PURIFICATION AND STRUCTURE DETERMINATION OF MOSQUITO LARVICIDAL COMPOUNDS FROM THE BASIDIOMYCETE JO5182

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

DECLARATION

I declare that this is my original work and has not been presented for the award of any other degree elsewhere.

Signature……Date…….

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APPROVAL

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DEDICATION

To my father Rev. Kendagor, Husband Josiah and Son Kim

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ABSTRACT

Mosquitoes transmit devastating diseases that kill millions of people each year. The adverse effects on human health, well being and high economic losses inflicted by mosquitoes, the malaria vector, is an important stimulant for the search for new, safe and effective mosquito larvicides. The pool of natural products is an important source of such larvicidal metabolites. Fungi have extensive secondary metabolites at their disposal, and some could be active against the mosquito larvae. This project involved searching for larvicidal compounds from the submerged cultures of a basidiomycete JO5182. Initial screening of the crude extract from the basidiomycete showed good larvicidal activity (LC_{50} 3.33 ppm, LC_{90} 59.33 ppm). On purification using chromatographic methods, thirteen fractions from the crude extracts of the basidiomycetesJO5182 were obtained. Fractions: JO5182M03N (LC_{50} 71.45 ppm, LC_{90} 167.77), JO5182M06N (LC_{50} 154.26 ppm, LC_{90} 248.50 ppm) JO5182M07N (LC_{50} 61.15, LC_{90}) and JO5182K12N (LC_{50} 129.49, LC_{90} 230.26) displayed good larvicidal activity against the *Aedes aegypti* larvae. Three compounds; Methyl 2-hydroxy-4-methoxybenzoate (JO5182M10N), 3, 4-Dimethoxyphenol (JO5182K14N) and [(R)-(-)-8-hydroxy-3-methyl-3, 4-dihydro-1H-2-benzopyran-1-one] (JO5182K15N), were isolated and their structures determined using NMR spectroscopy. The larvicidal activity displayed by the extract from the basidiomycete JO5182 can be useful in the reduction of mosquito population which is on the rise and in the long run help in the elimination of malaria.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FUGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

^{13}C NMR	Carbon – 13 Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
2D	Two dimensional
AICAD	African Institute for Capacity Development
AIDS	Acquired Immunodeficiency Syndrome
CI	Chemical Ionisation
COSY	Correlation Spectroscopy
DDT	DichloroDiphenylTrichloroethane
DEPT	Distortionless Enhancement by Polarization Transfer
DHF	Dengue Hemorrhagic Fever
DMAP	Dimethylaminopyridine
EI	Electron Impact Ionisation
GPR	General Purpose Reagent
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single-Quantum Correlation
IBRL	Integrated Biotechnology Research Laboratory
ITNs	Insecticide-Treated Nets
JE	Japan Encephalitis
Kex	Culture filtrate extract
LC	Liquid Chromatography
Mex	Mycelium extract
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser spectroscopy
TLC	Thin Layer Chromatography
UV/vis)	Ultraviolet Visible region
w/v	Weight by volume
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background to the study

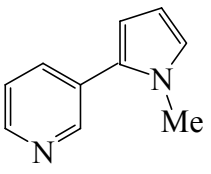
Despite recent significant advances in the techniques used for mosquito control during recent decades, it continues to pose serious public health problems (WHO, 2005). Malaria is by far the world's most important tropical parasitic disease, killing more children aged less than 5 years and pregnant mothers than any other disease, especially in Africa (Hammonda & Kubob, 1999). The battle for malaria control has been fought on several grounds, including efforts to implement improved diagnosis and prophylactic chemotherapy as well as integrated vector control through the use of insecticide-treated bed-nets and indoor residual spraying (Greenwood, 2005). However, prevalence as well as resurgence of malaria continues to be evident worldwide, much of it due to drug-resistant and insecticide resistant parasites and vectors respectively (Malkin *et al*., 2006).

The prime global choice to curb nuisance biting by mosquitoes or their transmission of parasitic or arboviral diseases continues to be the selective application of residual synthetic insecticides (Hemingway & Ranson, 2000). Mosquito-borne diseases, notably dengue, malaria, encephalitis, lymphatic filariasis, trypanosomiasis, and leishmaniasis remain endemic in many tropical areas. Chemical pesticides such as dichlorodiphenyltrichloroethane (DDT), gammaxane, malathion, and chlordane applied with the aim of eliminating mosquitoes have given rise to other serious problems. Not only has mosquito resistance against these chemicals been reported, but the pesticides themselves present threats to both human health and the ecosystem (Thompson *et al*., 2004). The public health benefit from synthetic insecticides, in tropical resource-poor settings, and in temperate zones, cannot be over-emphasized as they save thousands of lives each year. In these circumstances, the possibility of utilizing biopesticides as alternatives to synthetic chemicals is being examined. Biological control agents such as predatory fish (Legner, 1995), bacteria such as *Bacillus thuringiensis* serovar *israelensis* (Bti) and *Bacillus sphaericus* (Becker & Ascher, 1998), protozoa (Legner, 1995), nematodes (Kaya & Gaugler, 1993) are being used in worldwide field tests designed to

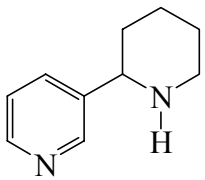
control the populations of mosquito larvae. It is therefore not surprising that interest in alternative non-synthetic chemical strategies has increased over the last decades (WHO, 2003).

Prevention of malaria encompasses a variety of measures that may protect against infection or the development of disease in infected individuals (Cymborowski, 1992). Measures that protect against infections are directed against the mosquito vector (Al Dakhil & Morsy, 1999). These can be personal (individual or household) protection measures (protective clothing, repellents, bed nets) or community/population protection measures (insecticides) or environmental management to control transmission. Measures which protect against disease development but not against infection include chemoprophylaxis and vaccination (Ciccia *et al.*, 2000).

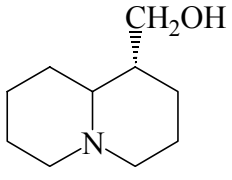
The treatment of mosquito breeding places with larvicides is potentially a good option for vector reduction and, thus for a decrease in malaria transmission. Larvicides from biological origin are particularly good candidates (Kroeger *et al*, 1995). Treatment of mosquito breeding habitats with larvicides helps reduce the adult mosquito population in nearby areas. Larvicides used to control mosquito larvae include biological insecticides, such as the microbial (*Bacillus sphaericus* and *Bacillus thuringiensis* var *israelensi*s (*Bti)*) and botanical larvicides like: nicotine (**1**) obtained from tobacco *Nicotiana tabacum* leaves, anabasine (**2**) and lupinine (**3**), the alkaloids extracted from Russian weed *Anabasis aphylla*, rotenone (**4**) from *Devris eliptica* (Campbell *et al*., 1993) and pyrethrins (**5**) from *Chrysanthemum cinerorifolium* flowers (Chandre *et al*., 1999). These larvicides had been used as natural insecticides even before the discovery of synthetic organic insecticides like DDT. Killing mosquito larvae before they emerge as adults can reduce or eliminate the need for ground or aerial application of pesticides (adulticides) to kill adult mosquitoes (Lacey & Cynthia, 1990).



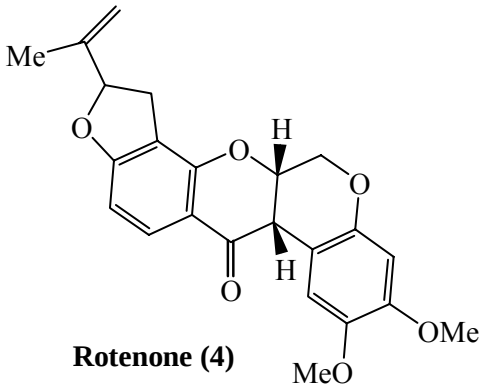
Nicotine(1)



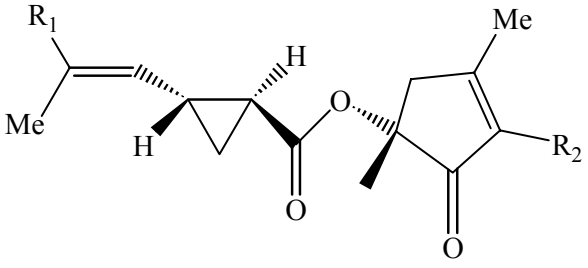
Anabasine (2)



Lupinine (3)



Rotenone (4)



	R_1	R_2
Pyrethrin I	CH_3	$CH_2CH{=}CHCH{=}CH_2$
Pyrethrin II	CH_3OOC	$CH_2CH{=}CHCH{=}CH_2$

Pyrethrins (5)

A large number of different flora from various geographical areas around the world have been shown to possess chemicals that are capable of causing a range of acute and chronic toxic effects on insects (Hung *et al*., 2003). Not only have many botanical extracts been shown to cause remarkable deleterious effects on the fecundity and hatchability of mosquito eggs, but they have been shown to have significant and promising anti-larval properties that include growth regulating effects. Any of these effects taken alone is usually not impressive, but the combined ovicidal, larvicidal and insecticidal effects possessed by many phytochemicals can produce impressive results. When joint-action is considered, the application possibilities for vector control increase significantly. Indeed, joint-action may well prolong the usefulness of synthetic insecticides that will eventually be unusable due to resistance development (Shaalan *et al*., 2005).

Little if any, research has been conducted on possibility of utilizing the biologically and chemically diverse secondary metabolites from fungi especially the higher ones for mosquito control. This study involved finding potential compounds from a local basidiomycete JO5182 with larvicidal activity.

1.2 Hypothesis

That basidiomycete JO5182 has larvicidal activity and can be good candidate from which larvicidal compound(s) can be isolated and structure determined.

1.3 Statement of the problem

The population of mosquitoes that are responsible for the increase in deaths due to malaria is on the rise. Mosquito control methods used since 1970 have not reduced mosquito populations enough to control malaria. To effectively deal with the malaria problem, it will be good to target the mosquito vector that transmits *Plasmodium* parasite rather than the disease. The best stage to target mosquito vector is at the larval stage rather than the adult stage because at the larval stage, the mosquitoes are resident in one place. There is therefore need to find larvicides that can kill the mosquito larvae. Initial screenings of extracts from the basidiomycete have shown good larvicidal activity. It is in this context that this research was geared towards finding compounds from a basidiomycete JO5182 which have larvicidal activity against mosquitoes.

1.4 Objectives of the Study

1.4.1 General objective

The aim of the study was to elucidate the structure of compounds from the basidiomycete JO5182 with mosquito larvicidal activity.

1.4.2 Specific objectives

i) To cultivate the basidiomycete JO5182.

ii) To obtain crude extracts from the culture of the basidiomycete JO5182.

iii) To screen for the larvicidal activity on crude extracts from the basidiomycete JO5182.

iv) To isolate and purify larvicidal compounds from the crude extracts.

v) To elucidate the chemical structures of the purified compounds.

1.5 Justification of the study

Malaria contributes the highest mortality of all infectious diseases in Kenya. Female anopheline mosquitoes when biting for blood meal normally transmits *Plasmodium* parasites that causes malaria. This vector may be targeted at its larval stage in order to interrupt its life cycle. For this reason compounds from natural sources that are environmental friendly need to be researched on in order to come up with mosquito larvicides that can be useful in combating spread of malaria by mosquito. Extracts from the basidiomycete JO5182 show good larvicidal activities and could be good candidates for developing larvicides to combat malaria.

CHAPTER TWO

LITERATURE REVIEW

2.1 Mosquito-Borne Diseases

Mosquitoes cause more human suffering than any other organism. Not only can mosquitoes carry diseases that afflict humans, they also transmit several diseases and parasites that infect dogs and horses. Mosquito transmitted diseases include protozoan diseases like malaria, filarial diseases such as dog heartworm, and viruses such as dengue, encephalitis and yellow fever (Collins & Paskewitz, 1995; Burfield & Reekie, 2005).

Malaria is an ancient disease probably originating in Africa. The malaria parasite (*Plasmodium*) is transmitted by female anopheline mosquitoes. Approximately 40% of the world's population, mostly in the tropical and sub-tropical areas of the world is susceptible to malaria (Gilles & Warrell, 1993). Human malaria is the most important parasitic disease of man in the tropics causing about 350–500 million infections (WHO, 2003) and approximately 1.3–3 million deaths annually which represent at least one death every 30 seconds (Campbell, 2005). The vast majority of cases occur in children under the age of 5 years, and pregnant women are also vulnerable (Greenwood, 2005). The death rate is expected to double in the next twenty years (Kevin, 2006). Precise statistics are unknown because many cases occur in rural areas where people do not have access to hospitals or the means to afford health care and therefore remain unreported (Kevin, 2006).

In Kenya, malaria contributes the highest morbidity and mortality of all infectious diseases. It is estimated that malaria kills 34,000 children every year (92 deaths a day) and is responsible for 30% of outpatient cases and 19% of hospital admissions (WHO, 2003). It is estimated that 170 million working days are lost each year because of malaria illness, which in turn affects the country's economy leading to increased poverty (Hoeck *et al.*, 2003). All Kenyan households are affected by the financial hardship caused by malaria.

The impacts of malaria on household economic status unfold slowly over time. Coping strategies adopted can have negative implications, weakening household ability to withstand malaria and other contingencies in future. To protect the poor and vulnerable, malaria control policies need to be integrated into development and poverty reduction programmes.

2.2 Species of mosquitoes that transmit malaria

Malaria is caused by the protozoan parasite *Plasmodium*, which is transmitted by the bite of the female mosquito of any of the 50 species of *Anopheles* mosquitoes, of which the most efficient is *Anopheles gambiae* (Riehle *et al.*, 2006). Four species of *Plasmodium* can cause human malaria: *P. falciparum*, responsible for the greatest number of deaths, *P. vivax*, which has the widest geographical distribution, *P. ovale* and *P. malariae* (Girard *et al.*, 2007). Various species have been found to transmit malaria in different parts of the world. *Anopheles gambiae* Complex is the main vector in Eastern Africa and *Anopheles freeborni* in North America. About 45 species of the *Anopheles* mosquito have been found in India and are implicated for the transmission of malaria (Thayer & Houston, 2005). These include *Anopheles culicifacies*, *Anopheles fluviatilis*, *Anopheles minimus*, *Anopheles philippinensis*, *Anopheles stephensi*, *Anopheles sundaicus* and *Anopheles leucosphyrushave*. Species like *Anopheles stephensi* are highly adaptable and are found to be very potent vectors of human malaria (Wakeline, 1996). The distribution of malaria and its vectors follows rainfall patterns. Several factors have contributed to the persistence of a severe worldwide malaria problem (Wilairatana *et al*., 2003).

2.3 Research on effective mosquito control

Mosquito control is an important component of malaria control strategy, although elimination of malaria in an area does not necessarily require the elimination of all *Anopheles* mosquitoes (White & Breman, 1998). In North America and Europe for example, although the vector *Anopheles* mosquitoes are still present, the parasite has been eliminated (Tracy & Leslie, 1996). Socio-economic improvements (houses with screened

windows, air conditioning) combined with vector reduction efforts and effective treatments have led to the elimination of malaria without the complete elimination of the vectors (Park, 1997). On the other hand, controlling these highly adapted, flying and hiding vectors is indeed a formidable task. Development of resistance to insecticides has compounded the problem (WHO, 1984). Ban on non-biodegradable and non-eco-friendly insecticides like DDT also may have contributed to the resurgence of malaria (Tracy & Leslie, 1996).

Methods used to prevent the spread of disease, or to protect individuals in areas where malaria is endemic, include prophylactic drugs, mosquito eradication, and the prevention of mosquito bites (White, 1987). Many researchers argue that prevention of malaria may be more cost-effective than treatment of the disease in the long run, but the capital costs required are out of reach of many of the world's poorest people (Shuit *et al*., 2003). It has been argued that, in order to meet the Millennium Development Goals, money should be redirected from HIV/AIDS treatment to malaria prevention, which for the same amount of money would provide greater benefit to African economies (Kevin, 2006).

2.3.1 Source reduction of larval habitats

One of the simplest ways to reduce larval mosquito populations is to drain any unnecessary containers/pools of water which allow mosquito larval development (Ciccia, *et al*., 2000). If it is not possible to drain areas, then treating them with larvicides if they are supporting larvae is an alternative. Many mosquito larvicides in the market are very host specific (Berenbaum, 1989) and only disrupt the larval stages of mosquitoes and do not harm non-target species.

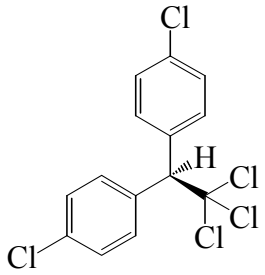
The extensive use of synthetic organic insecticides during the last five decades has resulted in environmental hazards and also development of physiological resistance in the major vector species (ICMR Bulletin, 2003). Moreover the present occurrence of the diseases caused by mosquitoes is due to high number of breeding places in today's throwaway society resulting in increasing resistance of mosquito to contemporary insecticides (Carvalho *et al*., 2003). Before the discovery of synthetic compounds, essential oils and their mixtures formed the basis of most commercial repellent

formulations and this is where research is going back to in its bid to find lasting solution to mosquito problems (Curtis, 1990).

2.4 DDT (DichloroDiphenylTrichloroethane)

Malaria was by and large eradicated in the temperate area of the world during the 20th century with the advent of DDT and other organochlorine and organophosphate mosquito control insecticides. However, more deaths are still reported annually in the developing world especially in Africa (Burfield & Reekie, 2005). DDT (**6**) was first synthesized in 1873 but its use increased worldwide after World War II because of its effectiveness against the mosquito that spreads malaria and lice that carry typhus. The World Health Organization estimates that during the period of its use approximately 25 million lives was saved (WHO, 1984). DDT seemed to be the ideal insecticide since it is cheap and of relatively low toxicity to mammals (oral LD_{50} is 300 to 500 mg/kg). Many species of insects developed resistance to DDT which was also found to have high toxicity to fish (White & Olliaro, 1996).

The chemical stability of DDT and its fat solubility compounded the problem. Animals do not metabolize DDT very rapidly; instead it is deposited and stored in the fatty tissues. The biological half life of DDT is about eight years. If ingestion continues at a steady rate, DDT builds up within the animal over time (Ndungu *et al.,* 2003).



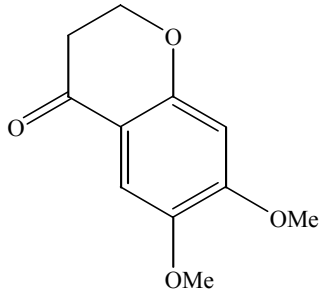
DDT (6)

2.5 Mosquito larvicides and insecticides from plants

Mosquito control using various products from plants has been well established (Evans & Kaleysa, 1988). Many plants have been shown to produce pesticidal

compounds (Ciccia *et al*., 2000), as a chemical defense mechanism against predators or infection. Plants have almost limitless ability to synthesise primary and secondary metabolites. Secondary metabolites include alkaloids, phenolic compounds, glycosides, essential oils, steroids, coumarins, sugar and many others. Studies have focused on natural products for controlling mosquitoes at the larval stage although few plant products have been exploited as insecticides or larvicides (ICMR Bulletin, 2003).

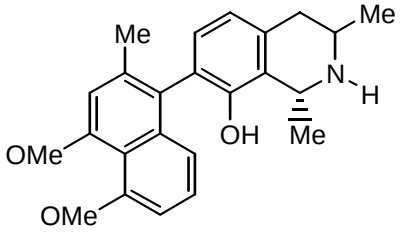
In the search for compounds with larvicidal and pesticidal activities from plants, the larvicidal activities of rotenoids isolated from the seeds of *Millettia dura* Dunn have been reported to have larvicidal and pesticidal activity (Yenesew *et al*., 2005). Rotenoids are also known to occur in *Derris*, *Lonchocarpus* and *Tephrosia* species of the family Leguminosae (Dewick, 1994). A compound identified as 6, 7-Dimethoxy-4-chromanone (**7**) from methanol extract of the seeds of *Derris trifolia,* showed potent larvicidal activity against the 2nd instar larvae of *Aedes aegypti* (Yenesew *et al*., 2006).



6,7-dimethoxy-4-chromanone (7)

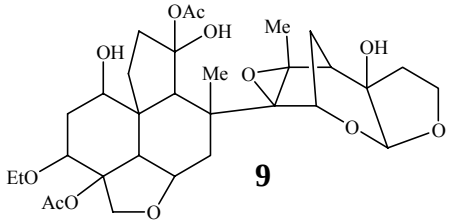
Methanol extract of fresh vegetative aerial parts of *Spilanthes mauritiana* after repeated chromatographic separations and mosquito larvicidal bioassays, afforded a potent mosquito larvicide which gave 100% mortality against 3rd instar larvae of *Ae. aegypti* ($LC_{50}10^{-5}$ mg/ml) (Jondiko, 2001). *Melicope subunifoliolata* (Stapf) T.G. Hartley (Rutaceae) extracts have showed good larvicidal activity against *Ae. aegypti* (Hung, *et al*., 2003).

Dioncophylline A (**8**) a representative of the naphthylisoquinoline alkaloids, a new class of natural compounds from species belonging to the Ancistrocladaceae and the Dioncophyllaceae, isolated from the carnivorous West-African liana *Triphyophyllum peltatum* (Dioncophyllaceae) (Bringmann *et al*., 1990)) is a promising mosquito larvicide candidate against *Anopheles stephensi*. The compound has a specific activity against the early instar stages of *A. stephensi* ($LC_{50} < 1$ mg/1) (Franqois *et al*., 1996).



Dioncophylline A (**8**).

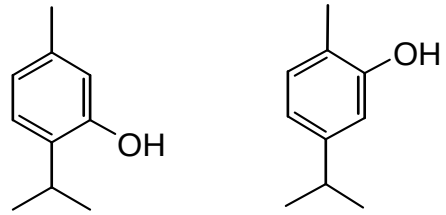
One of the most studied plants for control of mosquitoes is *Azadirachta indica* (Meliaceae) commonly known as neem tree in India, (ICMR Bulletin, 2003). This plant was investigated and was found to have mild antimalarials activity (Nkunya *et al*., 1995). Azadarachtin (AZA) (**9**) is an insecticidal compound, isolated from the plant *Azadirachta indica* (Addae-Mensah, 1991).



9

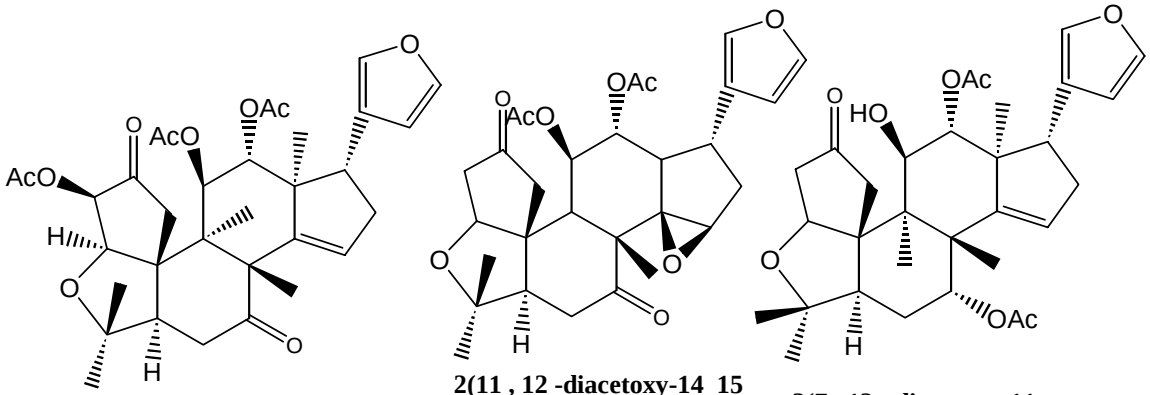
Though neem products show a high larvicidal activity, which is attributed to the epoxide ring, they do not show adulticidal action (ICMR bulletin, 2003). It has been suggested that azadirachtin acts as an anti-ecdysteroid and thus kills the larvae by growth inhibition effect (Zebitz, 1984).

A study carried out on essential oils obtained from the shoots of *Lippia edoides* in which co-distilled water called hydrolate was produced reveled tha pure hydrolate caused larval mortality of *Ae. aegypti* and it was revealed that its main constituents were carvacrol (**10**) and thymol (**11**). It was also found that thymol is the active componed ot the essential oil hydrolate (Carvalho *et al*., 2003). Hence, the use of plant essential oils in insect control is an alternative pest control strategy for minimizing the noxious effect of some pesticide compounds on the environment (Fatope *et al*., 1993).



Carvacrol (10) **Thymol (11)**

Some limonoids were isolated from the root back of *Turraea wakefieldii* and were characterized as tecleaninoid derivatives. However, there was a five membered ring, which was discovered and proposed the name neotecleanin. It was suggested that this compound (neotecleanin) could serve as an intermediate in the pathway for synthesis of tecleanin and related compound. Among these compounds 1(11β, 12α-diacetoxyneoetecleanin), (**12**) 2(11β, 12α-diacetoxy-14β 15β-epoxyneotecleanin (**13**) and 3(7α, 12 α-diacetoxy-11β-hydroxyneoteclanin), (**14**) showed strong larvicidal activity against *Anopheles gambiae* (Ndungu *et al.,* 2003).

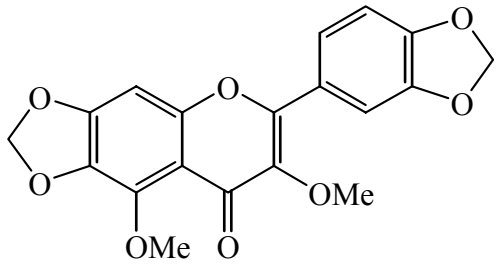


1(11 , 12 -diacetoxyneoetecleanin) (12)

2(11 , 12 -diacetoxy-14 15 -epoxyneotecleanin (13)

3(7 , 12 -diacetoxy-11 -hydroxyneoteclanin) (14)

From over 60 Malaysian plants that were screened against two species of insects *Melicope subunifoliolata* (Rutaceae) was shown to have strong feeding deterrent activity against *Sitophilus zeamais* and very good larvicidal activity against *Ae. aegypti.* From this plant one insecticidal and feeding deterrent compound meliternatin (3, 5-dimethoxy-3′, 4′, 6, 7-bismethylendioxyflavone) (**15**) and six other polyoxygenated flavones were isolated.



meliternatin (15)

Acetone extract from *Taegetes errecta* (Compositae) is reported to act as a growth regulator against *Culex quinquefasciatus* (Pathak *et al*., 2000). Essential oil extracted from *Mentha piperita* have also been used as larvicides and mosquito repellents against *An. stephensi, Ae. aegypti* and *C. quinquefasciatus* (Ansari *et al*., 2000). Fruit peel of *Citrus* species (Rutaceae), crude leaf extract from *Solanum nigrum* Linn (Solanaceae) and methanolic leaf extract of *Ferronia elephantum* (Rutaceae) have good larvicidal activity against *An. stephensi, Culex. pipiens, C. quinquefasciatus,* and *Ae. aegypti* (Ezenou *et al*., 2001; Singh *et al*., 2002; Venkatachalam & Jebanesan, 2001). Many other plants have been studied and have shown anti-mosquito activity.

2.6 Biological activity of basidiomycetes a class of fungi

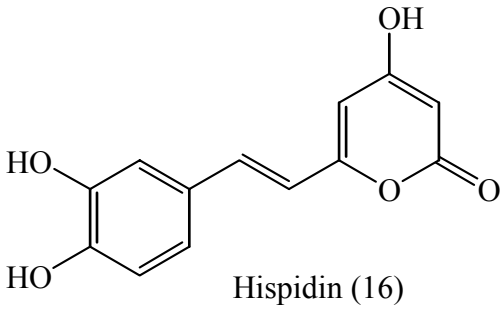
Fungi are an extremely diverse group of heterotropic micro-organisms that are exploited for various biotechnological applications. They are used in the production of foods, enzymes, polysaccharides, and anti-biotics and other pharmaceuticals; as agents of biological control of pest insects, fungi and weeds; and also in biomass conversion (Roberts, 1981). There is evidence that fungi, being simple but multicellular eukaroyotic systems have similar biochemical mechanisms to those found in mammals (Pedras *et al.,* 2002). The phylum basidiomycota commonly known as the club fungi or basidiomycetes, produce meiospores called basidiospores on club-like stalks called basidia. Basidiomycota contains about 30,000 described species which represent 37% of the described species of true fungi (Kirk *et al* 2001). The most conspicuous and familiar basidiomycota are those that produce mushrooms which are sexual reproductive structures. They also include yeast and asexual species. Most common mushrooms belong to this group, as well as rust and smut fungi, which are major pathogens of grains.

They are found in virtually all terestiral ecosystems as well as fresh water and marine habitat (Hibbett & Binder, 2001).

It is quite probable that highly differentiated fungi such as basidiomycotina will produce a rich array of secondary metabolites. 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. Most fungal metabolites have been reported from fermentations and have attracted attention for several reasons (Stadler & Sterner, 1997).

Secondary metabolites are not essential for growth and tend to be strain specific. For example destruxins isolated from *Oospora destructor*, the entomopathogenic fungus exhibit a wide variety of biological activities, but are best known for their insecticidal and phytotoxic activities (Pedras *et al*., 2002). They have a wide range of chemical structures and biological activities that could be novel candidates for controlling mosquitoes.

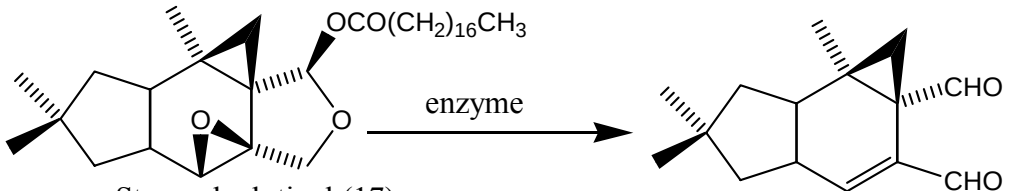
Lnonotus hispidus (Bull. ex Fr.) Karst. (Basidiomycetes) is a parasitic fungus preferably living on a variety of deciduous trees like *Fraxinus, Quercus, Sorbus* and *Malus*. It produces considerable quantity of yellow-brown pigment, hispidin (**16**) which is known to exhibit anti-microbial activity (Nasser *et al*., 1996).



Hispidin (16)

Most fungal metabolites have been reported from fermentations, but also compounds formed in fruit bodies have attracted attention for several reasons. The fruit body has a distinct role in the life of a fungus; it is normally short-lived and forms an entity that is separated from the rest of the organism. Therefore, the metabolites of the fruit bodies may be formed for completely other reasons than those found in the mycelium, and one possibility is that they protect the fruit body from parasites and predators.

The fruit bodies of several species of fungi have been shown to convert their secondary metabolites enzymatically to new compounds as a response to injury. Striking examples are the pungent species belonging to *Lactarius*, in which the biologically inactive precursor stearoylvelutinal (**17**) is converted to a strongly antibiotic and pungent sesquiterpenoid dialdehydes such as isovelleral in seconds (**18**) as a response to injury (Sterner *et al*., 1985).



Stearoylvelutinal (17) Isovelleral (18)

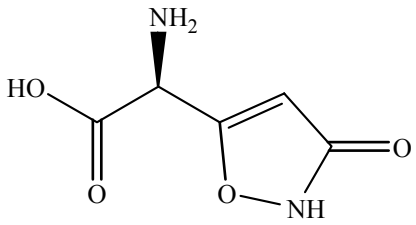
2.7 Larvicidal and insecticidal secondary metabolites from basidiomycetes

The phylum basidiomycota consists of fungi that produce spores that are formed outside a pedestal-like structure, the basidium (Roberts, 1981). The members of this phylum, known as basidiomycetes, include all the fungi with gills or pores, including the familiar mushrooms and bracket fungi. 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.

Many fungi compete with other organisms, or directly infect them (Lacey & Undeen, 1986). Some of these fungi are considered beneficial because they can restrict, and sometimes eliminate, the populations of noxious organisms like pest insects, mite, weeds, nematodes and other fungi (Lucarotti & Shoulkamy, 2000).

Insecticidal secondary metabolites have been reported from cultures of basidiomycetes (Lacey & Undeen, 1986). Insecticidal activity against houseflies was described for ibotenic acid (**19**) isolated from fruiting bodies of *Aminata muscaria*, *Aminata strobiliformis* and *Aminata pantheriana* (Krohn *et al*., 1997) A cyclopeptide, beauvericin was also isolated from basidiomycetes *Polyporus sulphureous* (Deol *et al*., 1978) and from entomapathogenic fungi *Beauveria bassiana* and *Paecilomyces fumosoroseus* on which insecticidal activity was exhibited against mosquito larvae, brine

shrimps, houseflies and cockroaches, cardiac cells *in vitro* (Roberts, 1981). It is evident that basidiomycetes provide a rich yet quite untapped source of bioactive compounds.



Ibotenic acid **(19)**

CHAPTER THREE

METHODOLOGY

3.1 Chemicals and reagents

Analytical grade chemicals, solvents and other reagents were used. Where general purpose (GPR) solvents were used, distillation was performed before use. The chemicals and reagents that were used include: ethyl acetate, methanol, dichloromethane, acetone, cyclohexane, potato dextrose, silica gel, sulphuric acid and anisaldehyde.

3.2 Equipments

The following pieces of equipments were used for sample preparation analysis and structure elucidation:

- Standard basic bench top equipments: Weighing balance (EK-1200GD max 1200g d=0.1g), analytical balance (PRECISA 310 SWISS QUALITY) vacuum pump (type 349\2) Mill (MILWAUKEE WINSOONSIN 53207 MODEL NO. 311) Refrigerator, pH meters (Fishes Accument ® model 610A), incubators (Carbolite Sekinic pocketcorder SK-50P), Autoclaves (Danofass 59407-3 NO 375 2 ATO PROVET 1977) etc.), rotary evaporator type 349\2) and oven (Struess Electrolux)
- Assorted plastic and glass ware
- Chromatographic apparatus and materials (glass column, TLC plates (10X10 Machary Nogel Duren), TLC chamber and Silica gel etc.)
- Infrared spectrophotometer (IR)
- Nuclear magnetic resonance spectrometer (NMR)

3.3 Apparatus and other materials

Bunsen burner, Erlenmeyer conical flasks, glass vials Buchner funnel, Petri dishes, measuring cylinders, round bottomed flasks, aluminium foil, cotton wool, parafilm, scissors, adjustable volume (analogue) micropipette and Pasteur pipette.

3.4 Collection of JO5182 and preparation of crude extract

The basidiomycete that was used had been collected as a fruiting body colonizing a dead wood from Mt. Kenya Forest and kept both as herbarium material and agar slant in the Integrated Biotechnology Research Laboratory (IBRL). It was sub-cultured in PDA and cultivated in liquid malt media before extraction with several solvents.

3.4.1 Preparation of liquid malt media

Liquid malt media was used in this project as the growth medium. It was prepared by dissolving 10.0 g of molasses (Chemelil Sugar Company, Molasses C), 4.0 g of glucose (Kobian Kenya Limited, Nairobi) and 4.0 g of yeast extract in 1 litre of tap water as per Anke et al., 1989 guidelines. The pH of this media was adjusted to 5.5 using 1.0 M NaOH and 1.0 M HCl after which the media was sterilized by autoclaving twice at a temperature of 121°C and pressure of 3 bars for 15 minutes.

3.4.2 Preparation of potato dextrose agar media (PDA)

The basidiomycete JO5182 from agar slant cultures was grown in PDA plates before culturing them in liquid submerged cultures. The plates were prepared by autoclaving 9.75 g PDA suspended in 250 ml distilled water, cooling to 40°C followed by dispensing 15 ml per sterile Petri dishes in sterile lamina flow hood.

3.4.3 Cultivation of the basidiomycete JO5182

A well grown pure culture of the basidiomycete JO5182 on PDA plate was cut into agar plugs (1x1 cm) of a well grown plate. This was then inoculated into 250 ml liquid media in 500 ml Erlenmeyer flasks. These served as starter cultures that were

transferred into 1 liter scale liquid nutrient media in 2 liter Erlenmeyer flasks. They were then allowed to grow with occasional agitation at ambient conditions. All this work was done in an integrated biological safety cabinet lamina flow hood under extremely sterile conditions. The growth of the culture was closely monitored and evaluated daily to check for glucose depletion using glucose testing stripes, biomass build up and presence of any contamination. The growth was stopped when there was no more apparent biomass build-up. The basidiomycete JO5182 took a period of twenty eight (28) days to complete its growth.

3.4.4 Preparation of the crude extract from culture broth and mycelium

Filtration method using a Buchner filtration system was used to separate the mycelium and culture filtrate immediately growth was stopped. The culture filtrate was extracted using liquid-solid adsorption technique. The resin was packed in a vertically mounted column of glass (diameter 2.2 cm and height 60 cm). The culture filtrate (1 l) was passed thrice through Mitsubishi HP21-DIAION resin packed in a glass column. The extract trapped in the column was eluted with 300 ml of acetone, followed by 300 ml of methanol into Erlenmeyer flasks. The acetone and methanol eluents were then concentrated under reduced pressure using rotary evaporator to recover acetone and methanol. The aqueous residue obtained after concentration was extracted 5 times using ethyl acetate. In this case a separating funnel (BS-2021) was used where the top organic layer was collected and the bottom aqueous layer discarded. Each time extraction using ethyl acetate was done, it was done in the ration 1:1 ethyl acetate to the concentrate. The ethyl acetate extract obtained was dried with anhydrous sodium sulphate, filted to remove the hydrated sodium sulphate before recovering ethyl acetate using a rotary evaporator at 50°C. This process was repeated for 30 litres of the culture filtrate that had been obtained after cultivation. The concentrate was transferred into screw-capped vials, dried under nitrogen atmosphere and was kept at 4°C awaiting further analysis.

Mycelium (500g) was soaked in acetone for 8 hours with constant agitation before filtering. The resulting solution was concentrated using a rotary evaporator after which the aqueous concentrate was extracted four times using equal volume ratio of ethyl acetate (1 : l) using a separating funnel. The combined ethyl acetate extract was dried

using anhydrous sodium sulphate before filtering and concentrating to dryness. The obtained extract was dried under nitrogen atmosphere at 45°C to a constant weight.

3.5 Larvicidal bioassays

The guiding biological activity in this research work was larvicidal activity. Both the crude extracts and the purified samples were subjected to mosquito larvicidal activity against late 3rd and early 4th instar larvae of *Aede. aegypti*. The bioassays were conducted in the IBRL at Egerton University using *Ae. aegypti* mosquito larvae obtained from Pyrethrum Board of Kenya. Standard methods for larvicidal activity assaying as recommended by the World Health Organisation (WHO, 2005) were followed in all experiments. Preliminary bioassays for the crude extract of the submerged cultures of the basidiomycete JO5182 were performed with late 3rd instar and early 4th instar larvae of *Ae. aegypti* and were carried out in triplicate using 10 larvae for each replicate assay. The larvae were placed into 200 ml universal bottles containing 4 ml of water with varying concentration (1-1000 ppm) of the test solution and kept at 27.8°C. Larvae were considered dead when they were unable to reach the surface of the solution when the cups 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 larval mortality. An aqueous solution of methanol was employed as the control experiment. Treatments that showed at least 50% mortality within 24 h were subjected to further bioassays. The purified sample from the active crude extracts were assayed at different concentrations (25, 50, 75, 125 and 250 ppm) to determine the concentration of purified fractions required to kill 50% (LC_{50}) and 90% (LC_{90}) of the larvae after 24h.

3.6 Statistical data analysis

The analysis of larvicidal assay data was carried out using Probit analysis from Genstat statistical software (Wim *et al*., 2004). The LC_{50} and LC_{90} values which are the concentration values (in ppm) for killing 50% and 90% respectively of the mosquito larvae in 24 hours were obtained from regression analysis.

3.7 Column chromatography

Silica gel (Merck 9385) was used as the adsorbent and elution with solvent was allowed to proceed by gravity. Different sized columns with diameter ranging from 1-4 cm were used in the isolation procedure depending on the amount of sample available. Solvents used in the column chromatography process include; dichloromethane, ethyl acetate, methanol and cyclohexane. In each case, fractions were collected differently for each solvent gradient system as much as possible and purity traced using analytical thin layer chromatography analysis. Similar fractions were combined and concentrated to dryness using rotary evaporator under reduced pressure at 50^{o}C.

3.7.1 Extraction and isolation of compounds from the mycelia extract

The mycelia crude extract (5.99 g) was subjected to column chromatograph on silica gel (150 g) eluted with two solvent system of cyclohexane to ethyl acetate (ratio 4:1 and 9:1) which resulted two to major fractions (fig 1). The Two major fractions were purified using varying systems of dichloromethane cyclohexane and methanol to obtain eight fractions (table 1).

Table 1: Repeated column chromatography for mycelia extract

Extract	**Fraction**	**Solvent system for repeated column chromatography**
Mycelia	JO5182M03N	E:CH 9:1, 100% D
	JO5182M04N	E:CH 9:1, 100% D
	JO5182M05N	E:CH 9:1, 100% D, 100%D
	JO5182M06N	E:CH 1:4, D:M 1:1
	JO5182M07N	E:CH 1:4, D:M 1:1
	JO5182M08N	E:CH 1:9, 100%D,
	JO5182M09N	E:CH 1:4, 100%D, 100%D
	JO5182M10N	E:CH 1:4, D:M 2ml:2d

Key: E - ethyl acetate CH - cyclohexane, D – dichloromethane, d-drops

The fractions that were collected are fraction JO5182M03N, JO5182M04N, JO5182M05N, JO5182M06N, JO5182M07N, JO5182M08N, JO5182M09N and JO5182M10N. All of them gave different yield as shown in figure 1.

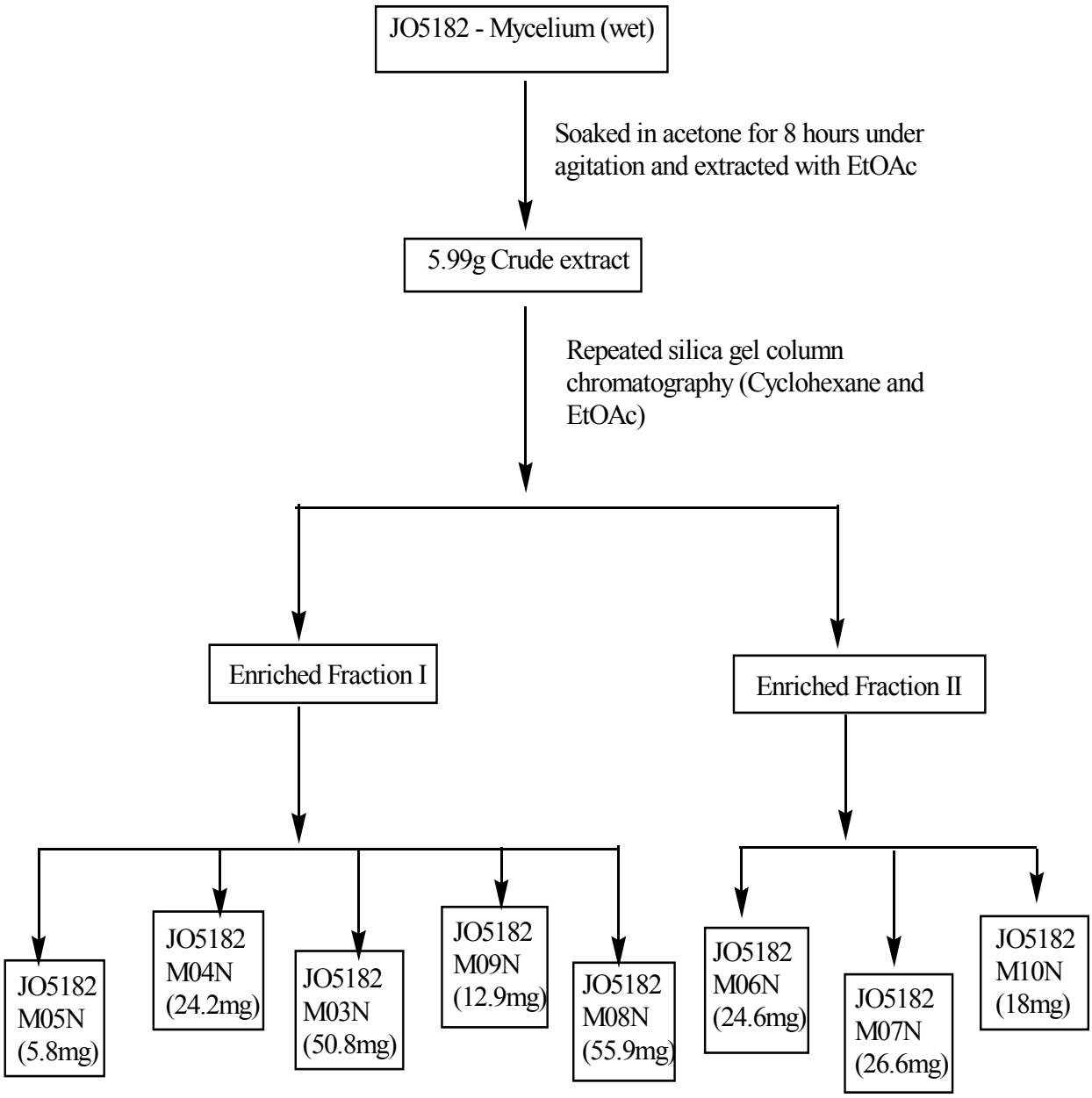


Figure 1: Purification for the fractions from the mycelia filtrate

3.7.2 Extraction and isolation of compound from the culture filtrate crude extract

On the other hand 30 liters of culture filtrate was passed through Mitsubishi resin, extracted using ethyl acetate and concentrated to yield 7.96 g of culture filtrate crude extract. Silica gel column chromatography of this crude extract using cyclohexane and ethyl acetate (Ethyl acetate: cyclohexane, 1:5 and 1:1) yielded two enriched fractions. The enriched fractions were further purified using silica gel column chromatography eluted with different solvent systems of dichloromethane cyclohexane and methanol (table 2). This yielded five fractions, JO5182K11N, JO5182K12N JO5182K13N JO5182K114N and JO5182K15N in different amount (fig. 3).

Table 2: Repeated column chromatography for culture filtrate extract

Extract	**Fraction**	**Solvent system for repeated column chromatography**
Culture filtrate	JO5182K11N	E:CH 1:5 100%D
	JO5182K12N	E:CH 1:1, D:M 2 ml:5d, D:M 2ml :4d
	JO5182K13N	E:CH 1:1, 100%D
	JO5182K14N	E:CH 1:5, 100%D
	JO5182K15N	E:CH 1:5, 100%D

Key: E - ethyl acetate CH - cyclohexane, D – dichloromethane, d - drops

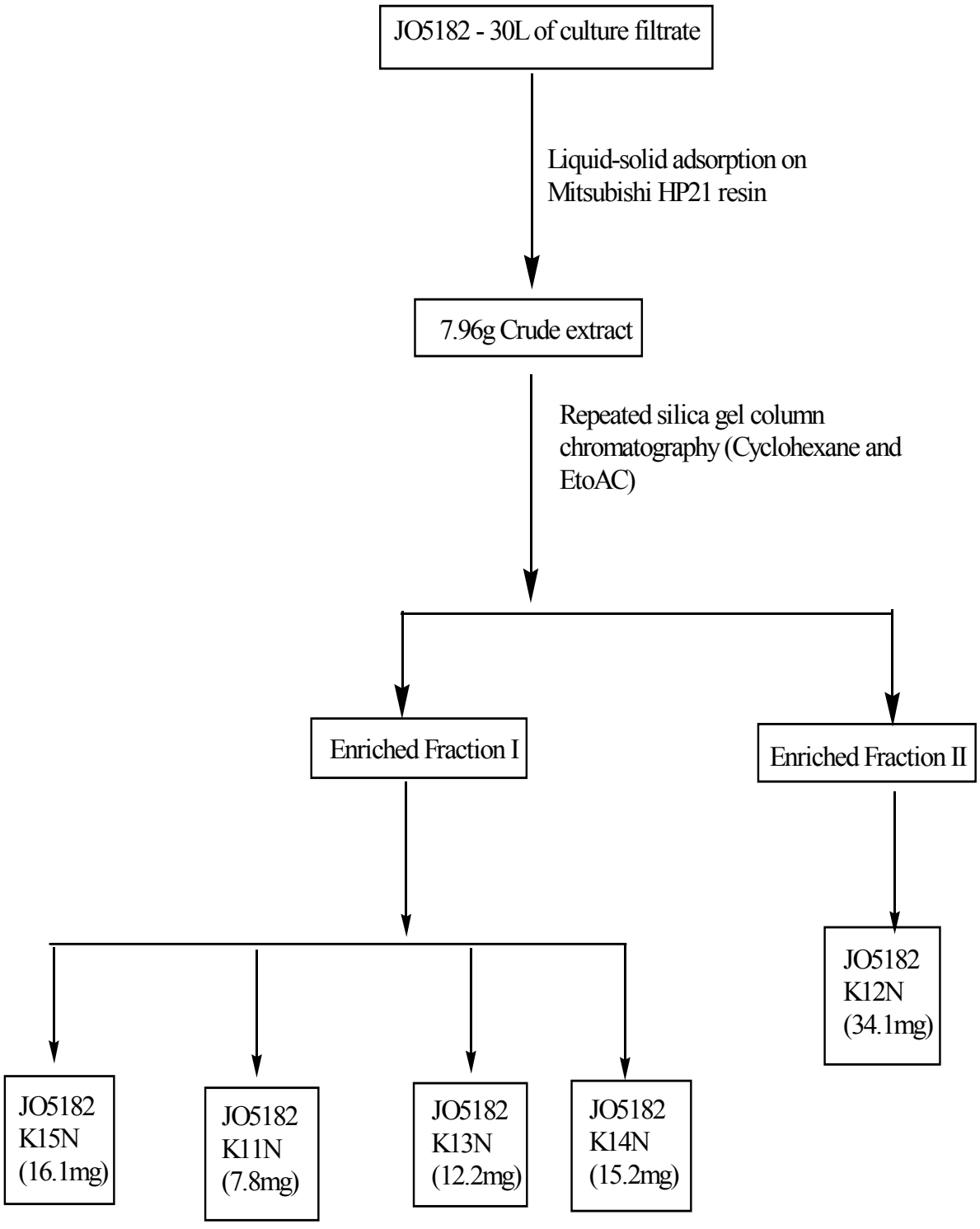


Figure 2: Purification scheme for the culture filtrate

The fractions were then transferred into a screw-capped vial and then kept at 4^{o}C for further analysis. The purified fractions collected were subjected to NMR spectroscopic analysis and larvicidal activity tests.

3.8 Thin layer chromatography TLC

Analytical thin layer chromatography was used to monitor the fractions from column chromatography. Analytical TLC was performed using silica gel (0.2 mm thick) on aluminium coated plates (Merck Art. 5554) which contained a fluorescent indicator (F254). The plates were first observed under UV lamp and then developed with a spray reagent consisting of anisaldehyde: conc. H_2SO_4: methanol in a ratio of 1:2:97 followed by heating in an oven. The fractions that were obtained recorded R_f values ranging between 0.2-0.7 on the TLC plates.

3.9 Structure determination of compounds

3.9.1 Nuclear magnetic resonance spectrometer (NMR)

One dimension (^{1}H-NMR and ^{13}C-NMR) and two dimension (COSY, HMBC, NOESY HSQC) NMR experiments were performed. NMR spectroscopy was carried out on a Bruker AV 300MHz or 500MHz NMR spectrometer. All spectra were recorded at room temperature using deuterated chloroform $CDCl_3$ or deuterated methanol CD_3OD. The chemical shifts were all recorded in ppm relative to tetramethyl silane.

3.9.2 Infrared spectrophotometer (IR)

IR spectra were recorded using a Nicolet impact 400 D spectrometer. Non-crystalline samples were dissolved in dichloromethane and analyzed on a NaCl window. The spectrometer was calibrated against an air background.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Taxonomic classification of the basidiomycete JO5182

The taxonomic classification was done using morphological (microscopic) and molecular techniques. The hyphal strands in a well grown mycelium indicated presence of clamp connections, which is a characteristic of basidiomycetes.

4.2 Cultivation of the basidiomycete JO5182

Growth in any organism is defined intuitively as the general tendency to increase in size. The basidiomycete grew as white mycelia and covered an area of radius 90mm on a PDA plate. The basidiomycete JO5182 grew in liquid malt media for 28 days as still cultures at room temperature. Glucose was included in the medium to act as immediate source of energy for the cultivated mycelia before they could be able to digest the complex cellulose in molasses. During the growth process there was evident increase in the biomass of the mycelia implying there was growth. Other than increase in biomass, glucose level in the liquid malt medium was used as the growth monitoring parameter the glucose level that was being checked daily showed a steady decrease. The basidiomycete JO5182 exhausted the entire glucose source in the culture within the first 28 days (Fig 1).

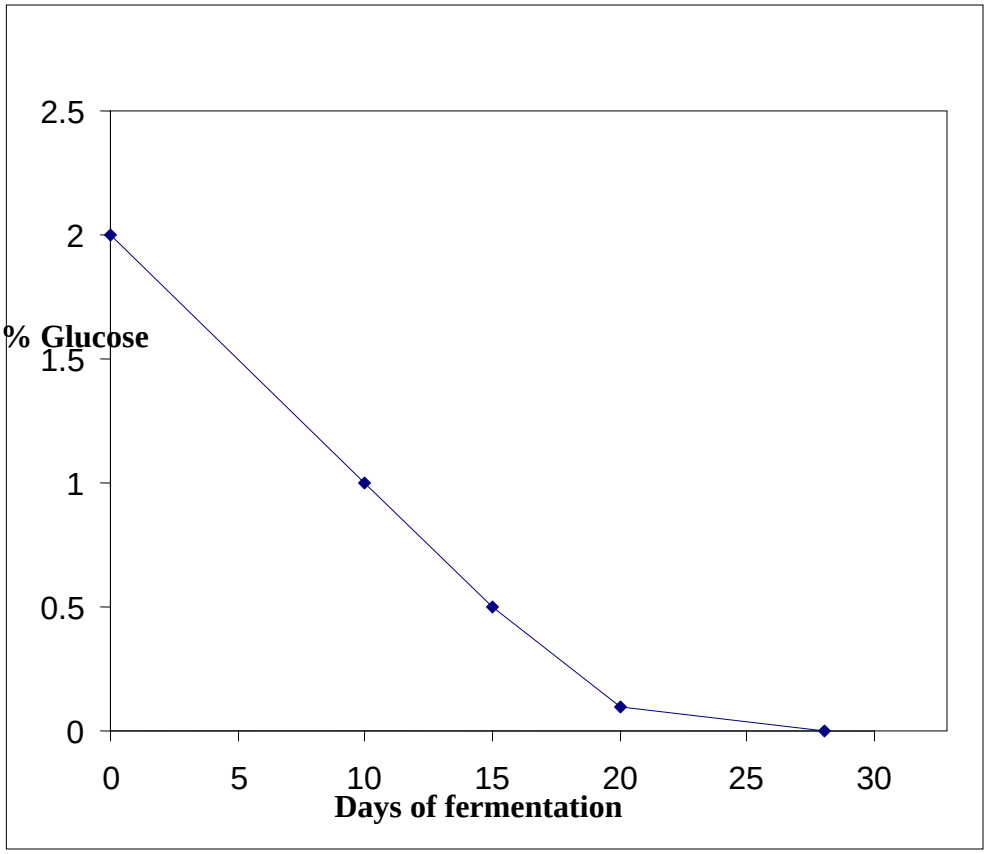


Figure 3: Glucose depletion trend as basidiomycete growth process progressed

Glucose uptake correlates positively with growth of fungi in defined media fermentation system. Fungi utilize glucose as the sole source of carbon and energy. Because glucose plays a vital role in the energy and material economies of organisms, its metabolism is the large regulatory phenomenon in fungal metabolism. Glucose is metabolized by glycolysis and a strong fermentation takes place even under aerobic conditions. It is from glucose that most secondary metabolites in organisms are directly derived (Yang & Illman., 1999).

4.3 Larvicidal activity test

4.3.1 Screening for larvicidal activity on the crude extracts

The mortality of *Ae. aegypti* larvae following the exposure by different concentrations of crude culture (Kex) and mycelia (Mex) extract of the basidiomycete JO5182 is presented in Table 3.

Table 3: Mortality (%) of *Aedes aegypti* mosquito larvae treated with crude extract of the submerged cultures of basidiomycete JO5182 after 24 hour exposure

Concentration (ppm)	Acetone culture extract (Kex)			Methanol culture extract (Kex)			Mycelia extract (Mex)		
	2 h	4 h	24 h	2 h	4 h	24 h	2 h	4 h	24 h
0 (control)	0	0	0	0	0	0	0	0	0
20	100	100	100	0	0	0	0	0	70
40	100	100	100	0	0	0	0	60	80
60	100	100	100	0	0	0	80	100	100
80	100	100	100	0	0	0	100	100	100
100	100	100	100	0	0	0	100	100	100

Values are the mean of 3 (n=3)

From the results the data show a dose-dependent effect on mortality. The larvicidal bioassay results show high activity against the late 3rd and early 4th instar larvae of *Ae. aegypti*. The crude extracts obtained from the submerged cultures of the basidiomycete JO5182 showed varying activity levels. The culture extract obtained using methanol was not activite against late 3rd and early 4th instar larvae but the one extracted using acetone showed 100% mortality against the larvae of *Ae. aegytpti* after 2 h at very low concentration of 20 ppm. For the mycelia extract (Mex), no larval mortality was observed below a concentration of 20 ppm during the first 4 h. However, 70% mortality was observed after 24 h. At 40 ppm there was no mortality in the first 2 h while 60 and 80% mortality was recorded after 4 h and 24 h respectively. Mortality at 60 ppm was 80% after 2 h and 100% after 4 h. At 80 and 100 ppm 100% larval mortality was observed after 2 h of exposure to extract (Table 3).

The absence of larval mortality in negative control up to 24 h of exposure (Table 3) shows that the real cause of the mortality is due to the treatment with the crude extract from the submerged cultures demonstrating that the extracts from the basidiomycete JO5182 are potential sources of larvicides. The enhancement of larval mortality with increasing concentration also confirms this observation.

The parameters that were used to measure the lethality of the samples was LC_{50} and LC_{90} values. LC_{50} describes how lethal the sample is while LC_{90} describes the concentration of the sample that can be kill a majority of the larvae within 24 h. The acetone culture filtrate (Kex) and mycelium (Mex) extract of the submerged culture of the basidiomycete JO5182 showed potent larvicidal activity against the late 3rd and early 4th instar larvae of *Ae. aegypti* with LC_{50} of 3.33 and LC_{90} 59.33 ppm, respectively after 24 hours (Table 4). The low values of LC_{50} (3.33) and LC_{90} (59.33), encouraged further investigation on this crude extract. By the end of 2 h, the acetone culture filtrate had killed all the ten mosquito larvae with LC_{50} at 3.33 ppm and LC_{90} at 59.33 ppm showing that the extract takes a very short time to kill the mosquito larvae.

Table 4: LC_{50} and LC_{90} for the crude acetone culture filtrate (Kex) and Mycelia crude extract

	LC_{50} (ppm)				LC_{90}(ppm)			
Extract	2 h	4 h	8 h	24 h	2 h	4 h	8 h	24 h
Acetone culture filtrate (Kex)	3.33	3.33	3.33	3.33	59.33	59.33	59.33	59.33
Mycelia extract (Mex)	31.27	28.92	23.07	21.31	79.38	77.26	71.12	67.21

4.3.2 Larvicidal activity for the purified fractions (compounds)

The results obtained in the preliminary assays of the crude extracts from the submerged cultures of the basidiomycete against Ae. *aegypti* larvae (Table 3 & 4) shows that only two were active and gave 100% mortality after 24 h. These active crude extracts were subjected to column chromatography and a total of 13 relatively purified fractions were isolated. Larvicidal activity tests were carried out on four of the fractions that gave enough material for both structure elucidation and bioassay (Table 5 & 6).

Table 5: Mortality (%) of *Aedes aegypti* larvae treated purified fractions

Sample	Time (h)	25 ppm	20 ppm	30 ppm	50 ppm	100 ppm
JO5182M03N	2	0	0	40	90	100
	4	0	10	70	100	100
	8	0	20	80	100	100
	24	0	30	90	100	100
JO5182M06N	2	0	0	0	0	0
	4	0	0	0	0	0
	8	0	0	0	10	90
	24	0	0	0	30	100
JO5182M07N	2	0	0	10	20	80
	4	0	30	100	100	100
	8	10	50	100	100	100
	24	30	60	100	100	100
JO5182K12N	2	0	0	0	0	0
	4	0	0	10	0	0
	8	0	0	20	20	60
	24	0	0	50	50	100

Fractions JO5182M04N, JO5182M05N, JO5182M08N, JO5182M09N JO5182M10N, JO5182K11N, JO5182K13N, JO5182K14N, and JO5182K15N were isolated in small amounts and were not tested for larvicidal activities. Mosquito larvicidal test of submerged culture fractions showed that fractions JO5182M03N, JO5182M06N, JO5182M07N and JO5182K12N were toxic to *Aede aegypti* larvae with LC_{50} of 71.446, 154.257, 61.149 and 129.487 ppm, respectively. Fraction JO5182M07N (LC_{50} 61.15 ppm, LC_{90} 161.53 ppm) recorded the highest toxicity *Aede aegypti* larvae (Table 6).

Fraction JO5182K12N originated from the culture filtrate (Kex) of the basidiomycete JO5182 meaning that it contributes to the activity seen in the crude extract. Mycelia extract (Mex) with LC_{50} of 21.31 ppm yielded fractions JO5182M03N, JO5182M06N and JO5182M07N which exhibited activity against the *Ae. aegypti* larvae. The activities of the isolated fractions show lower lethality against the *Ae. aegypti* larvae when compared to the crude extracts (Table 4 & 6). This suggests that the activity displayed by the crude extract acted synergistically thus the activity reduces on purifying.

Table 6: LC_{50} and LC_{90} index of *Aede aegypti* larvae exposed to fractionated crude extracts from submerged cultures of the basidiomycete JO5182

	LC_{50} (ppm)				LC_{90} (ppm)			
Fraction	2 h	4 h	8 h	24 h	2 h	4 h	8 h	24 h
JO5182M03N	112.47	98.64	121.62	71.45	198.09	187.76	198.09	167.77
JO5182M06N	-	-	178.47	154.26	-	-	287.64	248.50
JO5182M07N	143.22	75.72	62.31	61.15	237.21	169.96	163.08	161.53
JO5182K12N	-	-	196.67	129.49	-	-	334.56	230.26

Results on the larval mortality of the extract from the submerged cultures of the basidiomycete JO5182 reported in the present study, confirm their potential as sources of mosquito larvicidal compounds. Since compounds from natural source have little if any effect on the environment, the extracts from the submerged cultures of the basiomycete JO5182 can be exploited to protect the immediate environment of the user or the user from the bites of harmful mosquito, which is a vector of the malaria disease. The results illustrate that fraction JO5182M03N, JO5182M06N, JO5182M07N and JO5182K12N are promising candidate as mosquito larvicides.

4.4 Thin layer chromatography analysis

Fraction JO5182M07N (R_f 0.36-0.41) elicited the best activity of all the tested fractions. The other fractions that depicted reasonable activity (table 6) had R_f values ranging between 0.33-0.67. The two derivatives of carboxylic acid compound JO5182M10N and Compound JO5182K14N registered Rf value of 0.35 (Dichloromethane – methanol 2ml - 2 drops) and 0.39 (100% dichloromethane) respectively. The R_f values obtained for fraction JO5182K15N by thin-layer chromatography was 0.77 (Ethyl acetate-cyclohexane 1:5).

4.5 Structure elucidation

Fractions JO5182M04N, JO5182M05N, JO5182M06N JO5182M07N, JO5182M08N, JO5182M09N JO5182M10N, JO5182K11N, JO5182K12N, JO5182K13N, JO5182K14N and JO5182K15N were subjected to NMR experiments. Of these only three fractions; JO5182M10N, JO5182K14N and JO5182K15 had full NMR

spectral data for structure determination. Consequently, chemical structures of the compounds JO5182M10N, JO5182K14N and JO5182K15N were proposed from spectral and recorded data (Appendix I-XXII). From the observation of the respective ^{1}H-NMR (Appendix XXIII-XXX) for fractions JO5182M3N, JO5182M4N, JO5182M5N, JO5182M6N, JO5182M7N, JO5182M8N, JO5182M9N, JO5182K11N, JO5182K12N and JO5182K13N there was no enough information to determine their structural make-up. This could be due to low yield of extract or impurity. There is also the possibility that signals obtained when the compound is produced in low quantities are not easy to discern.

4.5.1 Compound JO5182M10N

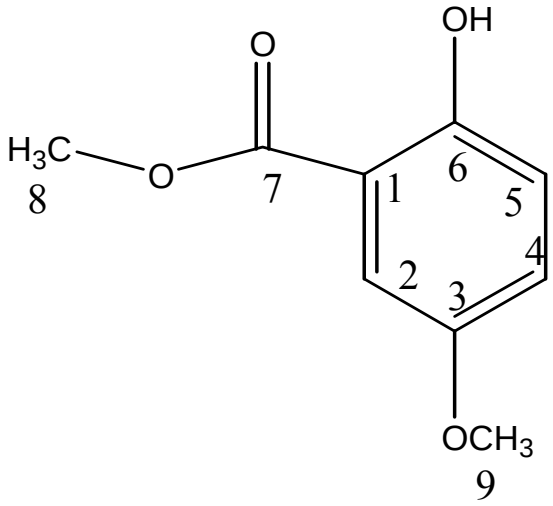
The ^{1}H NMR chemical shifts for the three aromatic protons indicate unsymmetrical substitution on the aromatic ring of compound JO5182M10N. Three types of aromatic protons integrating for one proton each illustrates that the ring is tri-substituted. ^{13}C-NMR and DEPT indicated that there are nine carbons comprising of six unique and characteristic aromatic (δ112.40, δ114.80, δ121.40, δ123.80, δ147.60, δ151.90) carbons, one characteristic carbonyl (δ167.60) carbon, one aromatic phenolic carbon bearing an hydroxyl group (δ151.80), one chemical shift for an aromatic carbon attached to a methoxy group (δ147.60) and two characteristic methoxy carbons (δ55.20, δ51.20). HSQC shows that C-2, C-4 and C-5 which are in the aromatic ring bear H-2 H-4 and H-5, respectively. There are two aliphatic carbons (C-8 and C-9) which respectively bear H-8 and H-9. ^{1}H NMR signal at δ 6.85 is ascribed to C-2 because of the *ortho* coupling constant of J=9 Hz. (Table 7). HMBC correlation was observed between H-5 and C-1 and H-2 and C-7.

Table 7: NMR (500 MHz) data for JO5182M10N in (Appendix I to VII)

Position	^{13}C	^{13}C Lit.	^{1}H	^{1}H Lit.	HMBC	COSY	NOESY	DEPT
1	121.40	108.32	-		-			-
2	112.37	101.84	7.56 d, (*J*=3 Hz)	7.71-7.73	C-1, C-4, C-3, C-6, C-7	H-4	H-9	CH
3	147.60	166.11	-		-	-	-	-
4	123.80	132.21	7.57 dd, (*J*=9 Hz, 3 Hz)	7.71-7.73	C-2, C-6	H-2, H-5	H-5	CH
5	114.60	106.15	6.85 d, (*J*=9 Hz)	6.52-6.54	C-1, C-6, C-3	H-4	H-4	CH
6	151.80	163.49	-		-	-	-	-
7	167.60	170.25	-		-	-	-	-
8	55.20	53.06	3.91 s	3.81 s	C-7	-	-	CH_3
9	51.20	56.49	3.88 s	3.87 s	C-2	-	-	CH_3

Lit.: Literature values as recorded by Legrand et al (2003)

Compound JO5182M10N was identified by comparison of its DEPT, HSQC, COSY, NOESY, HMBC, ^{1}H and ^{13}C spectroscopic data with published values. The ^{1}H and ^{13}C data obtained from NMR experiment for Compound JO5182M10N was found to agree with data found in literature as reported in table 7 (Legrand *et al*., 2004).



Compoud JO5182M10N (**20**)

Compound JO5182M10N is a carboxylic acid derivative whose IUPAC name is methyl 2-hydroxy-4-methoxybenzoate. It is commercially available and has been shown to have strong antifeedant effect on the pine weevil (Sunnerheim *et al.*, 2007).

4.5.2 Compound JO5182K14N

The structure of JO5182K14N was assigned from NMR data (Table 8).

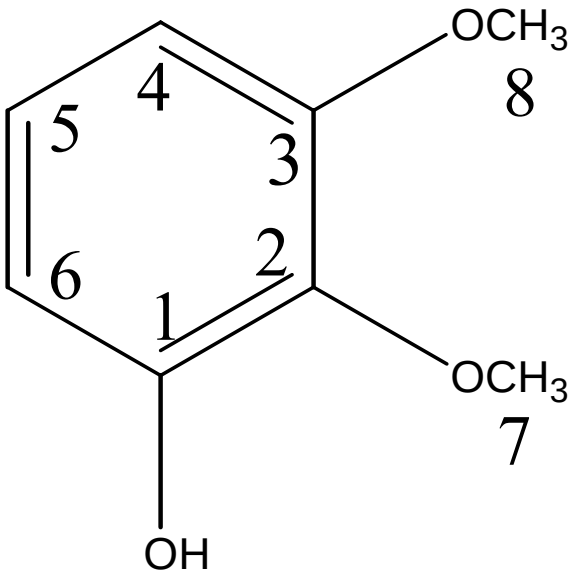
Table 8: Summarized NMR (500 MHz) data for ^{1}H-HSQC, HMBC, COSY, NOESY, DEPT and ^{13}C of JO5182K14N in CD_3OD (Appendix VIII-XIV)

No.	^{13}C	^{1}H	HMBC(H→C)	COSY	NOESY	DEPT
1	150.38	-	-	-	-	-
2	152.22	-	-	-	-	-
3	142.42	-	-	-	-	-
4	114.03	6.77, d, (*J*=9.0)	C-2,C-3	H-5	H-5	CH
5	105.79	6.32, dd, (*J*=9.0, 3.0)	C-3	H-4, H-6	H-4, H-6	CH
6	100.75	6.46, d (*J*=3.0)	C-2 ,C-5	H-5	H-5	CH
7	55.45	3.79, s	C-2		H-8	OCH_3
8	55.10	3.75, s	C-3		H-7	OCH_3

^{13}C NMR and DEPT experiment show that Compound JO5182K14N has 8 carbons; three quaternary (δ152.22, δ150.38, δ142.42), three methine (δ114.03, δ105.79, δ100.75) aromatic, and two characteristic methoxy (δ55.45, δ55.10) carbons. The 1H-NMR spectrum showed two *ortho*-coupled aromatic protons at δ 6.77 (d, *J*=9.0 Hz, H-5) and δ 6.32 (dd *J*=9.0 Hz, *J*=3.0 Hz, H-6) and one meta-coupled aromatic proton at δ 6.46 (d, *J*=3.0Hz) and their ^{13}C NMR signals were observed at δ114.03, δ105.79 and δ100.75 respectively. The COSY correlation indicates that H-5 is coupled with H-4 and H-6. The methoxy groups were assigned from the HMBC experiment that suggests that C-3

correlates with H-8 and C-2 correlates with H-7. The signal at δ150.38 is typical of hydroxyl group substituted aromatic carbon.

All the 1D and 2D NMR data for fraction JO5182K14N isolated from the culture filtrate of the basidiomycete JO5182 (Table 8) gave a structure consistent with the structure of 2, 3-Dimethoxyphenol as represented below.



Compound JO5182K14N (**21**)

4.5.3 Compound JO5182K15N

The structure of compound JO5182K15N was determined from ^{1}H, ^{13}C, COSY, DEPT, HSQC-DEPT, NOESY HMBC NMR and IR (appendix XV-XXII) data. The NMR experiment was run in deuterated chloroform ($CDCl_3$) as summarized in Table 7.

Table 9: NMR (300 MHz) data for ^{1}H-HSQC, HMBC, COSY, NOESY, DEPT and ^{13}C of JO5182K15N in $CDCl_3$ (Appendix XV to XXX)

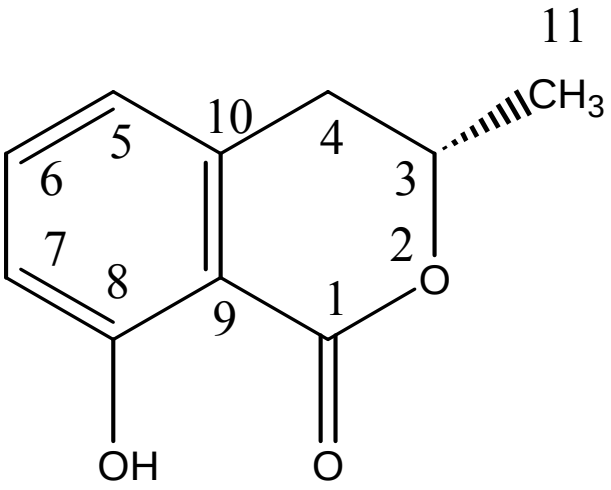
No.	^{13}C	^{13}C Literature	^{1}H	^{1}H Literature	HMBC (H→C)	COSY	NOESY	DEPT
1	170.15	170.1	-	-	-	-	-	-
2	-		-		-	-	-	-
3	76.29	76.9	4.72, m (*J*=6.3)	4.73, m	C-10, C-11	H-4, H-11	H-4,H-11	CH
4	34.81	34.8	2.93, d (*J*=7.5)	2.93, d (J=7.4 Hz)	C-9, C-5, C-1, C-3, C-11	H-3	H-11, H-3	CH_2
5	116.43	116.4	6.87, dd (*J*=7.5, 0.9)	6.89, d (J=8Hz)	C-8, C-7, C-10, C-1	H-5, H-7	H-5	CH
6	136.34	136.3	7.40, t (*J*=7.5)	7.41 (J=8.0 Hz)	C-8, C-9, C-6, C-10	H-6,	H-6	CH
7	118.09	118.0	6.70, dd (*J*=7.5 Hz, 3Hz)	6.69, d (J=8.0 Hz)	C-6, C-10, C-4, C-5, C-1, C-8	H-6	H-6	CH
8	162.40	162.4	-		-	-	-	-
9	139.59	139.5	-	-	-	-	-	-
10	108.49	108.4	-		-	-	-	-
11	20.96	20.9	1.54, d (*J*=6.3)	1.53, d (J=6.3Hz)	C-3, C-4	H-3	H-3, H-4	CH_3
-OH	-	-	11.03 bs	11.0 s	C-5, C-7, C-9, C-4	-	-	-

Assignment of the proton resonances were based on their intensity, multiplicity pattern and coupling constants. The number of protons calculated from the integral values in the spectra is in agreement with those calculated for the molecule. Signals for one hydroxyl unit (δ11.03, 1H;), three aromatic protons (δ7.40, t, *J*=7.5Hz 1H; δ6.75, d, *J*=5.4Hz 1H and δ6.87, dd *J*=7.5Hz, 0.9Hz), one methyl (δ1.54, d, *J* =6.3 Hz, 3H) one methylene (δ2.93, d, *J*=7.5 Hz 2H) and one methine (δ4.73, m, *J*=6.3Hz 1H) were detected in the ^{1}H NMR spectrum.

The ^{13}C and DEPT spectra of JO5182K15N exhibited a total of ten carbon resonances comprising of one carbonyl (δ170.15), six aromatic carbons three of which are aromatic methines (δ136.34, δ116.43, and δ118.04) and three are quaternary (δ162.40, δ139.59, and δ108.49). In addition the compound has one oxygenated methine carbon (δ76.29), one aliphatic methylene carbon (δ34.81) and one methyl group (δ20.96) carbon signals. The aromatic signal at δ162.40 is typical of hydroxyl substituted carbon. ^{1}H-HSQC-DEPT experiment indicated that H-5, H-6, H-7, H-3, H-4 and H-11 sits on C-

5, C-6, C-7, C-3, C-4 and C-11, respectively. COSY experiment indicated clearly that the proton signal at δ6.87 (H-5) correlated with H-6 and H-7, the multiplet proton signal δ4.72 (H-3) correlated with H-4 and the doublet proton signal H-11, δ7.40 (H-6) correlated with H-7 and δ2.93 (H-4) correlated the H-11. The COSY spectrum in conjunction with the coupling constants, showed *ortho* coupling between aromatic protons at δ7.40 (*J*=7.5Hz), H-5 and δ6.87 d (*J*=7.5 Hz), H-6. Further, HMBC correlations of the proton signal at δ7.40ppm (H-5) with the carbons C-8, C-9, C-6 and C-7, proton signal at δ6.70 ppm (H-7) with the carbons at C-6, C-10 & C-5 and finally the proton signal at δ6.87 ppm (H-6) with the carbons C-8, C-7 and C-10 which permitted unambiguous location of the aromatic carbons. HMBC experiments showed quite well correlations between the doublet proton signal at δ2.93 (H-4) with the carbons C-9, C-5, C-7, C-3 & C-11 and the multiplet proton signal at δ4.72 ppm (H-3) with C-9, the doublet proton signals at δ1.54ppm (H-11) with C-3 and C-4 permitted the location of the methyl group in the cyclic ring. All the above NMR data were interpreted and found to be consistent with the proposed structure of compound JO5182KN15 whose IUPAC name is (R)-(-)-8-hydroxy-3-methyl-3, 4-dihydro-1H-2-benzopyran-1-one. The assignment of the structure was found to agree with those reported in the literature reports (Islam *et al*., 2006, Efdi *et al*., 2007).

IR experiments show an aromatic C-H stretch for peak at 3046 $cm^{-1}$and C-H (-CH_3) bend peak at 1464cm^{-1}. There is also a characteristic C═O pick at 1662 cm^{-1} and a C—O peak at 1117cm^{-1}. Since this functional groups are in the proposed structure it goes further to confirm compound JO5182K15N.

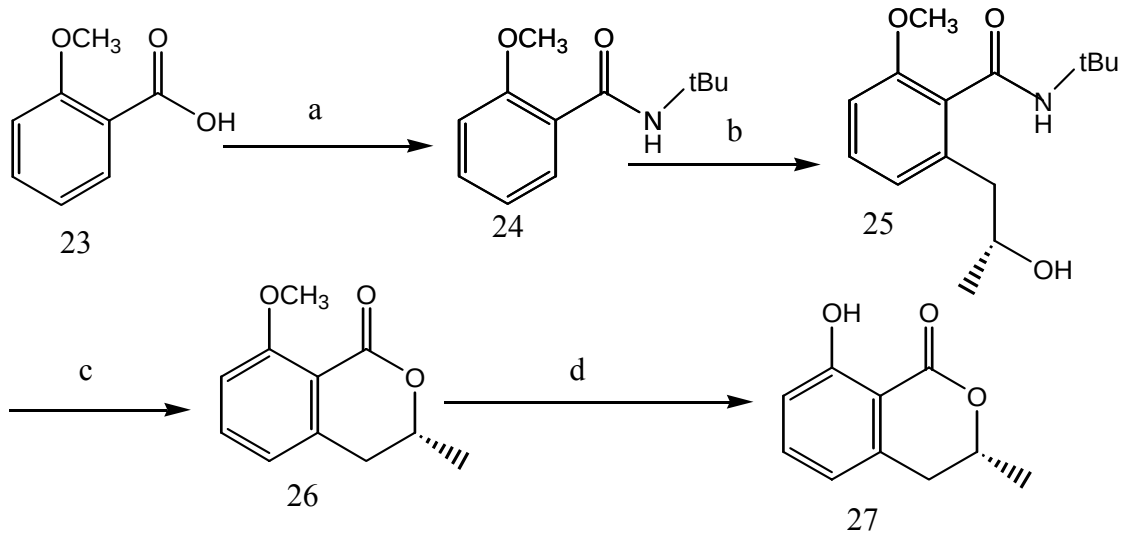


Compound JO5182K15N (**22**)

Compound JO5182KN15 has been isolated before and is commonly known as (R)-(-)-mellein (Flörke *et al.,* 2006). It is a naturally occurring dihyroisocoumarin which was first isolated as a metabolite of *Aspergillus melleus*. Since then the compound has been found in many fungal cultures and several insects in which it appears to play a pheromonal role (Krohn *et al*., 1997). More recently it has been isolated from fungus *Phomopsis oblonga, Microsphaeropsis sp.* marine sponge *Microsphaeropsis incrustans*. It has also been isolated from a group of endophytic fungi such as *Pezicula livida, Plectophomella sp*. and *Cryptosporiopsis malicoticis* (Claydon *et al*., 1985, Krohn *et al*., 1997). It is biosynthetically derived from a pentaketide and also occur in nature as the enantiomer (S)-(+)-mellein also named ochracin, isolated from *Fusarium larvarum* and showing insecticidal activity (Florke *et al*., 2006). (R)-(-)-mellein shows a number of interesting biological activity such as fungicidal, antibacterial, and algicidal in agar diffusion tests (Efdi *et al.*, 2007). Despite its long history, the first crystallographic characterization of mellein was performed only recently (Efdi *et al.*, 2007), reporting a triclinic cell, with six independent molecules in the asymmetric unit.

(R)-(-)-mellein has been synthesized by condensation of *o*-anisic acid (**23**) with *tert*-butylamine and DCC in the presence of DMAP to afford benzamide (**24**) in 75% yield. Treatment of **24** with two equivalence of *n*BuLi in the presence of TMEDA led to the formation of the orange coloured dianion, which opened (R)-(+)-propylene oxide to

afford secondary alcohol (-)-**25** in 61% yield. Acid induced (pTsOH.H2O) intramolecular attack of the newly generated alcohol at the amide group of (-)-**25** led to δ-lactone (-)-**26** in 72% yield. The methyl group in (-)-**26** was cleanly removed by exposure of (-)-26 to BCl_3 in CH_2Cl_2 to obtain (-)-mellein (-)-**27** 95% yield (Islam *et al*., 2006).



a) *t*BuNH$_2$ DCC DMAP/CH_2Cl_2 75%; b) *n*BuLi, TMEDA, (R)-(+)-propylene oxide/THF -78^{0}C, 61% (62% recovery of **23**); C) *p*TsOH.H_2O/toluene, reflux, 72%; d) BCl_3/CH_2Cl_3, -78^{0}C to 0^{0}C. 95%

4.5.4 Fraction JO5182M03N

Fraction JO5182M03N is soluble in water suggesting that it could be attached to a glycosidic linkage ^{1}H NMR peaks between 4.0 and 5.5 may also suggest this. Its ^{1}H NMR spectra suggested absence of aromatic hydrogens. The spectra show presence of aliphatic protons. There could be protons attached to carbons that are attached to highly electronegative atoms which most likely could be oxygen (Fig 4). The fraction showed good larvicidal activity with LC_{50} (71.45 ppm) against *Aedes aegypti* mosquito larvae. This suggests that a compound with larvicidal activity does not necessarily contain an aromatic ring.

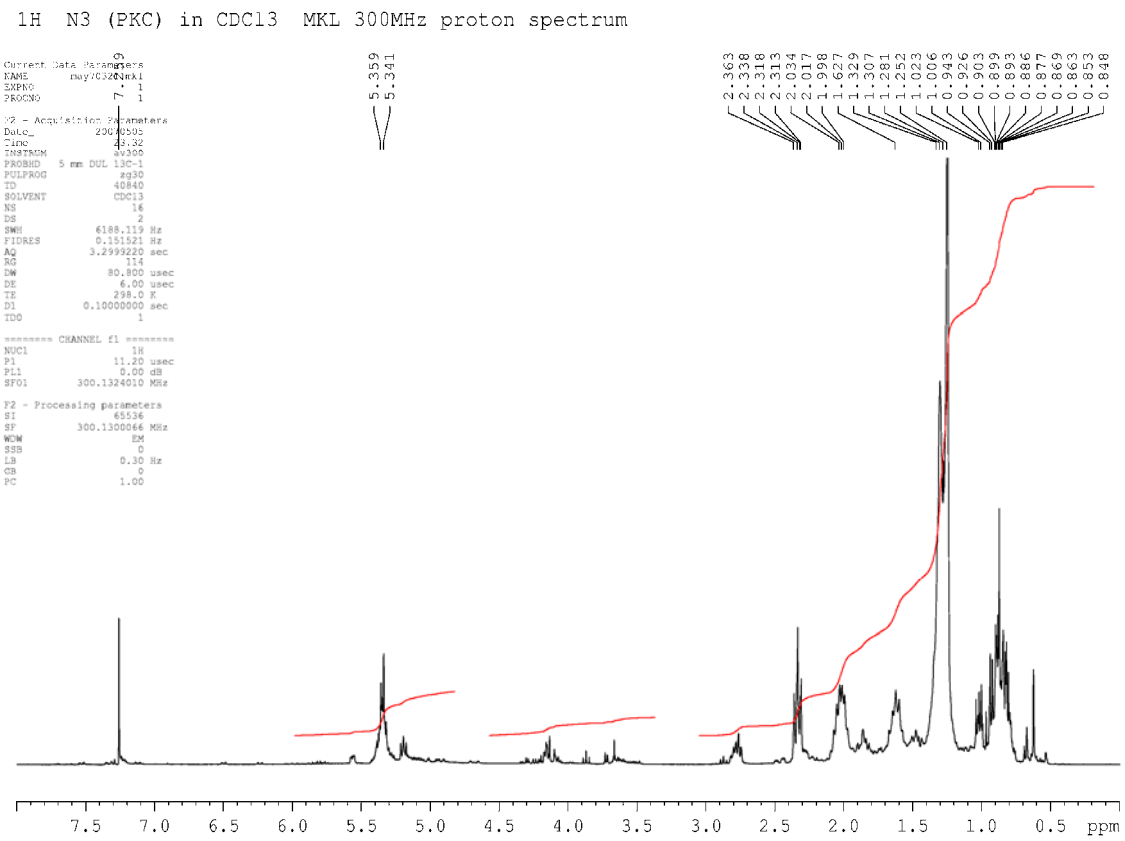


Figure 4: ^{1}H-NMR spectrum for fraction JO5182M03N

CHAPTER FIVE

CONCLUSION AND RECOMMEDATION

5.1 Conclusion

The Basidiomycete JO5182 takes a total of 28 days to grow in liquid malt media. This implies that the liquid malt media that was used to culture the basiomycete gives the basiomycete the necessary conditions for its growth.

Three crude extracts, from mycelium and culture filtrate, were isolated from the submerged culture of the basidiomycete using liquid-liquid and solid-liquid adsorption tequniques. The study established that the crude extracts of the basidiomycete JO5182 exhibit potent larvicidal activity against the *Aedes aegypti* larvae. The acetone culture filtrate was found to be highly lethal to the larvae with LC_{50} of 3.33 ppm and LC_{90} of 59.33 ppm. Further, the isolated fractions showed significant larvicidal activity. Of the tested fractions, fraction JO5182M07N recorded the highest lethality with LC_{50} value of 61.15 ppm and LC_{90} of 161.53 ppm implying that it is a good candidate as a mosquito larvicide.

It is evident from the LC_{50} results that the toxicity of the purified fractions is lower than that of the crude extract. The reason is that the compounds in the crude extract act synergistically to kill the mosquito larvae.

The chemical investigation of the submerged cultures of the basidiomycete JO5182 yielded three compounds whose structural make-up was determined. The compounds isolated include the isocoumarin, JO5182K15N and two carboxylic acid derivatives of JO5182M10N and .JO5182K14N.

Although these compounds were not tested for larvicidal activity owing to low yield of extract, they are known compounds with a wide range of biological activity. JO5182K15N (mellin) is known to inhibit Hepatitis C Virus protease with an LC_{50} value of 35 ppm (Islam *et al*., 2006). It is also reported to have atifungal activity (Dai *et al*., 2001), *in vitro* toxicity to mice and pheromonal activity (Efdi *et al*., 2007). On wheat

seedlings, mellin causes inhibition of root and coleoptile elongation as well as reduction of carbondioxide assimilation (Parisi *et al* ,1993)

These findings are sufficient to establish that the extract from the basidiomycete JO5182 is a potential source of mosquito larvicide.

5.2 Recommendation

Since fractions JO5182M3N, JO5182M6N, JO5182M7N, and JO5182N12N, were active against the mosquito larvae there is need to determine their structures. Larvicidal activity for the fractions not tested should be carried out to determine their activity. Larvicidal activity of the compounds isolated should also be determined. The extracts of the basidiomycete should be subjected to other biological activities to exhaust their potency.

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Yenesew, A., Mushibe, E. K., Induli, M., Derese, S., Midiwo, J. O., Kabaru, J. M., Heydenreich, M., Koch, A., Peter, M. G., (2005). 7a-Omethyldeguelol, a modified rotenoid with an open ring-C, from the roots of *Derris trifoliata. Phytochemistry,* **66**: 653–657.

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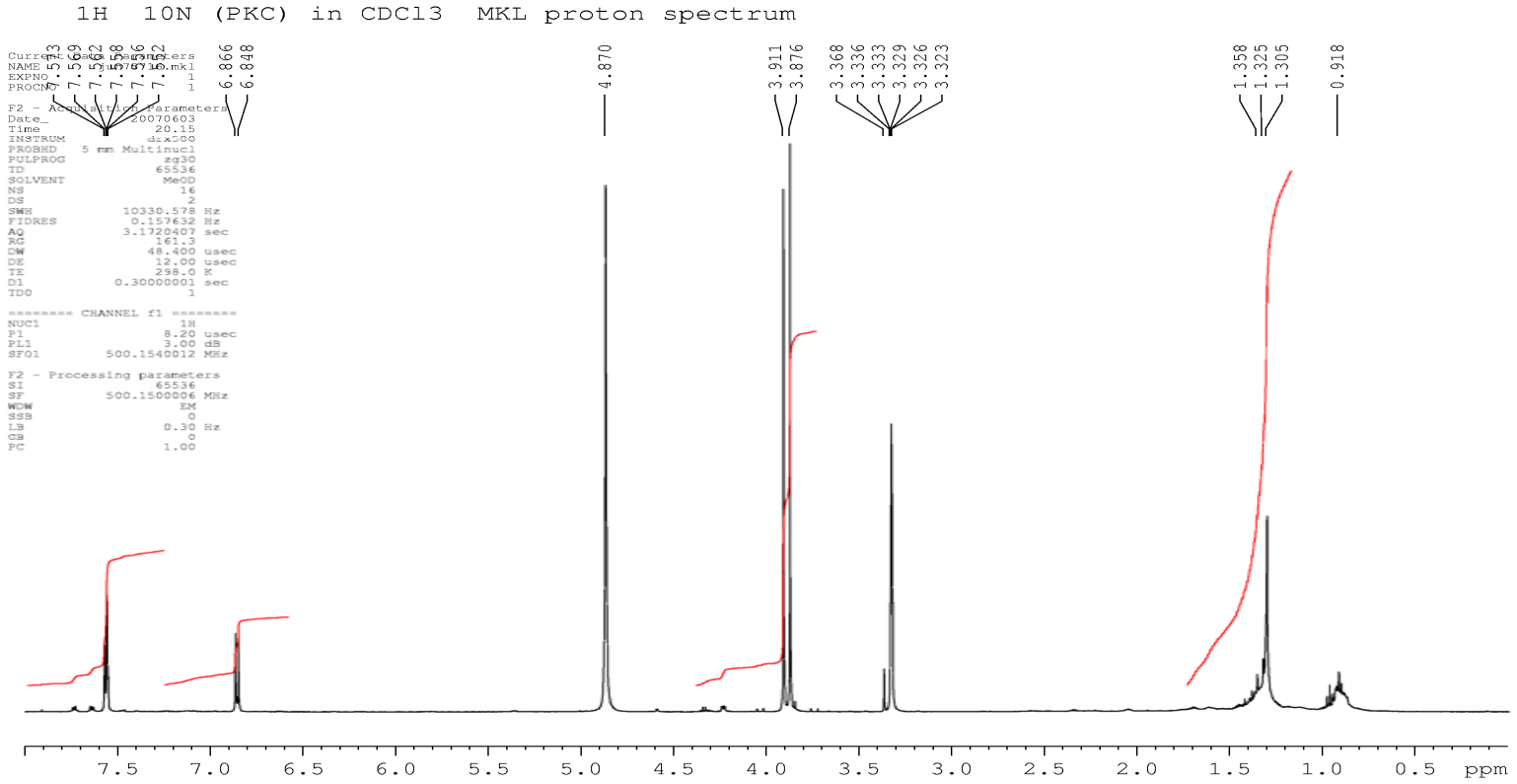
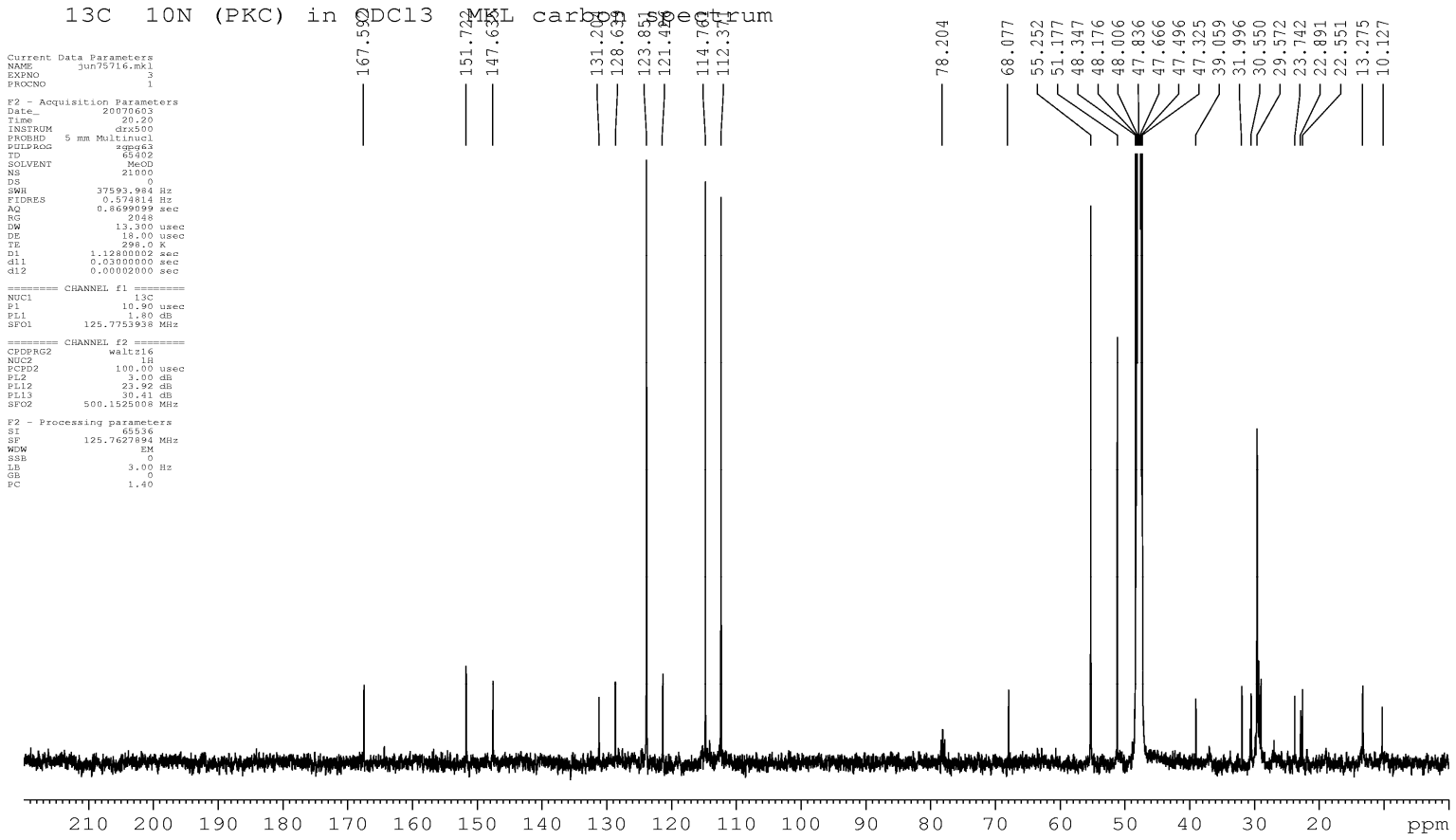
APPENDICES

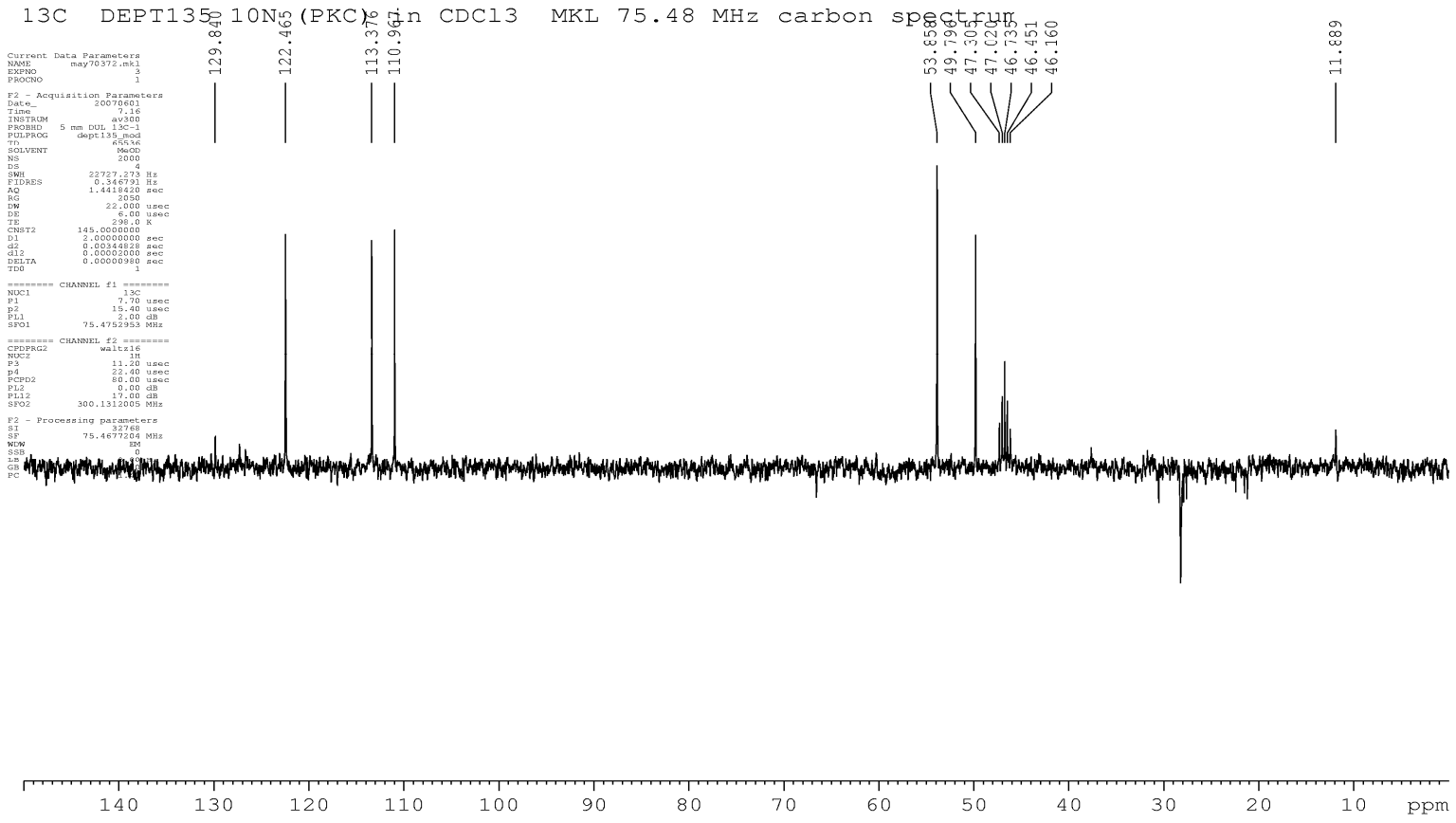
Appendix I: ^{1}H-NMR spectrum for JO5182M10N



Appendix II: ^{13}C- NMR spectrum for JO5182M10N

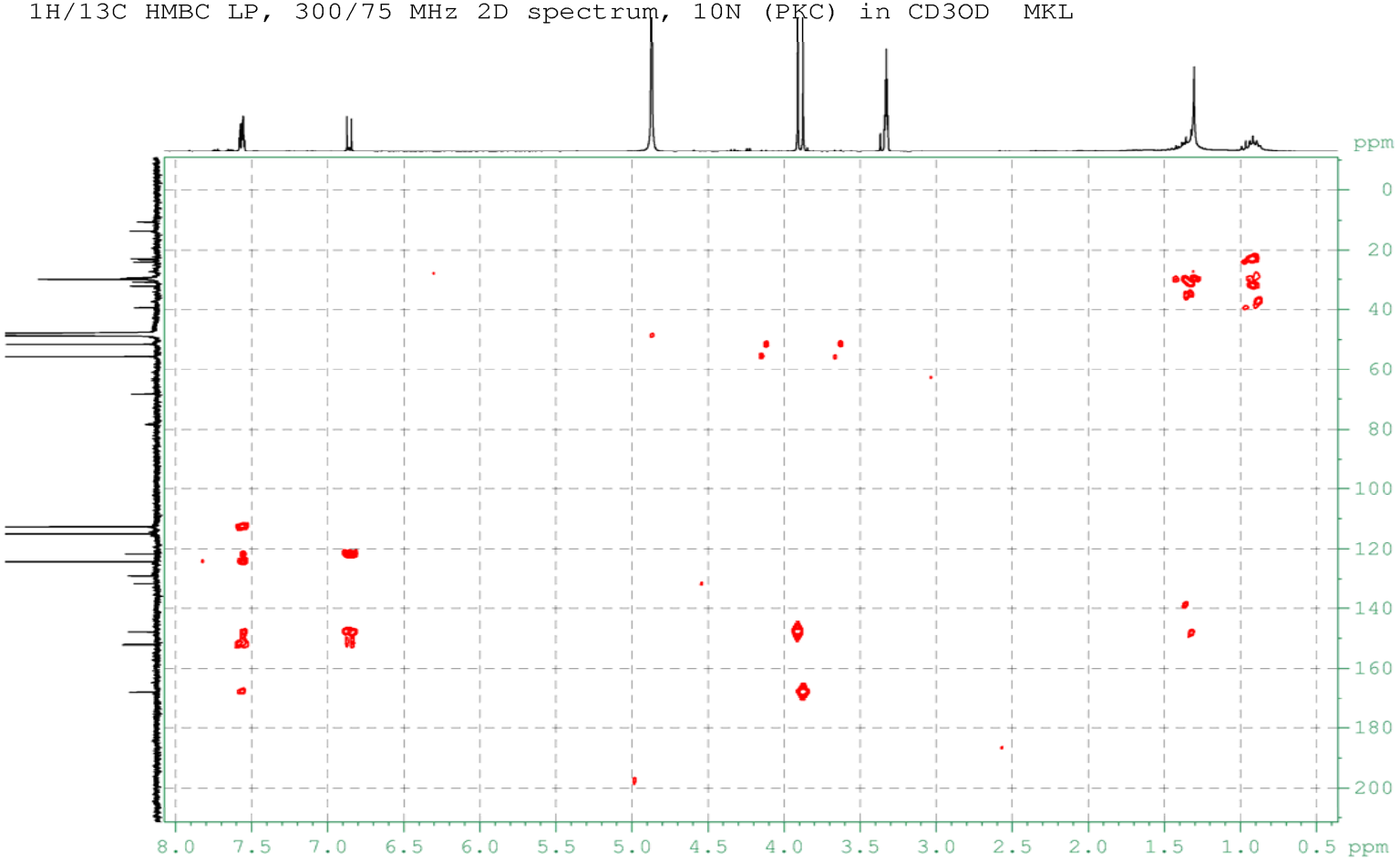


Appendix III: ^{13}C-DEPT spectrum for JO5182M10N

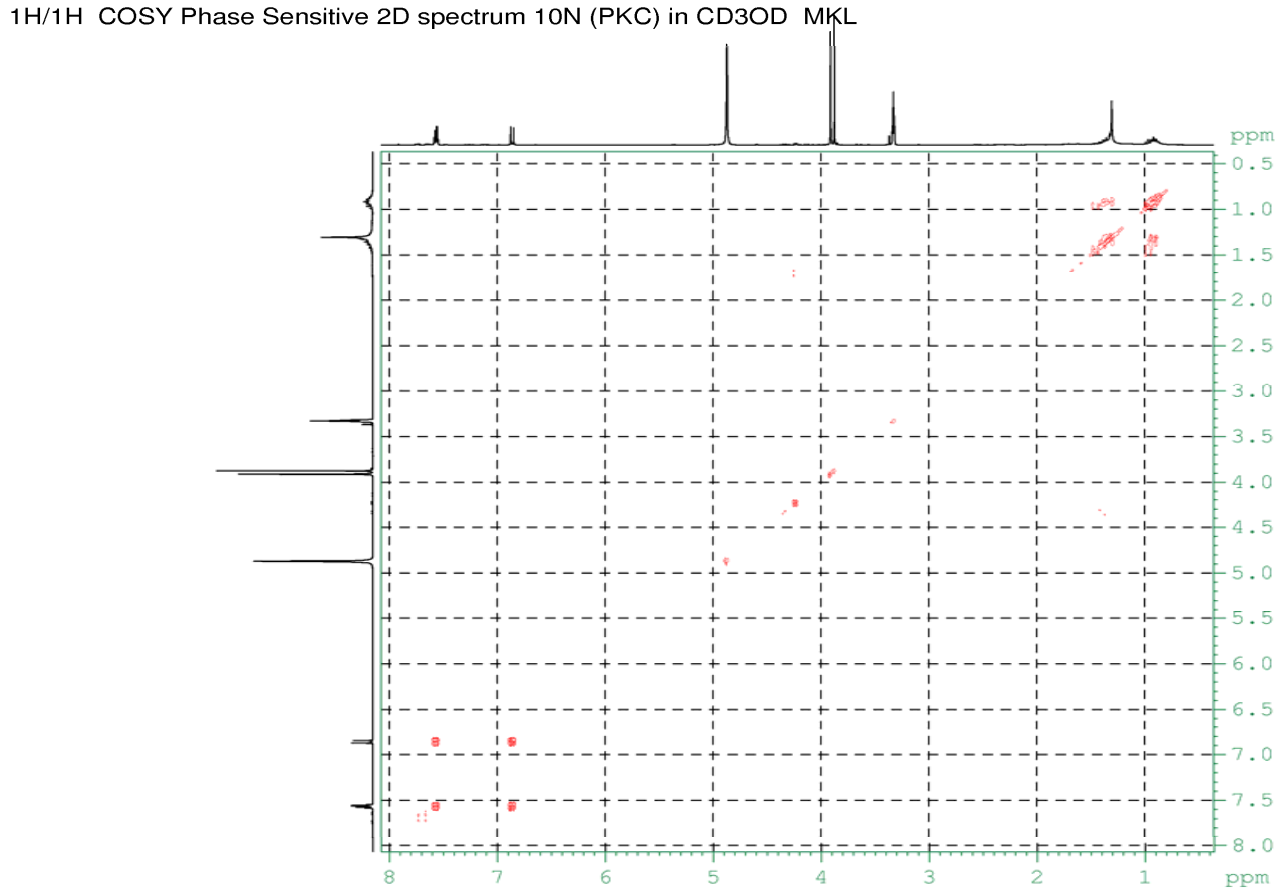


Appendix IV: HMBC spectrum for JO5182M10N

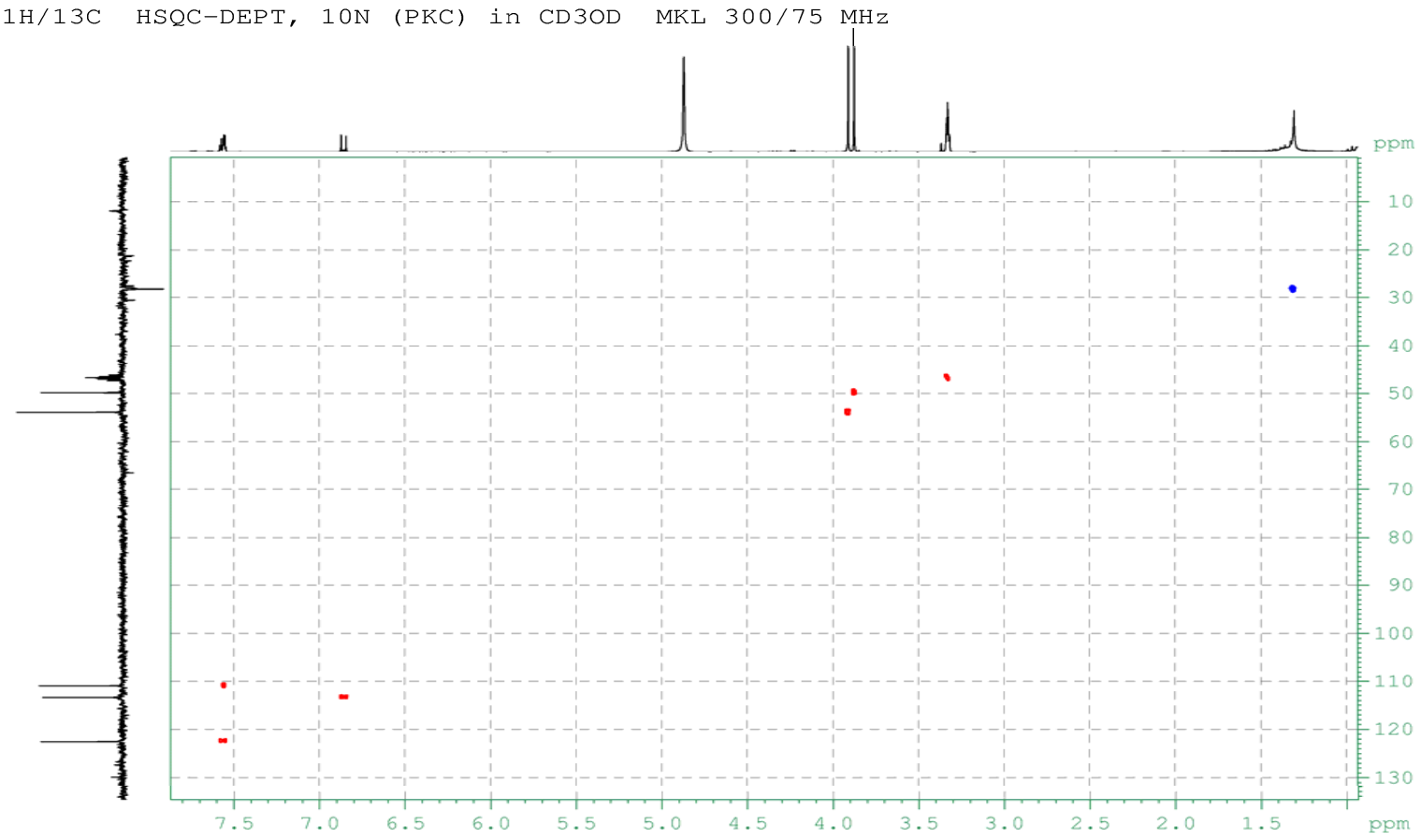
1H/13C HMBC LP, 300/75 MHz 2D spectrum, 10N (PKC) in CD3OD MKL



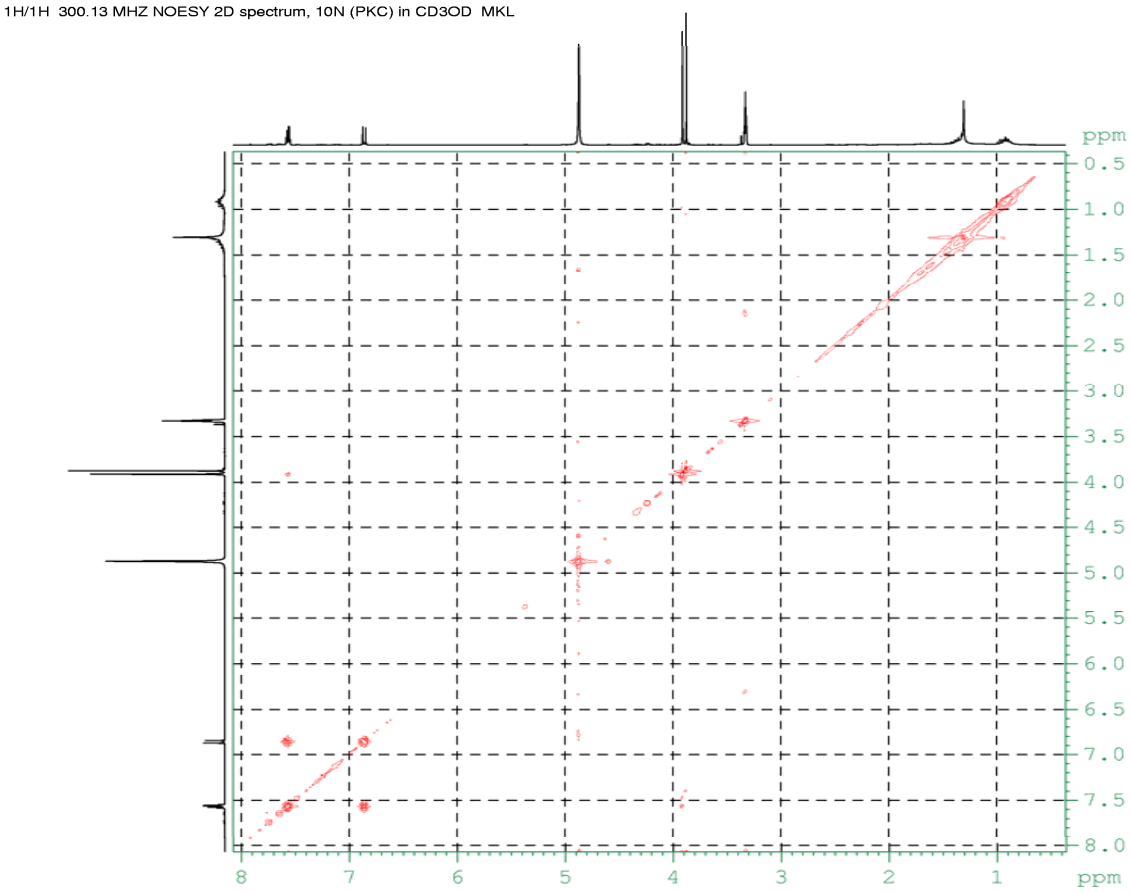
Appendix V: COSY spectrum for JO5182M10N



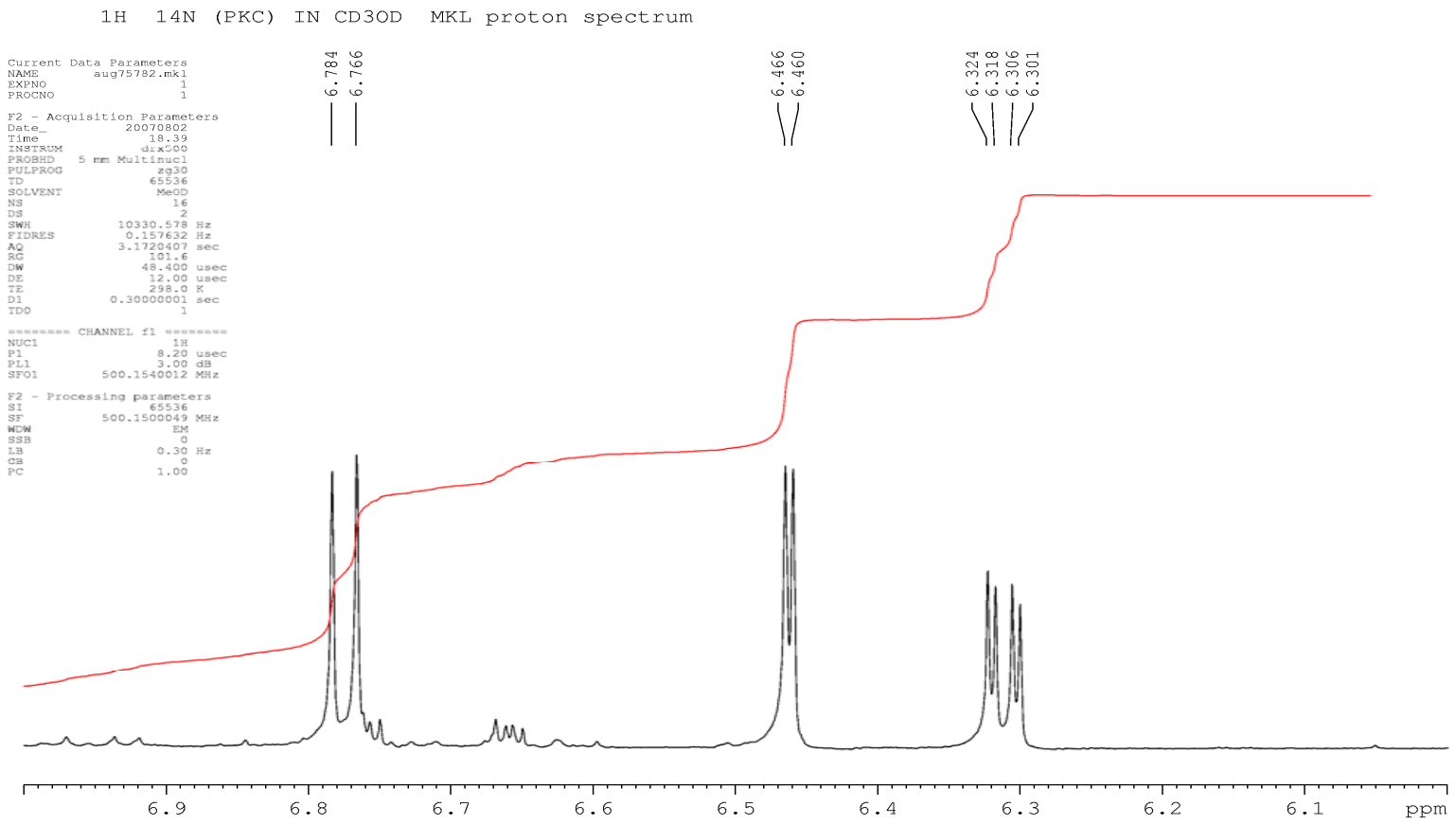
Appendix VI: HSQC-DEPT spectrum for JO5182M10N



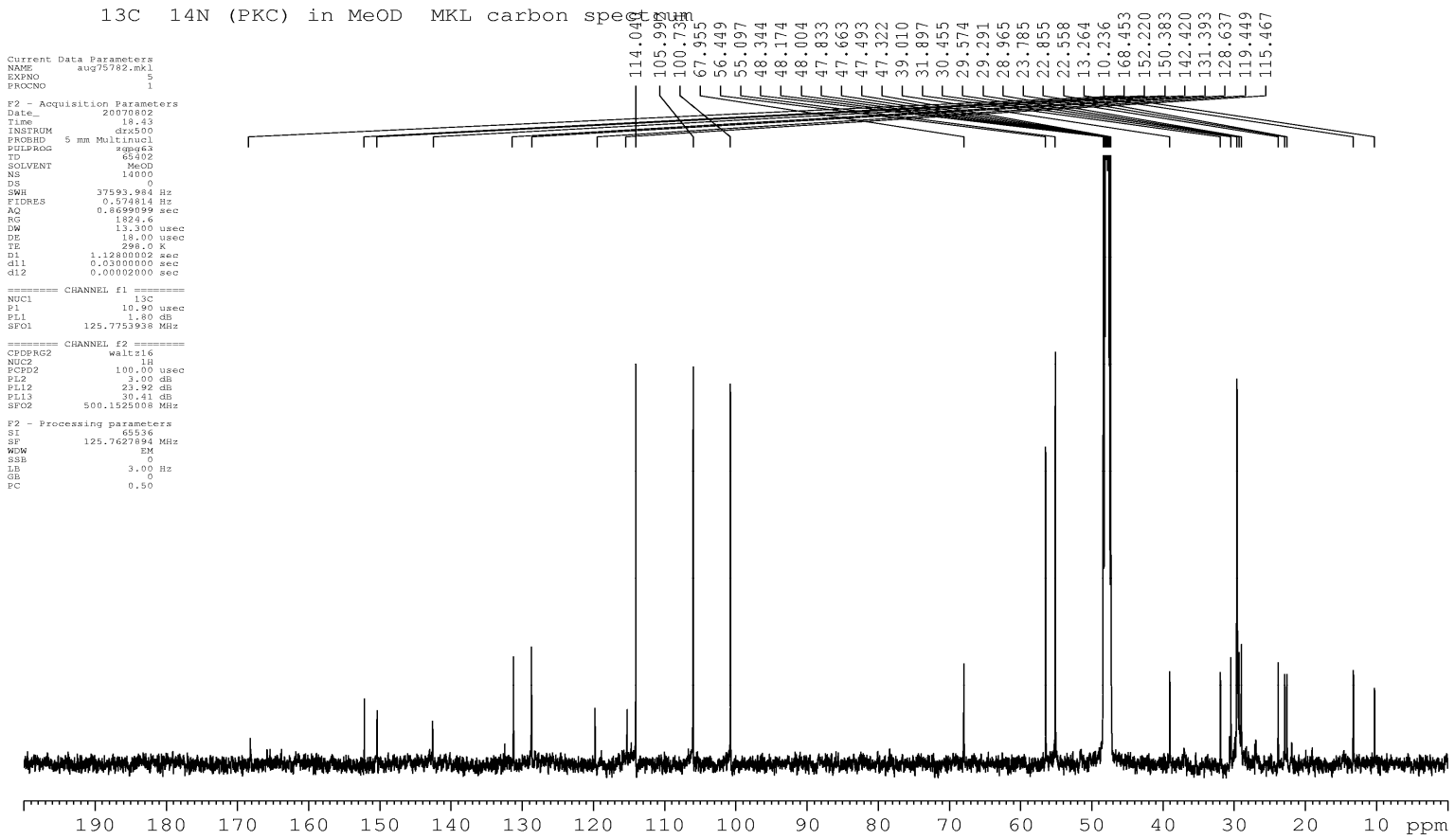
Appendix VII: NOESY spectrum for JO5182M10N

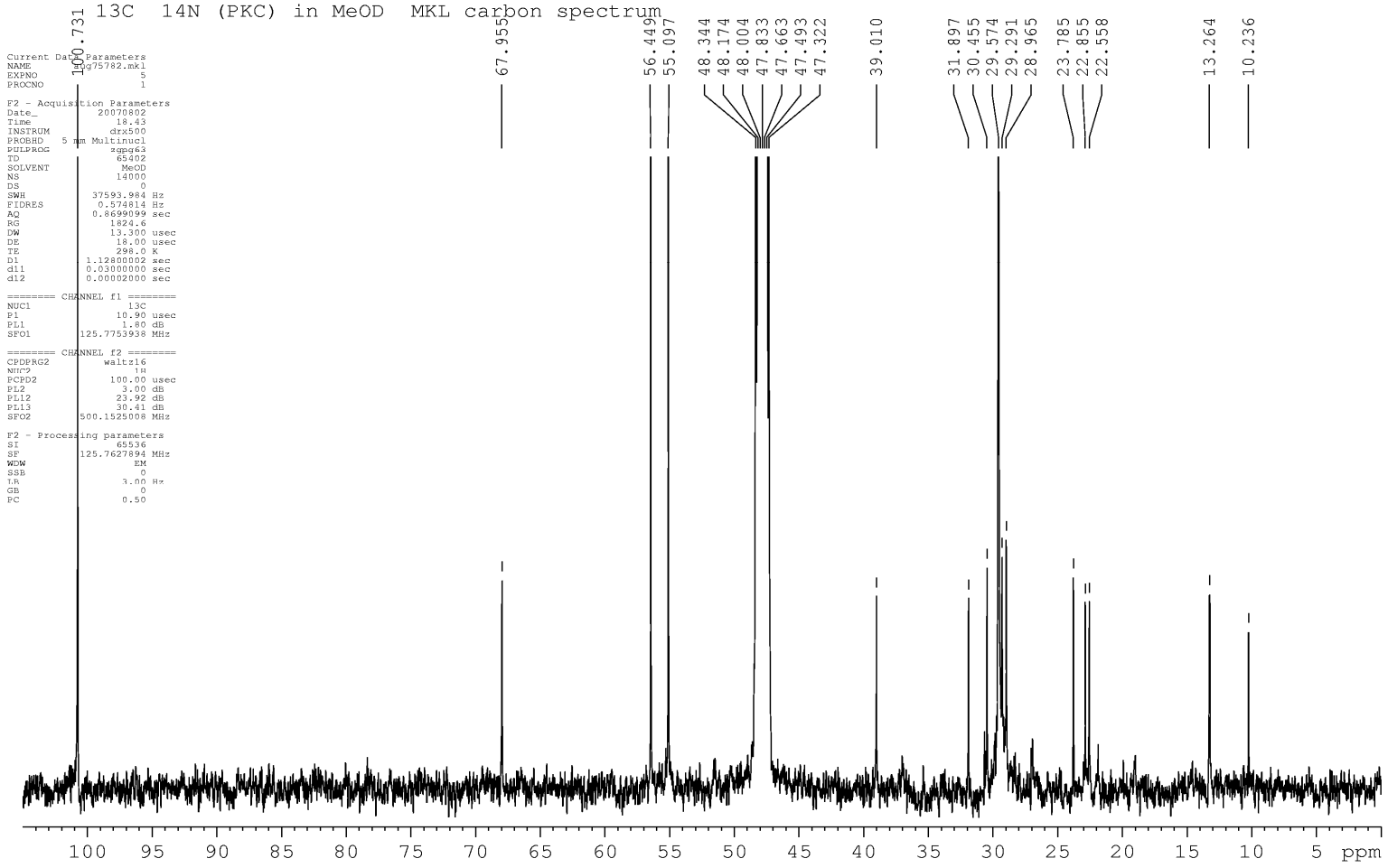


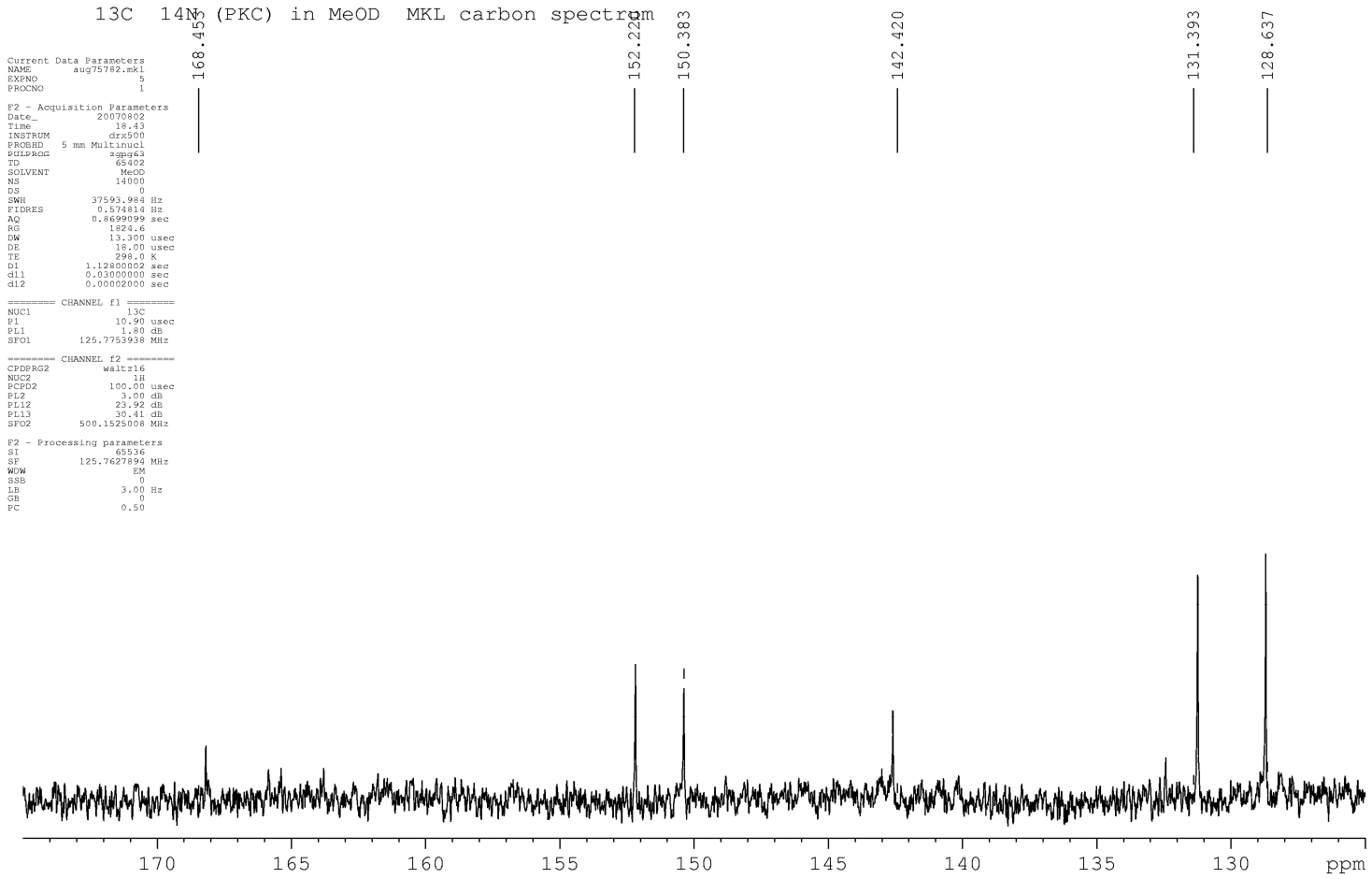
Appendix VIII: ^{1}H-NMR spectrum for JO5182K14N

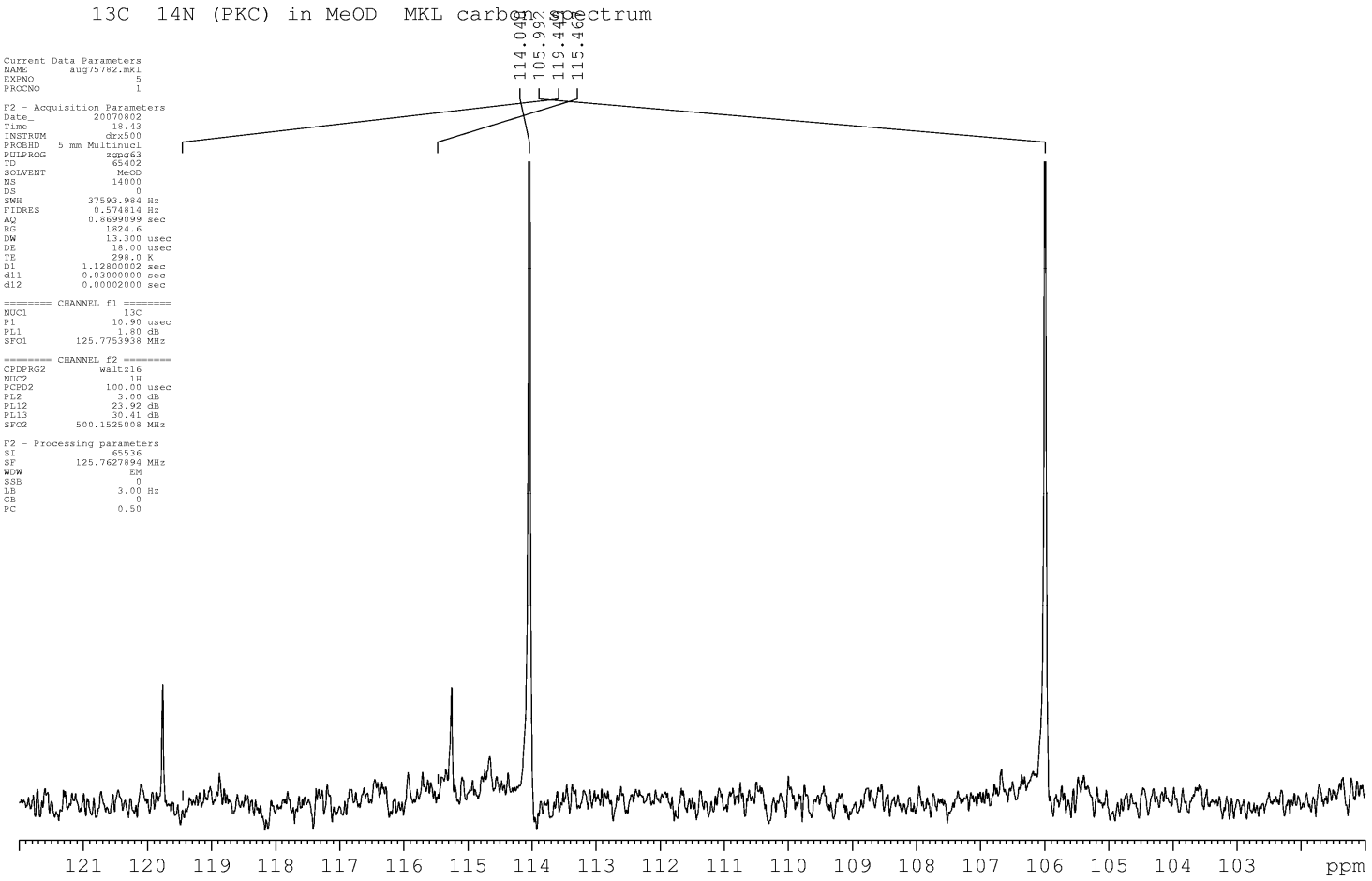


Appendix IX: ^{13}C-NMR spectrum for JO5182K14

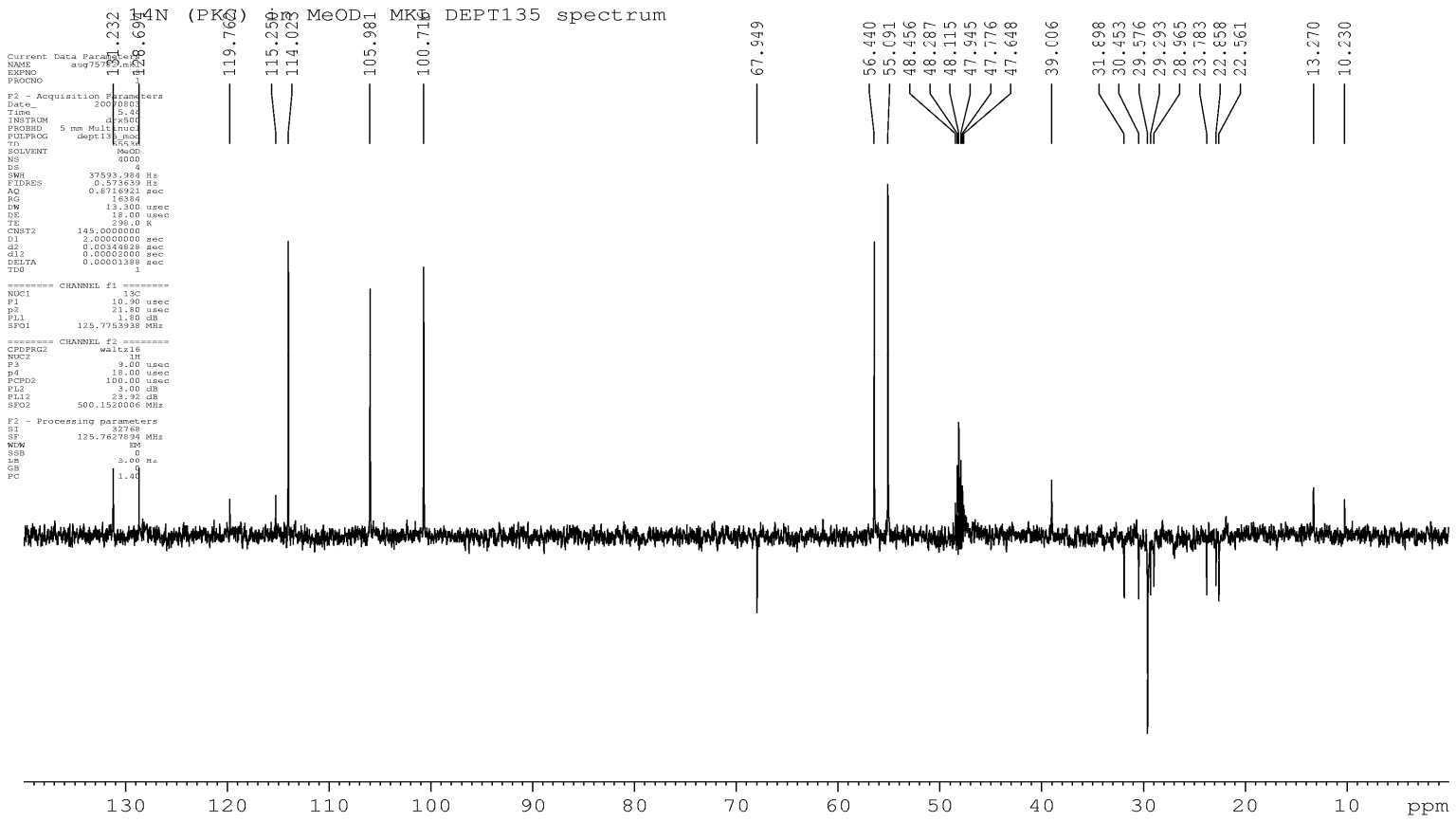



13C 14N (PKC) in MeOD MKL carbon spectrum
100.731
67.955
56.449
55.097
48.344
48.174
48.004
47.833
47.663
47.493
47.322
39.010
31.897
30.455
29.574
29.291
28.965
23.785
22.855
22.558
13.264
10.236
Current Data Parameters
NAME kg75782.mkl
EXPNO 5
PROCNO 1
F2 - Acquisition Parameters
Date_ 20070802
Time 18.43
INSTRUM drx500
PROBHD 5 mm Multinucl
PULPROG zgpg63
TD 65402
SOLVENT MeOD
NS 14000
DS 0
SWH 37593.984 Hz
FIDRES 0.574814 Hz
AQ 0.8699099 sec
RG 1824.6
DW 13.300 usec
DE 18.00 usec
TE 298.0 K
D1 1.12800002 sec
d11 0.03000000 sec
d12 0.00002000 sec
======== CHANNEL f1 ========
NUC1 13C
P1 10.90 usec
PL1 1.80 dB
SFO1 125.7753938 MHz
======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 100.00 usec
PL2 3.00 dB
PL12 23.92 dB
PL13 30.41 dB
SFO2 500.1525008 MHz
F2 - Processing parameters
SI 65536
SF 125.7627894 MHz
WDW EM
SSB 0
LB 3.00 Hz
GB 0
PC 0.50
100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 15 10 5 ppm

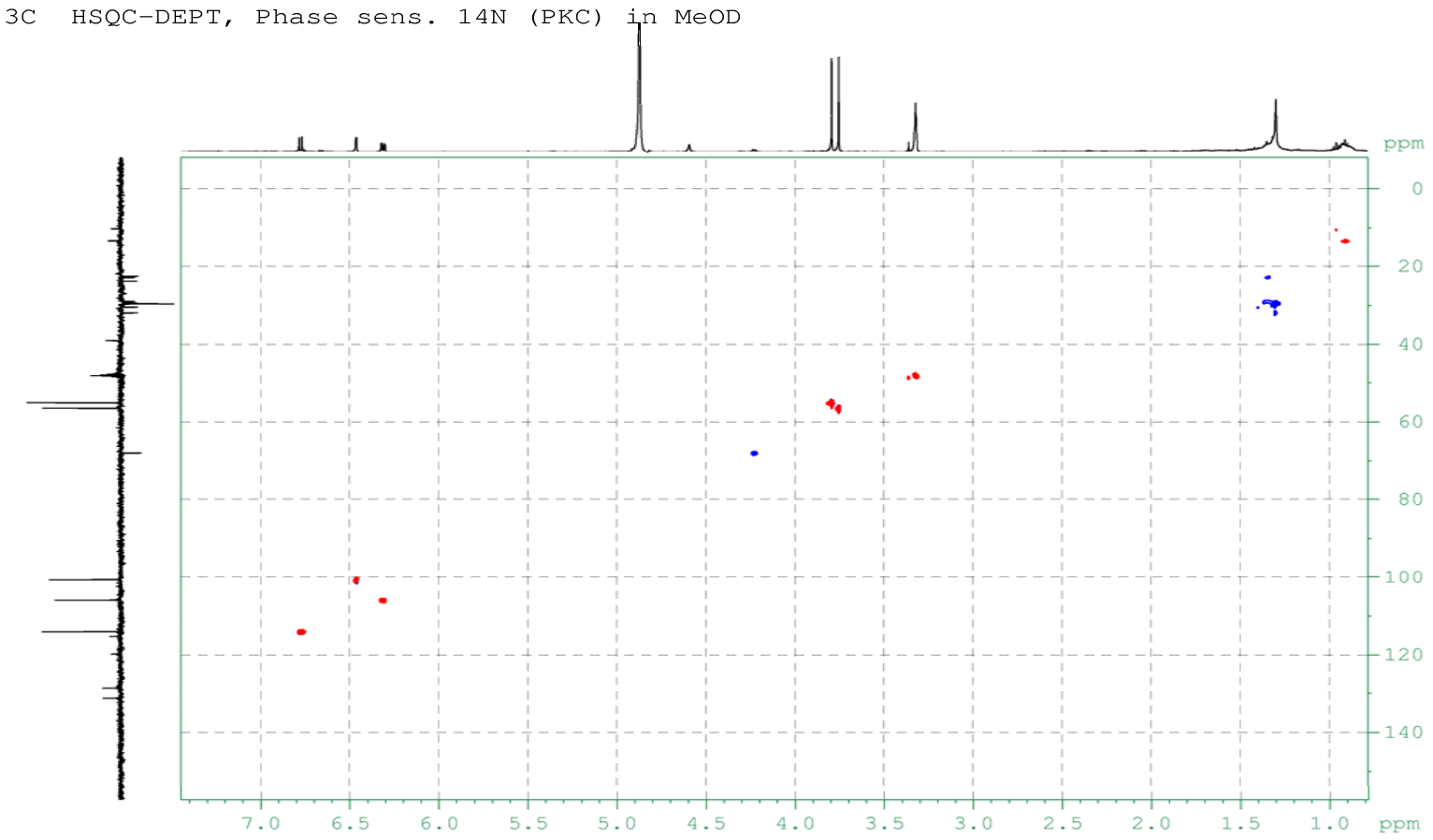

13C 14N (PKC) in MeOD MKL carbon spectrum
168.453
152.220
150.383
142.420
131.393
128.637
Current Data Parameters
NAME aug75782.mkl
EXPNO 5
PROCNO 1
F2 - Acquisition Parameters
Date_ 20070802
Time 18.43
INSTRUM drx500
PROBHD 5 mm Multinucl
PULPROG zgpg62
TD 65402
SOLVENT MeOD
NS 14000
DS 0
SWH 37593.984 Hz
FIDRES 0.574814 Hz
AQ 0.8699099 sec
RG 1824.6
DW 13.300 usec
DE 18.00 usec
TE 298.0 K
D1 1.12800002 sec
d11 0.03000000 sec
d12 0.00002000 sec
======== CHANNEL f1 ========
NUC1 13C
P1 10.90 usec
PL1 1.80 dB
SFO1 125.7753938 MHz
======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 100.00 usec
PL2 3.00 dB
PL12 23.92 dB
PL13 30.41 dB
SFO2 500.1525008 MHz
F2 - Processing parameters
SI 65536
SF 125.7627894 MHz
WDW EM
SSB 0
LB 3.00 Hz
GB 0
PC 0.50
170
165
160
155
150
145
140
135
130
ppm


13C 14N (PKC) in MeOD MKL carbon spectrum
114.042
105.992
119.442
115.462
Current Data Parameters
NAME aug75782.mk1
EXPNO 5
PROCNO 1
F2 - Acquisition Parameters
Date_ 20070802
Time 18.43
INSTRUM drx500
PROBHD 5 mm Multinucl
PULPROG zgpg62
TD 65402
SOLVENT MeOD
NS 14000
DS 0
SWH 37593.984 Hz
FIDRES 0.574814 Hz
AQ 0.8699099 sec
RG 1824.6
DW 13.300 usec
DE 18.00 usec
TE 298.0 K
D1 1.12800002 sec
d11 0.03000000 sec
d12 0.00002000 sec
======== CHANNEL f1 ========
NUC1 13C
P1 10.90 usec
PL1 1.80 dB
SFO1 125.7753938 MHz
======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 100.00 usec
PL2 3.00 dB
PL12 23.92 dB
PL13 30.41 dB
SFO2 500.1525008 MHz
F2 - Processing parameters
SI 65536
SF 125.7627894 MHz
WDW EM
SSB 0
LB 3.00 Hz
GB 0
PC 0.50
121 120 119 118 117 116 115 114 113 112 111 110 109 108 107 106 105 104 103 ppm

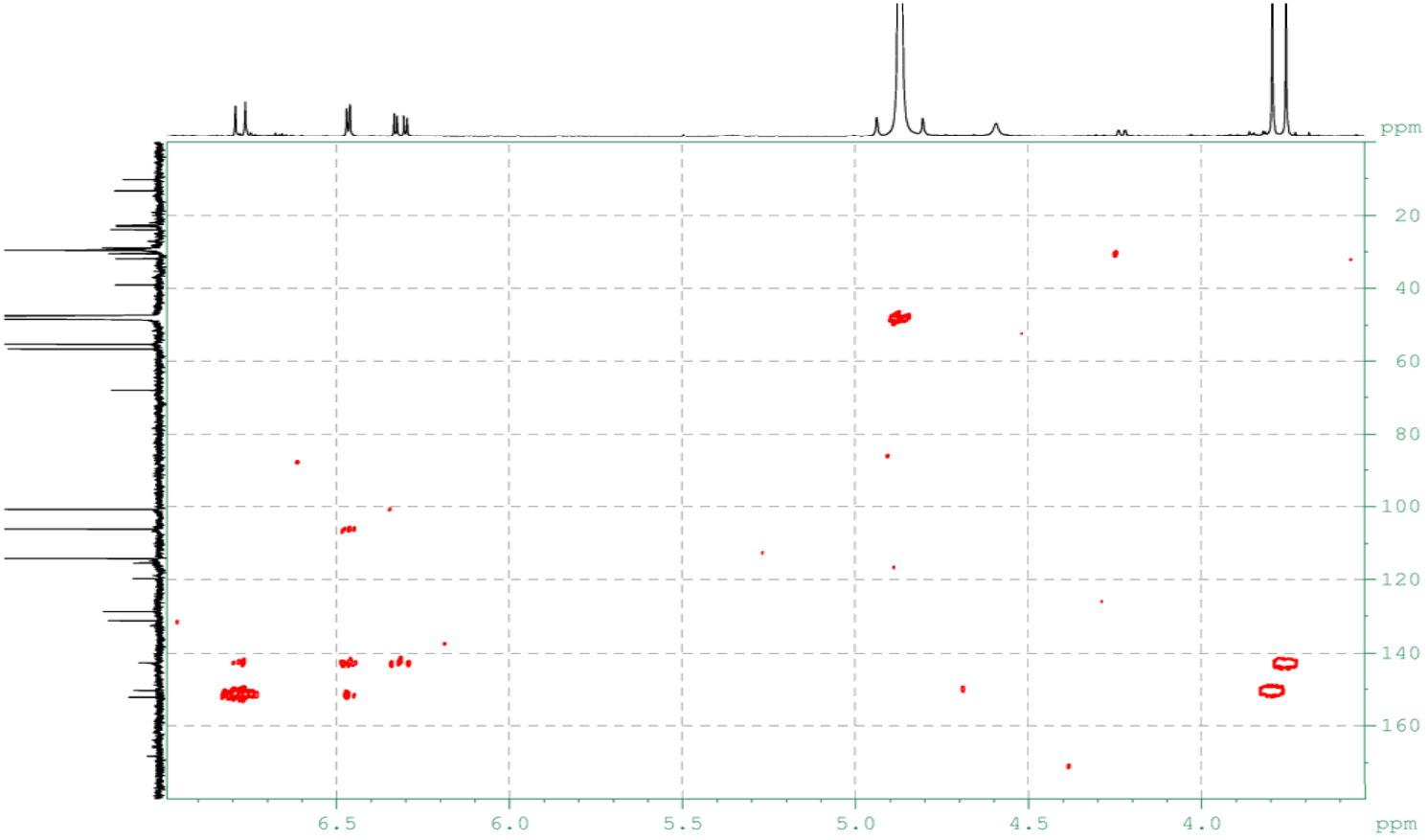
Appendix X: ^{13}C-DEPT for JO5182k14N



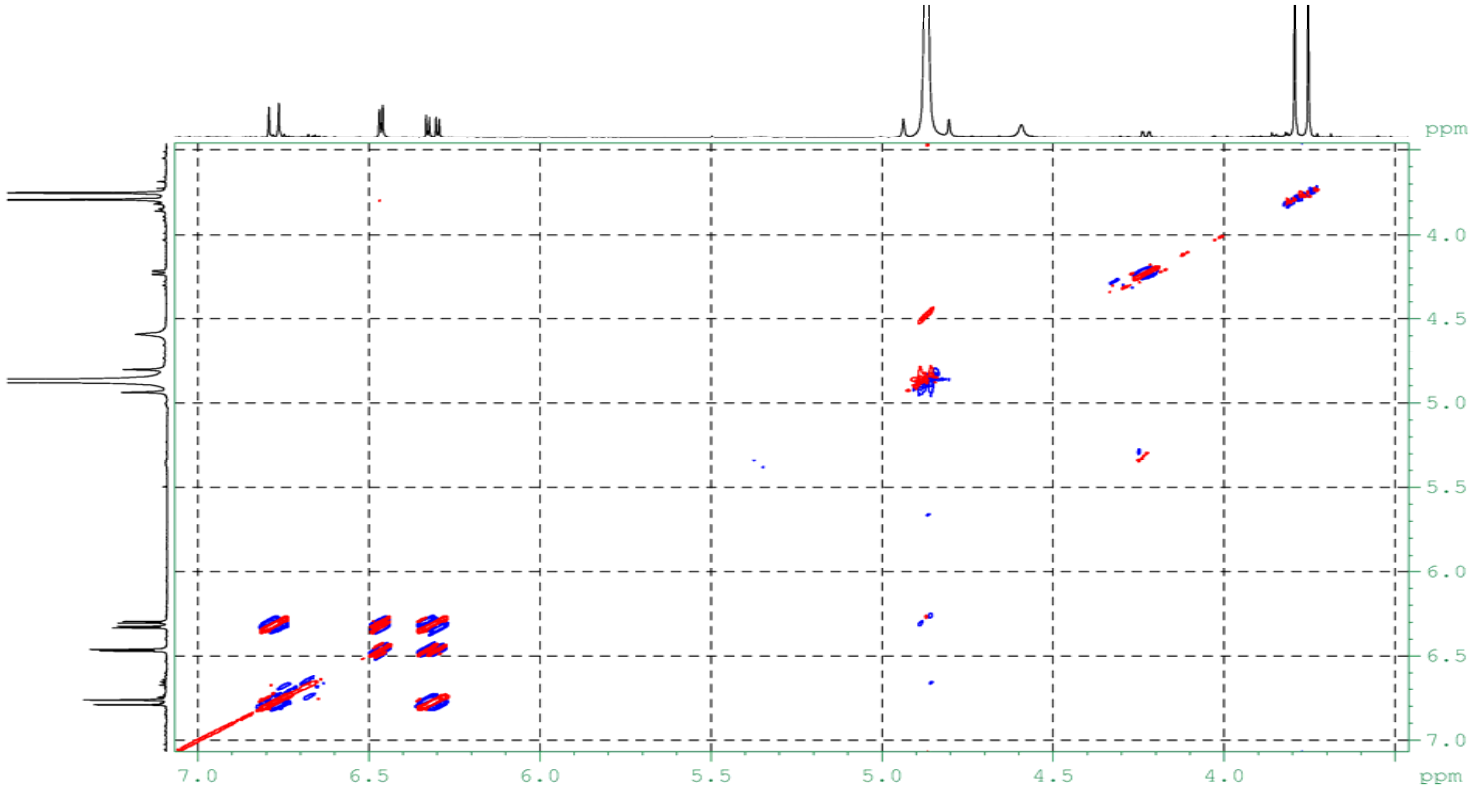
Appendix XI: HSQC – DEPT for JO5182K14N



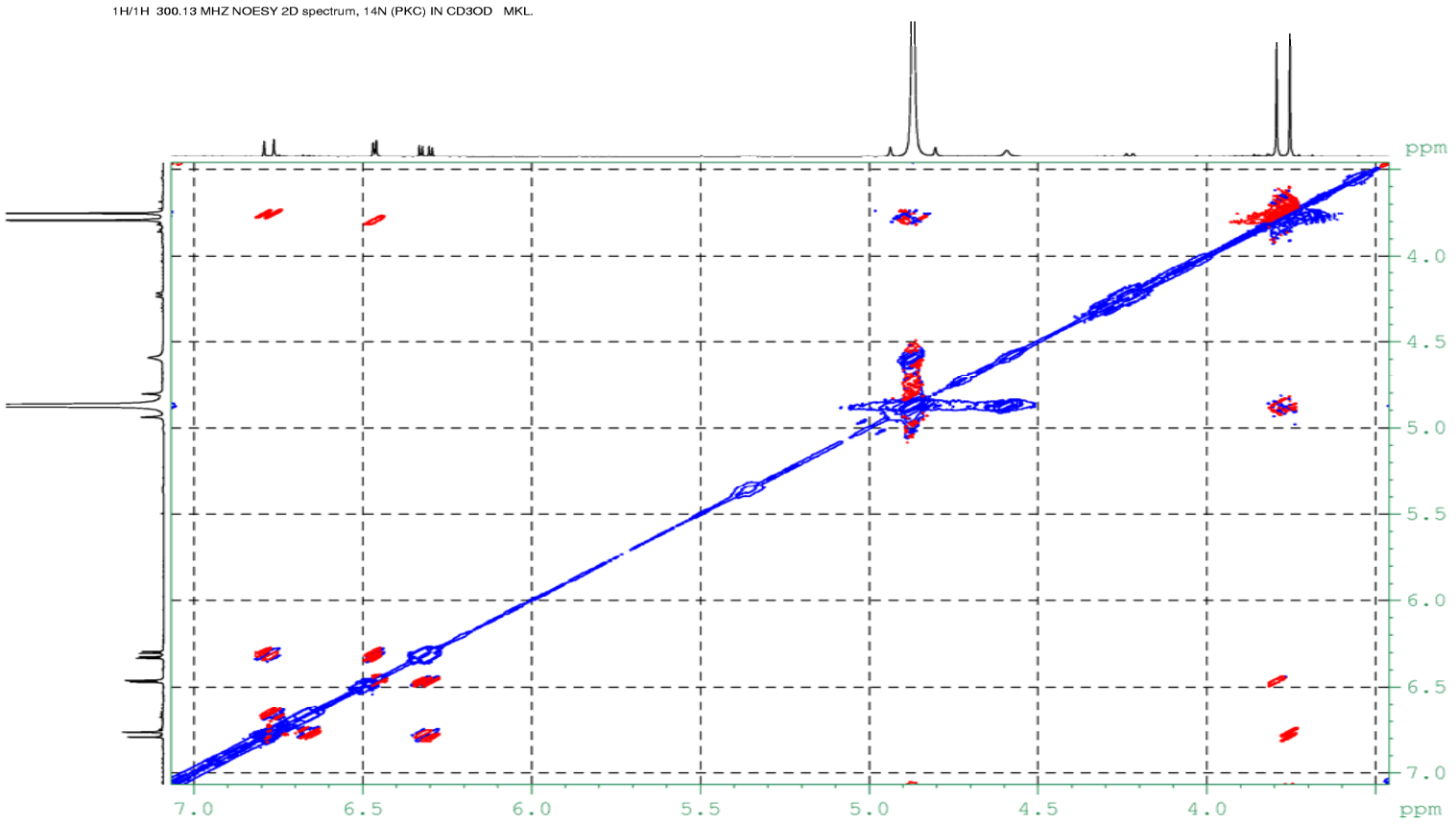
Appendix XII: HMBC spectrum for JO5182M14N



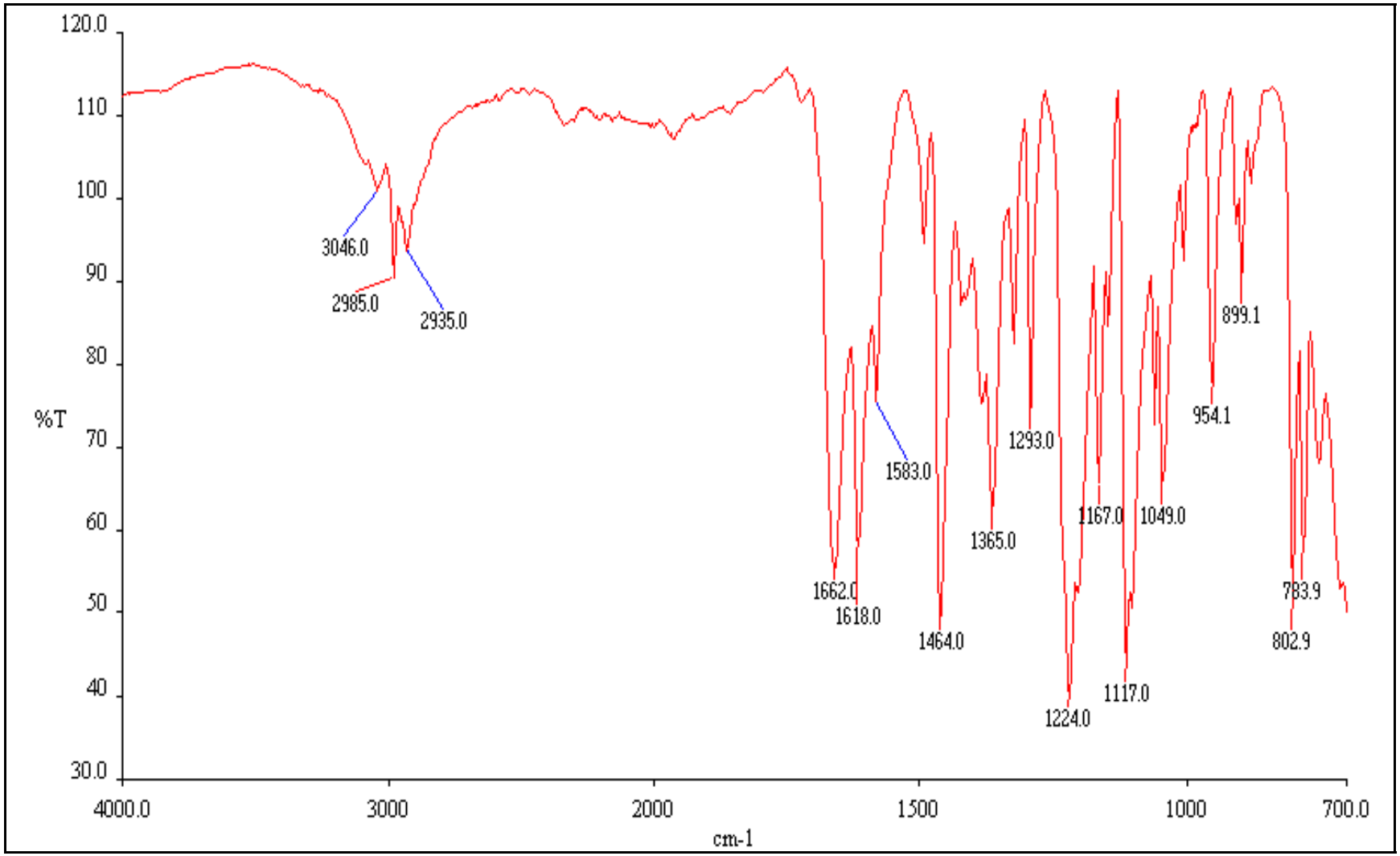
Appendix XIII: COSY spectrum for JO5182M14N



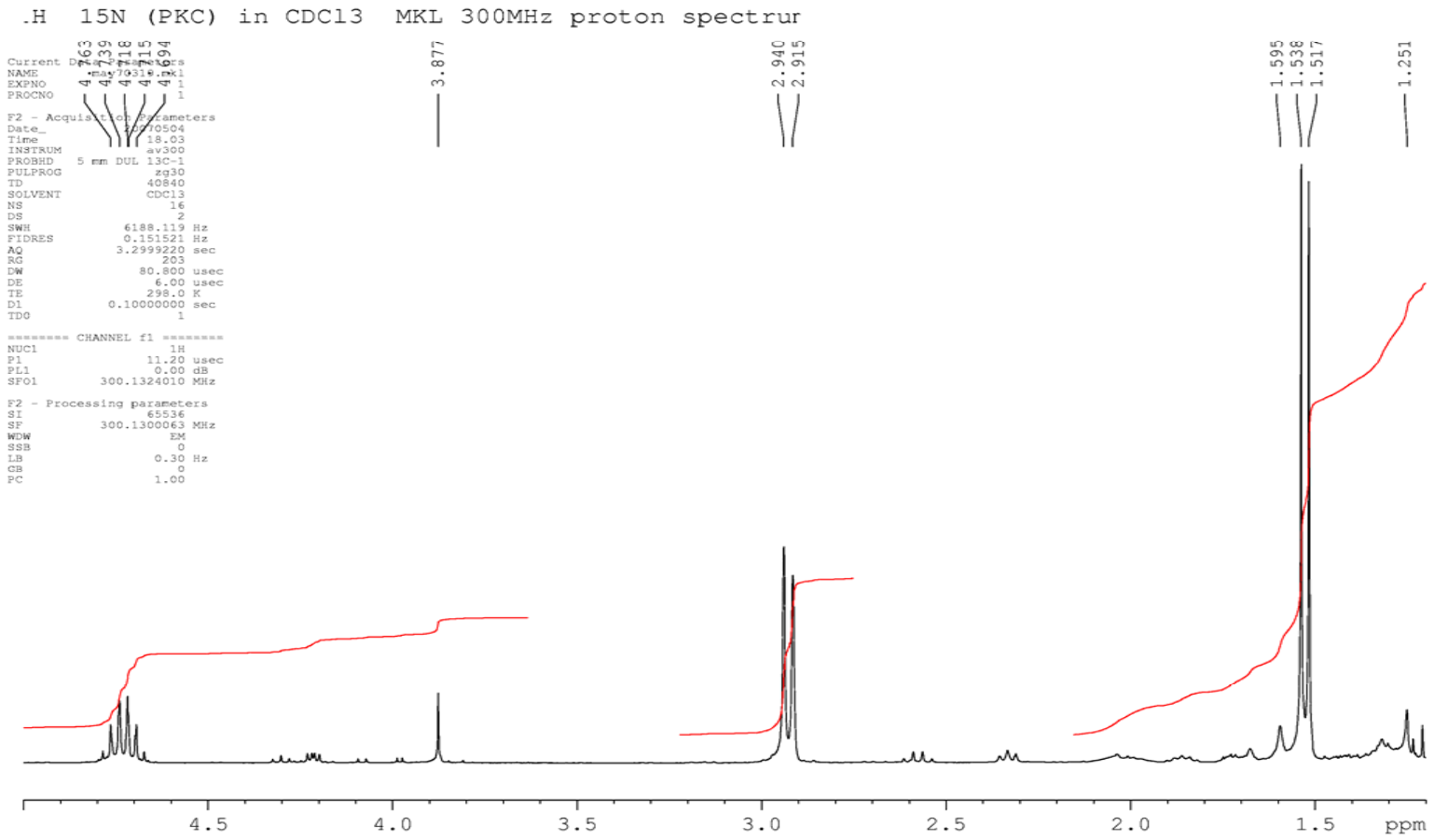
Appendix XIV: NOESY spectrum for JO5182M14N

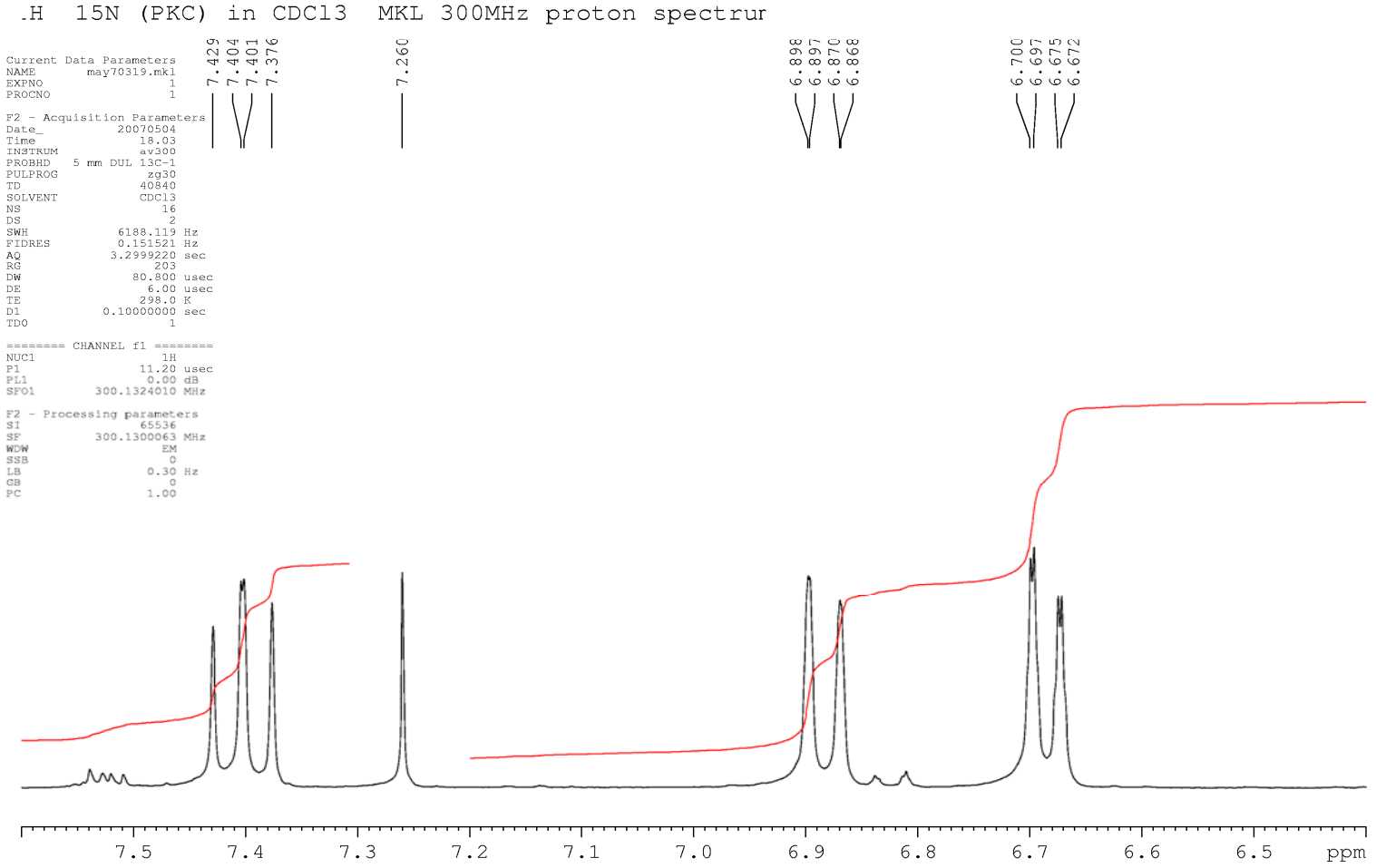


Appendix XV: IR spectrum for JO5182M15N

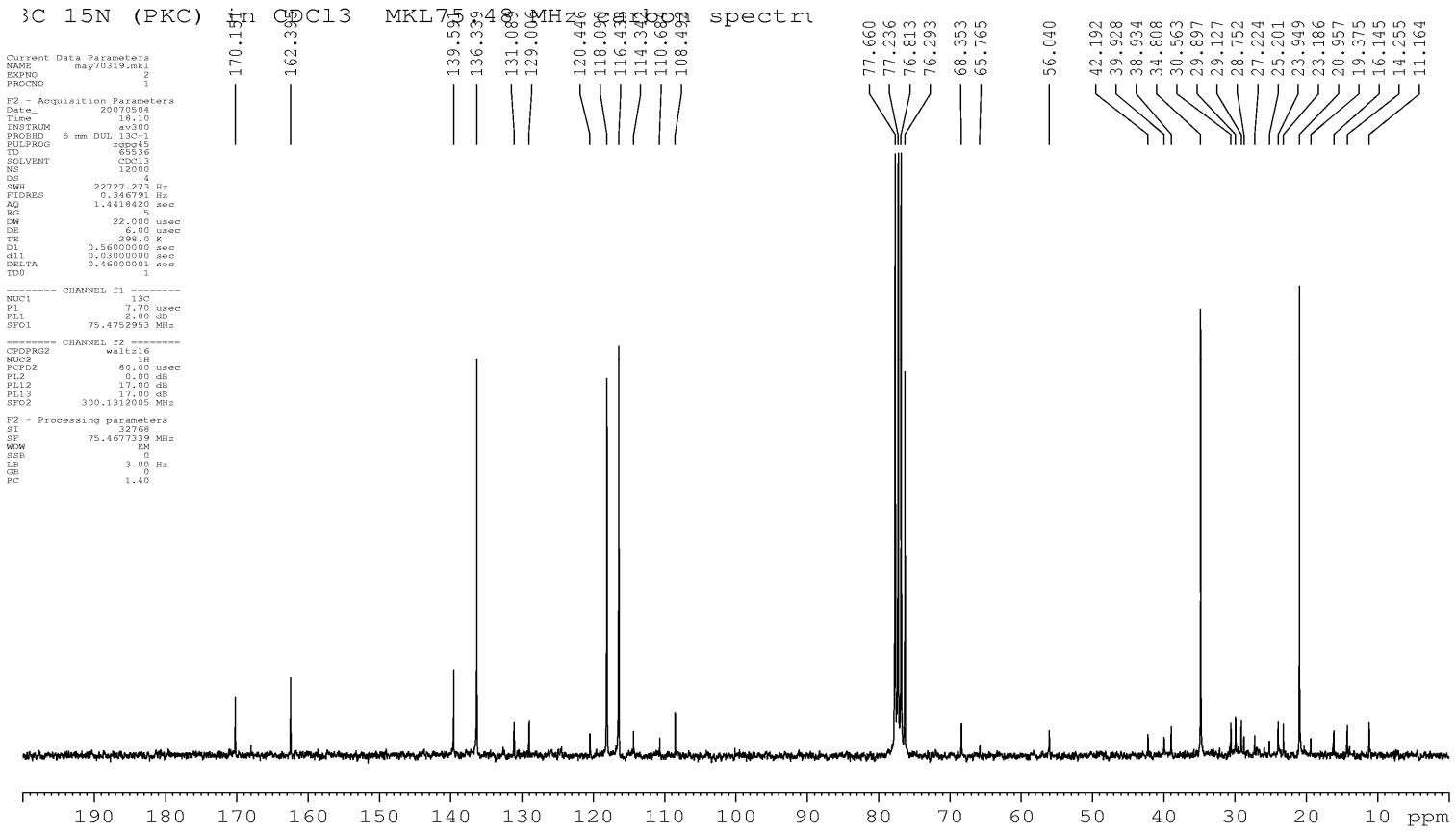


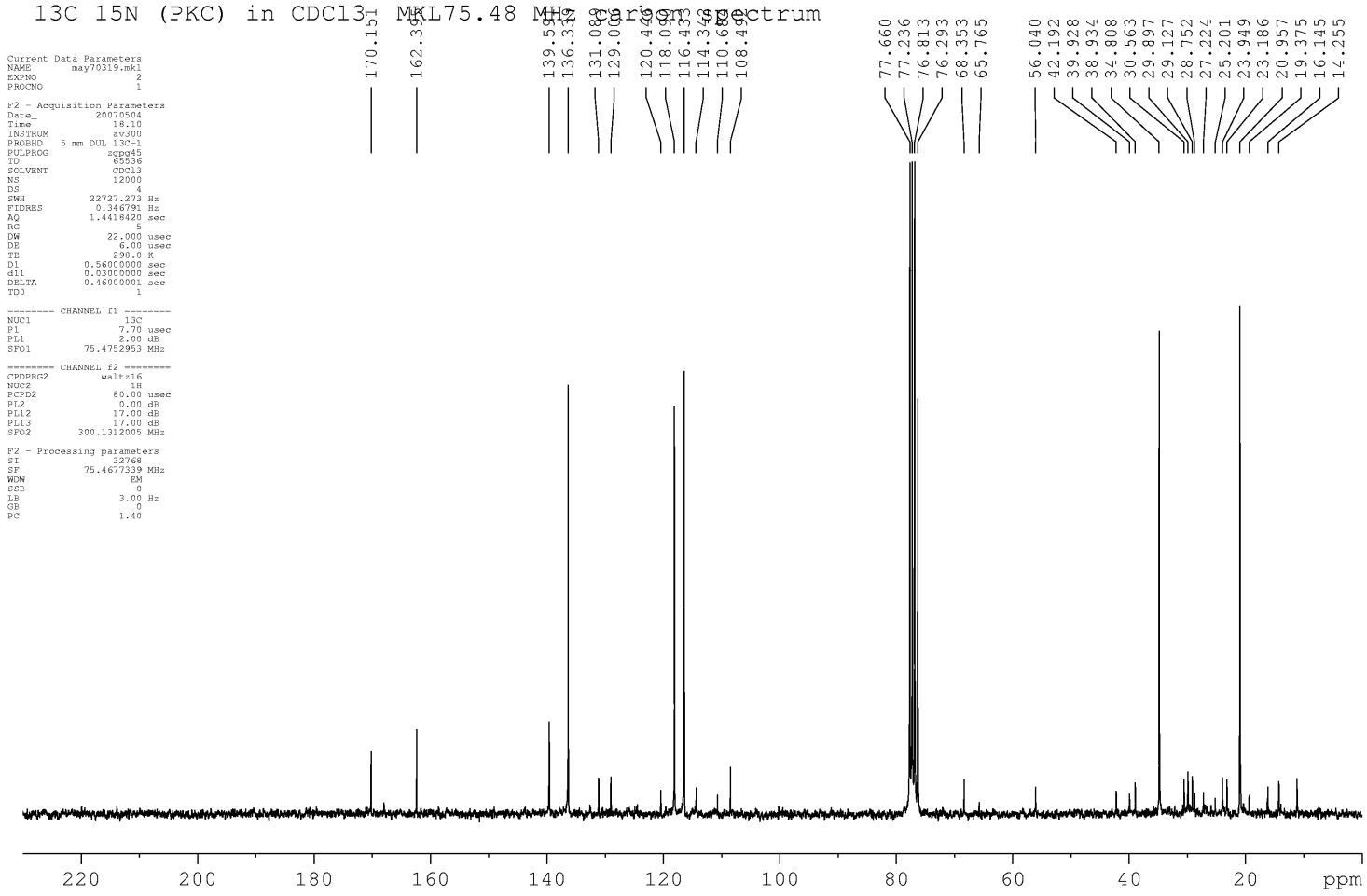
Appendix XVI: 1H-NMR spectrum for JO5182K15N



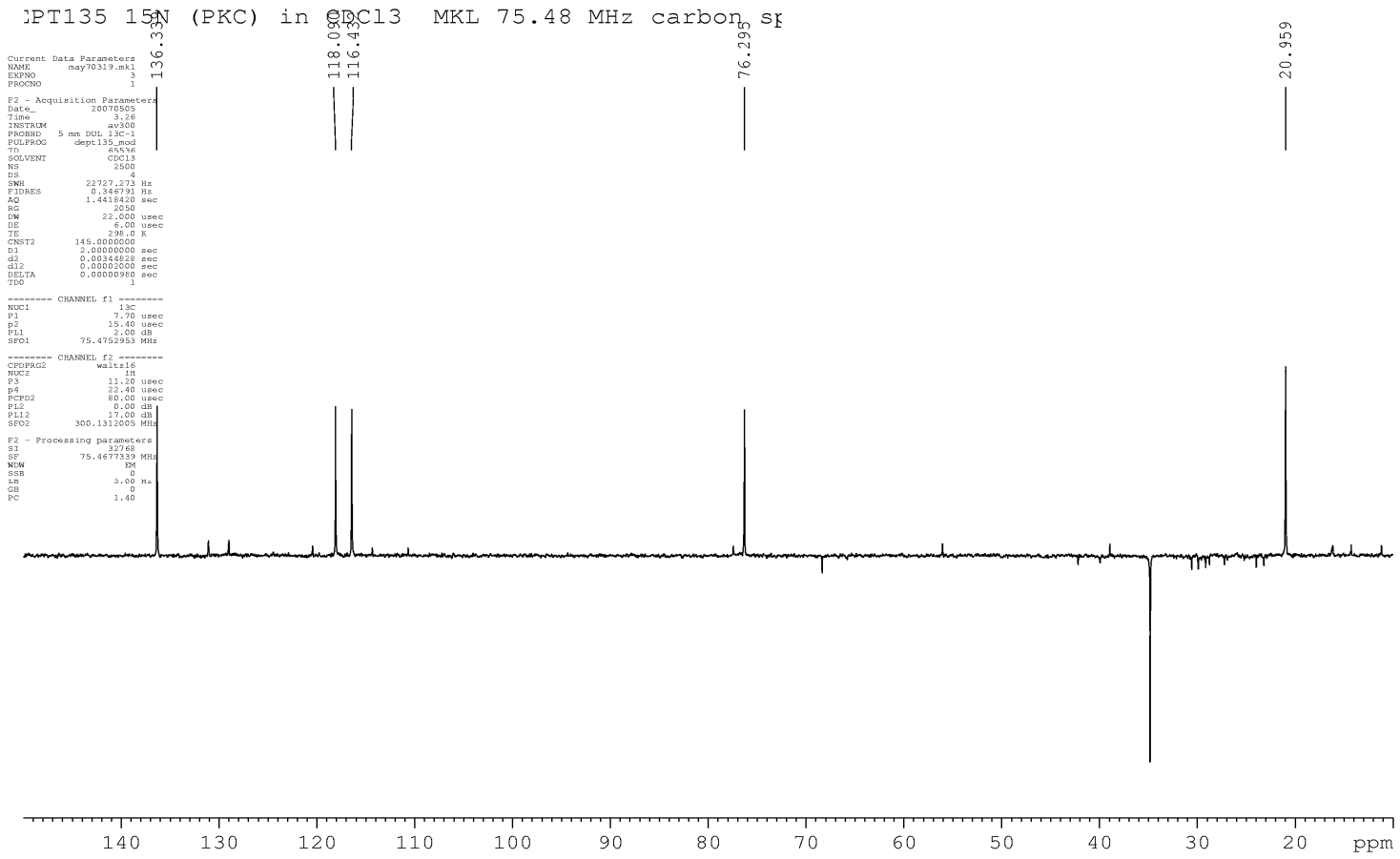

.H 15N (PKC) in CDCl3 MKL 300MHz proton spectrur
Current Data Parameters
NAME may70319.mkl
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20070504
Time 18.03
INSTRUM av300
PROBHD 5 mm DUL 13C-1
PULPROG zg30
TD 40840
SOLVENT CDCl3
NS 16
DS 2
SWH 6188.119 Hz
FIDRES 0.151521 Hz
AQ 3.2999220 sec
RG 203
DW 80.800 usec
DE 6.00 usec
TE 298.0 K
D1 0.10000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 11.20 usec
PL1 0.00 dB
SFO1 300.1324010 MHz
F2 - Processing parameters
SI 65536
SF 300.1300063 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
7.429
7.404
7.401
7.376
7.260
6.898
6.897
6.870
6.868
6.700
6.697
6.675
6.672
7.5
7.4
7.3
7.2
7.1
7.0
6.9
6.8
6.7
6.6
6.5
ppm

Appendix XVII: ^{13}C spectrum for JO5182K14N

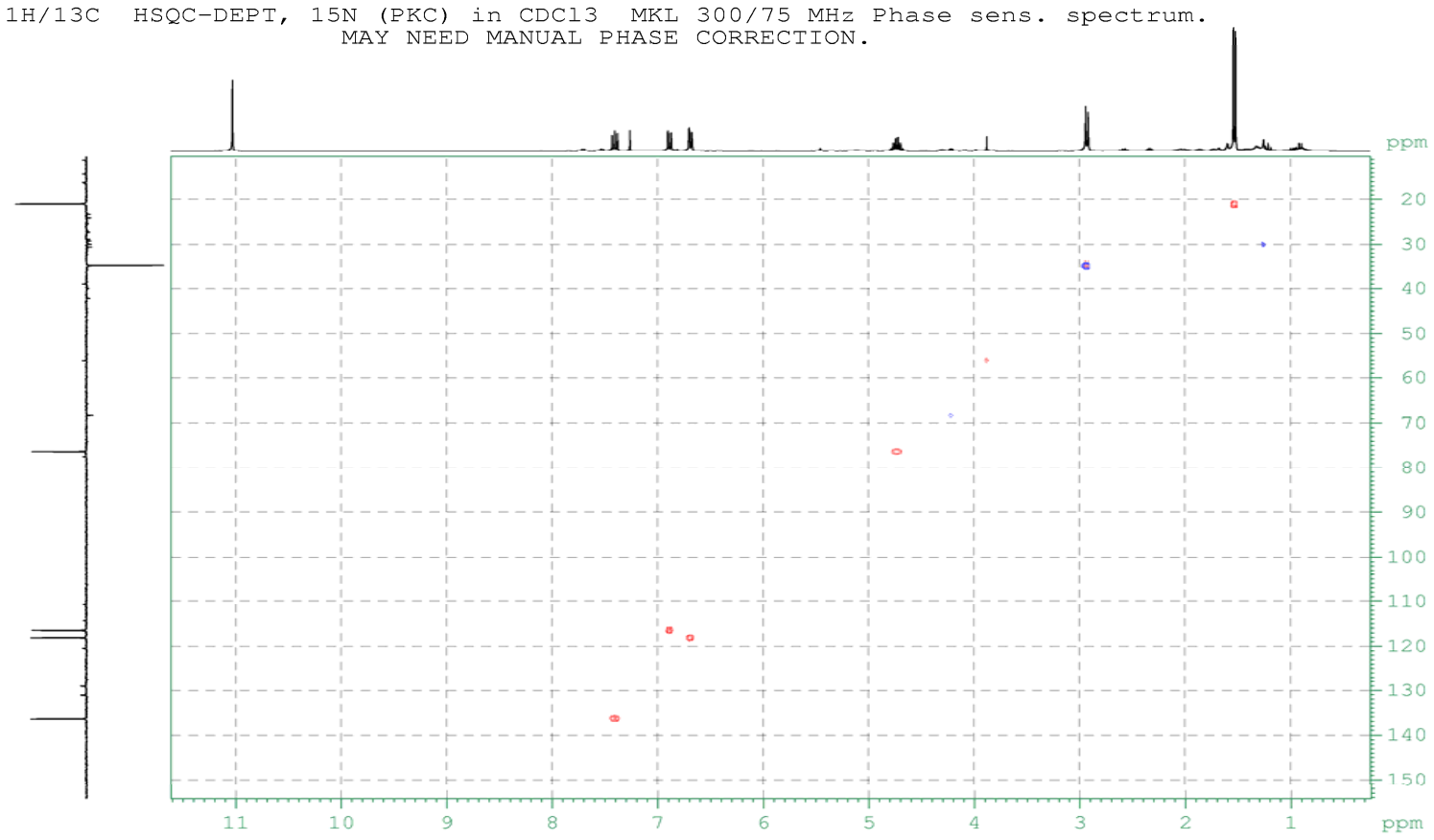



13C 15N (PKC) in CDCl3 MKL75.48 MHz carbon spectrum
Current Data Parameters
NAME may70319.mk1
EXPNO 2
PROCNO 1
F2 - Acquisition Parameters
Date_ 20070504
Time 18.10
INSTRUM av300
PROBHD 5 mm DUL 13C-1
PULPROG zgpg45
TD 65536
SOLVENT CDCl3
NS 12000
DS 4
SWH 22727.273 Hz
FIDRES 0.346791 Hz
AQ 1.4418420 sec
RG 5
DW 22.000 usec
DE 6.00 usec
TE 298.0 K
D1 0.56000000 sec
d11 0.03000000 sec
DELTA 0.46000001 sec
TD0 1
======== CHANNEL f1 ========
NUC1 13C
P1 7.70 usec
PL1 2.00 dB
SFO1 75.4752953 MHz
======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 0.00 dB
PL12 17.00 dB
PL13 17.00 dB
SFO2 300.1312005 MHz
F2 - Processing parameters
SI 32768
SF 75.4677339 MHz
WDW EM
SSB 0
LB 3.00 Hz
GB 0
PC 1.40
170.151
162.399
139.599
136.333
131.088
129.000
120.440
118.093
116.433
114.343
110.680
108.490
77.660
77.236
76.813
76.293
68.353
65.765
56.040
42.192
39.928
38.934
34.808
30.563
29.897
29.127
28.752
27.224
25.201
23.949
23.186
20.957
19.375
16.145
14.255
220
200
180
160
140
120
100
80
60
40
20
ppm

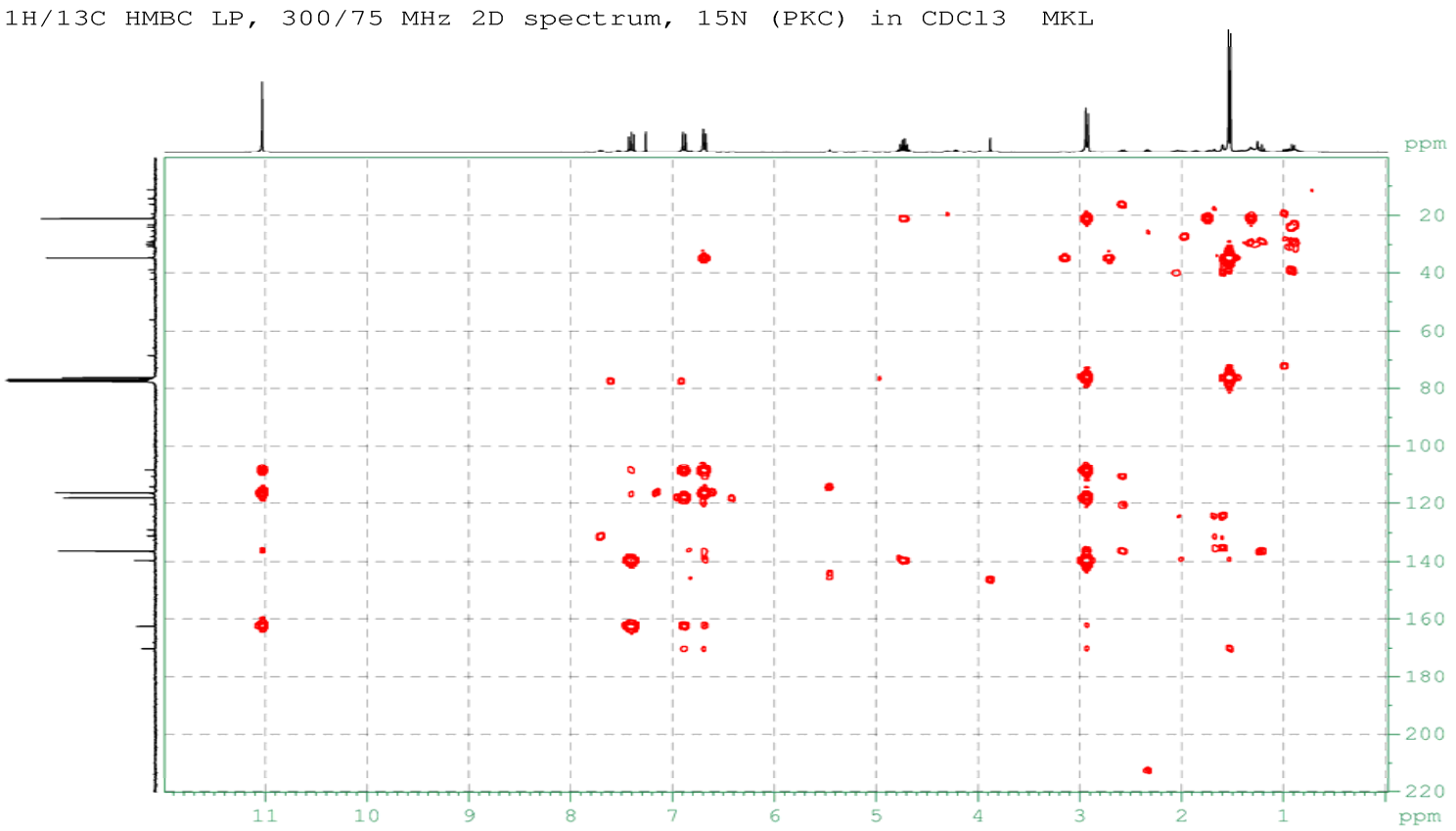
Appendix XVIII: DEPT spectrum for JO5182K15N



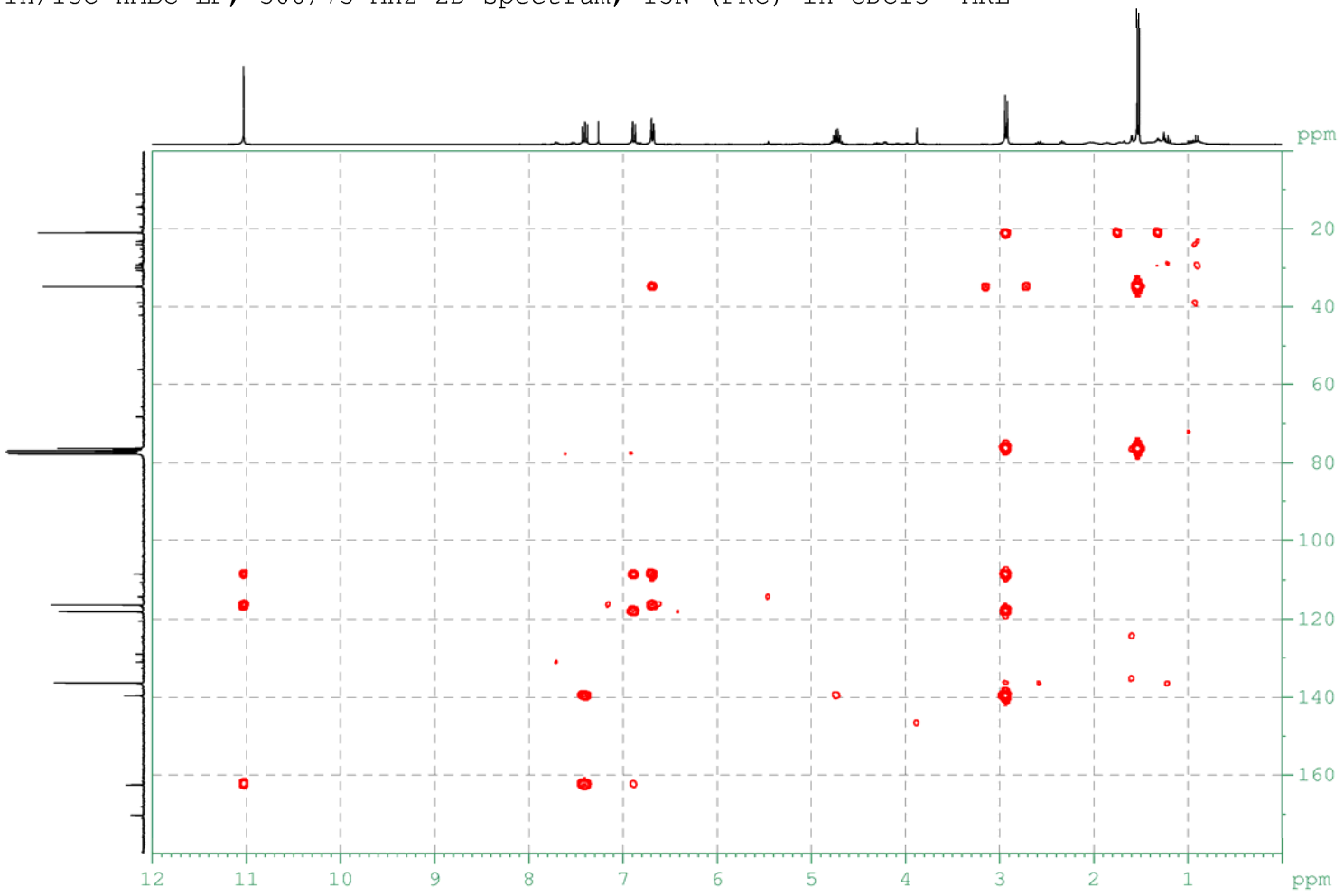
Appendix XIX: HSQC-DEPT for JO5182K15N

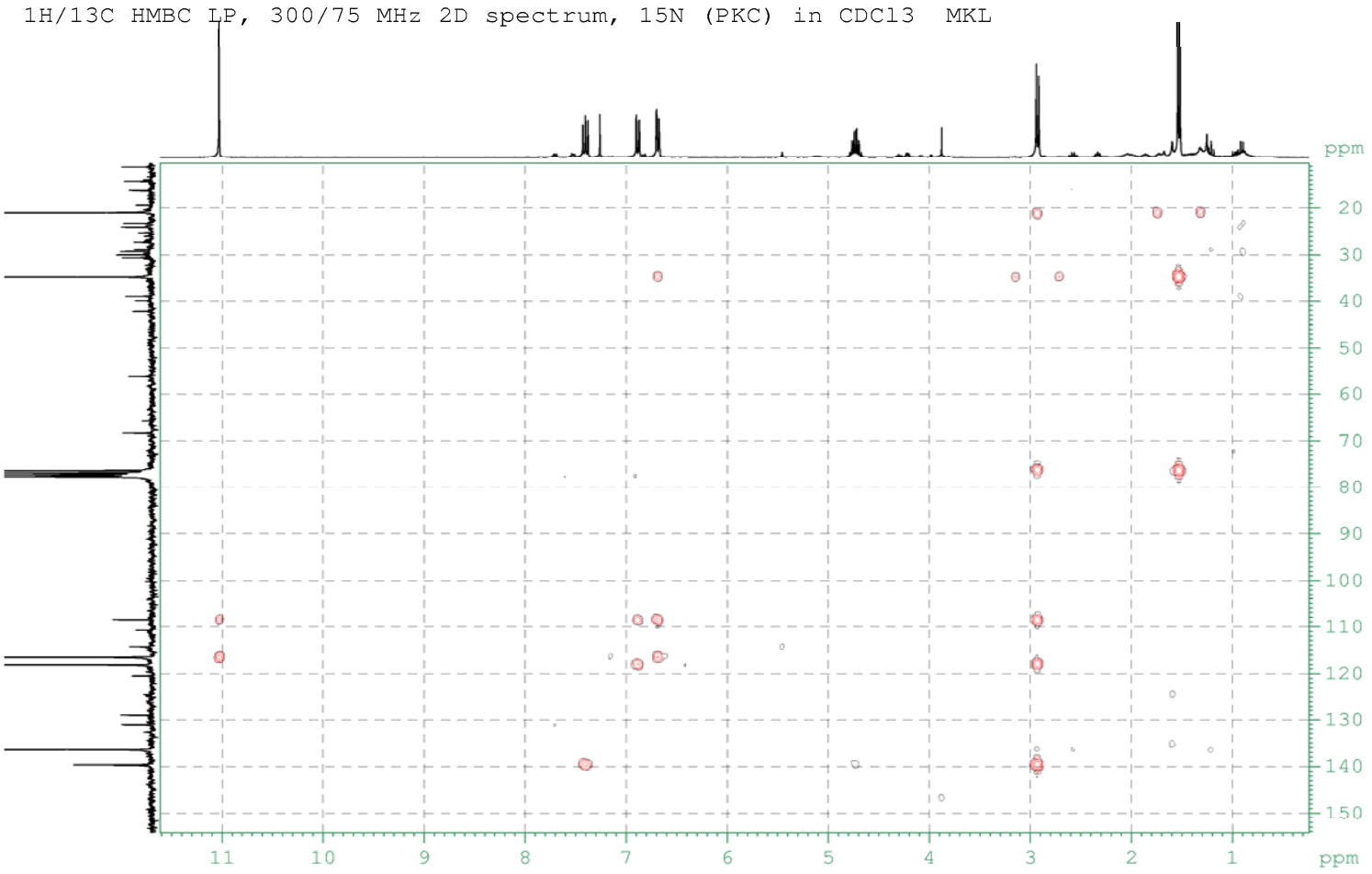


Appendix XX: HMBC spectrum for JO5182K15N

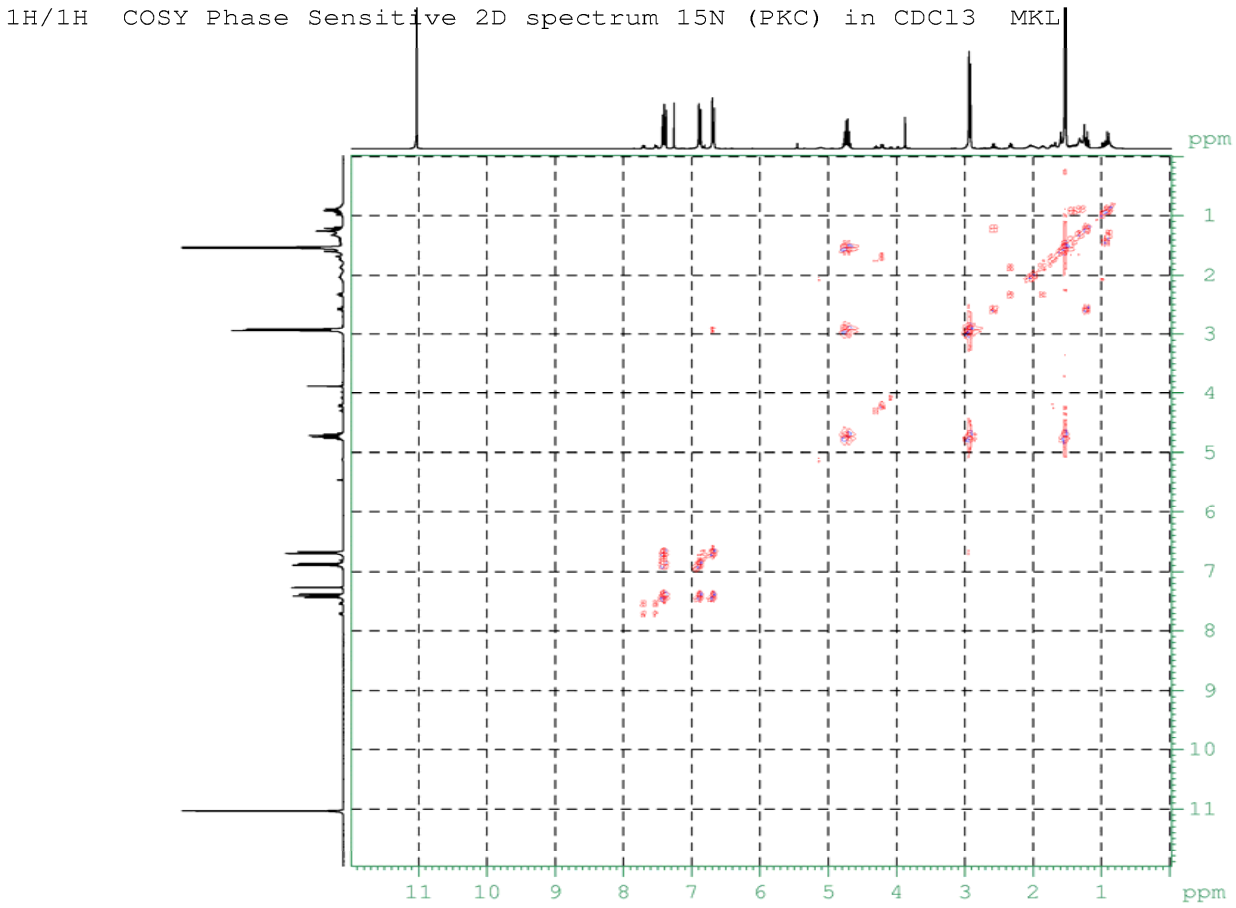


1H/13C HMBC LP, 300/75 MHz 2D spectrum, 15N (PKC) in CDCl3 MKL

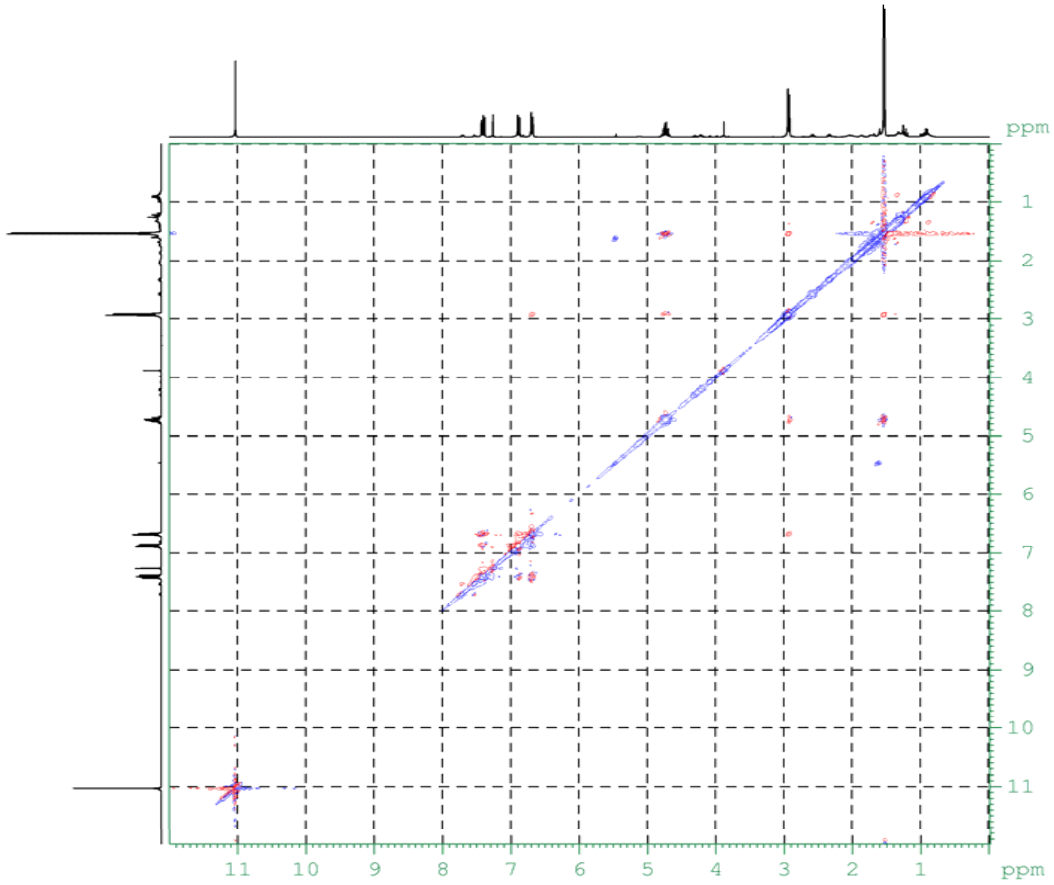



1H/13C HMBC LP, 300/75 MHz 2D spectrum, 15N (PKC) in CDCl3 MKL
ppm
20
30
40
50
60
70
80
90
100
110
120
130
140
150
11
10
9
8
7
6
5
4
3
2
1
ppm

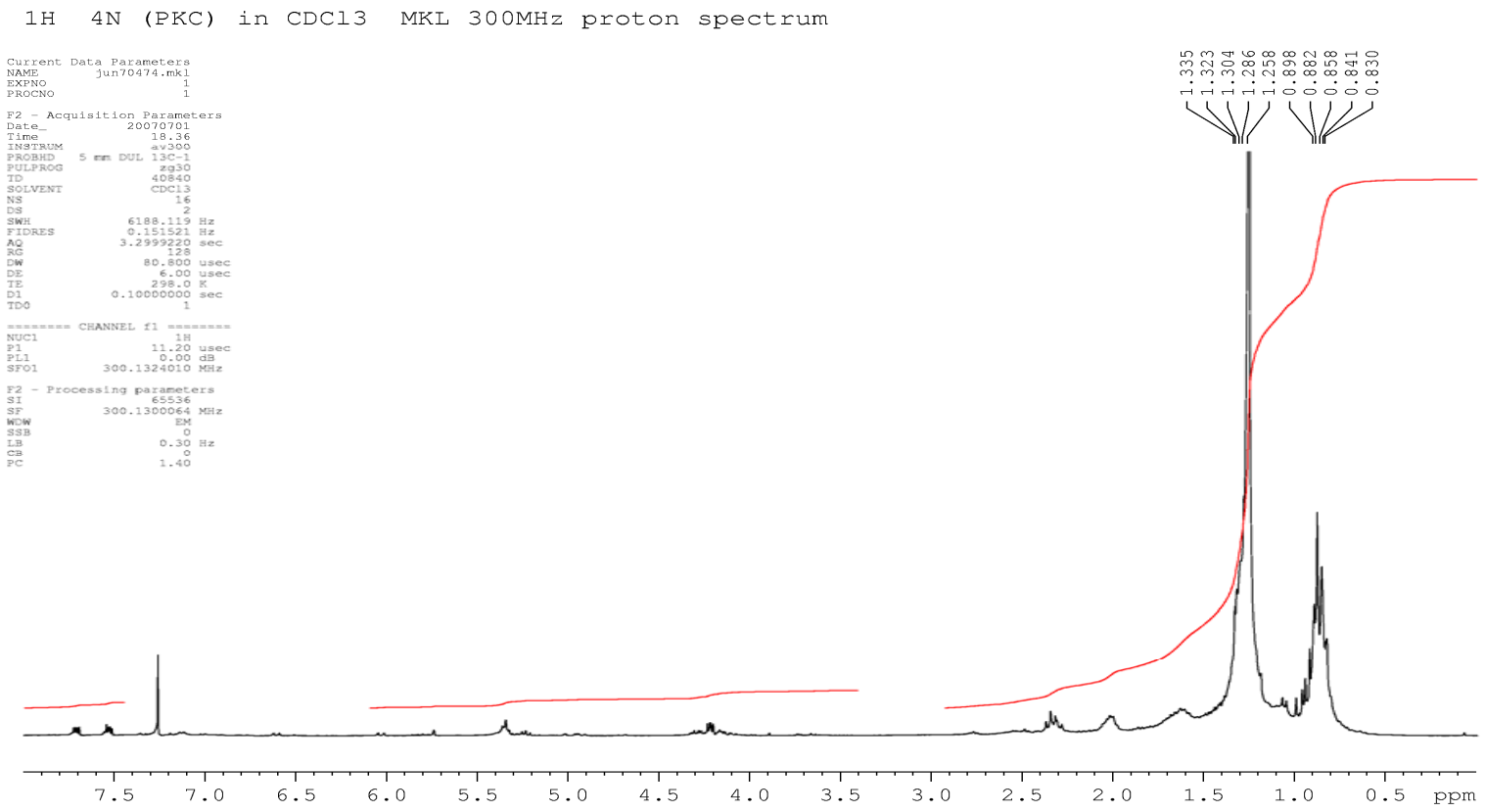
Appendix XXI: COSY spectrum for JO5182K15N



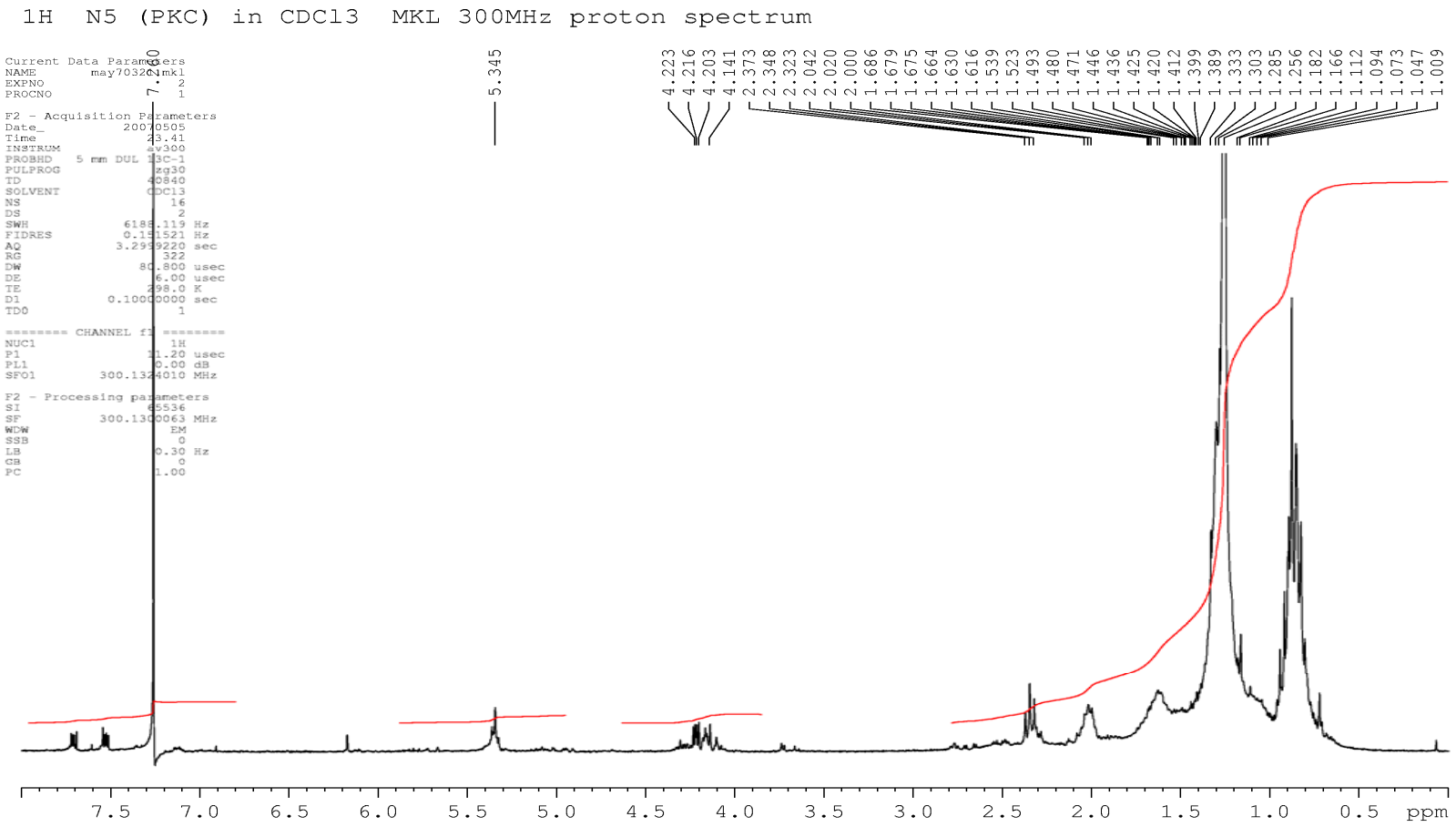
Appendix XXII: NOESY spectrum for JO5182K15N



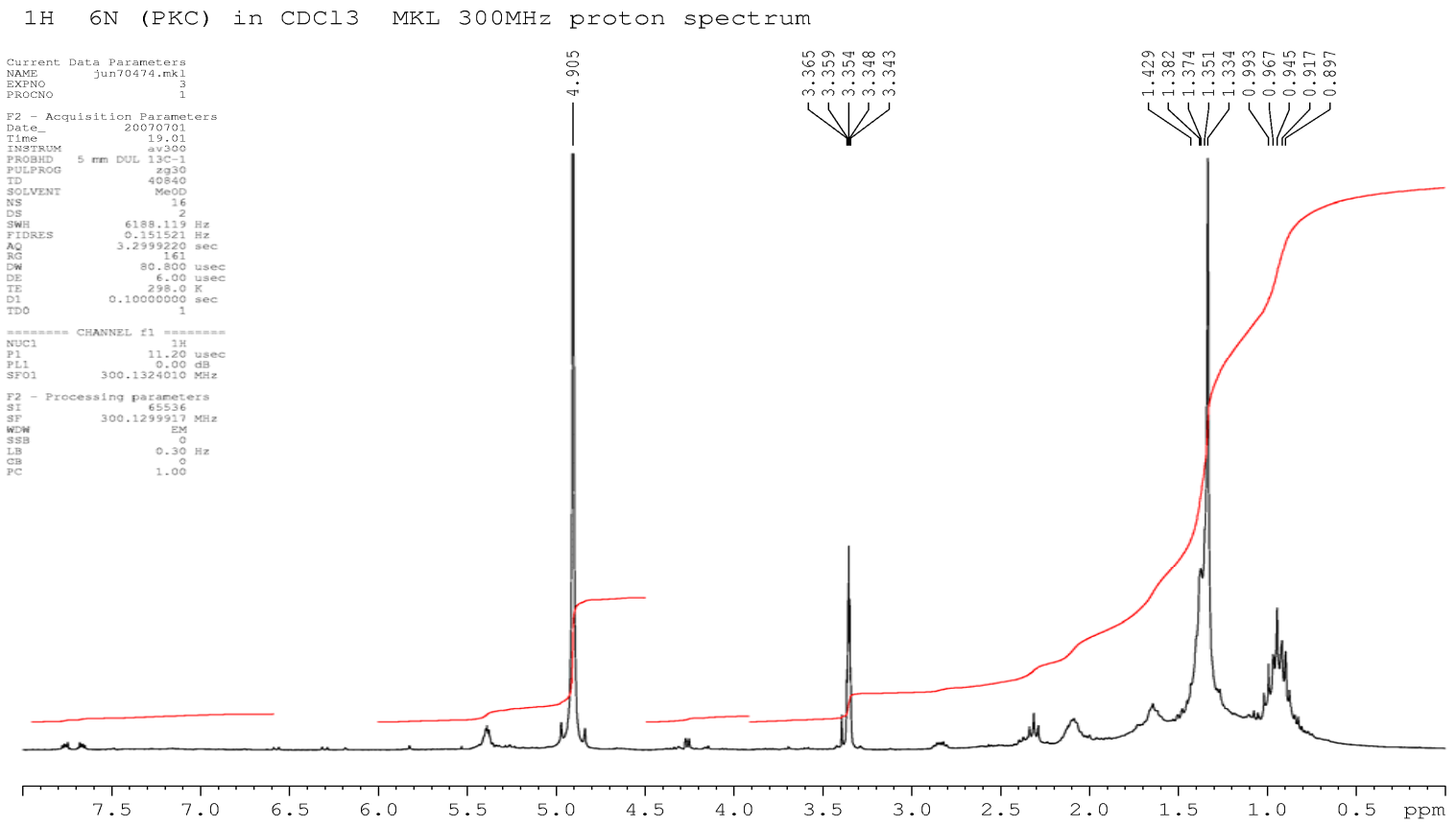
Appendix XXIII: ^{1}H NMR spectra for JO5182M04N



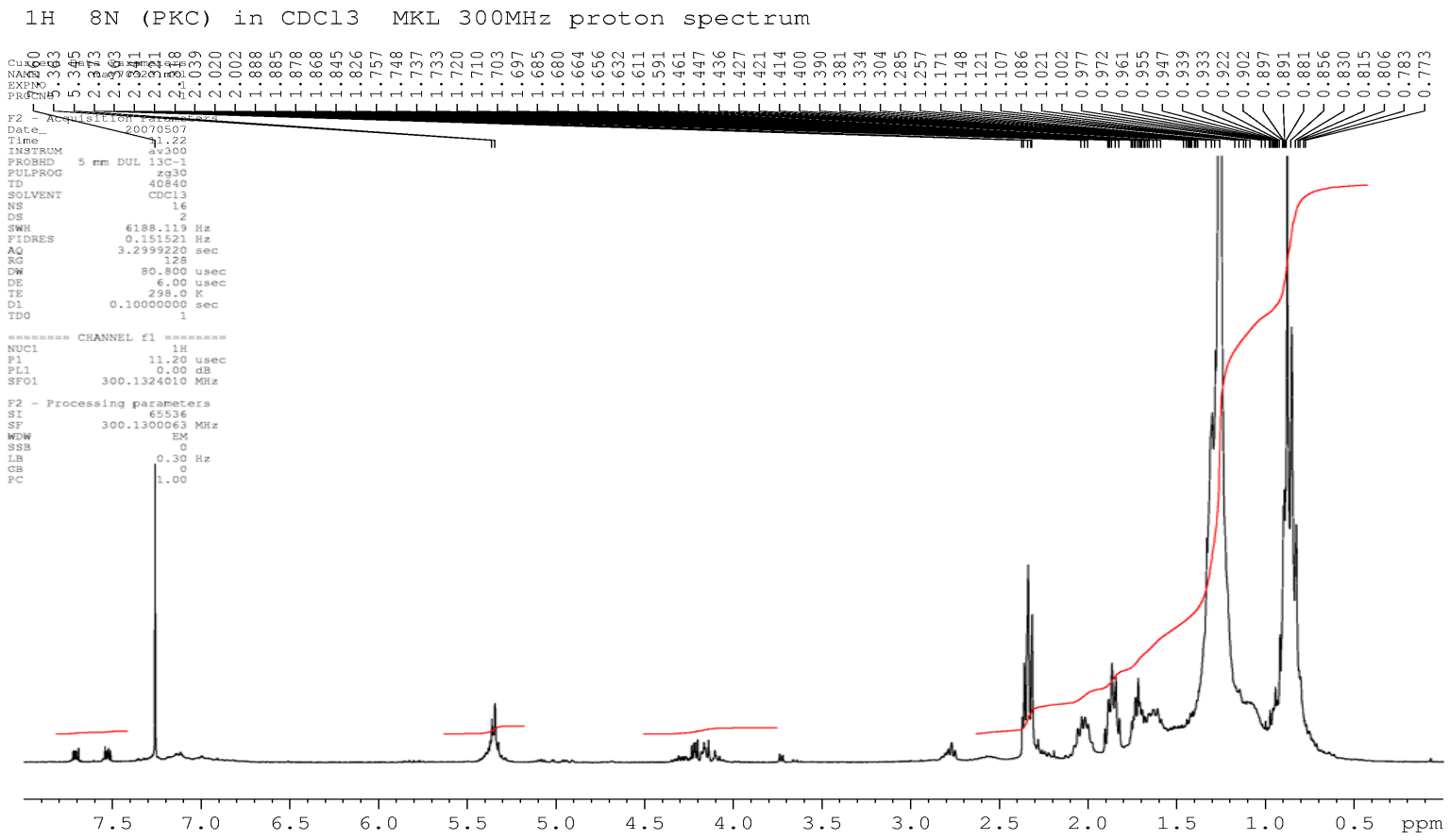
Appendix XXIV: ^{1}H-NMR spectra for JO5182M05



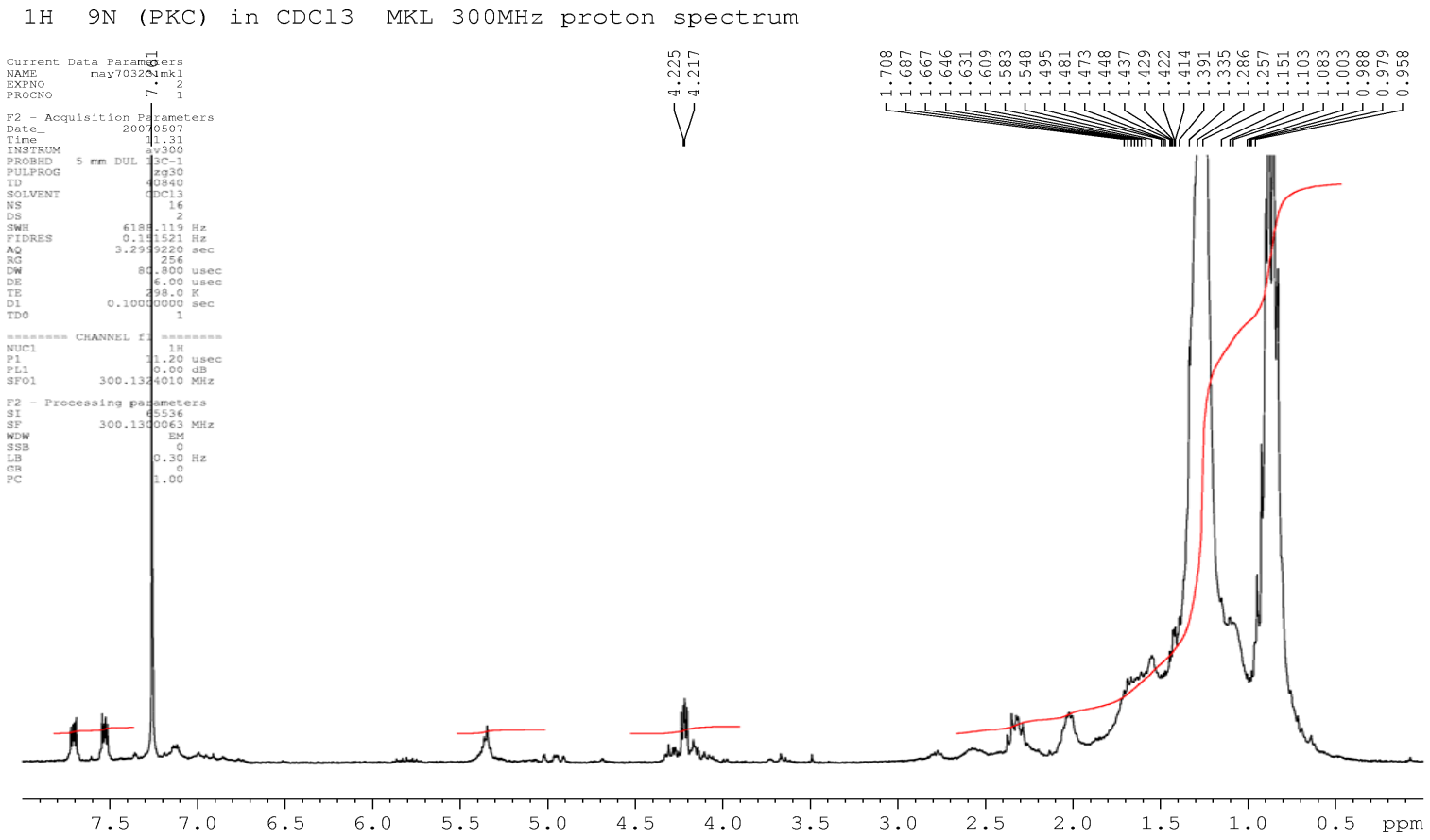
Appendix XXV: ^{1}H- NMR spectrum for JO5182M06N



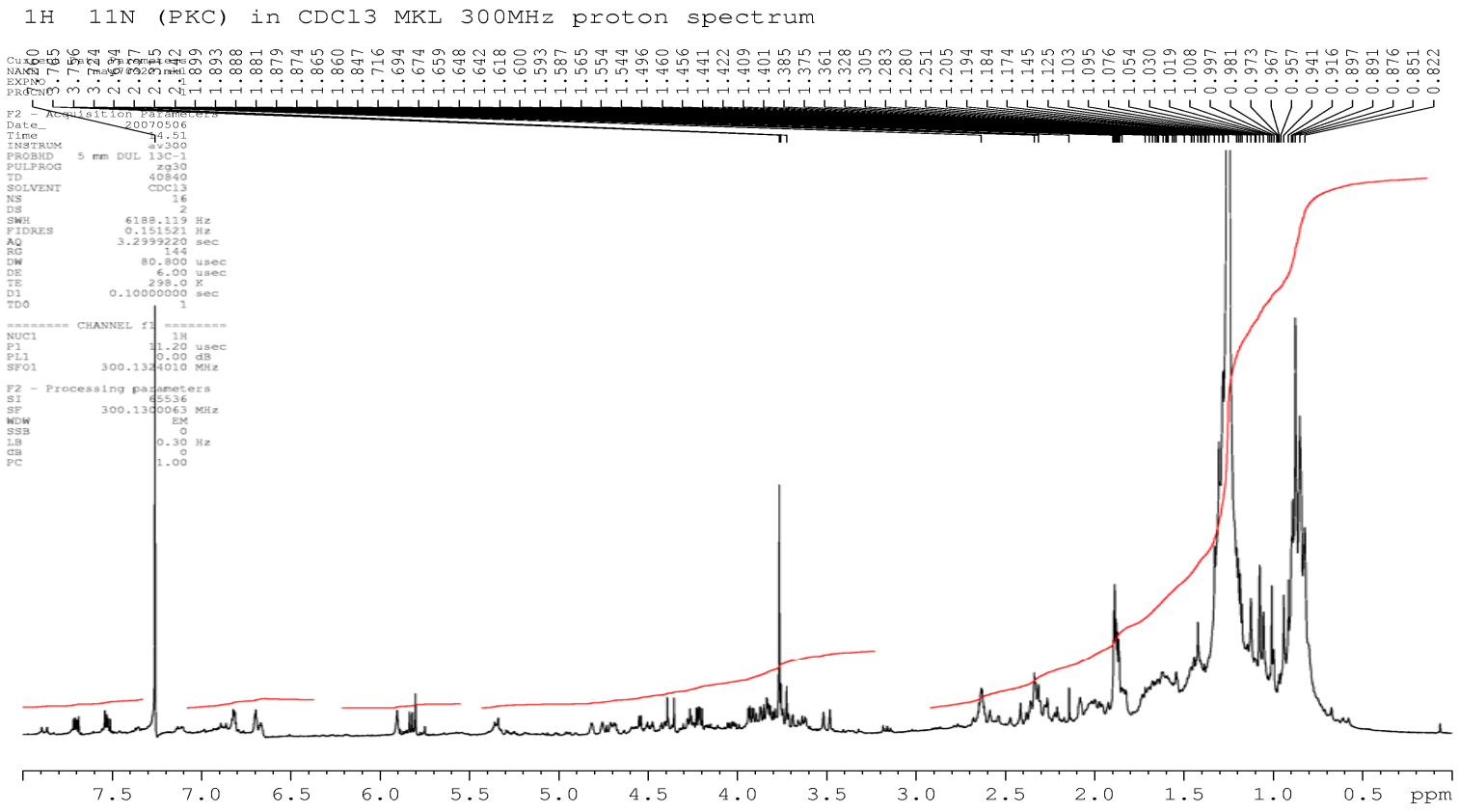
Appendix XXVI: ^{1}H-NMR spectrum for JO5182M08N



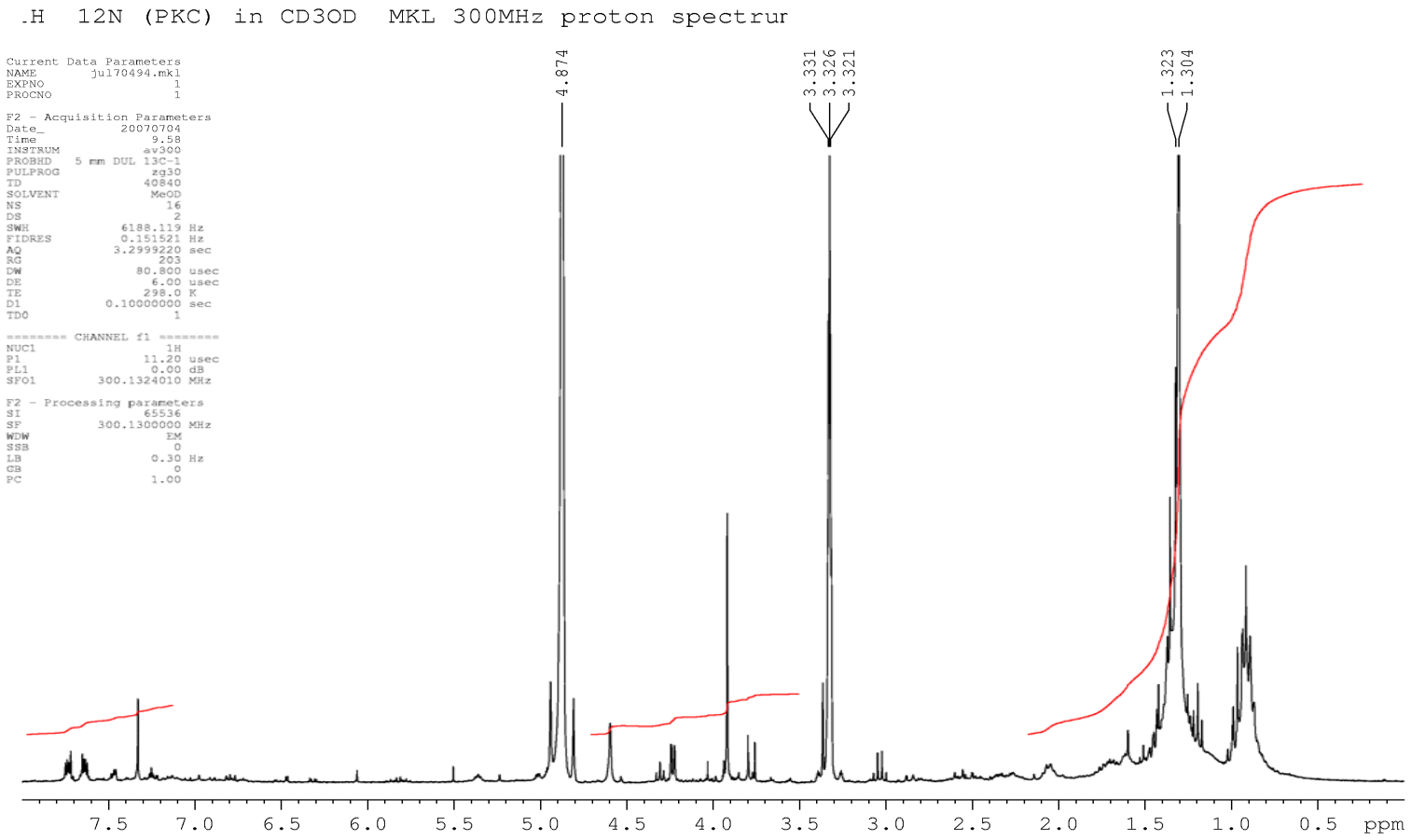
Appendix XXVII: ^{1}H NMR spectrum for JO5182M09N



Appendix XXVIII: ^{1}H NMR spectrum for JO5182M11N



Appendix XXIX: ^{1}H NMR spectrum for JO5182M12N



Appendix XXX: ^{1}H NMR spectrum for JO5182M13N