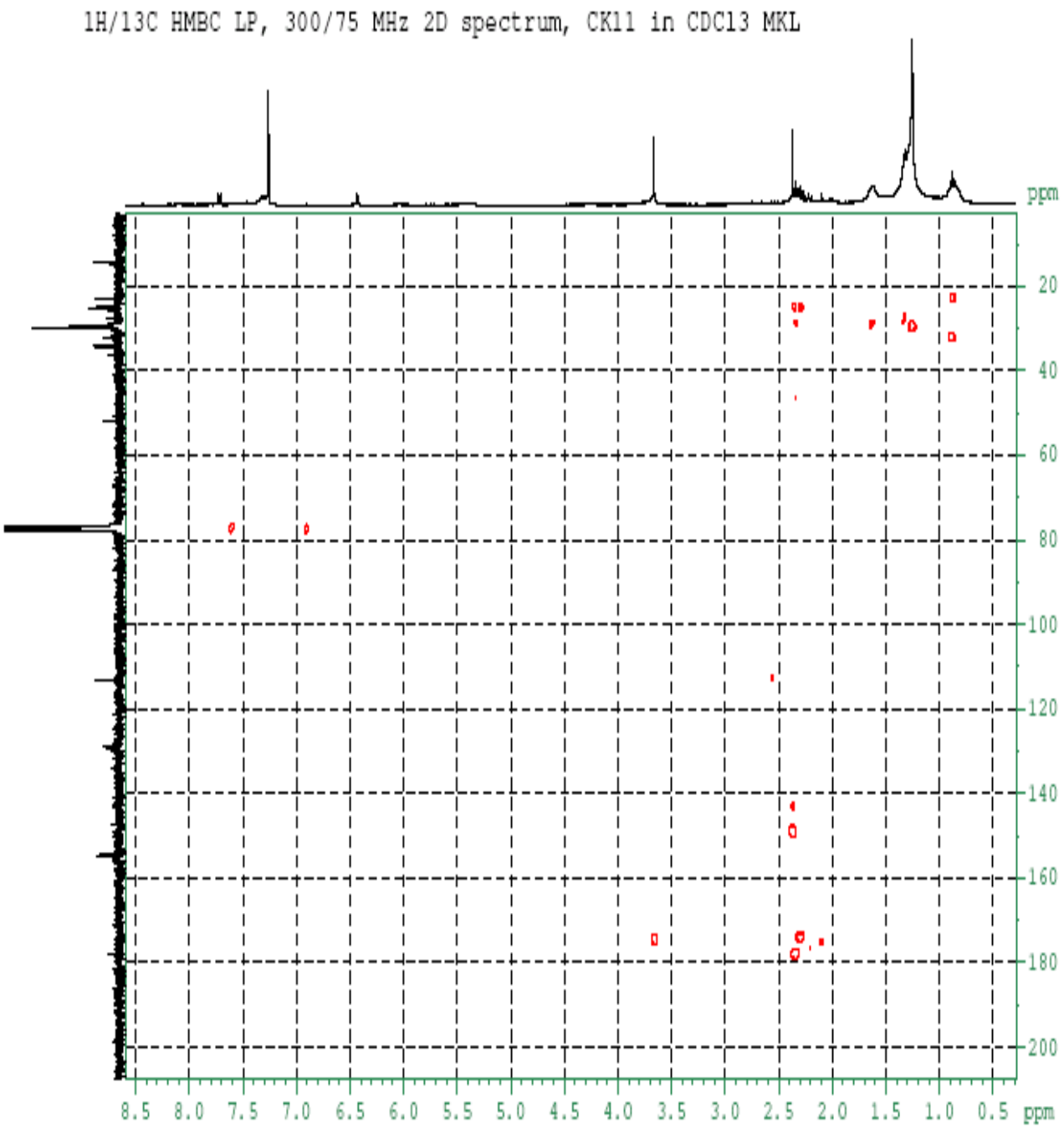
Appendix XVI: COSY NMR Spectrum CK11



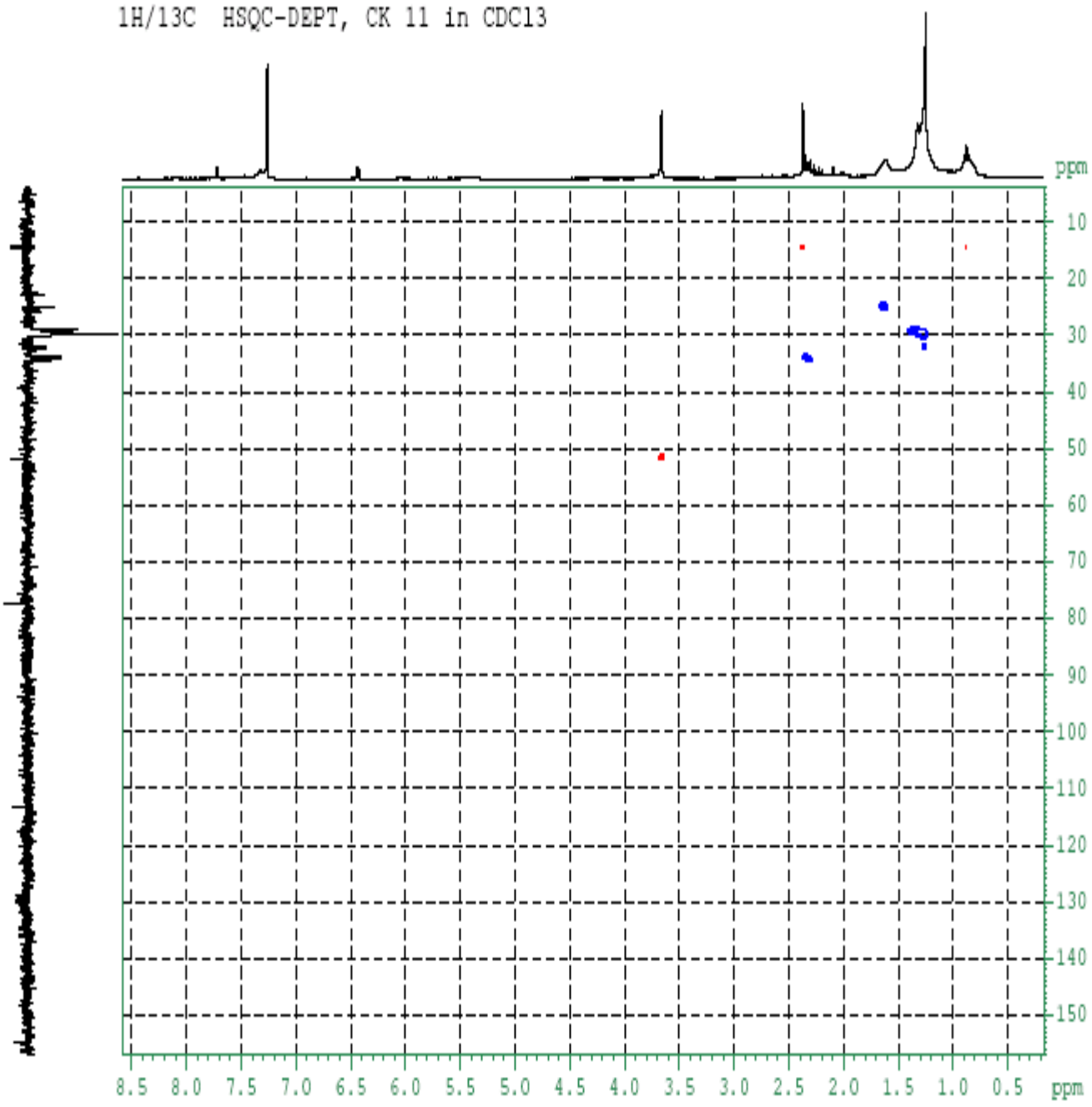
Appendix XVII: DEPT NMR Spectrum CK11



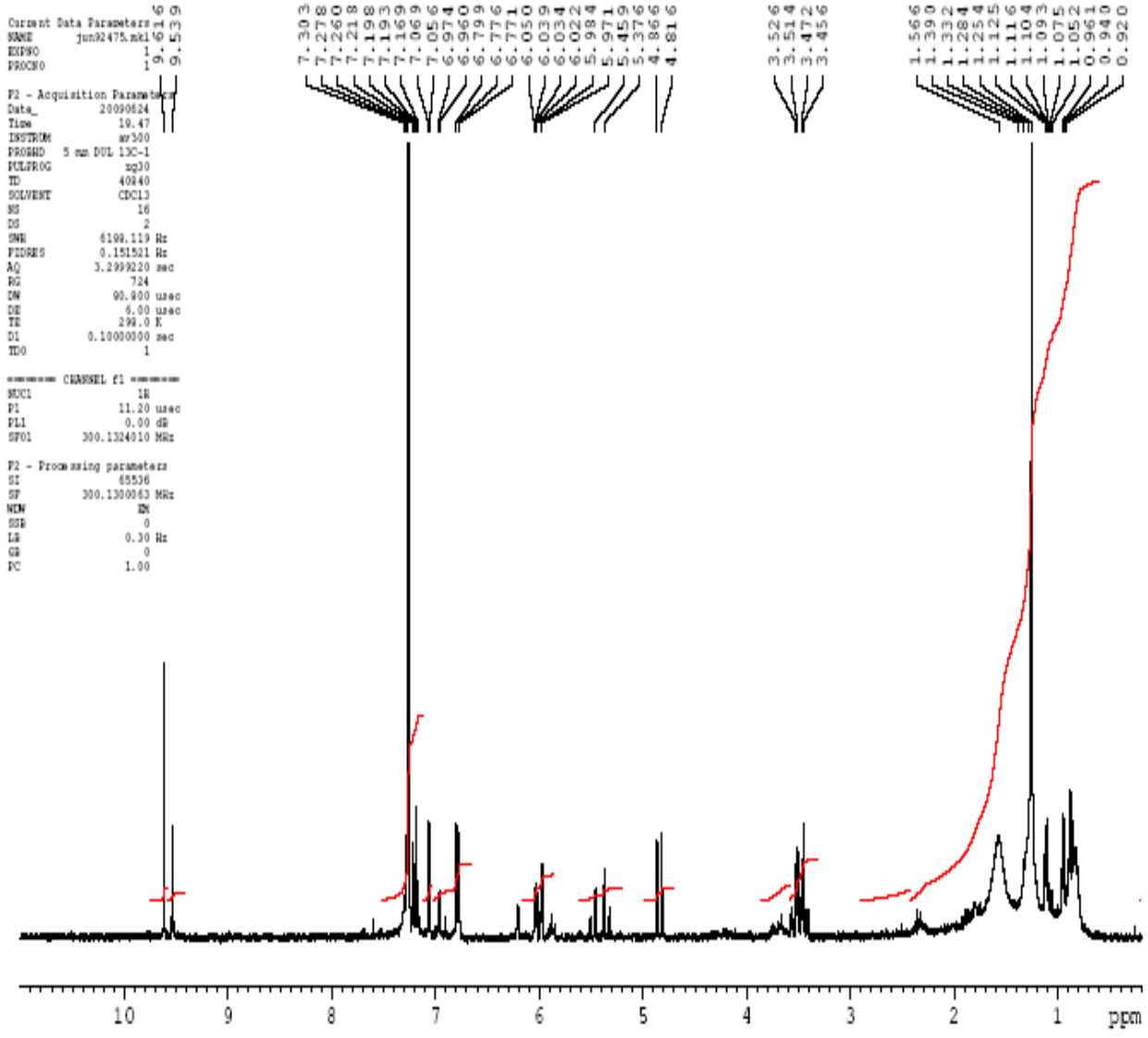
Appendix XVIII: HMBC NMR Spectrum CK11



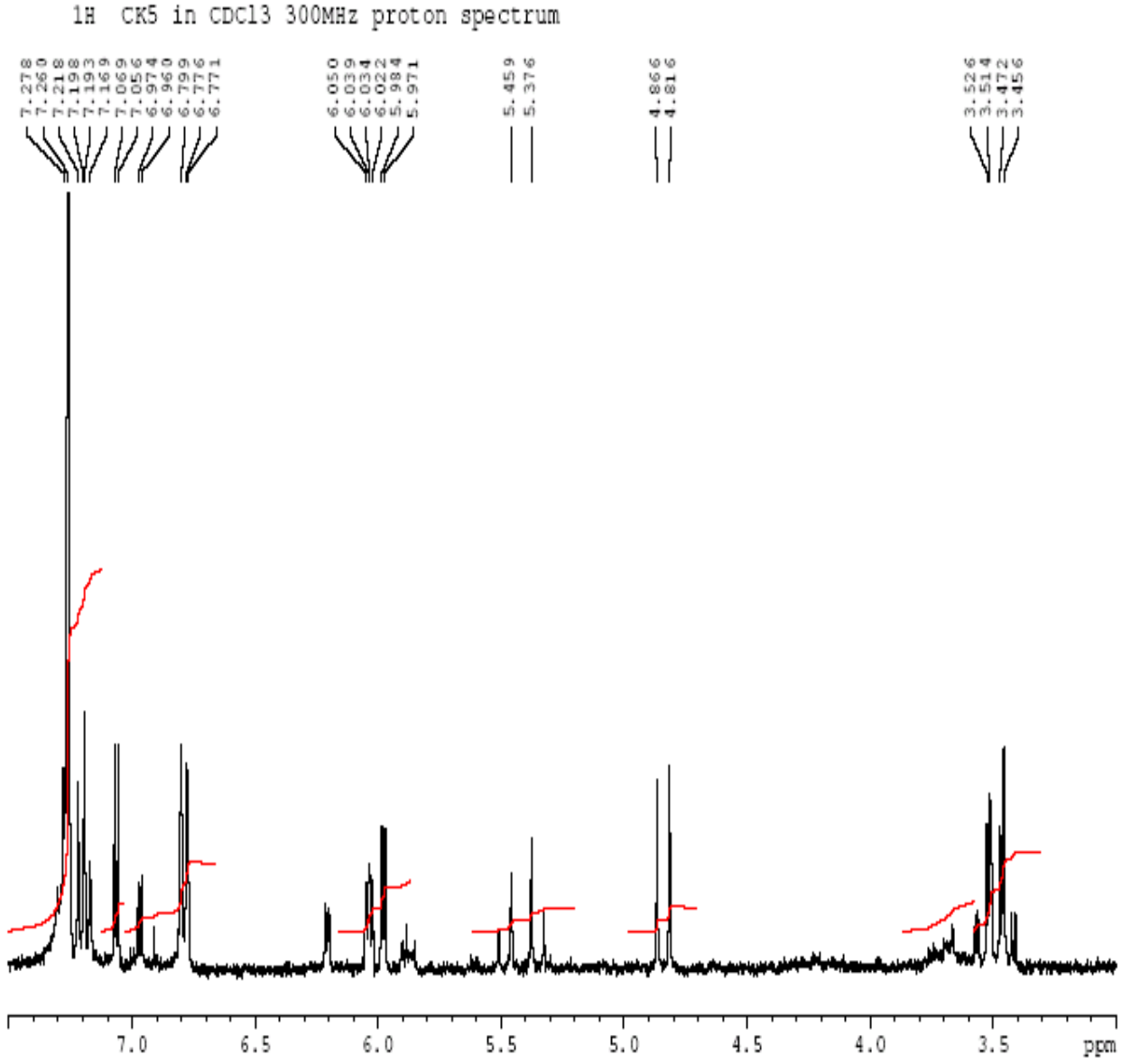
Appendix XIX: HSQC-DEPT NMR Spectrum CK11



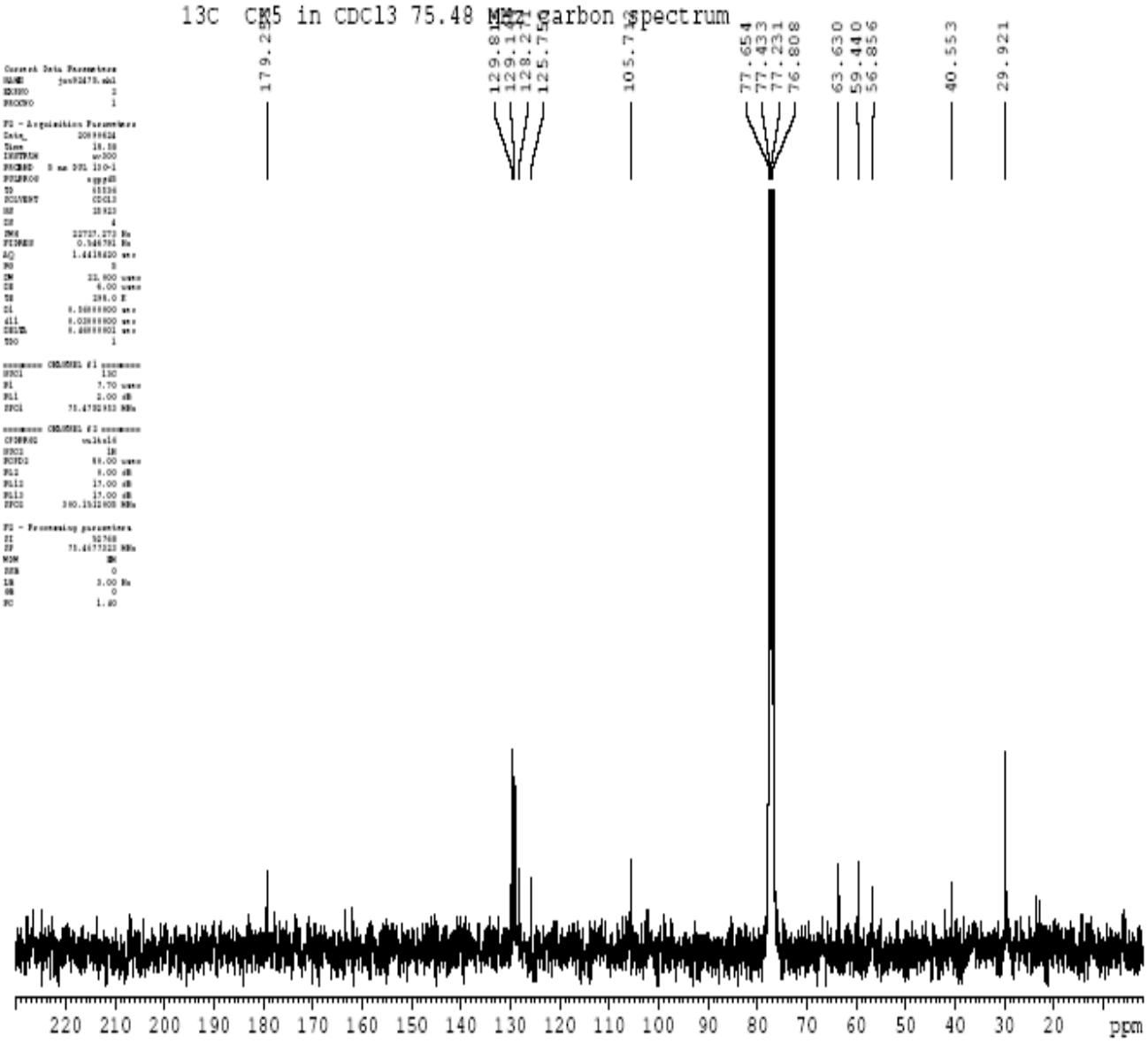
Appendix XX: ^{1}H NMR Spectrum CK5



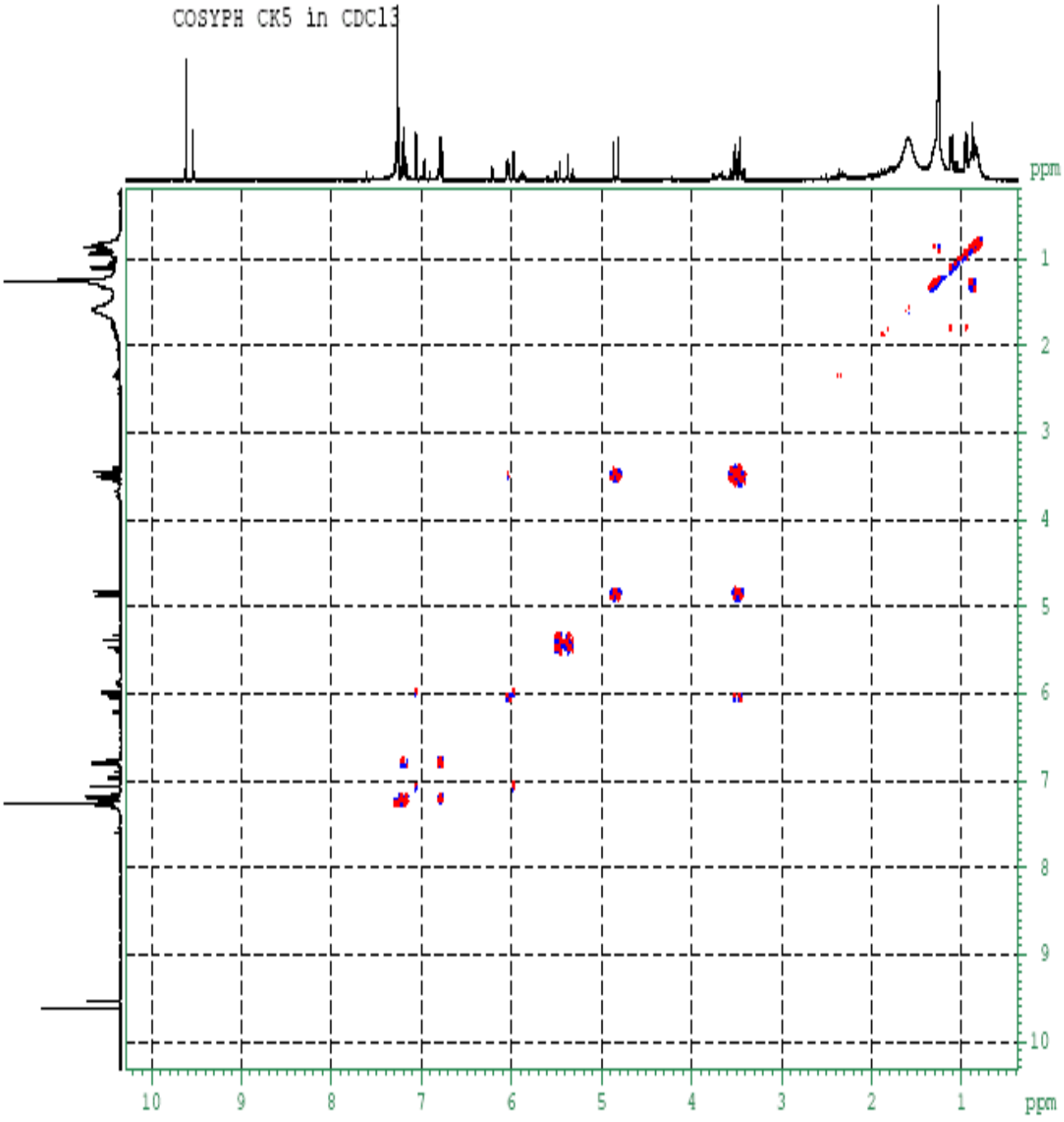
Appendix XXI: ^{1}H NMR Spectrum CK5



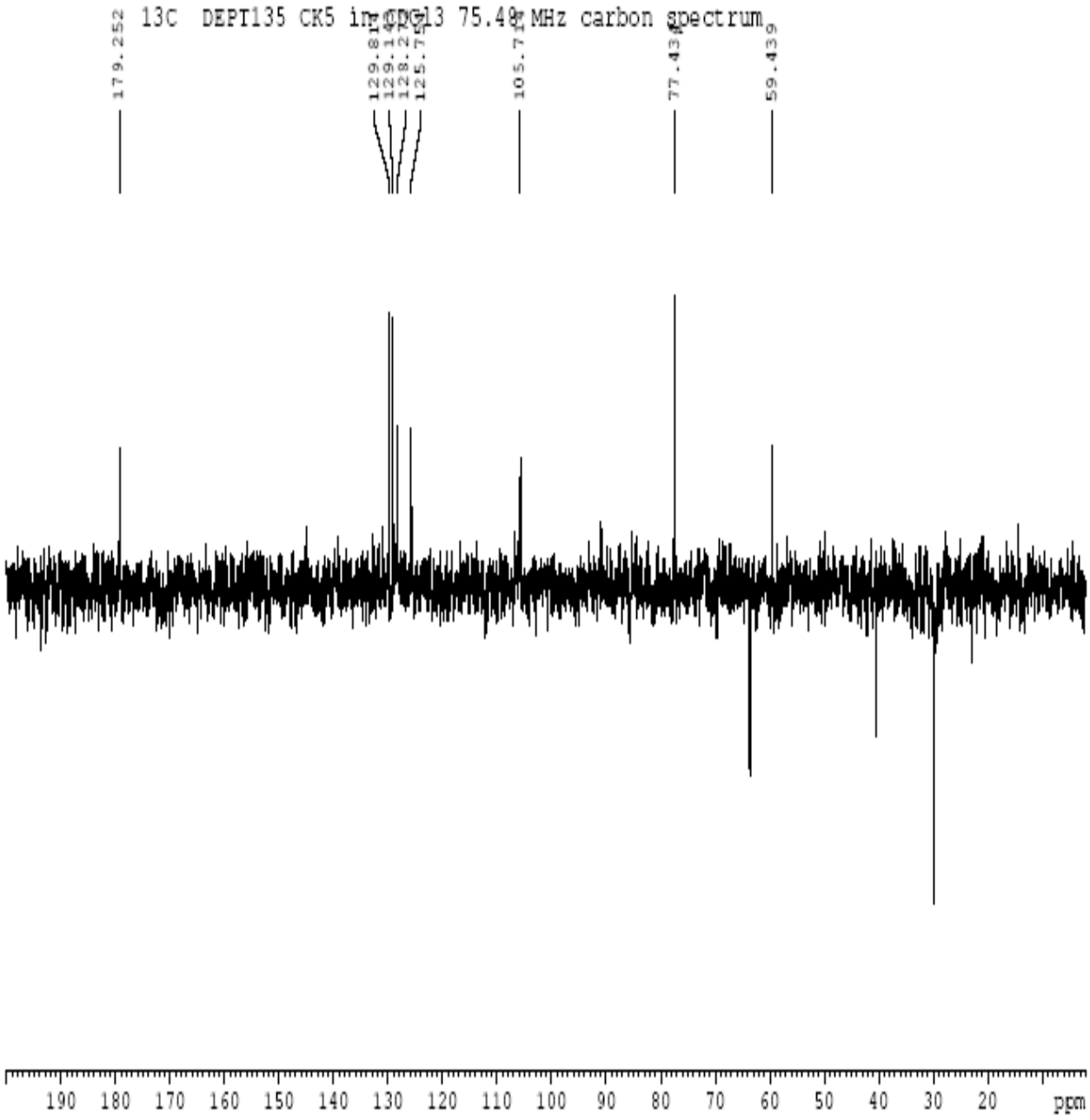
Appendix XXII: ^{13}C NMR Spectrum CK5



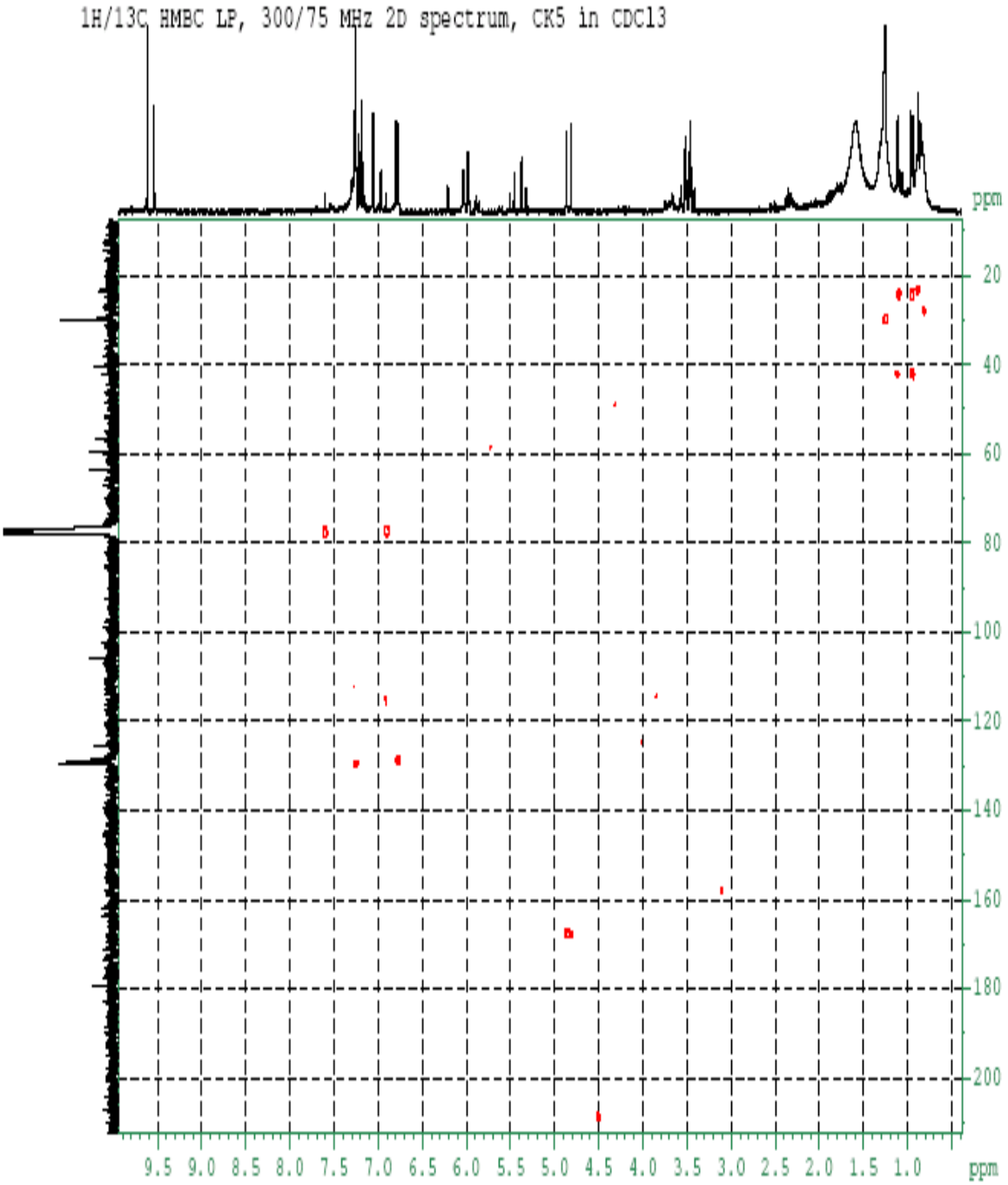
Appendix XXIII: COSY NMR Spectrum CK5



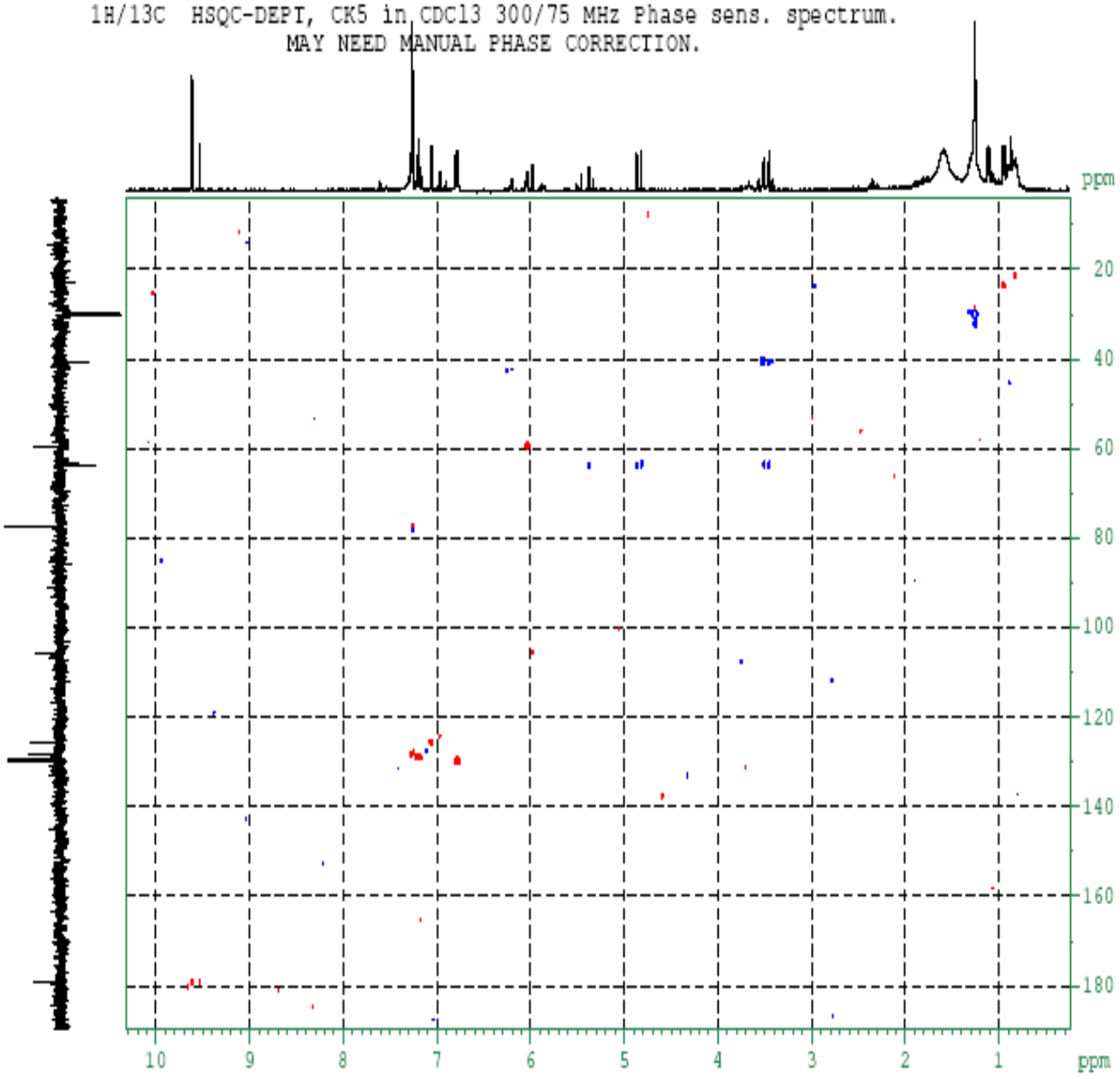
Appendix XXIV: DEPT NMR Spectrum CK5



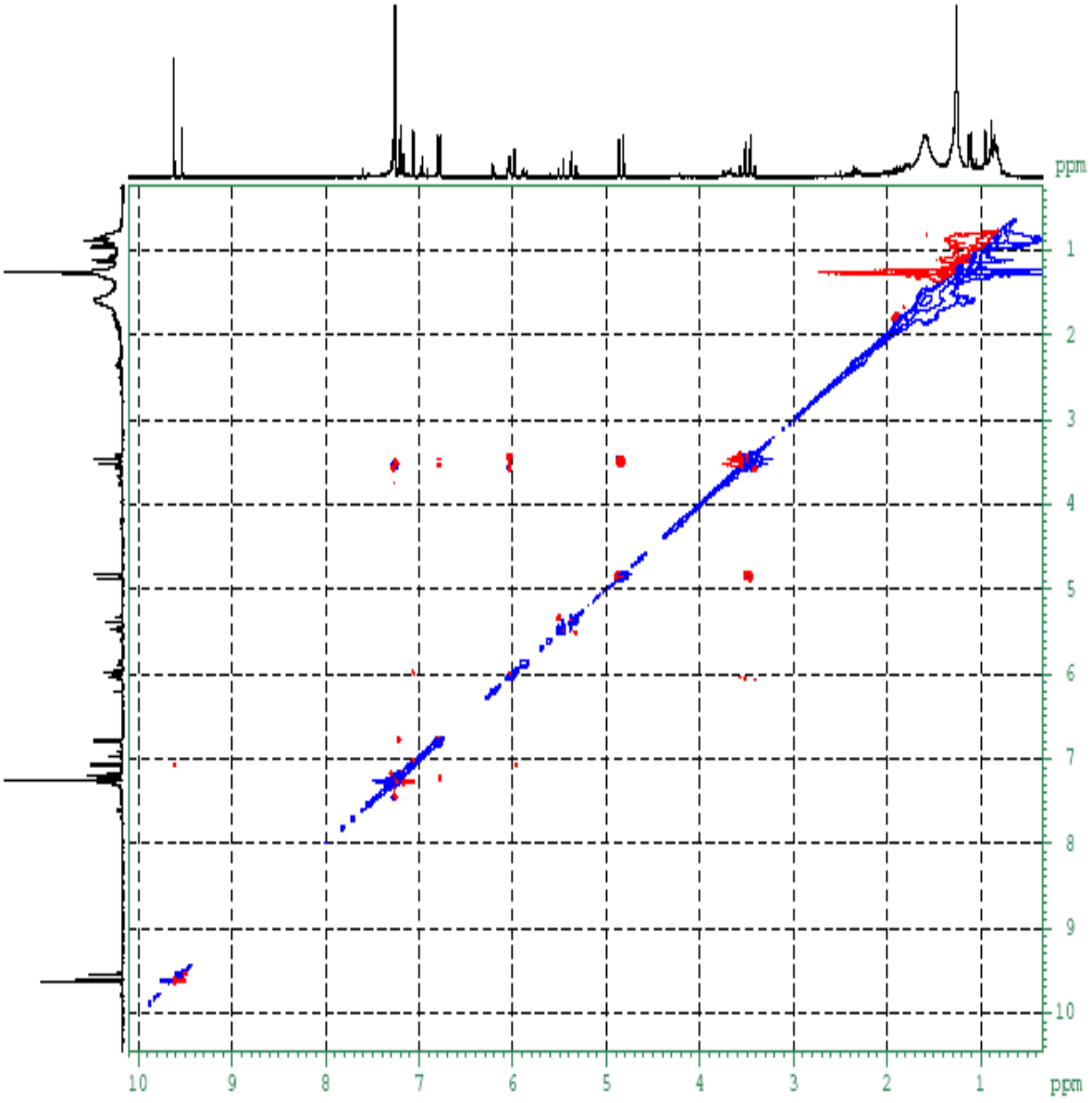
Appendix XXV: HMBC NMR Spectrum CK5



Appendix XXVI: HSQC-DEPT NMR Spectrum CK5



Appendix XXVII: NOESY NMR Spectrum CK5



Appendix XXVIII: Regression analysis