SEARCH FOR ANTIFUNGAL COMPOUNDS FROM EXTRACTS OF BASIDIOMYCETES AGAINST PHYTOPATHOGEN *Fusarium oxysporium* f. sp. *lycopersici*

NJUE ALICE WANJIKU

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

EGERTON UNIVERSITY

OCTOBER 2009

DECLARATION AND RECOMMENDATION

Declaration

I, Njue Alice Wanjiku, hereby declare that this research thesis is my original work and has not been submitted for an award in any other institution of learning to the best of my knowledge.

Signed:…………………………. Date:……………………….

SM11/1535/05

Recommendation

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

Signed:……………………………. Date:………………………

Dr. J. Ouma Omolo

Egerton University

Signed:……………………………. Date:………………………

Dr. P. K. Cheplogoi

Egerton University

Signed:……………………………. Date:………………………

Dr. D. O. Otaye

Egerton University

COPY RIGHT

All rights reserved. No part of this work may be reproduced, stored in a retrieval system, or transmitted by any means, mechanical, photocopying, electronic process, recording, or otherwise copied for public or private use without the prior written permission from Egerton University.

© Njue Alice Wanjiku

2009

DEDICATION

This work is dedicated to my husband David and my children Wambui, Njue and Njoki for their love and patience during my studies.

ACKNOWLEDGEMENT

I acknowledge and appreciate the following institutions, Departments and individuals.

1. Egerton University for allowing me to pursue the degree of Master of Science in Chemistry on part time programme.
2. Research and Extension Division of Egerton University for funding this research project (CTEE/DVC/RE/023) and for the permission to use the Integrated Biotechnology Research Laboratory (IBRL).
3. Chemistry Department for accommodating me while pursuing my Msc degree. Special thanks go to all the members of staff for their guidance, support and encouragement during my studies.
4. Crops, Horticulture and Soil Department for the permission to use their green houses to collect the infected tomato plants.
5. Prof. D. A. Mulholland and Mr. M. K. Langat of Surrey University, UK analysing the compounds using Nuclear Magnetic Resonance (NMR).
6. My supervisors, Dr. J. O. Omolo, Dr. P. K. Cheplogoi and Dr. D. O. Otaye for their guidance, advice, encouragement and their useful ideas, which were quite useful for this work.
7. Dr. Andy Foster of Institute for Biotechnology and Drug Research (IBWF) Kaisseislautern Germany for performing 18s RNA technique for taxonomic characterisation of the strain JO5125.
8. My family for their love, inspiration and encouragement, throughout my education endeavours.
9. To God who has been my source of strength throughout my studies and for enabling me to reach this far.

ABSTRACT

Tomato is one of the most widely cultivated horticutural crops but the production is affected by the pest problems like *Fusarial* wilt. *Fusarium oxysporium* f. sp. *Lycopersici*t is a destructive disease of the tomato and one of the main causes of the crop decline worldwide. The disease is currently managed using synthetic fungicides but there is a growing concern about the traces of the pesticide residues on the product and resistance to the current fungicides. Fungicides are applied at various stages of the tomato to protect the plant from fungal diseases but there is great concern due to environmental pollution and health problems. Therefore, alternative fungicides from naturally occurring compounds that are biodegradable offer a great potential for the control of crop fungal pathogens. Fungi are known to be prolific producers of secondary metabolites that have a wide range of beneficial biological activities, which can be researched on for crop protection. In this work, the fungi investigated were collected from different ecological niches in Kenya and kept on agar slants in the IBRL at Egerton University. For each strain collected, crude extracts were prepared from sterile submerged liquid nutrient media where growth conditions were manipulated to trigger production of secondary metabolites. In activity guided screening, *Fusarium oxysporium* isolate from a tomato plant, was used as the target organism in agar diffusion assay. From the ongoing screening of 400 crude extracts about 5% showed significant and interesting activity. One species, which showed significant and reproducible activity, was purified for natural antifungal compounds from its submerged cultures. The selected strain JO5125 was cultivated in constituted liquid nutrient media and crude extracts prepared from both the broth and mycelia at the end of the fermentation. The crude extracts were fractionated using both silica gel and reverse phase liquid chromatography to eventually lead to purification of the responsible compounds. The structure elucidation was determined using 1- and 2-D NMR spectroscopic techniques. The purified compounds were zearalenone and 3,4-dimethoxyphenol, which gave a minimum inhibitory concentration against the pathogen of about 550 and 500ppm, respectively. They are naturally occurring compounds which qualify as control agents of *F. oxysporium* with minimum impact in environment.

TABLE OF CONTENTS

DECLARATION AND RECOMMENDATION ii
COPY RIGHT iii
DEDICATION iv
ACKNOWLEDGEMENT v
ABSTRACT vi
TABLE OF CONTENTS vii
LIST OF FIGURES xi
LIST OF TABLES xii
LIST OF SYMBOLS ABBREVIATIONS AND ACRONYMS xiii
CHAPTER ONE 1
INTRODUCTION 1
1.1 Background Information 1
1.2 Fusarial wilt causative agent: *Fusarium oxysporium* 2
1.3 Secondary metabolism in fungi 3
1.4 Controlling *F. oxysporium* using antifungal compounds of fungi 4
1.5 Statement of the problem 5
1.6 Objectives 5
1.6.1 General objective 5
1.6.2 Specific objectives 6
1.7 Justification of the study 6
1.8 Hypothesis 6
CHAPTER TWO 7
LITERATURE REVIEW 7
2.1 Biological control of *Fusarial* wilt 7
2.2 Chemical control of *Fusarial* wilt 8
2.3 Synthesised fungicides 10
2.4 Natural antifungal compounds from plants 11
2.5 Antifungal compounds from fungi 12
2.6 Strobilurins - a new class of active substances 16
2.5 Application of research findings from the screened fungi 19

CHAPTER THREE **20**
MATERIALS AND METHODS **20**
3.1 Apparatus and materials 20
3.2 Preparation of liquid media 20
3.3 Preparation of test plates 20
3.3.1 Preparation of potato dextrose agar (PDA) media 20
3.3.2 Isolation and culturing of the test organism - *F. oxysporium* 21
3.3.3 Preparation of the test plates for antifungal activity 21
3.4 Cultivation of the selected strains in 250 ml submerged cultures 21
3.5 Preparation of crude extracts from mycelium and culture filtrate 22
3.5.1 Crude extracts from 250 ml scale initial cultivation 22
3.5.2 Cultivation of the selected strain JO5125 in 1 litre scale replicates 22
3.5.3 Liquid-solid adsorption resin extraction of the culture filtrate 23
3.6 Testing of the crude extracts for anti-fungal activities 23
3.7 Fractionation of the crude extract based on polarity using chromatography 24
3.7.1 Determination of the dry weight of the crude sample 24
3.7.2 Column chromatography 24
3.7.3 Thin layer chromatography(TLC) 25
3.8 Determination of minimum inhibitory concentration (MIC) 26
3.9 NMR spectroscopy 26
CHAPTER FOUR **27**
RESULTS AND DISCUSSION **27**
4.1 Isolation of the test organism - *F. oxysporium* from diseased tomato plant 27
4.2 Taxonomic identification of the strain JO5125 27
4.3 Screening of the crude extracts against the pathogen 27
4.4 Yields of crude extracts from the replicated 1.0L scale 30
4.5 Test cysteine-adduct formation 30
4.6 Determination of minimum inhibitory concentration (MIC) for crude extracts 31
4.7 Chromatographic fractionation and purification results 32
4.7.1 Fractionation and purification of mycelium crude extract 32
4.7.2 Fractionation and purification of culture filtrate (acetone) crude extract 33

4.7.3 Fractionation and purification of culture filtrate (methanol) crude extract........................35
4.8 Structure elucidation of the pure compounds 37
4.8.1 Structure elucidation of NA2, NA6 and NA7 37
4.8.2 Structure elucidation of NA3 42
4.9 DISCUSSION..44
CHAPTER FIVE 47
5.0 CONCLUSION AND RECOMENDATIONS 47
5.1 Conclusion 47
5.2 Recommendations..48
6.0 REFERENCES 49
APPENDICES..57
APPENDIX 1: ^{1}H NMR Spectrum for NA1..57
APPENDIX 2: ^{13}C NMR Spectrum for NA1..58
APPENDIX 3: ^{1}H NMR Spectrum for NA2..59
APPENDIX 4: ^{13}C Spectrum NMR for NA2..60
APPENDIX 5: 1H NMR Spectrum for NA3 61
APPENDIX 6: ^{13}C NMR Spectrum for NA3..62
APPENDIX 7: ^{1}H/^{1}H COSY NMR Spectrum for NA3 63
APPENDIX 8: DEPT Spectrum for NA3 64
APPENDIX 9: ^{1}H/^{13}C HMBC Spectrun for NA3 65
APPENDIX10: ^{1}H/^{13}C HSQC-DEPTSpectrum for NA3 66
APPENDIX 11: ^{1}H/^{1}H NOESY 2D Spectrum for NA 3 67
APPENDIX 12: ^{1}H NMR Spectrum for NA6..68
APPENDIX 13: ^{13}C NMR Spectrum for NA6..69
APPENDIX 14: ^{1}H NMR Spectrum for NA7..70
APPENDIX 15: ^{13}C NMR spectrum for NA7 71
APPENDIX 16: ^{1}H/^{1}H COSY Spectrum for NA7..72
APPENDIX 18: ^{1}H/^{13}C HMBC Spectrum for NA7 74
APPENDIX 19: ^{1}H/^{13}C HMBC Spectum for NA7..75
APPENDIX 20: ^{1}H/^{1}H NOESY Spectrum for NA7 76
APPENDIX 21: ^{1}H/^{1}H COSY Spectrum for NA 7..77

APPENDIX 22: $^{1}H/^{13}C$ HMBC Spectrum for NA7 78
APPENDIX 23: $^{1}H/^{13}C$ HSQC-DEPT Spectrum for NA7 79
APPENDIX 24: $^{1}H/^{1}H$ NOESY Spectrum for NA7 80

LIST OF FIGURES

Figure 4.1: Summary of preparation of culture and mycelium crude extracts 30
Figure 4.2: Flow scheme illustrating the fraction of mycelium crude extract and subsequent purification of compounds 32
Figure 4.3: Flow scheme illustrating the fraction of culture filtrate (acetone) crude extract and subsequent purification of compounds 34
Figure 4.4: Flow scheme illustrating the fraction of culture filtrate (methanol) crude extract and subsequent purification of compounds 35
Figure 4.5: Biosynthetic pathway of Zearalerone...42

LIST OF TABLES

Table 4.1: Antifungal activity for crude extracts from the 20 strains of fungal cultures ..29
Table 4.2: Bioassay results of JO5125 with cysteine against the *F. oxysporium*..............31
Table 4.3: The average MIC and standard deviation of the above crude extracts.............31
Table 4.4: The antifungal activity of the intermediate fractions and the purified compounds against the *F. oxysporium*..36
Table 4.5: ^{1}H NMR and ^{13}C NMR data for NA2, NA6, and NA7....................................38
Table 4.6: ^{1}H-NMR, ^{13}C-NMR' DEPT and HMBC data for compound NA7..................40
Table 4.7: ^{1}H-NMR, ^{13}C-NMR, DEPT and HMBC data for NA3...................................43

LIST OF SYMBOLS ABBREVIATIONS AND ACRONYMS

^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance Spectroscopy
^{1}H NMR	Proton Nuclear Magnetic Resonance Spectroscopy
Bc_1	Cytochrome bc_1
$CDCL_3$	Deuterated chloroform
COSY	Correlated Spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
GC	Gas chromatography
GC-MS	Gas chromatography-Mass Spectrometry
HMBC	Heteronuclear Multiple Bond Coherence
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High performance liquid chromatography
HREIMS	High Resolution Electron Impact Mass Spectrometry
HSQC	Heteronuclear Multiple Quantum Coherence
IBRL	Integrated Biotechnology Research Laboratory
KEX	Culture extract
MEX	Mycelium extract
MIC	Minimum inhibitory concentration
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance Spectroscopy
NOESY	Nuclear Overhauser Effect Spectroscopy
PDA	Potato Dextrose Agar
PPM	Parts per million
TLC	Thin layer chromatography
UV	Ultra-Violet radiations

CHAPTER ONE
INTRODUCTION

1.1 Background Information

In the last one hundred years, control of plant diseases and pests has depended increasingly on the extensive use of synthetic chemicals. In fact, these pesticides have become established as an essential input in growing of horticultural crops and without their use crop yields would be severely reduced to uneconomic levels. Many soil borne noxious organisms are microscopic making monitoring intrinsically difficult and thus costly. In general, soil-borne diseases are limiting factors in the production of many crops and accounts for 10 - 20% annul yield losses. Losses in individual fields can be as high as 100% (Fravel *et al.,* 2005). Management of soil-borne pathogens is necessary for a secure and stable supply of food as well as for maintaining agricultural exports and profit maximisation.

The structural, physical and biological complexity of the soil environment in which soil borne pathogens interact with plant roots inherently limits the options available for disease control. The already proven measures include conventional breeding and genetic engineering of disease-resistant plants, application of disease-suppressing cultural practices, and to some extent, use of antagonistic biological agents against the microorganisms that cause plant disease. Control through crop rotation or other cultural practices is not very feasible in most countries anymore and not always profitable for intensive use (Copping and Menn, 2000). Resistance in widely used agricultural fungicides has emerged as a significant problem. The situation is worsened further by lack of arsenal of fungicides that attack soil borne pathogens. Many chemical pesticides have been or are being phased out (like organochlorine insecticides, methyl bromide) either because of potential human risk, environmental pollution, effects on non-target organisms or the development of pest resistance. African agricultural products are still restricted in overseas markets just because the commodities or products are treated with synthetic fungicides and do no meet the basic requirement (Gullino *et al.,* 2000).

However, none of the fungicides used have been successful since there are unintended consequences highlighted above. The use of broad-spectrum fungicides further results in imbalances within the microbial community creating unfavourable conditions for

the activity of beneficial organisms leading to suppression of soil fertility (Ristaino and Thomas, 2000). Risk associated with implementation of alternative technologies is inherently higher due to climatic and pest crop variability. The global consensus to reduce inputs of chemical pesticides which are perceived as being hazardous by some consumers has provided challenges for the development of novel, benign, sustainable crop protection strategies. Therefore, development of alternatives to these synthetic fungicides can be sought from higher fungi like ascomyectes and basidiomycetes to discover selective and environmentally safe fungicides for use in agriculture.

1.2 Fusarial wilt causative agent: *Fusarium oxysporium*

The genus *Fusarium* is one of the most injurious and causes diseases that are found in a number of plants: cereals, grasses, legumes and horticultural crops. *F. oxysporium* is a species-complex that includes many host-specific plant pathogens. A common characteristic of *F. oxysporium* strains is the ability to parasitize plant roots, usually without inducing symptoms. The pathogen penetrates plants through root tips or wounds on roots and reaches the xylem. Vascular wilt diseases caused by the strains of these fungi are usually highly destructive whether they occur in cultivated crops or indigenous species. Under warm and conducive conditions the losses could reach 95% of production in some fields (Bailey and Lazarovits, 2003). The interaction with the host involves a specialized colonization of the vascular system setting in motion a series of biochemical and morphological reactions that culminate into the characteristic symptoms of these diseases - the wilting of the host plant.

The economic damage caused by *Fusarium* wilt diseases has inspired considerable research on those species affecting important crop species. Management of the disease is complicated by the speed at which it spreads. In corn, *Fusarium* wilts may reduce yield by 50% (Agrios, 1998). It has been reported that virtually any kernel of dried corn, anywhere, can be shown to contain *Fusarium*, if proper isolation techniques are used (Cavigelli *et al.,* 2005). Pathogenic strains of *F. oxysporium* are placed into **formae speciales** according to their host range. There about 150 host specific **formae speciales** described for this vascular wilt pathogen. They can also live as saprotrophs in the soils, so crop rotation is usually not sufficient for control. Fusarial wilts are most severe under warm soil conditions and in greenhouses.

Fusarium wilts are caused by pathogens that reside in the soil and survive on infected debris, in alternative host or as resistant propagules of which several can be produced in the life cycle of a species. The epidemiology of these pathogens is also extremely complex, with propagules being splash borne over short distances or carried on tools in the course of normal cultivation practices. The use of contaminated soil can also spread these pathogens to nurseries and shade houses whilst long distance spread is achieved through use of infected plant material such as seeds or vegetatively propagated material. Insect vectors can also be involved given their complex life cycle and epidemiology, the potential for variation within populations and their soil-borne nature, the management of diseases caused by these pathogens has always been a challenge to plant pathologists and is likely to continue to be so in the future (Flood, 2003). *Fusarium* is one of the most widely distributed pathogen and difficult to control because once an area becomes infected with it, it remains so indefinitely.

F. oxysporium f. sp. *lycopersici* is a highly destructive pathogen of both greenhouse and field grown tomatoes in warm vegetable production areas. The disease causes great losses, especially on susceptible varieties and under favourable weather conditions. Infected plants show pronounced vascular discoloration, severe defoliation and stunting which leads to the death of the plant. Occasionally entire field of tomatoes can be severely damaged before the crop is harvested. The disease, which is prevalent in Kenya's tomato growing zones, causes serious losses because soil and air temperatures are rather high during most of the season. Heavy damage to tomatoes often occurs in areas of continuous cultivation because monoculture practices could lead to pathogen build up and subsequent yield losses (Suleman *et al.,* 2003).

1.3 Secondary metabolism in fungi

Interactions of fungi with other microbes and non-microbial systems often include the production of biologically active metabolites that affect the potential competitors or predators. Such ecological phenomena have served as valuable lead to the discovery of secondary metabolites that have proven to be unlimited source of biologically active molecules. Using appropriate screening methods, molecules with almost any defined activity can be obtained, thus, secondary metabolite screening for compounds has been a main stay

of the pharmaceutical industry for many years (Demain, 1999) and has increased in importance in agricultural industry.

The starting point from primary metabolism is the basis of classification of secondary metabolites according to the biosynthetic precursors (Turner, 1971; Turner and Aldridge, 1983). The secondary metabolites are, however, low molecular weight, chemically and taxonomically extremely diverse compounds with obscure function, characteristic mainly to some specific, distinct types of organisms. The presently known secondary metabolites of microorganisms display a great number of diverse and versatile biological activities as well as chemical structures. Hence with application of appropriate fungal biotechnology, there is a great potential to find novel biologically active compounds and greatly improve the rate of exploration of uncommon and unreported groups of tropical fungi. Unique ecosystems may result in unique organisms with unique metabolic pathways (Lasure, 2000).

1.4 Controlling *F. oxysporium* using antifungal compounds of fungi

Tropical forest are endowed with enormous and a rich diversity of fungal genetic resources that can be exploited for new antifungal compounds (Berdy, 2003). Natural product resources including the microbial world are mainly unexplored both in dimension and in respect of geographic, ecological and environmental point of view. In some estimates the worldwide numbers of fungal species were 1.5 million (Hawksworth, 2001). Of these only 10% have been discovered and described as yet, and barely 1% examined for their spectrum of secondary metabolites (Arnold *et al*., 2000). Fungi in their natural habitats interact competitively and depending on the environment such a microorganism adapts appropriate survival mechanisms that confer selective advantage over their competitors. Mechanisms of fungal antagonism and defence often include the production of biologically active secondary metabolites by species that affects the potential competitors and/or predators. Laboratory studies clearly indicate that such ecological phenomena can serve as valuable lead to the discovery of novel natural products many of them potential targets for agrochemicals and biomedical development (Hostettman and Martson, 2000). Among the sources of bioactive metabolites less intensively investigated organisms like the higher fungi seem to hold an excellent promise for new structures with interesting biological activities.

Fungi inhabit most climatic zones from the arctic to the tropical rain forest. For more than 5 decades, interesting compounds of different biogenetic origin with antibacterial, antifungal, phytotoxic, nematocidal, cytostatic, antiviral and other pharmacological activities have been isolated from basidiomycetes (Thines *et al.,* 2004)

From the early 1990s the number of bioactive compounds isolated from various filamentous and higher fungal species had continuously increased up to more than 50% by the year 2000. The need for less toxic, more potent antifungal as well as non-antinfectives, the evolving resistance to existing fungicides and emergence of new fungal diseases, has posed a challenge to the researchers of the 1990s (Knight *et al.,* 2003). Especially, the resistance of pathogenic fungi to fungicides is a serious problem. Therefore, it is quite probable that highly differentiated fungi such as basidiomycetes will produce a rich array of secondary metabolites with a great potential to produce novel biologically active compounds against *F. oxysporium.* The potential chemical diversity of this vast untapped resource is surely one of the great driving forces behind today's search for new antifungal compounds.

1.5 Statement of the problem

Use of synthetic soil fumigants has been widely recommended in the management of this fungus. With the ban of methyl bromide, the commonly used soil fumigant there is a problem. Given the moist and warm climatic conditions of tropical soils, *F. oxysporium* the causative agent of tomato fusarial wilt, is bound to cause more crop loss. This scenario is impossible given the serious food and nutritional insecurity in most poor resource households. Naturally occurring fungicides from fungi, can be a possible alternative to these synthetic soil fumigants in controlling the fusarial wilt causative agent in tomato crop production.

1.6 Objectives

1.6.1 General objective

To search for antifungal compounds from submerged cultures of basidiomycetes against *F. oxysporium.*

1.6.2 Specific objectives

i. To isolate the pathogen from the infected plant.
ii. To screen crude extracts prepared from 400 strains of basidiomycetes against *F. oxysporium* using agar diffusion assays.
iii. To cultivate the selected basidiomycete in liquid submerged cultures.
iv. To determine the taxonomy of the selected basidiomycete.
v. To prepare crude extracts from the mycelium and culture filtrate of the selected basidiomycete using adsorption resin, Mitsubishi HP21 (DIAION).
vi. To carry out bioactivity-guided chromatographic fractionation of the mycelial and culture filtrate crude extracts, to purify the targeted antifungal compounds.
vii. To carry out structure elucidation of the purified compounds.
viii. To determine minimum inhibitory concentrations of the purified compounds.

1.7 Justification of the study

Control of fungal diseases, fusarial wilts included, has been almost exclusively controlled by synthetic chemicals. Despite the huge reduction on crops losses occasioned by these chemicals in the last decade, there are attendant problems especially the non targeted effects. Researches tailored to discovering environmentally benign compounds are as needed given that non-targeted effects are responsible for withdrawals of the hitherto effective synthetic chemicals. Given the unique tropical habitats and the high incidences of fusarial wilts, it's a worthwhile to investigate the natural arsenal of antifungal for the control of *F. oxysporium* in tomato.

1.8 Hypothesis

From the screening of the crude extracts of the 400 strains of basidiomycetes, a maximum of 5% will show significant and reproducible activity against *F. oxysporium.* Out of the 5% active lot, one basidiomycete will be selected, grown in submerged cultures and will be expected to produce the desired antifungal compounds.

CHAPTER TWO
LITERATURE REVIEW

2.1 Biological control of *Fusarial* wilt

Biological control of soil-borne plant pathogens is a potential alternative to the use of soil fumigation, which has been proved to be harmful to the environment (Elizabeth and Handelsman, 1999). Root of crops can be protected from pathogens to a significant degree by non-pathogenic root colonizing microorganism applied on the seed. For example, strains of the fungus *Trichoderma* have been found to be effective biocontrol agents of various soil-borne fungal plant pathogens under green house and field conditions (Herman, 2000). This approach has indeed led to the isolation of several strains of *T. harzianum* antagonistic to and effective in controlling plant pathogenic fungi, such as *F. oxysporium* under such conditions. In a study, tomato crop survival and yield were increased and disease decreased following application of *T. harzianum* either as a seed coating or as an amendment to the soil in the field (Sivan *et al*., 1987). Also isolates of *T. asperellum* from a suppressive mix prepared from sewage sludge compost showed high ability to suppress *Fusarium* wilt (Alabouvette *et al*., 2002).

Soil borne diseases have been controlled more recently by means of certain beneficial bacteria that are indigenous to the rhizosphere of plants. They rapidly colonize and suppress *F. oxysporium* at the root surface (Ahmed *et al*., 2007). Certain soils are naturally suppressive to *Fusarium* wilt a property due to the role of non-pathogenic *F. oxysporium* in the indigenous microbiota of these soils. One of these non-pathogenic *F. oxysporium* is Fo47, a strain isolated from a suppressive soil in Chateurenard, France has been shown to effectively suppress *Fusarium* wilt of the tomato (Fuchs *et al*., 1999). Another biocontrol fungus *F. oxysporium* strain CS-20 demonstrated potential for reducing the incidence of *Fusarium* wilt of the tomato both in the field and green houses. Strain CS-20 superficially colonizes the interior of the tomato roots, but does not penetrate the vascular system. It provides biocontrol of *Fusarium* wilt through a host-mediated mechanism (Fravel *et al.,* 2005).

Root dip applications of *Bacillus subtilis*, *Pseudomonas fluorescens*, *Aspergillus awamori,* and *Penicillium digitum* resulted in significant decline in the rhizosphere

population of *F. oxysporium*. Root dip treatment with the phosphate-solubilising microorganism tested resulted in significant increase in the yield of tomato being greatest with *A. awamori* and *P. digitum* in pathogen inoculated 36 and 33% (Mujeebur and Shahana, 2002). Compost obtained from heterogeneous vegetable wastes showed important suppressive effects against *Fusarium* species. *Fusarium* wilt reductions on tomato plant were generally between 30-65% (Larkin and Fravel, 2002). Crop damage due to disease in the field is rarely the result of the activity of one pathogen, but as a combination. Because of the complexity of such a situation, biocontrol studies are difficult to implement and thus a combination of both chemical and biological agents increase the protection of the crop. Elmer and McGovern (2004) combined commercial biological products with fungicides and found that the combination provided a higher degree of suppression of *Fusarium* wilt of tomato. The set back of these combinations is lack of knowledge of compatibility of biocontrol agents with pesticides, which may contribute to failure of biocontrol to perform as expected. Also fungal biological control agents have a narrow host range and that they act slowly therefore, giving limited protection. Another major setback in biological control strategy stems from the fact that these biological control agents have their activities dictated by various soil conditions such as soil moisture, pH, and even temperature (Copping and Menn, 2000).

2.2 Chemical control of *Fusarial* wilt

Chemical control of fungal diseases has a long history, starting with inorganic compounds such as copper salts and lime-sulfur in the 19th century and ending with sophisticated organic compounds such as imidazoles and triazoles in the 1970s and 1980s. Fungicides remain an important part of disease control strategy in plants. Fungicidal treatment can keep tomato crops healthy or suppress the infection sufficiently (Merterrhazy and Bartok, 1996). Various chemicals have been used to control the *F. oxysporium* disease such as imidazoles (prochlorax) and the trizoles (Strauss and Labuschagne, 1993) but Song *et al*. (2004) found prochlorax and carbendazim to be most effective fungicides in inhibiting mycelial growth of *F. oxysporium.* However, the effectiveness of fungicides is threatened by development of prochlorax and carbendazim resistance in the pathogen population. Allen *et al*. (2004) found that benomyl at all concentrations completely inhibited fungal growth of

Fusarium species. Calcium cyanide, a nitrogen fertilizer has also been found to reduce the incidence of the fusarial wilt disease to a certain percentage (Klasse, 2002). Common inorganic salts such as sodium and potassium bicarbonate have a potential use as an antifungal agent for controlling the mycelial growth of *Fusarium* species in crops but are not very effective (Hang and Woodams, 2003).

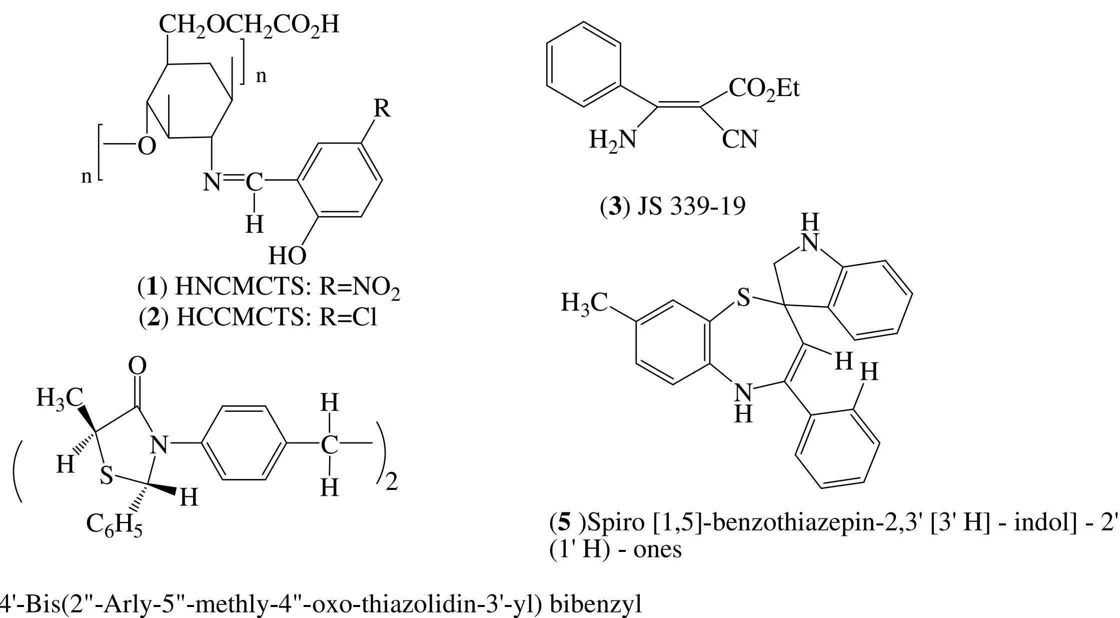
Fumigation of soil with Vorlex®, a commercial mixture of methyl isothiocyanate, dichloropropenes and dichloropropanes has been reported to reduce populations of *Fusarium* in soil (Chew and Hall, 1984). Fumigation of soil with chloropicrin or dazomet dramatically reduces the population of *Fusarium* species in fumigated plots but have environmental implications (Hansen *et al.,* 1996).

Several agricultural systems involving intensive production of high value crops have become dependent on the use of methyl bromide. The broad-spectrum activity and ease of application of these materials have led to their use as treatment of choice in a number of situations. However, the soil fumigation with synthetic organic pesticides has various hazardous environmental implications (Santos *et al*., 2006). Although methyl bromide is a most useful tool in specific instances, there are a number of technical and legislative limitations that have led to restrictions on its use. It has a substantial phytotoxicity that may accumulate in excessive levels in commodities that are fumigated several times and have been a cause of concern in ground water in some European countries. Of most concern is its ozone depleting potential. Soil fumigation with methyl bromide proved to be effective only as a short-term control measure in South Africa, due to the successful re-invasion of the pathogen back to soil after a period of approximately 2 years (Herbert and Marx, 1990). Both the resistance of the varieties and virulence of the fungal isolates also affect the fungicidal efficacy. There is also the fact that the regions capability and resources to sustain methyl bromide phase out beyond 2015 deadline are limited and is dependent on countries being able to unilaterally address and solve their own pest control problems. The Montreal protocol schedule required that developed countries phase out methyl bromide by 2005 and developing countries freeze consumption by 2002, achieve a 20% reduction by 2005 and a complete phase out by 2015. At least 50% of the countries in Africa have not met this challenge (U.S. Environmental Protection Agency).

Although farmers reported that tomatoes planted with agrochemicals were healthier, drought tolerant, resistant to diseases and gave better yields. There is need to for effective, sustainable and environmental friendly fungicides, which will be appropriate to local farmers. Currently no effective control measure is available for the control of *Fusarium* wilt except only prevention strategies (Nel *et al*., 2006). This concern has provided the impetus to investigate alternative control measures, like naturally occurring compounds from fungi.

2.3 Synthesised fungicides

Natural compounds have been a major source of bioactive derivatives and lead for development of synthetic drugs (Butler, 2005). Many naturally occurring compounds are synthesized so as to improve their physiochemical characteristics and bioactivities. One of these naturally occurring compounds is chitosan, which has limited activity due to its poor solubility. To improve its solubility, many derivatives of chitosan have been synthesised and carboxymethyl chitosan (CMCTS) is the most important one. Chitosan can reduce the growth of phytopathogenic fungi, which are harmful to field crops, fruit and vegetables. Antifungal activity of chitosan, CMCTS and the Schiff bases of CMCTS against *F. oxysporium* at 500ppm are as follows; chitosan gave inhibitory index of 14.3%, CMCTS gave 9.1%, 2-(2-Hydroxy-5-nitrobenzyllideneamino)-6-carboxyemthylchitosan (HNCMCTS) (**1**) gave 31.2% and 2-(5-chloro-2-hydroxybenzylideneamino)-6-carboxymethylchitosan (HCCMCTS) (**2**) gave 43.0%. Schiff bases have activity more than those of chitosan and CMCTS (Zhanyong *et al*., 2006). Synthesis of 2-cyano-3-phenylacrylic acetate (JS399-19) (**3**), which belongs to the cyanoacrylate fungicide group exhibited strong fungicidal activity against the plant pathogen *Fusarium* species *in vitro* (Hengkui *et al*., 2008). Synthesis of 4,4'-bis(2''-aryl-5''-methyl/unsubstituted-4''-oxo-thiazolidin-3''-yl) bibenzyl from benzil (**4**) gave a compound with significant fungitoxicity at 1000ppm against *F. oxysporium* (Ibadur *et al*., 2003). Large numbers of 1,5-benzothiazepine exhibit a variety of pharmacological activities. In the synthesis of new fluorinated spiro [1,5]-benzothiazepin-2, 3' [3'H] -indol] -2' (1 'H)–ones (**5**) increased the antifungal activity against *F. oxysporium* at 1000ppm by 98% (Anshu *et al*., 1998). The limiting factor of the synthesized fungicides is lack of combination or alternating application with other fungicides as a tool to limit fungicide resistance development.



(**1**) HNCMCTS: R=NO_2
(**2**) HCCMCTS: R=Cl

(**3**) JS 339-19

(**5**)Spiro [1,5]-benzothiazepin-2,3' [3' H] - indol] - 2' (1' H) - ones

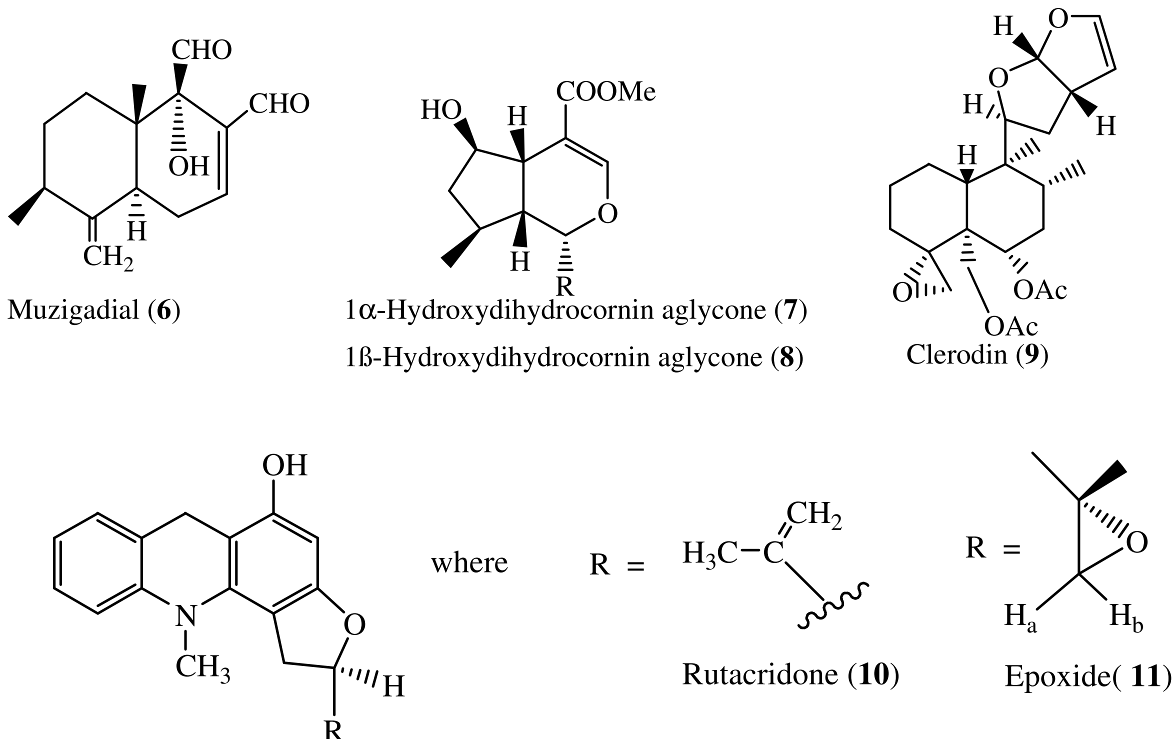
(**4**) 4,4'-Bis(2"-Arly-5"-methly-4"-oxo-thiazolidin-3'-yl) bibenzyl

2.4 Natural antifungal compounds from plants

Natural products have been shown to be ideal agrochemicals in a number of ways, especially given their high potency and specificity. Ever since the introduction of the large scale screening for natural compounds, they have been playing essentials roles as lead structures for the development of drugs, pesticides and as characterisation of new selective targets. In the last decade, the pharmaceutical industry has greatly sped up screening capacities with the use of Ultra High Throughput Screening (U-HTS) robotic systems, which can routinely screen thousands of molecules a day for biological activities. In this context, natural compounds are more and more revisited as rich source of promising model lead molecules (Piggutt and Karuso, 2004).

The use of botanicals in disease management is as old as humankind and transcends all stages of civilization. A great variety of plants have broad-spectrum bioactive organic compounds such as isocyanate, glucocynolate, glycosides, lipids and phenol with fungicidical and fungistatic properties (Chitwood, 2002). Higher plants such as *Warburgia ugandensis* have been explored as potential sources of antimicrobial agents. The aqueous methanolic extract of the barks of *W. ugandensis* displayed activity against *F. oxysporium.* Muzigadial (**6**) was isolated with minimum inhibitory concentration of 50ppm (Rugutt *et al.*, 2006). Many natural diterpenoids are very much functionalised and show good antifungal

activity. For instance, non-glucosidic iridoids were discovered in *Alibertia macrophylla* (Rubiaceae), two of which, 1α- and 1β- hydroxydihydrocornin aglycones (**7, 8**), showed fungi-toxicity against a range of *Cladosporium* and *Fusarium* species (Young *et al.,* 1992). A neo-clerodin diterpenoid from *Scutellaria* (Labiatae), clerodin (**9**) reduced growth and inhibited spore germination of *F. oxysporium* (Cole *et al.,* 1991). Rutacridone (**10**) and its epoxide derivative (**11**) were found to inhibit the growth of *F. oxysporium* at low concentration of 20ppm from ethyl acetate extracts of the roots of *Ruta graveolens* (Kumudini *et al*., 2005). The chemical investigation in volatile oils of *Curcuma longa* (rhizome) done by GC-MS was found to contain ar-lurmerone (51.8%) ar-lurmerol (11.9%) and others. The bioassays of these oils indicated *C. longa* as a good antifungal material and causes complete mycelial inhibition of *F. oxysporium* at 2000ppm (Gurdip *et al*., 2002).

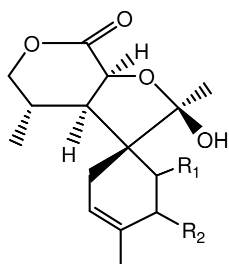


2.5 Antifungal compounds from fungi

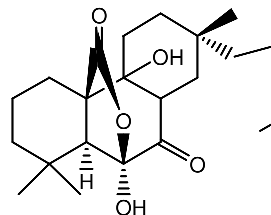
Fungi are the second largest group of eukaryotes and exceed the higher plants in terms of the number of possible existing species. The basidiomycetes (highly differentiated fungi) constitute a large class of fungi that is approximately one third of all fungi. The

extraordinary diversity of fungal species suggests virtually limitless potential for secondary metabolic variation. They are one of largest reservoir for isolating further bioactive metabolites. Despite their potential for development of biologically active compounds, few bioactive metabolites have been reported from fungi as compared with higher plants. The number of bioactive compounds isolated from various fungal species had continuously increased up to more than 50% by the turn of new millennium (Weber *et al.,* 2007).

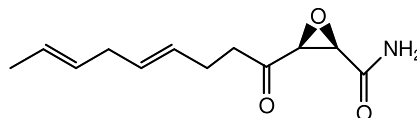
Fungi produce compounds that have various bioactivities and specific modes of action. Novel sesquiterpene name cycloclapinal (**12**) and O-acetylcycloclapin (**13**) has been isolated from the basidiomycete *Boletus calopus* (Dong-Ze *et al*., 2008a) and have antimicrobial activity. Sphaeropsidins (**14**) are a group of primarane diterpenes known from the anamorphic fungi *Sphaeropsis sapinea* f. sp. *cupressi* and *Diplodia Murila* (Sparapano *et al.,* 2004). Arundifungin (**15**) isolated from *Arthrinium arundis* by Cabello *et al*. (2001) was found to have antifungal activity and its mode of action was against β-(1,3)-glucan synthesis inhibitor. Erinacines and their xyloxides 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 and have been proposed as medicines for degenerative neuronal disorder such as Alzheimers disease and peripheral nerve regeneration. Erinacine R (**16**) has been isolated from mycelia of *Hericium erinacium*, a mushroom belonging to the family of Hericiaceae (Bing-Ji Ma *et al*., 2008). A new spiroaxane sesquiterpene (**17**) was isolated from the cultures of the basidiomycete *Pholiota adiposa*. *P. adiposa* is an edible and medicinal fungus, which has been found to show antimicrobial activities and can also prevent infection from *Straphylococcus aureus, Escherichia coli*, and *Mycobacterium tuberculosis* (Dong-Ze *et al*., 2008b).



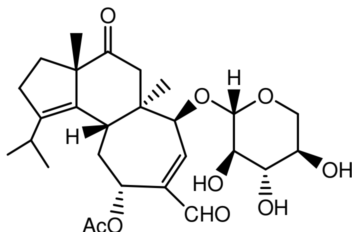
R_1=R_2=OH Cycloclapinal (**12**)
R_1=OH R_2=OAC O-Acetylcycloclapin (**13**)



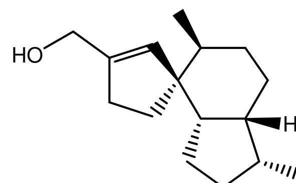
Sphaeropsidins (**14**)



Arundifungin (**15**)

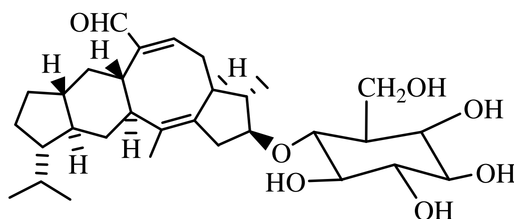


Erinacine R (**16**)

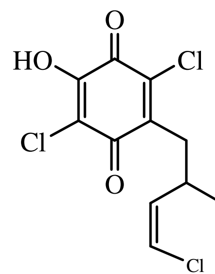


Spiroaxane (**17**)

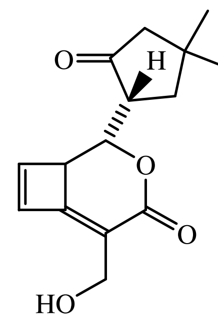
Some earlier screening for antifungal compounds from basidiomycetes against plant pathogens resulted in the isolation of aleurodiscal **(18)** from mycelial cultures of *Aleurodiscus mirabilis* (Lauer *et al*., 1989). A novel chlorinated benzoquinone derivative, mycenone (**19**), was isolated from cultures of a *Mycena* species (Hautzel *et al*., 1990). Mycenone inhibits isocitrate lyase preparations from plants, bacteria and fungi. Fomannosin (**20**) was isolated from cultures of wood rotting basidiomycetes *Fomes annosus* (*Heterobasidion annosus*). Fomannosin exhibits phytotoxic activity in assays with *Chlorella pyrenoidosa* and against *Pinus taeda* seedlings when applied to the stem base or a lateral root at a concentration of 88µg per seedlings (Basset *et al*., 1967).



Aleurodiscal (**18**)



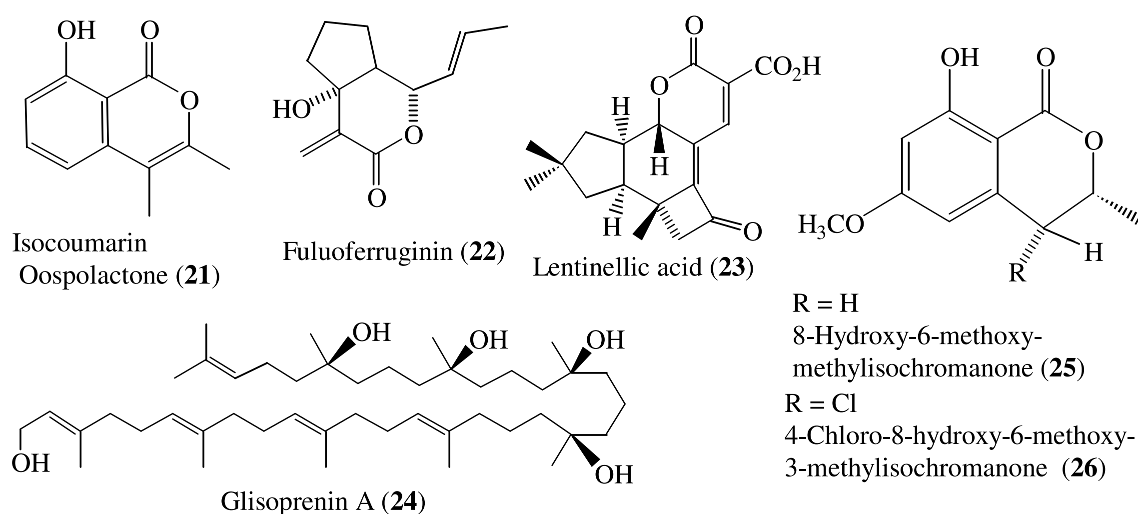
Mycenone (**19**)



Fomannosin (**20**)

Fermentation of wood rotting basidiomycete, *Gloephyllum sepiarium* lead to the isolation of the antifungal isocoumarin oospolactone (**21**) and the organisms most sensitive were *Alternaria* strains with minimal inhibitory concentration of 12.5-25ppm (Nakajima *et al*., 1991). Fulvoferruginin (**22**), an antifungal was isolated from *Marasmius fulvoferrugineus.* Lentinellic acid (**23**), a methyl ester that exhibits much higher antifungal activity and was isolated from *Lentinellus omphalodes* (Stark *et al*., 1988).

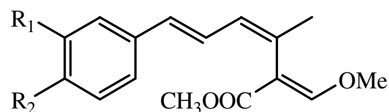
Glisoprenin A (**24**) isolated from submerged cultures of the deuteromycete *Glisocladium roseum* was found as inhibitor of melanin biosynthesis in the rice-blast fungus *Magnaporthe grisea,* have proven to be environmentally safe because of their low toxic potential (Thines *et al*., 1997). Basidiomycetes are known to secrete a number of chlorinated aromatic compounds. Chlorinated metabolites have some antibacterial and anti-fungal properties and are generally cytotoxic, inhibit enzymes and reduce seedling germination. The biosynthesis of chlorinated metabolites can be induced by the presence in the immediate environment of antagonistic fungi, implying that the production of these compounds is a form of chemical defence. The secretions of chlorinated metabolites by fungi play a role in suppressing plant pathogenic fungi. 4-Chloro-8-hydroxy-6-methoxy-3-methylisochromanone (**26**) isolated from *Gilmaniella humicola* has been reported to inhibit the growth of *Gaeumannomyces graminis* var. *tritici,* a wheat fungal pathogen. Another compound 8-hydroxy-6-methoxy-3-methylisochroman-1-one (**25**) isolated from the same fungi showed some cytotoxicity, phytotoxicity and antimicrobial activity (Holloway, 2003).



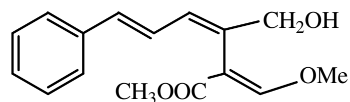
In search for natural products with novel uses particularly related to pest management, extracts of three endemic species of *Flourensia* spp. were found to have antifungal activity on *Fusarium oxysporium*. The ethanol extracts of *F. microphylla, F. cernua,* and *F. retniphylla* inhibited the mycelial development of *F. oxysporium.* Inhibition effect was observed from 10ppm although total inhibition was found at 1000ppm. The compounds reported to be present in these extracts are glycones of flavanoids 5, 7, 3'-trihydroxy-3-isobutyroylflavanonol; 5, 7, 3'-trihydroxyflavanone and 5, 7-dihydroxy-3'-methoxyflavanone (Rodriguez *et al*., 2007).

2.6 Strobilurins - a new class of active substances

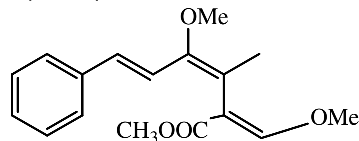
Strobilurin fungicides have become effective against several different plant pathogenic fungi. This group is unique in that these fungicides are the first natural lead synthetic, site-specific compounds to provide significant control of plant diseases caused by pathogens from all three major groups of fungi: Oomycota, Ascomycota, Basidiomycota. Fungi that produce strobilurins are found all over the world in all climate zones (Anke and Steglich, 2000). The discovery of strobilurins (**27-37**) led to intensive investigation of basidiomycetes in fermentation for different kinds of biologically active ingredients, chiefly in Europe, N. America and to some appreciable extent in the Orient (Kaeokamnerd, 1998). Strobilurins are strong antifungal compounds produced by many basidomycete genera (*Agaricus, Crepidotus, Cyphellopsis, Favolaschia, Filoboletus Hydropus, Mycena, Oudemansiella, Strobilorus* and *Xerula*).



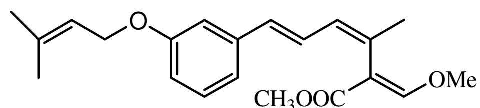
	R_1	R_2
Strobilurin A (**27**)	H	H
Strobilurin B (**28**)	OMe	Cl
Strobilurin F (**29**)	OH	H
Strobilurin H (**30**)	OMe	H
Strobilurin X (**31**)	H	OMe



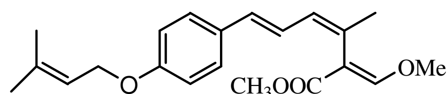
Hydroxystrobilurin A (**32**)



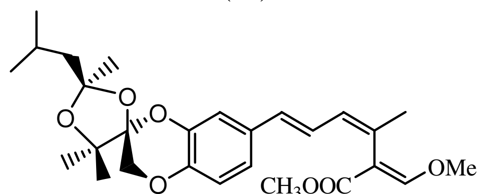
9-Methoxystrobilurin A (**33**)



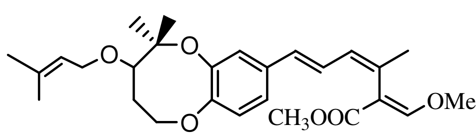
Strobilurin C (**34**)



Strobilurin F2 (**35**)



Strobilurin E (**36**)



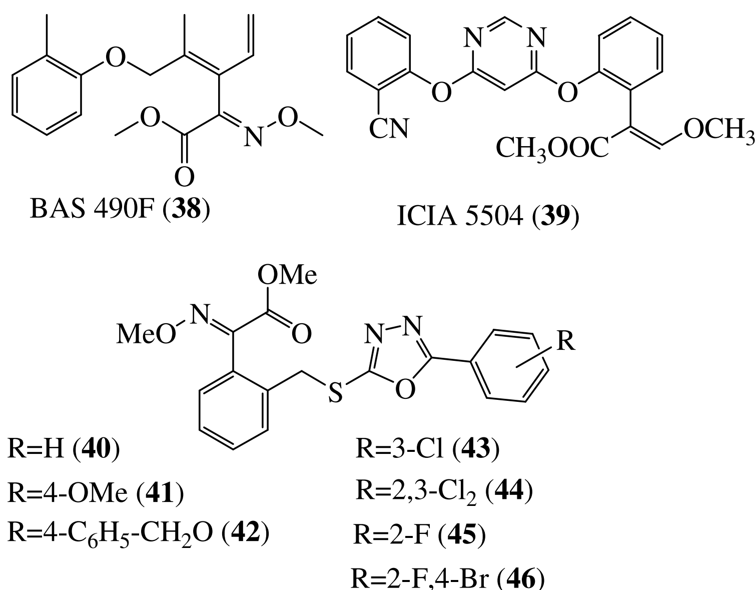
Strobilurin G (**37**)

Strobilurins are a fungicide group with a preventive mode of action frequently used worldwide. Strobilurins belong to the E-β-methoxyacrylate group of antibiotics and inhibit mitochondrial respiration by blocking electron transfer at the cytochrome-bc1 complex. The mode of action, which is novel for a fungicide, is based on inhibition of the cellular respiration of the fungal pathogen and thus valuable asset in the management of resistance to established fungicides (Tamwa and Mizutani 1999).

Their lack of toxicity towards mammals (Sauter *et al.*, 1996) made them interesting lead compounds for the development of commercial fungicides. Extensive synthetic effort lead to simple mimics and revealed that the E-β-methoxyacrylate unit is a prerequisite for the antifungal and respiration inhibiting properties of strobilurins (Schram *et al.*, 1982; Anke *et al.*, 1988). Continuous efforts by Steglich, and coworkers (Anke and Steglich, 1989) and by BASF resulted in compounds with improved activity and light stability. This eventually led to the development of BAS 490F (**38**) (Sauter *et al.*, 1996) and ICIA 5504 (**39**) (Clough, 1993). The strobilurins fungicides BAS 490F has been shown in several fungal species to completely inhibit spore germination, this accounts for the selectivity of their action against fungi as the target organism, a decisive factor for the generally very good environmental

tolerability of strobilurins. With their broad spectrum of activity, long duration of action, high activity at low rates of application, and outstanding environmental tolerability, the strobilurins have set new standards in the control of fungal diseases, thereby making a key contribution to integrated crop protection strategies (Reuveni, 2000). This shows that fungi have a great potential for producing agrochemicals, which are economically and environmentally friendly. This is untapped resource, especially in Africa where biodiversity is abound, search for more antifungal compounds that are compatible with local climatic conditions is a worthwhile pursuit.

Novel analogues of strobilurins which contain both (E)-methylmethoxyiminoacetate and 1,3,4-oxadiazole ring moieties, methyl (E)-α-(methoxyimino)-2-[(5-aryl-1,3,4-oxadiazole-2-mercapto)-methyl]-benzeneacetates were synthesised in order to obtain better fungicidal activities. About 5 novels (E)-α-methoxyimino-benzeneacetate derivative (**40-46**) were stereo selectively synthesized, and their activities were tested against *Rhizoctonia solani, Botrytis cinerea Gibberella zeae, Physalospora piricola* and *Bipolaris maydis.* All of the tested compounds showed potent fungicidal activity against all of the fungi tested; especially it was found that compounds **40–42** had higher fungicidal activities against *R. solani* (Yan *et al*., 2006).



The fungicides have been known to increase the greening effects which again are hypothesized to be due the potent effect of strobilurins on spore germination which avoids energy losses from the plant due to defence reactions, and a longer residual effect of

strobilurins (Jorgensen *et al.,* 1999). Apart from their fungicidal effect strobilurins can induce physiological and developmental alterations. It has been proposed that, this is due to inhibition of ethylene biosynthesis, increase of endogeneous cytokinins and reduction in the carbon dioxide compensation point (Grossmann and Retzlaff, 1997). These effects may contribute to delayed senescence and thus delayed ripening of the plant. They are site-specific compounds, which have often indicated a high resistance risk; however, they have a new mode of action that was difficult for fungi to overcome (Sudisha *et al.,* 2005).

2.5 Application of research findings from the screened fungi

Pathogens continue to be a major problem despite the fact that the world is rich in under exploited natural products. Although the use of synthetic fungicides in disease management has long been part of the practices, there is a growing concern due to their environment and health effects. Some farmers in Kenya currently practice organic farming aimed at producing healthy crops. The study was based on producing a high yield of healthy tomato crops using fungicides from naturally occurring compounds, which are environmentally friendly.

Effective environmental friendly means to prevent or reduce the damage caused by *Fusarium* wilt is valuable. The current trend to near zero market tolerance for pesticides residues in tomatoes provides an additional motivation to search for naturally occurring chemicals to control the disease. Although strobilurins have proved to be effective fungicides because of their widespread use on agriculture and originate from natural compounds, they are commonly used in developed countries. Their efficacies and great impact of the strobilurin fungicides on agriculture is reflected to very significant advancements in yield and quality in cereal production (McCartney *et al.*, 2007). Although they have served as lead compounds for the development of a new generation of industrial fungicides for crop protection, little evaluation has been done on horticultural crops such as tomatoes. With application of appropriate fungal biotechnology, there is a great potential to find novel biologically active compounds from Kenyan natural ecosystem which can be exploited for the development of safe and effective agrochemicals.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Apparatus and materials

Standard laboratory procedures were followed to sterilise media, glassware and benches. The working bench was continuously maintained sterile with 70% ethanol and a hot flame, which was also used to sterilise the inoculating needle, blades and wire loops as well as opening and closing corked sterile flasks.

Liquid and solid media as well as glassware were heat sterilized using an autoclave (Danfoss 59407-3 No. 375). The apparatus and materials were sterilized twice at a temperature of 121°C and a pressure of 1.5 bars for 15 minutes. Double sterilization was used to destroy used and contaminated old plates and other materials used before incineration. Plates containing *F. oxysporium* were used and kept in containment for a maximum of two weeks and later destroyed.

3.2 Preparation of liquid media

Liquid nutrient media was prepared by dissolving 10.0g of molasses, 4.0g glucose, and 4. g of yeast extract in 1.0L of tap water. The pH of the media was determined using a pH meters (Fishers Accument ® Model 610A) and adjusted to 5.5 using 1.0M sodium hydroxide and 1.0M hydrochloric acid solutions. The media was sterilized immediately by autoclaving twice at a temperature of 121°C and pressure of 1.5 bars for 15 minutes and left to cool on the sterile working bench.

3.3 Preparation of test plates

3.3.1 Preparation of potato dextrose agar (PDA) media

The PDA solid media were prepared by autoclaving 39g of the manufacturer's potato dextrose agar suspended in 1.0L of distilled water. This was then cooled to 40°C, before dispensing 15 ml per sterile Petri dish under sterile conditions in a lamina flow hood. PDA plates were used to grow the strains before being cultured in liquid submerged cultures, small sliced pieces of the infected tomato plant and culturing of spores of the *Fusarium* pathogen.

3.3.2 Isolation and culturing of the test organism - *F. oxysporium*

Infected tomato plants were collected in the green houses of Crops, Horticulture and Soil (CHS) Department, Egerton University. The infected parts of the plant (roots and stem) were washed using distilled de-ionised water and then sliced in small pieces. The pieces from the inner portions were sterilized with 10% of sodium hypochlorite and rinsed with sterilized distilled de-ionised water. The sterilisation of these pieces was repeated three times before the pieces were placed on freshly prepared PDA plates and left to grow at room temperature until the hyphal strands emerged. The cultures were periodically checked for purity and successively sub-cultured until pure cultures were obtained. The taxonomic identification of *F. oxysporium* was performed based on morphological and conidial characteristics. The mycelium was colourless at first, but at day 7-10 daysit becomes cream-coloured, pale yellow, pale pink or somewhat purplish. The spores are three to five celled, septate and with gradually pointed and curved ends.

3.3.3 Preparation of the test plates for antifungal activity

The sterilized PDA media was cooled to a temperature of 40°C in a water bath. About 10 ml of sterilized distilled water was poured into the plates containing the pure fully-grown culture of the test organism (*F. oxysporium*) so as to pick the spores and the suspension was thoroughly mixed. About 20ml of the spore suspension was added into 250ml of cooled media in the conical flask. The mixture was swirled thoroughly to ensure uniformity and later dispensed into sterile Petri dishes. This led to approximately 1.0 – 3.0 x 10^6 spores/ml per plate. Hemocytometer was used to determine concentration of the spores where the spore density was kept at 10^6 spores/ml. The antifungal test was carried out immediately as described later in section 3.6.

3.4 Cultivation of the selected strains in 250 ml submerged cultures

New cultures were prepared from the strains which gave positive results against the *F. oxysporium* from their corresponding agar slants, to follow up the reproducibility and selection of fungal strains for scale-up cultivation. Agar plugs from a well-grown culture of the fungi were used to inoculate the sterilized liquid malt media. The growth rate was

monitored by checking glucose levels using glucose testing strips (Diabur-test ® 5000 (Roche). The growth of the culture was monitored and evaluated daily for any contamination and biomass build-up.This indicated the progression of growth in the main culture and once glucose levels were depleted after 21 days, mycelium was separated from the culture broth by filtration.

3.5 Preparation of crude extracts from mycelium and culture filtrate

3.5.1 Crude extracts from 250 ml scale initial cultivation

Immediately the growth was stopped, mycelium was separated from culture filtrate by filtration. The crude extract from the culture filtrate were prepared using solvent-solvent extraction method. It was extracted twice with ethyl acetate in the volume ratio of 1:1. The combined ethyl acetate extract was dried using anhydrous sodium sulphate to remove residual water; then filtered and the organic filtrate concentrated under reduced pressure using a vacuum rotary evaporator at about 40-50°C. The concentrate was transferred into screw-capped vials and then kept at 4°C awaiting further analysis.

The mycelium was immediately suspended in acetone for 4 hours under constant agitation with a magnetic stirrer (Gallenkamp). The acetone filtrate was concentrated under reduced pressure using rotary evaporator to remove acetone and this left an aqueous solution. The aqueous solution was extracted thrice with equal volume of ethyl acetate. The combined ethyl acetate solution was dried using anhydrous sodium sulphate, then filtered and concentrated under reduced pressure using a rotary evaporator. The concentrate was transferred into screw-capped vials, and kept at 4°C awaiting further analysis.

The crude extracts prepared above were tested and checked for reproducibility of activity. The strain JO5125 whose extract showed the best reproducibility was selected for further cultivation on large-scale cultures in 1.0L scale replicates.

3.5.2 Cultivation of the selected strain JO5125 in 1 litre scale replicates

The media was prepared as described in section 3.2. From a well-grown plate of the selected strain JO5125, pieces of agar plugs (1cm x 1cm) were cut and used to inoculate the sterilized replicates of 250ml scale of the liquid media. Growth was monitored by

accumulation of mycelium and depletion of glucose levels. After evidence of large accumulation of mycelium the submerged cultures were aseptically transferred into sterilized 1L scale of the liquid media. The growth profile as a function of time was monitored by aseptically withdrawing aliquots of samples from the main culture broth and testing for glucose as previously described in section 3.4. Build-up of the biomass was used to monitor the rate of growth and as the rate asymptotically approached a constant, the growth was stopped. Once growth was stopped the mycelium was separated from the culture filtrate by filtration. The culture filtrates were combined while at the same time mycelia were also combined. From each of the crude extracts were separately prepared, the mycelium was processed by organic solvent extraction (refer section 3.5.1) and the culture filtrate using resin (section 3.5.3).

3.5.3 Liquid-solid adsorption resin extraction of the culture filtrate

A manufacturer's reverse phase (RP) resin (Mitsubishi HP21 DIAION) was pre-equilibrated in 1.0M HCl for 72 hours and thoroughly washed with distilled de-ionised water to neutral pH. The combined culture filtrate was passed thrice through the resin. The extract-laden column was rinsed with distilled-de-ionised water before elution with organic solvents. The column was eluted with 300ml of acetone, followed by 300ml of methanol and the eluents collected. The eluents were concentrated under reduced pressure using rotary evaporator to remove acetone and methanol, respectively. The aqueous remaining from the acetone extract was extracted thrice with ethyl acetate to give a dried crude extract, which was transferred into screw-capped vials and stored at 4°C awaiting further analysis.

3.6 Testing of the crude extracts for anti-fungal activities

Exactly 20µl of the crude extract was applied to a filter paper disc (Rundfilter, ∅6 mm, Schleicher & Schuell) and the solvent left to evaporate. The amount was delivered using an adjustable (analogue) Eppendorf micropipette. The dry paper disc was carefully placed onto the surface of the freshly prepared test plate as described in section 3.3.3 and incubated for duration of 96 hours at room temperature (Rugutt *et al*., 2006). The results were evaluated by scoring the inhibition zones around the paper discs and the diameter of the zones measured in millimetres (mm) and recorded. The larger the diameter of the zone

formed the better the activity of the extract. The same procedure was used to test for the activity of the fractions and pure compounds obtained from the chromatographic separation of the crude extracts (refer section 3.7.2) and to test for cysteine-adduct formation. This was done by adding 10µl of 1% cysteine onto a dried paper disk, which was already impregnated with 20µl of the crude extract. For each there was parallel screen of untreated crude extracts against *F. oxysporium*.

3.7 Fractionation of the crude extract based on polarity using chromatography

3.7.1 Determination of the dry weight of the crude sample

The crude extract was dissolved in minimal amount of methanol. About 2.0g of silica gel was weighed using analytical balance (Precision 310M Swiss Quality) in different beakers of known weight. To this, small amounts of acetone and methanol extract solution were slowly added and this was repeated until all the extract was adsorbed onto the silica gel particles. The solvent was left to evaporate in a running fume hood in a beaker of which was covered with a perforated aluminium foil to avoid spillage. The silica gel adsorbed extract was allowed to dry to constant weight and the mass of the crude extract determined.

3.7.2 Column chromatography

This technique was used to fractionate the crude extract into enriched fractions and eventually to purify the actual active compounds. A glass chromatographic column was mounted vertically on a fixed support. Fifty grams of silica gel was suspended in 150ml cyclohexane, and was swirled vigorously until homogenous slurry was obtained. The column was slurry packed with the silica gel suspension, ensuring that no air is trapped within the packed slurry. The dry sample adsorbed on to the silica gel was ground to fine particles, (refer to section 3.7.1) before it was loaded onto the column uniformly as a disc above the slurry. The incipient air bubbles introduced by the loaded sample were released by gently tapping the column with air filled rubber. The formed sample disc was anchored in place with acid-washed sand, which was introduced as a suspension in cyclohexane. This was done to control turbulence so as to avoid interfering with the formed and anchored

sample disc while adding the mobile phase. The column was then eluted with a mobile phase introduced as discrete solvent gradient system with increasing polarity.

The eluents from the column were collected into test tubes as sequenced fractions determined by volume and interval time of the collection. Each of the fractions were correspondingly spotted onto a silica gel pre-coated aluminium TLC plate and developed in a saturated TLC chamber with an optimal solvent system. To obtain the optimal solvent system different mixtures of different ratios of cyclohexane/ethyl acetate/methanol were prepared and evaluated. Once allowed to dry, the developed TLC plate was visualised by spraying with a freshly prepared *p*-anisaldehyde solution before heating at 115°C for 10 minutes and by UV lamp preset at fixed wavelengths, λ=254 and 365nm. The visualisation enabled observation of colour characteristics under the employed technique and determination of the retardation factor (R_f) values, which were used to pool the collected fractions into main fractions.

The pooled main fractions were concentrated by removing the organic solvents under reduced pressure using rotary evaporator. Each fraction was separately transferred to a screw-capped vial and labelled. Each of these fractions was re-dissolved in 2ml of methanol. Exactly 20µl of each of the methanol solution was tested against test organism according to the procedure outlined in section 3.6. The main fractions were further re-chromatographed but separately, on a smaller column using silica gel in the same solvent system as descried earlier in this section.

3.7.3 Thin layer chromatography(TLC)

The TLC plate used was silica gel pre-coated aluminium plate (20x20cm, Macherey-Nagel). The base line and solvent front were marked 1cm from the top and bottom of the TLC plate, respectively. The point of application of the spots were also marked 0.5cm apart. The extracts were spotted on the TLC and placed inside the chromatography tank that contained the saturated solvent systems. In the tank, the TLC plate was developed until the solvent front was reached. The plates were sprayed with *p*-anisaldehyde solution, heated to 115°C to visualize the separation. The solvent system that showed good separation on the TLC plates were noted and used as eluting solvents in the column chromatography. This

technique was used to combine the fractions that had the same retardation factor (R_f) values and to determine the purity of compounds.

3.8 Determination of minimum inhibitory concentration (MIC)

To be able to determine the extent of the observed antifungal activity accurately and precisely, the crude extracts and pure compounds were tested for minimum inhibitory concentration (MIC) in a serial dilution assay using modified published methods (Rugutt *et al*., 2006). This was done by setting up an array of sterile test tubes, in which 2ml of a suspension having 1 x 10^6 spores/ml in 2% (w/v) malt extract were added in each tube. A blank was prepared by omitting the test substances, while the negative ones contained the medium only (methanol) the solvent used to dissolve the crude extract and pure compounds. The extracts were delivered using an analogue micropipette at different concentrations in sterile tubes under sterile conditions and left to grow for 96 hours at room temperature. The concentration ranged from 50 to 1500ppm. The lowest concentration whose optical density corresponds to the blank was taken as the MIC.

3.9 NMR spectroscopy

The pure compounds which showed potent antifungal activity the pathogen were run to obtain the spectra data using Bruker ARX300 spectrometer. One and two dimension NMR experiments were carried out on the purified compounds but two dimensions NMR was only carried on purified compounds that reasonable quantity. The samples were either dissolved in deuterated chloroform ($CDCl_3$) and the solvent signals (δ_H 7.26 and δ_C 77.0) were used as reference or in deuterated methanol with solvent signals at (δ_H 4.90, δ_H 3.40 for the methyl hydrogen atoms of methanol and δ_C 49.0). Samples were prepared in 5ml of the solvent in a 5mm NMR tube and the data was processed using TOPSIN software where they were attached onto a PNG file. The chemical shifts were reported in ppm relative to the solvent peaks. From the spectroscopy experiments, structures were proposed based on the interpretation of the spectra and compared with known compounds in NMR spectroscopic data reported in literature.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Isolation of the test organism - *F. oxysporium* from diseased tomato plant

The symptoms of the diseased tomato plant was the plant drooping or wilting with brown discolouration of the vascular region visible in the cross section of infected stems or roots in the sections tangential to the xylem. The fresh diseased materials were cut in small piece, sliced off the outer layer and the piece placed on freshly prepared PDA plate. After 96 hours at 25°C the white mycelium grew sufficiently from the cut ends. The white mycelium was isolated and subcultured in PDA media to get pure cultures using monosporic techniques (Anguiz, 1989). The mycelium was colourless at first, but with age it became cream coloured, pale yellow, pale pink, or somewhat purplish.

4.2 Taxonomic identification of the strain JO5125

The fruiting bodies of strains used in this research were collected from indigenous forest in Kenya (Mt. Kenya and Londiani forests). They were placed on paper bags serialised using standard methods which included the collector initials, date and sequence in collection. They were different in morphological features, which indicated that they were different strains and were collected in the same year (2005). They were kept as herbarium materials and as pure cultures, which are stored as agar slants in the IBRL Egerton University. The selected strain JO5125 was collected from Mt. Kenya forest. Further mycological investigation was done on it using 18 SRNA technology and was identified as *Fusarium* species but the genus could not be unequivocally assigned. The mycelium gave a pink colouration when grown on a PDA plate. This suggested that a Fusarium species was producing compounds that can control F. oyxporium. This observation had been observed before at biological level (Fushs *et. al*., 1999).

4.3 Screening of the crude extracts against the pathogen

About 400 crude extracts prepared from the culture filtrate (Kex) and mycelium extracts (Mex) were screened against *F. oxysporium* using agar diffusion technique. This was done in triplicates to ascertain the results. The clear zone of inhibition around the disc

was measured and expressed as inhibition diameter. Twenty of these extracts were found to give significant activity against the pathogen with zone of inhibition of diameters between 10-40mm shown in table 4.1 below, which was about 5% of the crude extracts screened. The results significant were as expected since about 5% of the crude extracts screened would show significant activity against the *F. oxysporium* which is accepted in screening research (Rosa, 2005).

The first column in table 4.1 below gives the serial numbers of the basidiomycete, arranged in ascending order according to their strain code. The letters in the code are the initials of the collector, the first number gives the year of collection while the remaining numbers are the sequential position numbers by collection. The third table column gives the place of collection while the fifth shows the average zone diameter. The extracts that gave significant activity were recultivated from agar slants and the procedure produced flesh extracts to check for reproducibility of activity. The outcomes of activity of the crude extracts are shown in column six which was less than the first activity in column five. This may be due to difference in secondary metabolites produced by the fungi after recultivation. Changes in ambient and metabolic conditions cause different enzymatic reaction, which produces different concentrations of the secondary metabolites. The strain JO50125 was chosen because it was much more viable than the other strains and both the mycelium and culture extract were active. It was noted that there was much stronger antifungal activity in the crude extracts prepared from culture filtrate as compared to those prepared from the mycelium for strains of fungi that had both types of crude extracts.

Table 4.1: Antifungal activity for crude extracts from the 20 strains of fungal cultures

SNo	Strain (code)	Collection location	Crude extract	Screening results	Reproducibility
1	JO5057	Mt. Kenya	Kex	26±1.7	<10
			Mex	20±0.6	<10
2	JO5064	Mt. Kenya	Kex	14.3±1.2	Not observed
	JO5106	Mt. Kenya	Kex	20±1.3	12±2.6
3	JO5115	Mt. Kenya	Kex	10±1.2	Not observed
4	JO5125	Mt. Kenya	Kex	32±1.4	20±1.7
			Mex	20±1.5	10±1.8
5	JO5185	Mt. Kenya	Mex	20±1.4	15±1.4
6	JO5301	Mt. Kenya	Kex	10±1.6	Not observed
7	JO5304B	Mt. Kenya	Kex	33±1.6	20±0.8 diffuse
8	JO5315B	Mt. Kenya	Kex	39±1.2	14±1.2
9	JO5319	Mt. Kenya	Kex	19±0.8	22±1.6
10	JO5447A	Londiani	Kex	12±1.6	<10
11	JO5447B	Londiani	Kex	23±1.0	Not observed
12	JO5460A	Londiani	Kex	32±0.6	Not observed
13	JO5460B	Londiani	Kex	32±1.4	Not observed
14	JO5469	Londiani	Mex	15±1.6	<10
15	JO5505A	Londiani	Kex	30±1.2	Not observed
16	JO5506	Londiani	Kex	13.5±1.3	Not observed
17	JO5511A	Londiani	Kex	30±0.6	<10

Kex: crude extract prepared from extracellular secondary metabolites in the culture filtrate and Mex: crude extract prepared from intracellular secondary metabolites from mycelia.

4.4 Yields of crude extracts from the replicated 1.0L scale

The yields of the crude extracts prepared from the combined culture filtrate and the mycelium of the cultivated replicates on 1.0L scale of basidiomycete JO5125 are summarised as shown in figure 4.1. From Mitsubishi (DIAION) resin elution, it was noted that the crude extract from the methanol eluent (5.46g) had the highest yield in comparison to the acetone eluted crude extract (2.36g). This is expected since methanol being polar, extracts up the abundant compounds in the cultures.

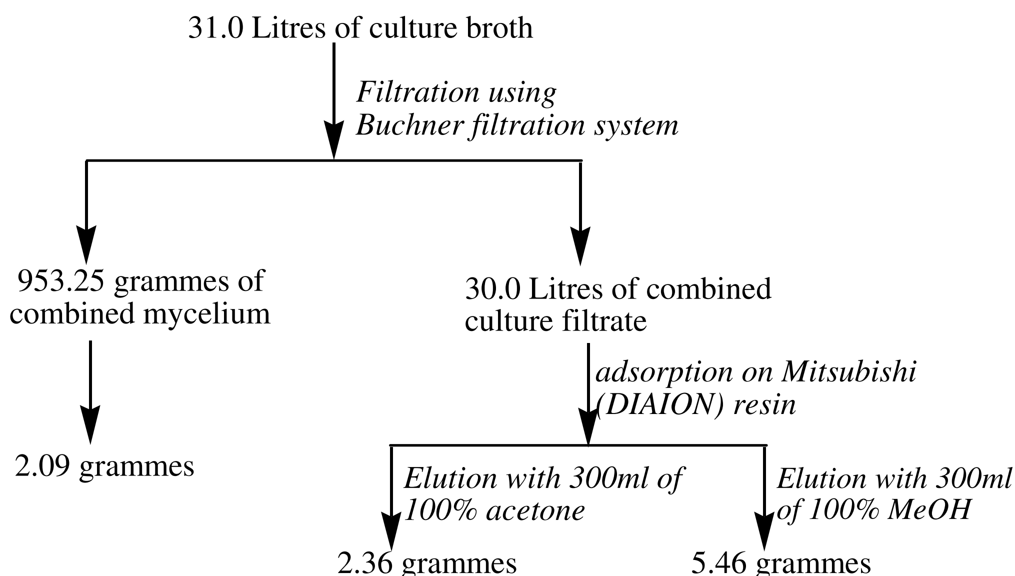


Figure 4.1: Summary of preparation of culture and mycelium crude extracts

4.5 Test cysteine-adduct formation

Nature of activity of the crude extracts was verified using cysteine to check for easy adduct forming functional groups (like polyacetylene). The results showed that the zone diameter of extracts with cysteine was the same as the diameter of extracts without cysteine. This shows that there could be absence of adduct forming functional groups in the compounds present in the crude extracts since the zone diameter remained the same after addition of cysteine. The following results were obtained as shown in Table 4.2:

Table 4.2: Bioassay results of JO5125 with cysteine against the *F. oxysporium*

Strain	Zone diameter of extracts with Cysteine (mm)	Zone diameter of extracts without Cysteine (mm)
JO5125 Kex (Acetone)	15±1.6	15±1.6
JO5125 Kex (Methanol)	10±1.2	10±1.2
JO5125 Mex	14±1.0	14±1.0

4.6 Determination of minimum inhibitory concentration (MIC) for crude extracts

Evaluation of MIC was carried out for the crude extracts in a serial dilution assay. Stock solutions of the crude extracts were prepared by dissolving the extracts in methanol. The different amounts of the crude extracts dissolved in 1 ml of methanol were 54.1mg of the strain JO5125 Mex, 31.8mg of JO5125 Kex methanol and 137.6mg of JO5125 Kex acetone. For each of the stock solutions prepared, a concentration range was tested from 50 to 1000 ppm. This was done at intervals leading to 7 concentrations prepared namely; 50, 100, 200, 300, 400, 500 and 1000ppm. This series were set up in 9 replicates, average MIC and standard deviation were calculated and the results are shown in the table 4.3 below:

Table 4.3: The average MIC and standard deviation of the above crude extracts

Crude extract	Mean±Std (ppm)
JO5125 Mex	433.3±50.0
JO5125 Kex acetone	222.2±44.1
JO5125 Kex methanol	455.6±52.7

From the Mitsubishi resin liquid-adsorption extraction process, the crude extract for acetone eluent was 222.2±44.1ppm, methanol eluent was 455.56±52.7ppm and for the mycelium prepared (Mex) extract activity was 433.3±50ppm. From the table the crude extract (JO5125 Kex) coming with the acetone eluent was found be more active than the mycelium crude extract. This indicated that the active compounds were relatively less polar.

4.7 Chromatographic fractionation and purification results

4.7.1 Fractionation and purification of mycelium crude extract

The mycelium crude extract, weighing 2.09g, was initially chromatographed on silica gel to afford three intermediate fractions I, II and III weighing 880, 620 and 560mg, respectively (figure 4.2). The intermediate products I and II were not analysed further because the activity was insignificant and had more components which made them difficult to separate with the manual column chromatography. To achieve any meaningful purification, it would have required an automated-pressurised chromatographic system like an HPLC. When tested for antifungal activity, fraction III showed significant activity and was further chromatographed twice successively leading to purification of two compounds coded JO5125M31 (NA1) and JO5125M32 (NA6) as summarised in the flow scheme (figure 4.2) below;

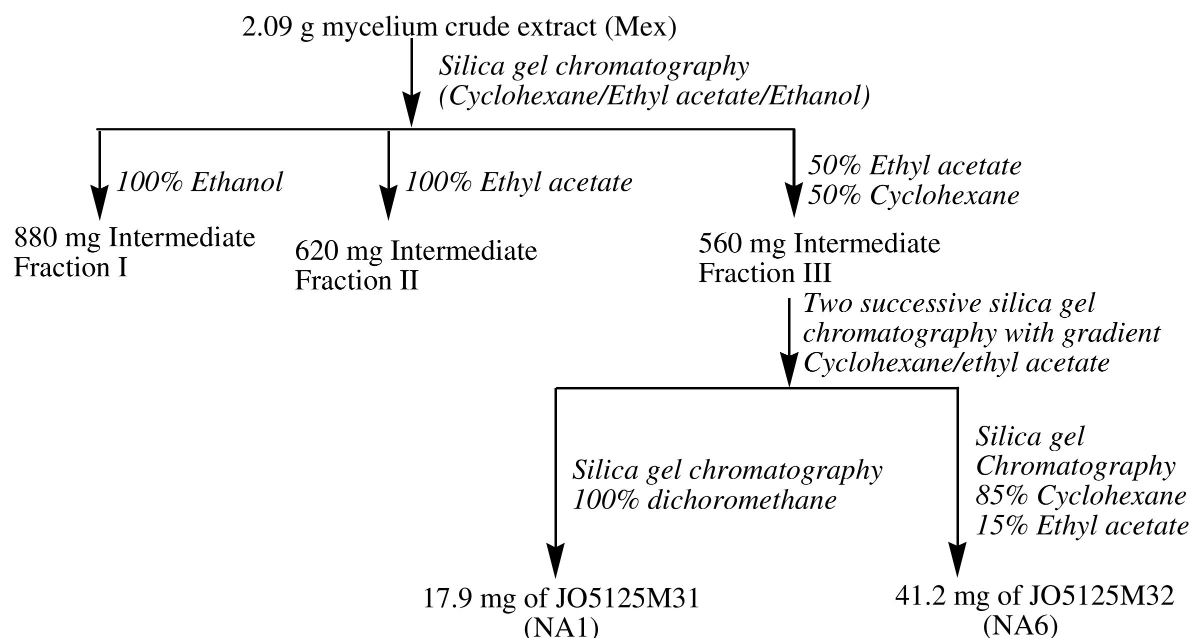


Figure 4.2: Flow scheme illustrating the fraction of mycelium crude extract and subsequent purification of compounds

From figure 4.2 (above) the compound NA1 was obtained using 100% dichloromethane to yield of 17.9mg. NA1 gave single spots on TLC analysis and auto-fluoresced as light bluish spots when viewed under longer UV wavelength (365nm). NA1

was further subjected to NMR experiments and the spectra are attached in appendices 1 and 2. From the spectral data it was easily discerned that the compound was an unsaturated fatty acid. The ^{13}C spectrum had peak at 179.29ppm, which is a typical carbonyl carbon in carboxylic acid, and two olefinic carbons that gave carbon signals at 129.9 and 130.2ppm confirming mono unsaturated fatty acid. The other carbon signals were appearing in the aliphatic carbon range between 10-40ppm. The results of structure elucidation corroborated the fact that this compound did not show any significant activity.

From intermediate fraction III, another compound NA6 (41.2mg) was found to have single spot when analysed on TLC. It gave a yellow colour when sprayed with *p*-anisaldehyde. NA6 was found to have significant antifungal activity and was tested in serial dilution assay in 9 replicates and it gave a minimum inhibitory concentration of 550±35.4ppm. The detailed discussion of the NMR spectra for NA6 will be presented and discussed in section 4.8.1.

4.7.2 Fractionation and purification of culture filtrate (acetone) crude extract

The 2.36g crude extract, prepared from acetone elution of Mitsubishi (DIAION) resin (refer figure 4.1), was chromatographed to give three intermediate fractions IV, V and VI weighing 680, 820 and 420mg, respectively. The intermediate fractions IV and VI were further chromatographed twice successively because they both had good separation profiles on TLC plated and had significant activity. Intermediate product IV gave two pure compounds JO5125K41 (NA2) and JO5125K42 (NA3) weighing 12.5 and 11.5mg respectively (see figure 4.3 below). Similarly intermediate fraction VI gave three pure compounds (see figure 4.3) JO5125K61 (NA7), JO5125K62 (NA8) and JO5125K63 (NA9) weighing 15.0, 6.5 and 2.9mg, respectively.

One of the compounds purified from intermediate fraction IV, NA2, gave a single spot and yellow colour on TLC analysis. The compound was found to show significant activity and was tested in a serial dilution assay in 9 replicates to give a minimum inhibitory concentration (MIC) value of 550±35.4ppm. This compound (NA2) was further analysed in NMR experiments and the spectra obtained are attached as appendices 3 and 4. The detailed discussion of the NMR spectra for NA2 will be presented and discussed in section 4.8.1.

From the intermediate fraction IV, another compounds NA3 was purified and gave a single spot on TLC analysis. This spot gave a red colour when spayed with *p*-anisaldehyde. The 17.8mg was divided into two portions 11.8mg for NMR experiments and 6.0mg was used for antifungal activity testing. Given that the amount left for antifungal testing in serial dilution assay was low, the experiment was conducted in duplicate that gave MIC value of 500ppm. With only two data generated for the MIC value, only the mean is reported and standard deviation was not calculated.

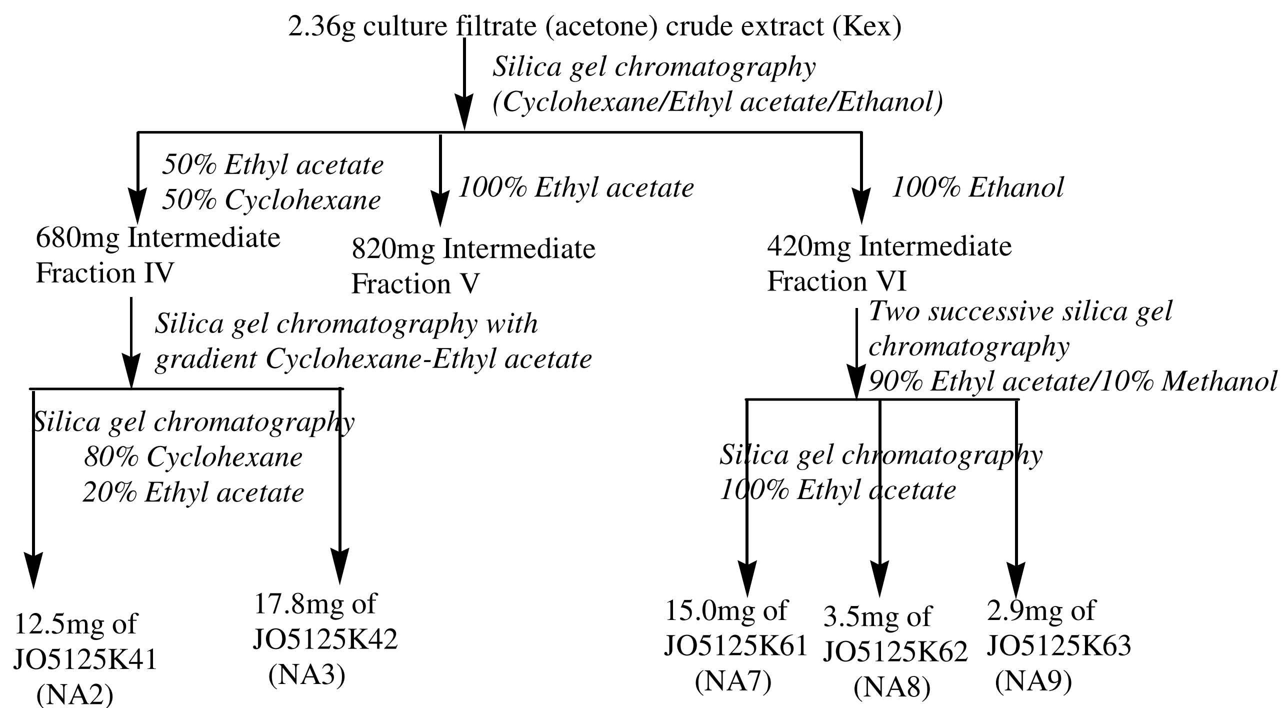


Figure 4.3: Flow scheme illustrating the fraction of culture filtrate (acetone) crude extract and subsequent purification of compounds

The intermediate fraction V (820mg), though obtained high yields, was not processed further because the TLC profiles showed that there were compounds that were not easily separable. The fraction also showed very insignificant antifungal activity and further purification of the active compounds was not prioritized.

Intermediate fraction VI was further chromatographed using 100% ethyl acetate on silica gel to afford 3 pure compounds NA7, NA8 and NA9 as shown in the flow scheme in figure 4.3. Whereas NA7 was obtained in substantial amounts sufficient for both MIC

determination and NMR analysis, the yield for NA8 and NA9 were quite low. All the amounts that were obtained for each of the latter two were sent for NMR analysis and only gave ^{1}H-NMR spectra. The detailed discussion of the NMR spectra for NA7 will be presented and discussed in section 4.8.1. In addition to low yield for NA8, there were also impurities that could not allow further experiments. NA9 was not enough for further experiments to be performed.

4.7.3 Fractionation and purification of culture filtrate (methanol) crude extract

The methanol 5.46g crude extract was chromatographed as shown in figure 4.4 to afford three intermediate fraction VII, VIII and IX weighing 1020, 1360 and 1450mg, respectively. Fractions VII and IX showed weaker activity with the former having better separation profile on TLC, hence prioritised for further purification. This was motivated by the fact that the yield was high, there were lots of non-active compounds whose presence were masking the activity of minor components when definite concentrations were prepared. This led to fraction VII giving two pure compounds JO5125K71 (NA4) and JO5125K72 (NA5) was weighing 6.2 and 7.5mg, respectively.

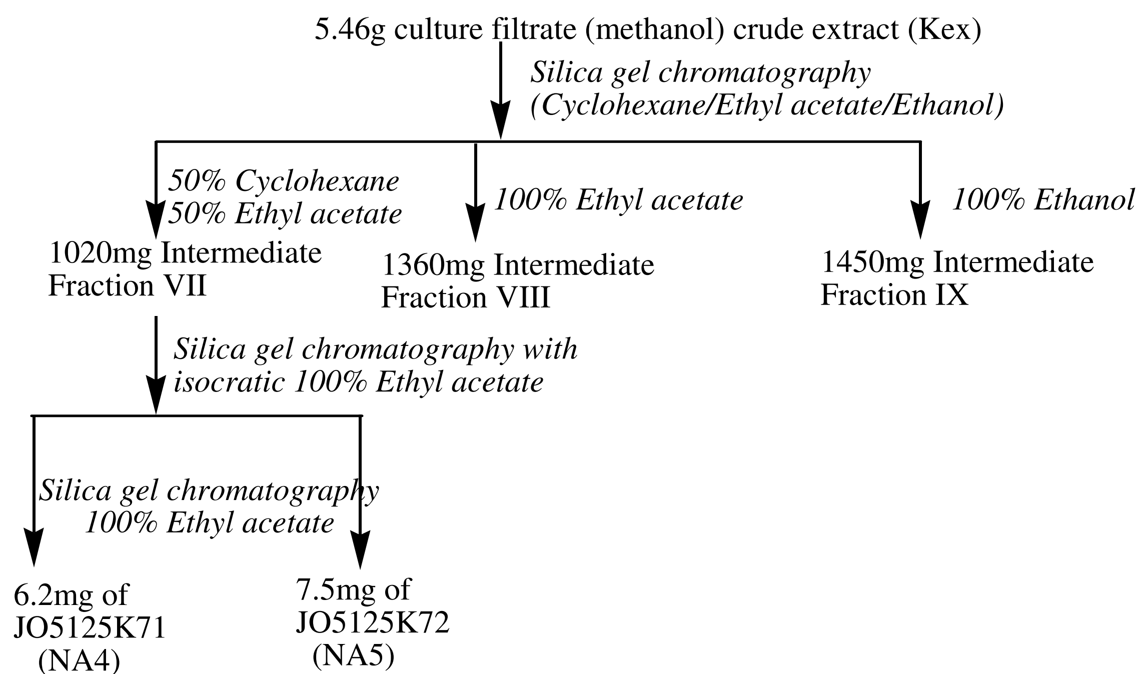


Figure 4.4: Flow scheme illustrating the fraction of culture filtrate (methanol) crude extract and subsequent purification of compounds

NA4 and NA5 were obtained in low yields and were only analysed using ^{1}H NMR experiments. The yields were not sufficient enough for further experiments to be conducted given the power of the NMR machine. The intermediate fractions VIII and IX were not chromatographed further when the activity and the separation profiles were considered.

Table 4.4: The antifungal activity of the intermediate fractions and the purified compounds against the *F. oxysporium*

Intermediate Fraction	**Antifungal activity (mm)**	**Corresponding purified compound**
I	10±1.3	None
II	9±0.9	None
III	15±1.6	JO5125M31 (NA1)
		JO5125M32 (NA6)
IV	12±0.6	JO5125K41 (NA2)
		JO5125K42 (NA3)
V	<10	None
VI	15±1.2	JO5125K61 (NA7)
		JO5125K62 (NA8)
		JO5125K63 (NA9)
VII	10±2.1	JO5125K71 (NA4)

Each of the intermediate factions (I – IX) was tested for activity using agar diffusion assay with the results presented in the second column in table 7 against the corresponding fraction in the first column. From each fraction the corresponding purified compounds are listed in the third column. When the NMR experiments were conducted on the purified compounds, compounds NA2, NA6 and NA7 were found to be same compound, a result that is in agreement with the physic-chemical characteristics observed on TLC analysis. Once this result was obtained, the three were pooled together to give 55mg and a solution of known concentration was prepared. The solution was serially diluted for testing against the

test organism in a serial dilution assay in 9 replicates. From this a minimum inhibitory concentration (MIC) value of 550±35.4ppm was found.

4.8 Structure elucidation of the pure compounds

4.8.1 Structure elucidation of NA2, NA6 and NA7

The three compounds were found to be the same as shown in table 4.5 below which represents the chemical shifts of protons and carbon-13 in their respective NMR spectra. The chemical shifts were serialised according to the IUPAC naming system of the structure and HSQC was used to assign the protons attached to carbons. The ^{13}C NMR spectra of NA2 and NA7 showed the presence of 18-carbon compound while NA6 showed 17 carbons. They were only slight differences for ^{13}C NMR and ^{1}H chemical shifts, a proof that the compounds were the same. There were two carbonyl carbon in each compound serialised 1 and 7, quaternary carbon (S/No's 14, 16, 17, 18.) olefinic carbons (11, 12), oxygenated carbon (3), methylene carbon (4, 5, 6, 8, 9, 10) and one methyl carbon (19). Structure elucidation was done on compound NA7 since the peaks on the spectra were much clearer than others (see appendices 16-25).

Table 4.5: ^{1}H NMR and ^{13}C NMR data for NA2, NA6, and NA7

Serial Nos	**NA2**		**NA6**		**NA7**	
	^{13}C	**^{1}H**	**^{13}C**	**^{1}H**	**^{13}C**	**^{1}H**
1	171.5	-	171.5	-	171.8	-
3	73.6	5.01	73.6	5.00	73.6	4.91
4	34.9	1.73	34.9	1.72	35.0	1.43
5	22.5	1.73	22.4	1.78	22.4	1.72
6	43.1	2.60 2.22	43.1	2.61 2.18	43.0	2.56 2.23
7	211.4	-	212.9	-	213.5	-
8	36.8	2.79 2.35	36.9	2.62 2.16	36.7	2.61 2.18
9	21.2	2.18 1.49	21.1	2.17 1.53	21.1	1.89 1.42
10	31.2	2.18 2.23	31.1	2.29 2.17	31.2	2.29 2.18
11	132.0	5.69	132.4	5.70	132.0	5.70
12	133.3	7.04	133.4	7.02	133.4	6.78
13	108.3	6.35	108.8	6.37	108.6	6.78
14	160.5	-	161.3	-	162.9	-
15	102.6	6.34	102.6	6.38	102.0	6.18
16	165.7	-	165.4	-	165.2	-
17	104.2	-	-	-	103.3	-
18	144.2	-	144.0	-	143.8	-
19	21.0	1.42	20.9	1.34	20.8	1.36

The ^{1}H NMR showed the presence of two meta-coupled aromatic protons at δ 6.3 (d, *J*=2.4 Hz, H-15) and δ 6.1 (d, *J*=2.4 Hz, H-13) and their ^{13}C NMR signals were observed at δ 108.6 and δ 102.6 respectively. The NOESY spectrum confirmed the presence of a proton at C-13 through correlation with H-12. The HMBC spectrum also showed correlation between C-11 and H-13. Two downfield resonances at δ 162.9 and δ 165.2 had their signals

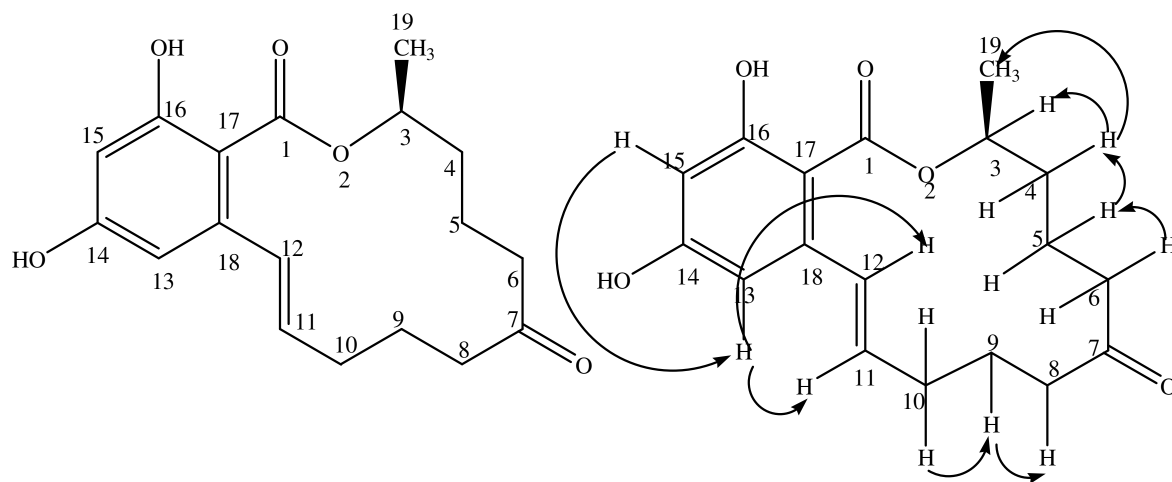
observed in ^{13}C NMR but were absent in DEPT spectrum therefore, quaternary carbons and given positions C-14 and C-16, respectively. These were confirmed by HMBC spectrum, which showed correlation between C-16 and H-15 and C-14 with two protons H-13 and H-15. The ^{13}C NMR spectrum further showed two quaternary carbons at δ 103.3 (C-17) and δ 143.8 (C-18). The assignments were confirmed by HMBC spectrum correlations between C-17 and proton H-15 and C-18 with two protons (H-11 and H-12). The ^{1}H NMR of NA7 also showed characteristic olefinic proton absorptions at δ 5.7 (*J*=7.2 Hz, H-11) and δ 6.9 (d, *J*=15.1 Hz, H-12) and were found to be *trans* protons substituted on the double bond since they commonly have values of approximately 17 Hz. Their ^{13}C NMR signals were observed at δ 132.3 and δ 133.4 respectively. The DEPT spectrum revealed six methylene groups, which were seen in the COSY spectrum to be linearly bonded. The difference in their ^{13}C NMR signals observed in methylene carbons showed the presence of the carbonyl carbon C-7 in between them since methylene protons next to carbonyl group appear at δ 2-3 the region expected for protons on carbon next to unsaturated centre. This was also confirmed by HMBC spectrum correlations between C-7 and five protons (2H-6, 2H-8 and H-5). Two non-equivalent protons were observed on C-6, C-8 and C-10, showing that the structure was rigid cyclic. The ^{1}H NMR spectrum revealed a methyl group proton resonance at δ 1.3 (d, *J*=6.9 Hz, 3H-19) and its ^{13}C NMR signal occurred at δ 20.18. The adjacent methine group showed ^{1}H and ^{13}C NMR signals at δ 4.9 and 73.65, respectively, indicating bonding to oxygen. HMBC correlations were observed between C-3 and 3H-19. The carbon resonance at δ 171.7 (C-1) was ascribed to the ester carbonyl group. NOESY spectrum (figure 4.5) confirmed the structure by showing the correlations between the protons. The structure was supported by the literature values, which are given in the table below.

Table 4.6: ^{1}H-NMR, ^{13}C-NMR' DEPT and HMBC data for compound NA7

Position	^{1}H	^{1}H literature	^{13}C	^{13}C Literature	DEPT	HMBC (H→ C)
1	-	-	171.81	171.77	C	
3	4.9	4.72	73.65	73.38	CH	
4	1.4	1.44	35.04	34.65	CH_2	5
5	1.7	1.28	22.42	22.25	CH_2	
6	2.2 2.5	2.02 1.78	43.0	42.69	CH_2	4, 5
7	-	-	213.54	211.02	C	
8	2.3 2.6	1.58 2.34	36.71	36.29	CH_2	9, 10
9	1.4 1.9	1.14 2.09	21.14	21.25	CH_2	10
10	2.2 2.28	2.14 2.18	31.22	31.23	CH_2	9
11	5.7	5.38	132.30	132.44	CH	18
12	6.9	6.97	133.44	133.55	CH	13, 17, 18
13	6.3	6.34	108.62	108.80	CH	12, 14, 15
14	-	-	162.97	161.35	C	
15	6.1	6.27	102.06	102.79	CH	13, 16, 17
16	-	-	165.20	166.54	C	
17	-	-	103.35	104.14	C	
18	-	-	143.85	144.37	C	
19	1.3	0.97	20.18	20.50	CH_3	3, 4

From this interpretation the structure was identified as Zearalenone (common name) while IUPAC name is 3,4,5,6,9,10-hexahydro-14,16-dihydroxy-3-methyl-[S-(E)]-1H-2-benzoacyclotetradecin-1,7-(8H)-dione. The compound belongs to the family of macrolides

and had been isolated from a *Fusarium* species (Pohland *et al*., 1982). The identity of this compound was established by comparison of ^{1}H and ^{13}C NMR spectroscopic data with those reported from literature. The ^{1}H was operated at 500MHz while ^{13}C at 125MHz (Cordier *et al*., 1990). The molecular formula from ^{13}C and ^{1}H data of NA7 was $C_{18}H_{22}O_5$, which gives eight as the unsaturation number. This information complements to the structure of the compound shown below and can be accounted for by the aromatic ring, one double bond, two carbonyls and one lactone ring.



Zearalenone (**47**) and NOESY correlations

The proposed biosynthetic pathway of Zearalenone shown in figure 4.5 is a relatively simple structure, which is derived entirely from acetate-malonate units. Head to tail condensation of nine acetate units synthesizes it via the acetate-malonyl-coenzyme enzyme system. The aromatic ring is derived through the polyketide synthesis pathway where it catalysis the biosynthesis of polyketides which are structurally diverse class of natural products such as antibiotics, toxins and pigments (Blackwell *et al.,* 1985). The cyclization product formed from a poly-β-keto ester, requiring a variety of reduction processes and formation of an aromatic ring by aldol condensation near the carboxyl terminus.

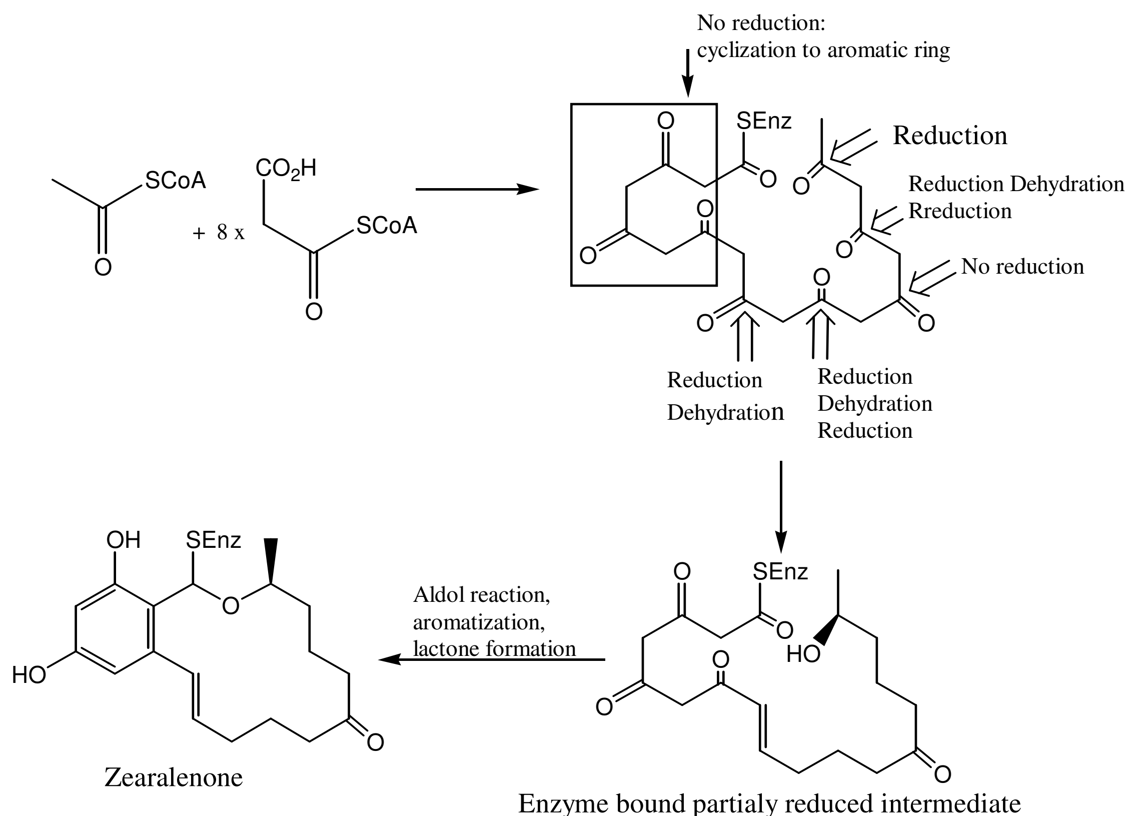


Figure 4.5: Biosynthetic pathway of Zearalerone

4.8.2 Structure elucidation of NA3

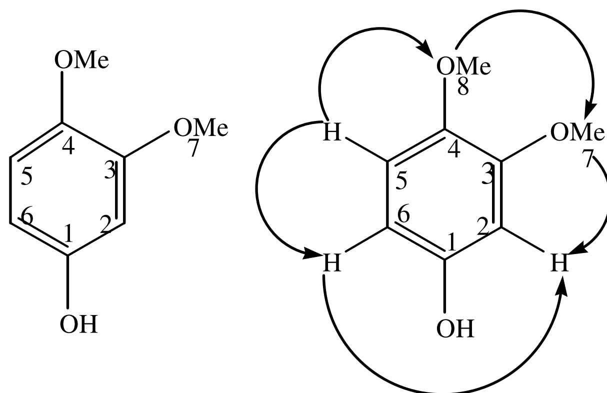
The NMR spectra of 1- and 2 dimension of NA3 are shown in appendices 5-11. From the spectra data it showed the presence of three quaternary and three methine carbon in the aromatic region and two oxygenated methyl groups. The ^{1}H NMR of NA3 showed the presence two *ortho-coupled* aromatic protons, at δ 6.74 (d, *J*=9Hz, H-5) and δ 6.3 (d, *J*=11 Hz, H-6) and *meta-coupled* aromatic proton at δ 6.48 (d, *J*=2.7Hz, H-1). Their ^{13}C NMR signals were observed at δ 112.5, 106.0 and 100.8 and were ascribed as C-5, C-6 and C-2, respectively. The HMBC spectrum confirmed correlation between H-2 and C-6, H-5 and C-6, and H-6 and C-5. The COSY spectrum also showed correlations between H-5 and H-6 and H-6 and H-2. Three downfield resonances were observed at δ 143.4, 150.3 and 150.1 and were described as C-1, C-3 and C-4 respectively. Their signsls were absent in DEPT spectrum, confirming that they were quaternary carbons. The HMBC spectrum showed correlation between H-2 and C-1, H-2, and C-4, H-5 and C-1, H-5, and C-4, and lastly H-6

and C-1, H-6 and C-4. The proton NMR spectrum revealed $–OCH_3$ signal that appeared as a sharp singlet at δ3.9. These were confirmed by DEPT spectrum, which revealed two oxygenated carbon groups at δ 56.0 and 56.7. The HMBC spectrum showed correlation between 3 x protons (7) and C-3, and 3protons (8) and C-4 and confirmed their positions in the aromatic ring. NOESY spectrum also showed correlations between the protons of the methoxy groups. The spectra data are given in the table 4.7 below.

Table 4.7: ^{1}H-NMR, ^{13}C-NMR, DEPT and HMBC data for NA3

Position	1H	13C	DEPT	HMBC (H →C)
1	-	143.41	C	
2	6.48 d	100.83	CH	1,4,6
3	-	150.35	C	
4	-	150.14	C	
5	6.74 d	112.59	CH	1,4
6	6.48 d	106.0	CH	1,2,4
7	3.82 s	56.77	CH_3	1,3,8
8	3.81 s	56.03	CH_3	4,7

From this interpretation the structure was therefore proposed and identified from literature as 3,4-dimethoxyphenol. The molecular formula from ^{13}C and ^{1}H NMR data of NA3 was $C_8H_{10}O_3$ and unsaturation number was proposed to 4. The aromatic ring accounts for the unsaturation number.



3,4-Dimethoxyphenol (**48**) and NOESY correlations

4.9 DISCUSSION

In the screening of the 400 crude extracts of both mycelium and culture filtrate against the pathogen, thirteen of them gave a zone diameter between 20-40mm in the bioassay analysis. The activity of the fungal extracts was quite significant as compared with other crude extracts extracted from native Kenyan plants against *F. oxysporium*. The methanol extract of *Warbugia ugandensis* leaves, *Azadirachta indica* leaves and seeds, and *Tagetes minuta* aerial part gave a zone diameter of 12.0mm, 11.5mm, 10.0mm, 9.0mm and 10.0mm respectively (Rugutt *et al*., 2006). This shows that the fungi have a potential of controlling the pathogen. After checking for reproducibility of activity only two crude extracts (culture extract of strain JO5125 and JO5319) gave a zone diameter greater than 20mm. The strain JO5125 was selected because both culture and mycelium extracts were active with culture having significant activity.

The strain JO5125 was found to be *Fusarium* species after molecular markers technology investigation using 18 SRNA. The difference between strain JO5125 and the phytopathogen *F. oxysporium* was that on sub-culturing on PDA media, the mycelium of strain JO1525 formed pink pigmentation on the plate while the *F. oxysporium* formed a purple pigmentation. Also the **formae speciales** of fungus JO5125 has not been identified. This is possible as confirmed from literature where Fuchs *et al*. (1999) and Fravel *et al.* (2005) found that non-pathogenic *F. oxysporium* could effectively suppress *Fusarium* wilt of the tomato through a host-mediated mechanism.

Three crude extract were prepared from submerged cultures of fungus JO5125 which were JO5125 Mex, JO5125 Kex methanol, JO5125 Kex acetone and their MIC concentrations was found to be 433.3±50ppm, 455.56±52.7ppm and 222.2±44.4ppm respectively. The acetone eluent culture extract was more active giving the lowest MIC concentrations as compared to other extracts. Also activity of the intermediates fractions of the acetone eluent extract were more active and had good separation thus more compounds were purified from them compared to fractions of the mycelium and methanol eluent extracts. The MIC from these extracts was quite significant compared with other crude extracts such as from the essential oils from *Curcuma longa* that gave complete mycelia

inhibition at 2000ppm (Gurdip *et al*., 2002) and from *Flourensia* species, which gave total inhibition at 1500ppm.

The compounds isolated from strain JO5125 were 3,4-dimethoxyphenol and zearalenone. The two compounds were isolated from acetone eluent extract and labelled as NA2 for zearalenone and NA3 for 3,4-dimethoxyphenol although zearalenone was also isolated from mycelium extract labelled as NA6 and as NA7 for methanol eluent extract. Zearalenone is a white crystalline solid, produced by fermentation of various *Fusarium* species such as *F. graminearum, F. culmorum*, and *F. crookwellence.* 3,4-dimethoxyphenol has been produced by various plant species including *Croton*, *Passiffora, lysimachia* and from a range of marine microorganism. The average MIC for 3,4-dimethoxyphenol was 500ppm while for zearalenone was 550±35.4ppm. Comparing the results with the fungicides used to control the pathogen, Song *et al*. (2004) found that MIC of the fungicides screened *in vitro* on tomato wilt pathogen for seven fungicides, prochlorax, carbendazin, thiram, toclofos-methyl, hymezazol, azoxystrobin and carboxin were 0.019, 0.235, 26.292, 53.606, 69.961, 144.58, and 154.03ppm respectively. Other antifungal compounds isolated from plants such as muzigadial (**6**) was isolated from *Warbugia ugandensis* gave a MIC of 50ppm (Rugutt *et al*., 2006). Rutacridone (**10**) and its epoxide derivative (**11**) were found to inhibit the growth of *F. oxysporium* at low concentration of 20ppm from ethyl acetate extracts of the roots of *Ruta graveolens* (Kumudini *et al*., 2005). Although the results of the MIC of the zearalenone and 3,4-dimethoxyphenol are not as significant as the above antifungal compounds, they also have a potential to control the plant pathogen.

However, zearalenone is a mycotoxin but only becomes injurious to mammals upon ingestion. From the MIC results obtained zearalenone has been found to be active against the phytopathogen *F. oxysporium.* The pathogen infects the tomato plant by penetrating through root tips or wounds on roots. Therefore if zearalenone has to be applied to control the pathogen it must be applied onto the soil. Therefore, there is a high probability that bioaccumulation of the mycotoxin might not appear in the fruits of the crop since it's not sprayed directly on the plant but absorbed through the roots. The plant system also has a mechanism of degrading the toxins since the pesticide residual found in the harvested crop are of low concentrations. Zearalenone qualifies as environmentally friendly fungicide since it is from nature, thus biodegradable and can easily be degraded by plants mechanism. The

activity can be associated with the lactone ring found in structure of zearalenone. The lactone rings are major pharmacophoric substructures found in compounds isolated from fungi. These compounds are fomannosin (**20**), isocoumarin oospolactone (**21**), fuluoferruginin (**22**), lentinellic acid (**23**) and 4-chloro-8-hydroxy-6-methoxy-3-methylisochromanone (**25**) that have antifungal activity against other plant pathogens. The other compound 3,4-dimethoxyphenol was also found to be effective in to controlling *F. oxysporium* as shown from the MIC results. It is biodegradable since it is from nature and not toxic. Zearalenone and 3,4-dimethoxyphenol can be potentially used for the development of new fungicides if zearalenone will have low residual effect on the product and can be effective agrochemicals.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMENDATIONS

5.1 Conclusion

On screening of the crude extracts from both the mycelium and culture broth of the strains against *F. oxysporium,* 20 of the extracts screened gave significant activity. This was as expected that in a random hit rate analysis about 5% of the extracts screened would show significant and interesting results. The active strains were recultivated again to check for the reproducibility of activity. One strain serialized JO5125 was selected from the recultivated strains and crude extract were prepared from the mycelium and culture filtrate using different solvents. From the Mitsubishi resin liquid-adsorption extraction process for culture filtrate the acetone eluent crude extract was more potent against *F. oxysporium* (an *in vitro* MIC of 222.2±44.4ppm) compared to that of methanol (MIC 455.56±52.7ppm). This shows that the extract eluted with less polar solvent (acetone) was more effective in controlling the pathogen than the extract eluted with more polar (methanol). The mycelium crude extract showed low efficacy (MIC 433.3±50ppm) against *F. oxysporium.*

The fractions that had good separation were purified further to give pure compounds. NMR analysis was done on pure compounds and their spectral data has been provided but some were in very low yields only the proton NMR studies were possible. They were only two compounds (3,4-dimethoxyphenol and zearalenone) whose yield were high therefore 1 and 2 dimension NMR spectra analysis was done on them. MIC was determined for 3,4-dimethoxyphenol and zearalenone where they gave average *in vitro* MIC of 500 and 550±35.4ppm respectively against the pathogen. The MIC from the pure compounds was higher as compared to that of the crude extracts. The crude extracts especially the acetone extract where the two compounds were purified from was more effective due synergistic effects from the compounds present. These antifungal compounds were found to be mid-polar from their chemical nature and evidence that the agents responsible for activity are organic compounds. The results obtained from the crude extracts and pure compounds supports the use of fungi to produce antifungal compounds. The antifungal compounds produced can be of low cost, biodegradable and selective in activity and thus fit as agents of

food protection. Also the findings that the compounds inhibit *F. oxysporium* can be harnessed for the control of tomato fusarial wilts especially in soil interactions to inhibit xylem blockage. This is possible as in the chemical defence theory in the wild can be inferred, *Fusarium* species can inhibit another *Fusarium* species. Finally, this study further reaffirms the assertion that there are plenty of untapped resources in the higher fungi, which could be of great utility to mankind.

5.2 Recommendations

Following recommendations were made in relation to the study:

i. The other fungal strains that showed positive results during screening should be investigated for the production of compounds that have activity against *F. oxysporium.*
ii. Broad screening of more basidiomycetes against the *F. oxysporium* test organism
iii. *In vivo* analyses of the pure compounds on pot experiments and evaluate the bioaccumulation of these compounds in the tomato fruits.

6.0 REFERENCES

Agrios, G. N. (1998). Plant pathology. Academic press Ltd., 4th edition New York.

Ahmed, I. H., Labuschagne, N. and Korsten, L. (2007). Screening rhizobacteria for Biological control of *Fusarium* root and crown rot of sorghum in Ethiopia. *Biol. Cont.* **40**: 97-106

Alabouvette, C. Steinberg, C., Trillas-Gay, M. I., and Cotxarrera, L. (2002). Use of sewage sludge compost and *Trichoderma asperellum* isolates to suppress *Fusarium* wilt of tomato. *Soil Bio. and Biochem*. **34**: 467-476.

Allen, T. W., Enebak, S. A. and Carey, W. A. (2004). Evaluation of fungicides for control of species of *Fusarium* on long leaf pine seed. *Crop Prot*. **23**: 979-982.

Anguiz, R. (1989). Anastomosis group pathogenicity and other characteristics of *Rhizoctonia solani* isolated from potatoes in Peru. *Plant Dis.* **73**: 199-201.

Anke, T. and Steglich, W. (1989). β-Methoxyacrylate antibiotics from biological activity to synthetic analogues: Biologically active molecules – identification, characterization, and synthesis (SCHLUNEGGAR, U. P., Ed), pp. 9-25. Berlin- Heidelberg: Springer-Verlag.

Anke, T. and Stelglich, W. (2000). Strobilurins a success story. *Angew Chem. Int. Ed*. **38**: 408-412.

Anke, T., Schram, G., Stelglich, W. and Von Ja Gow, G. (1988). Structure-activity relationship of natural and synthetic E-β-Methoxyacryalates of the strobilurin and oudemansin series, in: The roots of modern biochemistry (Kleinkauf, H., Von Dohren, H., Janicke, L., Eds.), pp. 657-662. Berlin.

Anshu D., Mani U., Babita R., Pant U. C. and Gupta I. J. (1998). Synthesis and antimicrobial evaluation of some new fluorinated spiro [[1,5] -benzothiazepin-2,3' [3'H] -indol] -2'-(1 'H) –ones. *J. Flourine Chem*. **91**: 171-174.

Arnold, A. E., Maynard, Z., Gilbert, G. S., Coley, P. D. And Kursar, T. A. (2000). Are tropical fungal endophytes hyperdiverse? *Ecol. Lett*. **3**: 267-274.

Bailey, K. L. and Lazarovits, G. (2003). Suppressing soil borne diseases with residue management and organic amendments. *Soil Tillage Res*; **72**: 169-180.

Basset, C., Sherwood, R. T., Kepler, J. A. and Hamilton, P. B. (1967). Production and biological activity of fomannosin, a toxic sesquiterpene metabolite of *Fomes annosum*. *Phytopathol.* **57**: 1046-1052.

Berdy, J. (2003). Are actinomycetes exhausted as a source of secondary metabolites? In Biotechnologija (ISSN0234-2751) Prot. 9th Int. Symp. On the Biology of Actinomycetes. Debanov VG. pp. 13-34.

Bing-Ji, M., Yan, Z., Lian-Zhen, L., He-Min, L., Zhi-Mong, G. and Yuan, R. A. (2008). New Cyathane-xyloside from the mycelia of *Hericium erinaceum*. *Z. Naturforsch.* **63**: 1241-1242.

Blackwell, D. A., Greenhalgh, R. and Miller, J. D. (1985). ^{13}C NMR study of the biosynthesis of toxins by *Fusarium graminearum*. *J. Biol chem*. **260**: 4243-4247.

Butler, M. S. (2005). Bioactive molecules from natural products. *Nucl. Acid. Res*. **34**: 678-683.

Cabello, M. A., Platas, G., Collado, J., Diez, M. T., Martin, I. Vicente, F. Meinz, M., Onishi, J. C., Douglas, C., Thompson, J., Kurtz, M. B., Schwartz, B. E., Bills, G. F., Giacobbe, R. A., Abruzzo, G. K., Flattery, A. M., Kong, L. and Pelaez, F. (2001). Arundifungin, a novel antifungal compound produced by fungi; biological activity and taxonomy of the producing organism. *Int. Microbial*. **4**: 493-102.

Cavigelli, M. A., Lengnick, L. L., Buyer, J. S., Fravel, D. R., Handoo, Z. A., McCarty, G. W., Millner, P. D., Sikora, L. J., Wright, S. E., Vinyard, B. T. and Rabenhorst, M. (2005). Landscape level variation in soil resources and microbial properties in a no-till corn field. *Appl. Soil Ecol.* **29**: 99-123.

Chew, P. S. and Hall, R. (1984). Effect of *Pythium* root rot on yield of white bean *Phaseolus vulgaris*. *Crop Prot*. **3**: 423-429.

Chitwood, D. J. (2002). Phytochemical based strategies for nematode control. *Ana. Rev. Phytopathol*. **40**: 221-249.

Clough, J. M. (1993). The strobilurins,oudemansins, and myxothiazols, fungicidal derivatives of β-methoxacrylic acid. *Nat. Prod. Rep*. **10**: 565-574.

Cole, M. D., Bridge, P. D., Dellar, J. E., Fellows, L. E., Cornish, M., C. and Anderson, J. C. (1991). A neo-clerodane balcalensis diternoid from *Scutellaceae. Phytochem.* **31**: 3433-3435.

Copping, L. G. and Menn, J. J. (2000). Biopesticides, their action application and efficacy. *Pest Manag. Sci*. **56**: 651-676.

Cordier, C., Gruselle, M., Jaouen, G., Hughes, D. W. and McGlinchey, M. J. (1990). Structures of Zearalenone and Zearalenone in solutions: A High-Field NMR and molecular modeling study. *Magn. Reson. Chem*. **28**: 835-845.

Demain, A. L. (1999). Pharmacologically active secondary metabolites of microorganism. *Appl. Microbial Biotehnol*. **52**: 455-463.

Dong-Ze, L., Rui-Rui J., Fei, W. and Ji-Kai, L. (2008b). A new spiroaxane sesquiterpene from cultures of the basidiomycetes *Pholiata adiposa*. *Z. Naturforsch* **63b**: 111-113.

Dong-Ze, L., Rui-Rui, J., Fei, W. and Ji-Kai, L. (2008a). A novel sesquiterpene from cultures of the Basidiomycetes *Boletus calopus*. *Z. Naturforsch* **63b**: 114-116.

Elizabeth, A. B. and Handelsman, J. (1999). Biocontrol of plant diseases. *FEBS Microbiol Lett*. **171**: 1-9.

Elmer, W. H. and McGovern, R. J. (2004). Efficacies of integrating biologicals with fungicides for the suppressions of *Fusarium* wilt of tomato. *Crop Prot.* **23**: 909-914.

Flood, J., (2003). *Fusarium* wilt of tropical perennial crops: challanges to management. CABI. Bioscience Egham surrey, United Kingdom.

Fravel, D. R., Deahl,. L., and Stommel, J. R. (2005). Compatibility of the biocontrol fungus. *Fusarium oxysporium*. Strain. CS-20 with selected fungucides. *Biol. Cont*. **34**: 165-169

Fuchs, J. G. Moenne-Locioz, Y. and Defago, G. (1999). Ability of non-pathogenic *Fusarium oxysporium* Fo47 to protect tomato against *Fusarium* wilt. *Biol. Cont*. **14**: 105-110.

Grossmann, K. and Retzlaff, G. (1997). Bioregulatory effect of the fungicidal strobilurin kresoxim-methyl in wheat (*Triticum aestivum*). *Pest. Sci*. **50**: 11–20.

Gullino, M. L., Leoux, P. and Smith, C. M. (2000). Uses and challenges of novel compounds for plant disease control. *Crop Prot*. **19**: 1-11.

Gurdip, S., Prakash, S. and Sumitra, M. (2002). Chemical and biocidal investigations on essential oils of some Indian *Curcuma* species. Progress in Crystal Growth and Characterization of Materials 75-81.

Hang, Y. D. and Woodams, E. E. (2003). Control of *Fusarium oxysporium* by baking soda. *Lendensm.-Wiss. u-Technol.* **36**: 803-805.

Hansen, E. M., Myrold, D. D. and Hamm, P. B. (1996). Effects of soil fumigation and cover crops on potential pathogens, microbial activity, nitrogen availability, and seedling quality in tomato nurseries. *Phytopathol.* **80**: 698-740.

Hautzel, R., Anke, H. and Sheldrick, W. S. (1990). Mycenone, a new metabolite from *mycena* species TA 87202 (Basidiomycetes) as an inhibitor of isocitrate lyase. *J. Antibiot.* **43**: 1240-1244.

Hawksworth, D. L. (2001). The magnitude of fungal diversity; the 1.5million species estimate revised. *Mycol. Res.* **105**: 1422-1432.

Hengkui, L., Yamei, D., Jianxin, W., Changjun, C., Juping, N. and Mingguo, Z. (2008). JS399-19, a new fungicide against wheat scab. *Crop Prot.* **27** 90-95.

Herbert, J. A. and Marx, D. (1990). Short term control of Panama diseases in South Africa. *Phytophylactica.* **22**: 339-340.

Herman, G. E. (2000). Myths and dogmas of biocontrol: changes in perception derived from research on *Trichoderma harzianum* T-22. *Plant Dis*. **84**: 377-393.

Holloway, P. (2003). Potential of fungal chlorinated metabolites. ARDI Project 00-459. *Phytochem.* **36**: 35-38.

Hostettman, K. and Martson, A. (2000). Chemistry and pharmacology of natural products. *J. Antibiot***. 53**: 1153-1166.

Ibadur, R .S., Pravin, K. S., Jaya S. and Jagdamba, S. (2003). Synthesis and Fungicidal Activity of Novel 4,4'-Bis(2''-aryl-5''-methyl/unsubstituted-4''-oxo-thiazolidin-3''-yl) Bibenzyl. *J. Agric. Food Chem*. **51**: 7062-7065.

Jorgensen, L. N., Henriksen, K. E. and Nielsen, G. C. (1999). Adjusting thresholds for *Septoria* control in winter wheat using strobilurins. In: Van Ginkel, M., Mc Nab, A., Krupinsky, J. (Eds.), *Septoria* and *Stagonospora* Diseases of Cereals: A Compilation of Global Research. CIMMYT, Mexico, DF, pp. 173–175.

Kaeokamnerd, W. (1998). Khao, Yae National Park and its diversity; the importance of inventory. *Proceedings of the Asia-Pasific Mycological conferences on Biodiversity and Biotechnology, July 6-9. Hua Hin, Thailand.*

Klasse, H. J. (2002). Calcium cyanide – An important tool in methyl bromide replacement strategies. SKW. Trostberg AG, Germany.

Knight, V., Sanglier, J., DiTullio, D., Braccili ,S., Bonner, P., Weaters, J., Hughes, D. and Zhang, L. (2003). Diversifying microbial natural products for drug discovery. *Appl Microbiol* **23**: 1146-1152.

Kumudini, M. M., Kevin, K. S., David, E. W. and Stephen, O. D. (2005). Algicidal and Antifungal compounds from roots of *Ruta graveolens* and synthesis of their analogs. *Phytochem.* **66**: 2689-2695.

Larkin, R. P. and Fravel, D. R. (2002). Efficacy of various fungal and bacterial biocontrol organism for *Fusarium* wilt of tomato. *Plant Dis*. **83**: 1022-1028.

Lasure, L. L. (2000). Screening microbial metabolites for new drugs – Theoretical and practical issues. *J. Antibiot*. **53**: 1123-1129.

Lauer, U. Anke, T., Sheldrick, W. S., Scherer, A. and Stelglich, W. (1989). Antibiotics from basidiomycetes. Aleurodiscal: an antifungal sesterpenoids form *Aleurodiscus mirabilis* (Berk and Curt.) Hohn. *J. Antibiot*. **42**: 875-882.

McCartney, C., Mercer, P. C., Cooke, L. R. and Fraaije, B. A. (2007). Effects of a Strobilurins based on disease control, green leaf area, yield and development of fungicide resistance in *Mycosphaerella graminicola* in Northern Ireland. *Crop Prot*. **26**: 1272-1280.

Merterrhazy, A. and Bartok, T. (1996). Control of *Fusarium* by fungicides by fungicides and its effect on toxincontamination of the grains. *Pflanzenschutz-Nachrichten* **49**: 181-198.

Mujeebur, R. K. and Shahana, M. K. (2002). Effects of root dip treatment with certain phosphate solubilizing micro organism on the *Fusarium* wilt of tomato. *Biores. Techn.* **85**: 213-215.

Nakajima, M., Itoi, K., Takamatsu, Y., Sato, S. and Takada, M., (1991). A new fungal metabolite with herbicidal activity. *J. Antibiot*. **48**: 1389-1395.

Nel, B., Stanberg, C., Labuschagne, N. and Viljoen, A., (2006). Evaluation of fungicides and sterilants for potential application in management of *Fusarium* wilt of banana. *Crop Prot.* **26**: 697-705.

Piggut, A. M. and Karuso, P. (2004). Quality, not quantity: The role of natural products and chemical proteomics in modern drug discovery. *Comb. Chem High throughput series.* **7**: 607-630.

Pohland, A. E., Schuller, P. L. and Steyn, P. S. (1982). Physiochemical Data for some selected mycotoxins. *Pure and Appl. Chem*. **54**: 2219-2284.

Reuveni, M. (2000). Efficacy of trifloxystrobin (Flint), a new strobilurin fungicide, in controlling powdery mildews on apple, mango and nectarine, and rust on prune trees. *Crop prot.* **19**: 335-341.

Ristaino, J. B. and Thomas, W. (2000). Agriculture, methyl bromide and the ozone hole. *Plant Dis.* **81**: 964-977.

Rodriguez, J. D., Hernandex-castillo, D. Angulo-Sanchez, J. L., Rodriguez-Garaa, R. Villarreal, Q. J. A. and Lira-Scldiwar, R. H. (2007). *Indust. Crops and Prod.* **25**: 111-116.

Rosa, H. L., Machado, K. M. G., Jacob, C. C., Capaleri, M., Rosa, C. A. and Zani, L. C. (2003). Screening of Brazillian basidiomycetes for antimicrobial activity. *Mem. Inst. Owswaldo Cruz, Rio de Janeiro.* **98**: 967-974.

Rugutt, J. K, Ngigi, A. N., Rugutt, K. J. and Ndalut, P. K. (2006). Native Kenyan plants as possible alternatives to methyl bromide in soil fumigation. *Phytomedicine* **13**: 576–583

Santos, B. M., Gilreath, J. P., Motis, T. N., Noling, J. W., Jones, J. P. and Norton, J. A. (2006). Comparing methylbromide alternatives for soilborne diseases nematodes and weed management in fresh market tomato. *Crop Prot*. **25**: 690-695.

Sauter, H. Ammermann, E, Benolt, R. B., Gold, R. E., Wingert, H. and Schirmer, U. (1996). Mitochondrial respiration as a target for antifungal: Lessons from research on strobilurins, in antifungal agents; Discovery and mode of action, pp. 173-191. Oxford: Bios Scientific Publishers.

Schram, G., Stelglich, W. and Anke, T. (1982). Structure-activity relationship of stobilurins, oudemansin, and synthetic analogues, Abstact 457, in: Abstracts 13th Int. Congr. Microbial. Boston.

Sivan, A., Uoko, O. and Chet, I. (1987) Biological control of *Fusarium* crown rot of tomato by *Trichoderma harzianum* under field conditions. *Plant dis*. **71**: 587-592

Song, W., Zhou, L., Yang, C., Cao, X., Zhang, L. and Liu, X. (2004). Tomato *Fusarium* wilt and its chemical control strategies in a hydroponic systems. *Crop Prot.* **23**: 243-247.

Sparapano, L., Bruno, G., Fierro, O. and Evidente, A. (2004). Studies on structure-activity relationship of sphaeropsidins A-F, phytotoxins produced by *Sphaeropsis sapinea* f. sp. *Cuppressi. Phytochem* **65**: 189-198.

Stark, A., Anke, T., Moeck, U., Steglich, W., Kirtel, A. and Will, G. (1988). Lentinellic acid, a biologically active protoilludane derivative from lentinellus species (basidiomycetes). *Z. Naturforsch*. **43**: 177-183.

Strauss, J. and Labuschagne, N. (1993). *In vivo* and *in vitro* effect of fungicides on *Fusarium solani* associated with citrous roots. *Plant Sci*. **7**: 56-59.

Sudisha, K. N., Amruthesh, S. A., Deepak, N. P., Shetty, B. R., Sarosh, H. and Sheckar, S. (2005). Comparative efficacy of strobilurin fungicides against downy mildew disease of pearl millet. *Pesticide Biochem. Physiol*. **81**: 188-197.

Suleman, P., Tohamy, A. M., Saleh, A. A., Madkour, M. A. and Straney ,D. C. (2003). Variation in sensitivity to tomatine and rishitin among isolates of *Fusarium oxysporum* f. sp. *lycopersici*, and strains not pathogenic to tomato. *Physiol Mol Plant Pathol*. **48**: 131–144.

Tamwa, H. and Mizutani, A. (1999). Mode of action of strobilurins fungicides. *J. Pesticide sci*. **24**: 189-196.

Thines, E., Anke, H., and Weber, R. W. (2004). Fungal secondary metabolites as inhibitors of infection-related morphogenesis in phytopathogenic fungi. *Mycol. Res*. **108**: 14-25.

Thines, E., Eilbert, F., Sterner, O. and Anke, H. (1997). Glisoprenin A, an inhibitor of the signal transduction pathway leading to appressorium formation in germinating conidia of *Magnaporthe grisea* on hydrophobic surfaces. *FEMS Microbiol. Lett.* **151**: 219-224.

Turner, W. B. (1971). Fungal metabolites I. Academic press inc., London. United Kingdom.

Turner, W. B. and Aldridge D. C. (1983) Fungal metabolites II. Academic press, New York. Vol 2: 631.

U. S. Environmental protection Agency, office of Atmospheric programs Washington DC. Methyl bromide use background. http://www.epa. Gov/docs/ozone/mbr/background. Html. (accessed June 2009) path.

Weber, R. W. S., Kappe, R., Paululat, T., Mosker, E. and Anke, H. (2007). Anti-candida metabolite from endophytic fungi. *Phytochem.* **68**: 886-892.

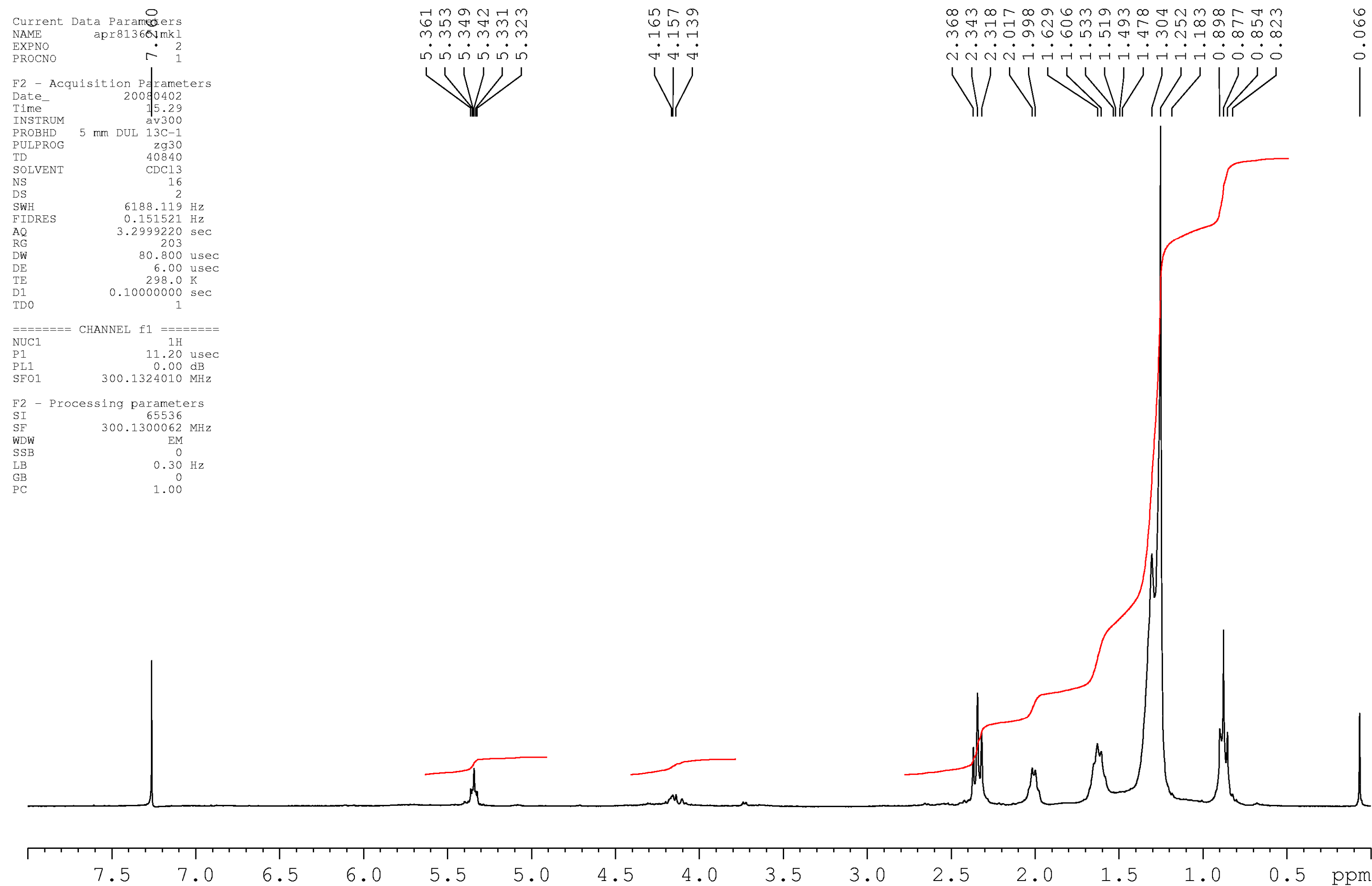
Yan, L., Liu J., Zhang, H. ,Xiangping, Y. and Zhaojie , L. (2006). Stereoselective synthesis and fungicidal activities of (E)-a-(methoxyimino)-benzeneacetate derivatives containing 1,3,4-oxadiazole ring. *Bioorg. & Med. Chem. Letts*. **16**: 2278–2282.

Young, M. C., Braga, M. R., Dietrich, S. M., Gottlied, H. E., Trevisan, L. M. and Bolzani, V. (1992). Fungitoxic non-glycoside iridoids from *Alibesta macrophylla*. *Phytochem.* **31**: 3433-3435.

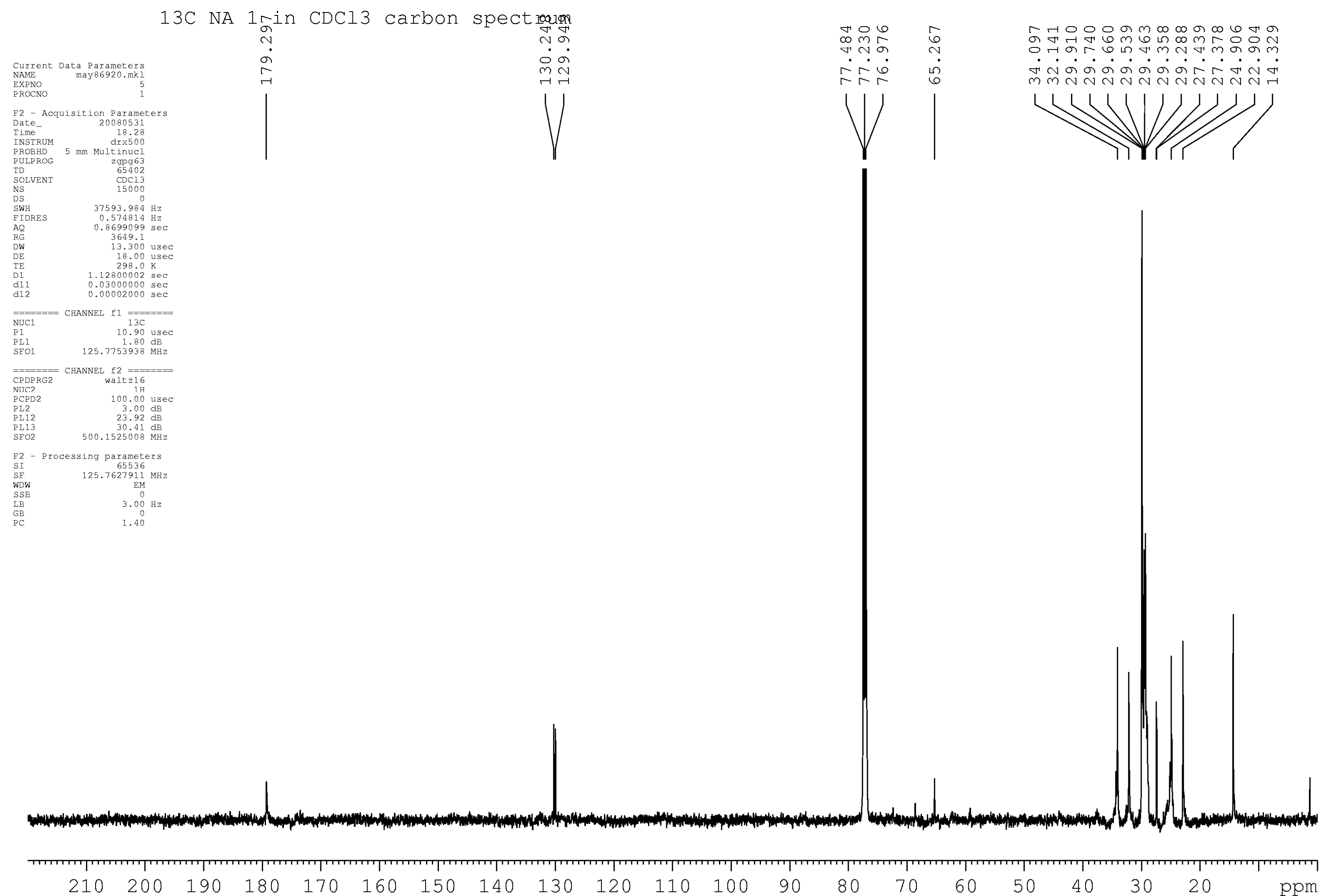
Zhanyong, G., Rong, C., Ronye, X., Song, L., Huahua, Y., Wang, P., Cuiping, L. and Pengcheng, L. (2006). Novel derivatives of chitosan and their antifungal activities in vitro. *Carbohydrate Res.* **341**: 351-354.

APPENDICES

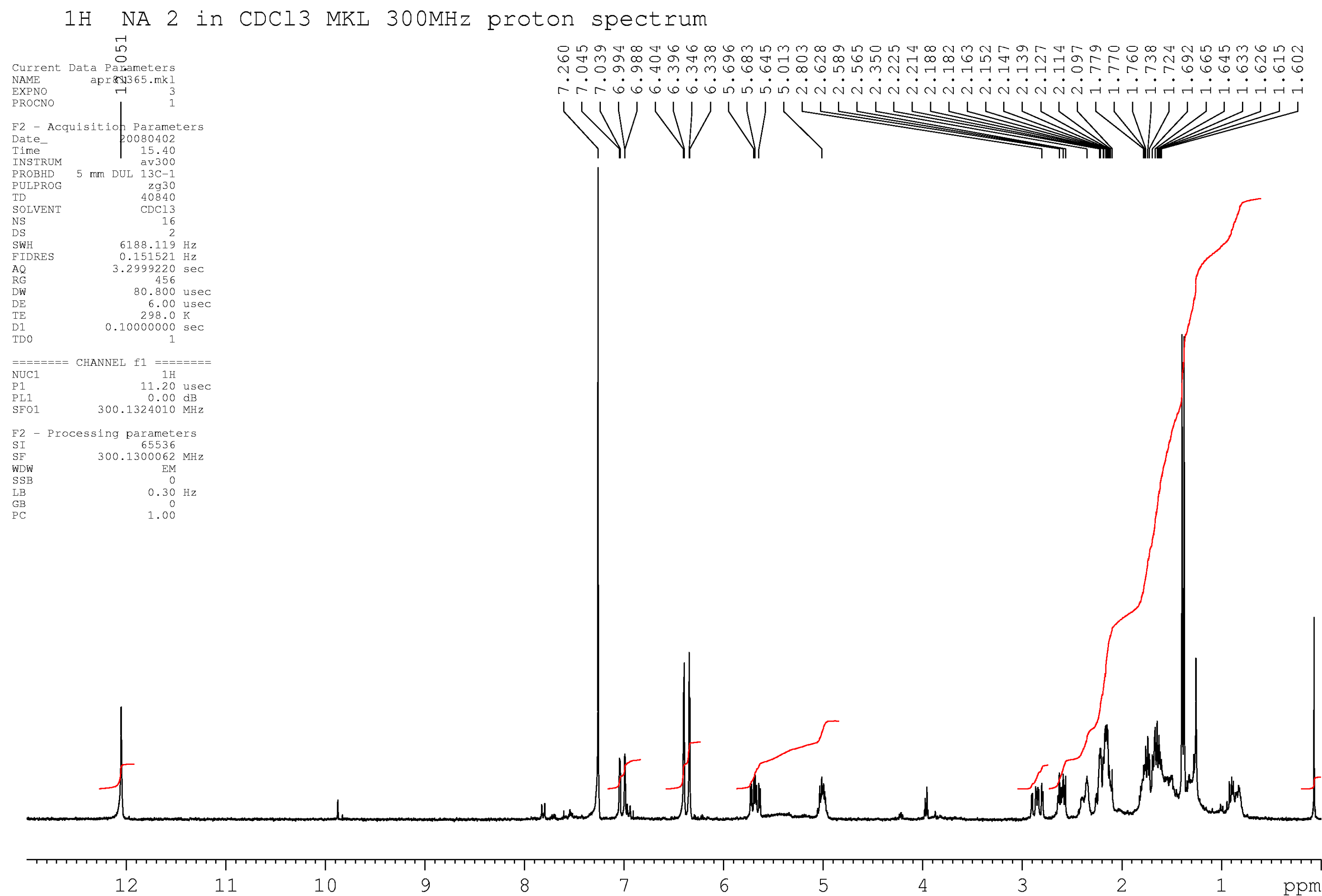
APPENDIX 1: ^{1}H NMR Spectrum for NA1



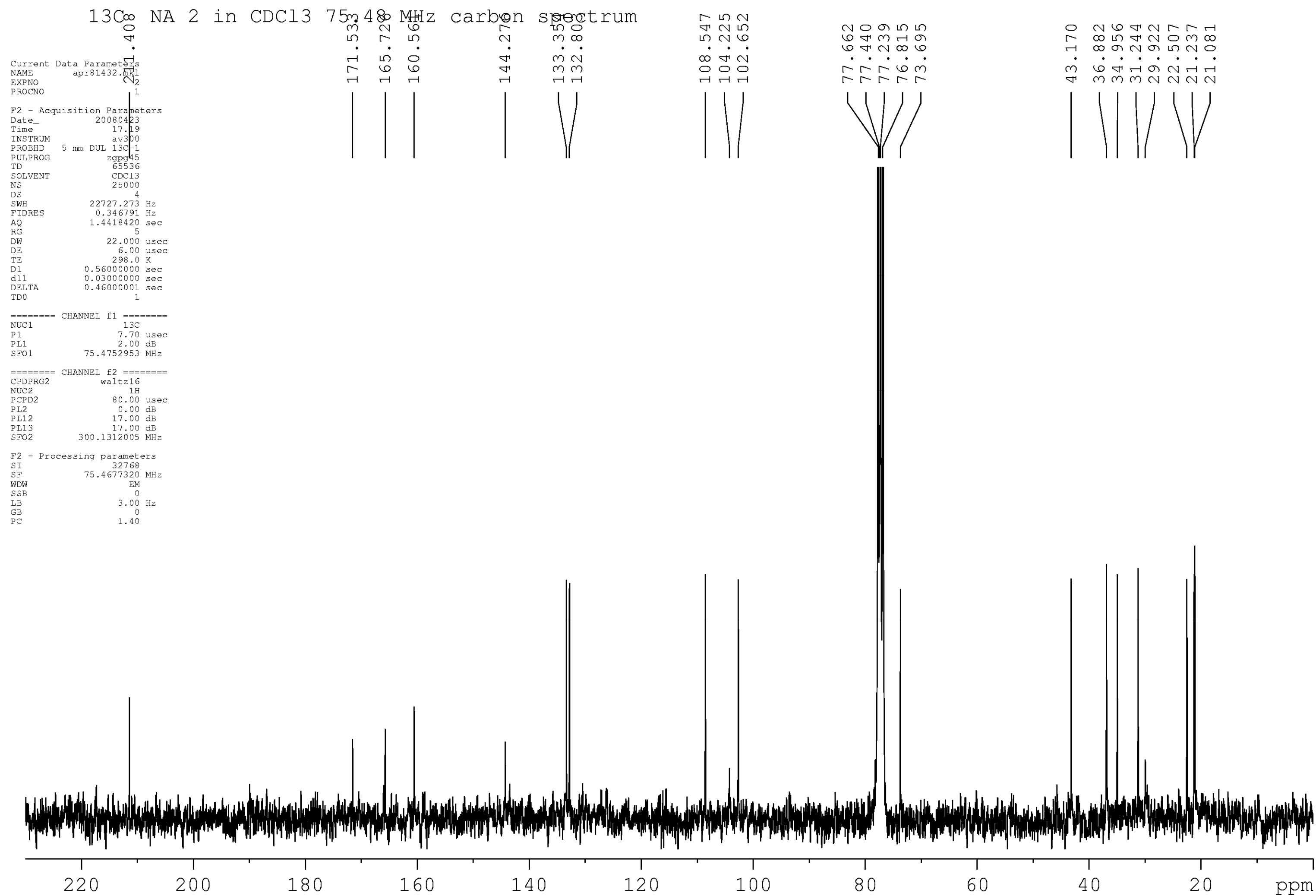
APPENDIX 2: ^{13}C NMR Spectrum for NA1



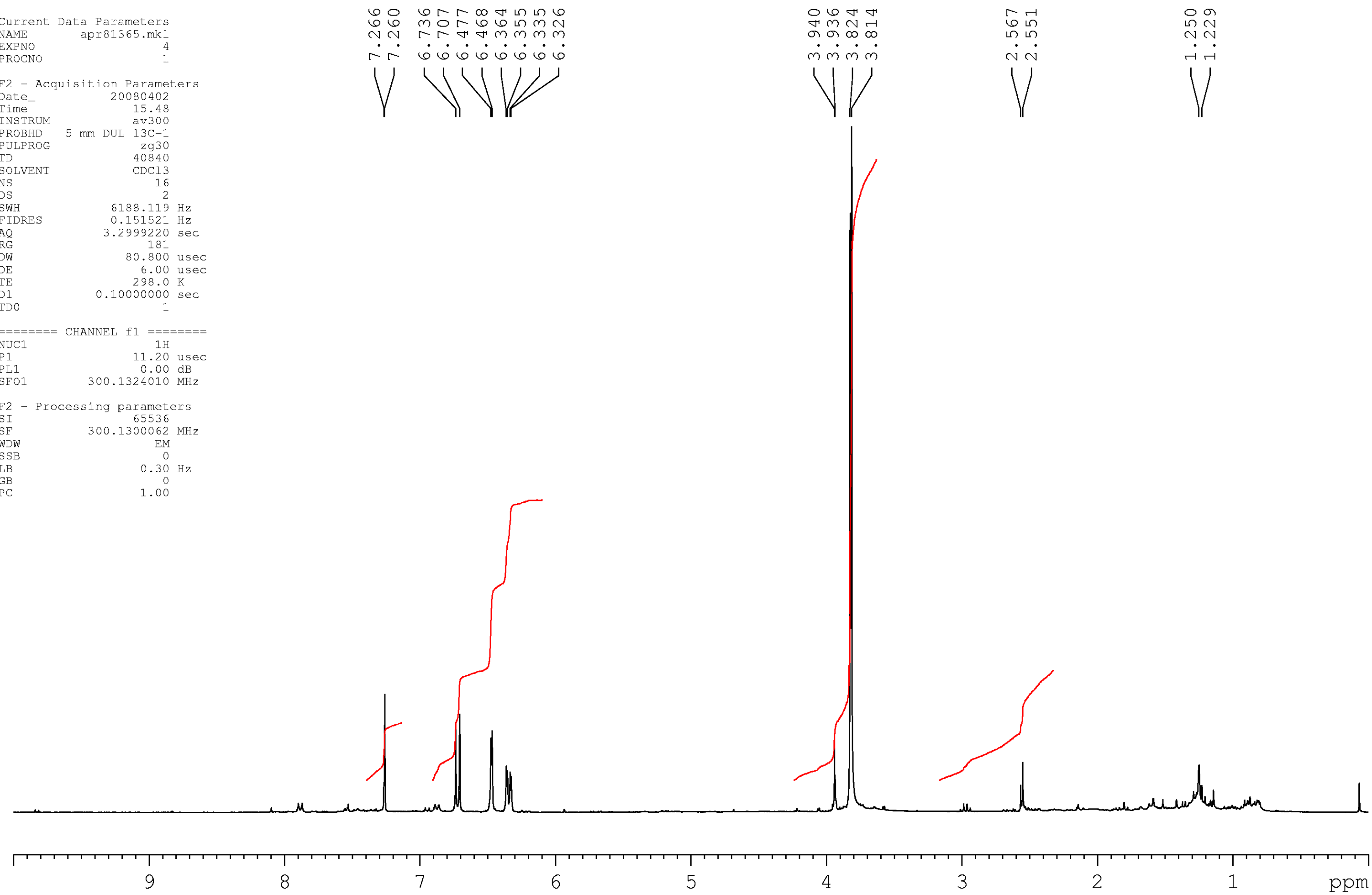
APPENDIX 3: ^{1}H NMR Spectrum for NA2



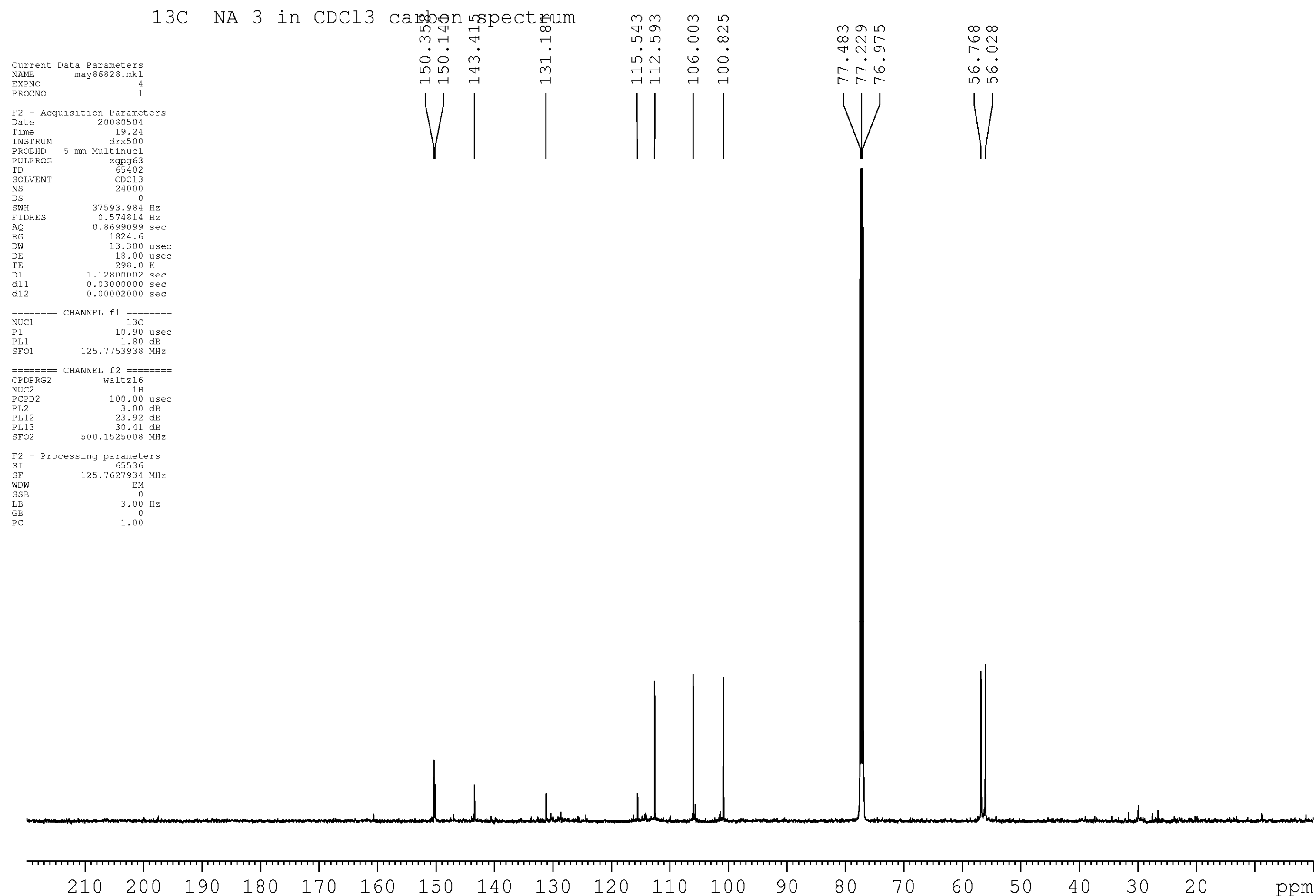
APPENDIX 4: ^{13}C Spectrum NMR for NA2



APPENDIX 5: 1H NMR Spectrum for NA3

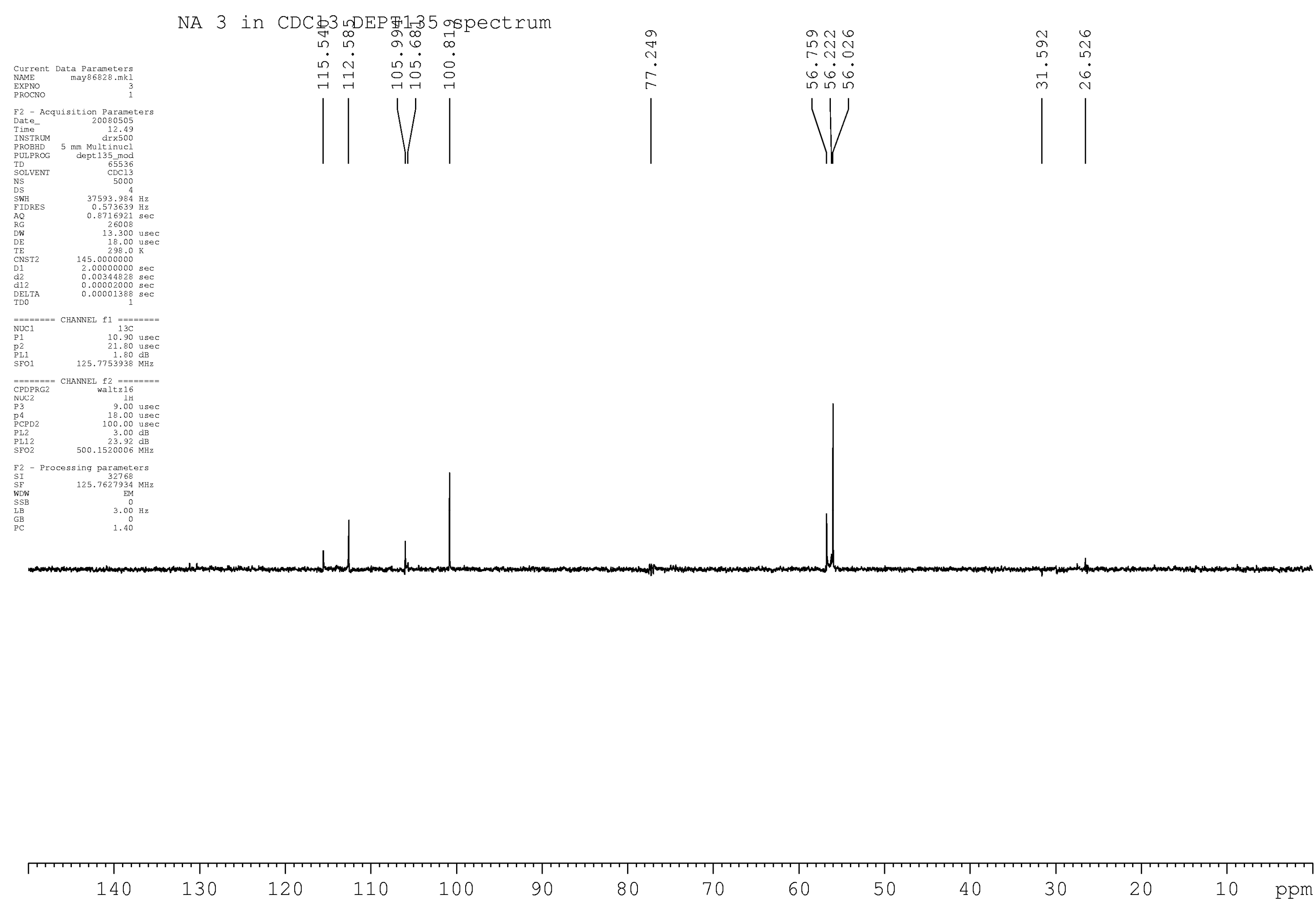


APPENDIX 6: ^{13}C NMR Spectrum for NA3



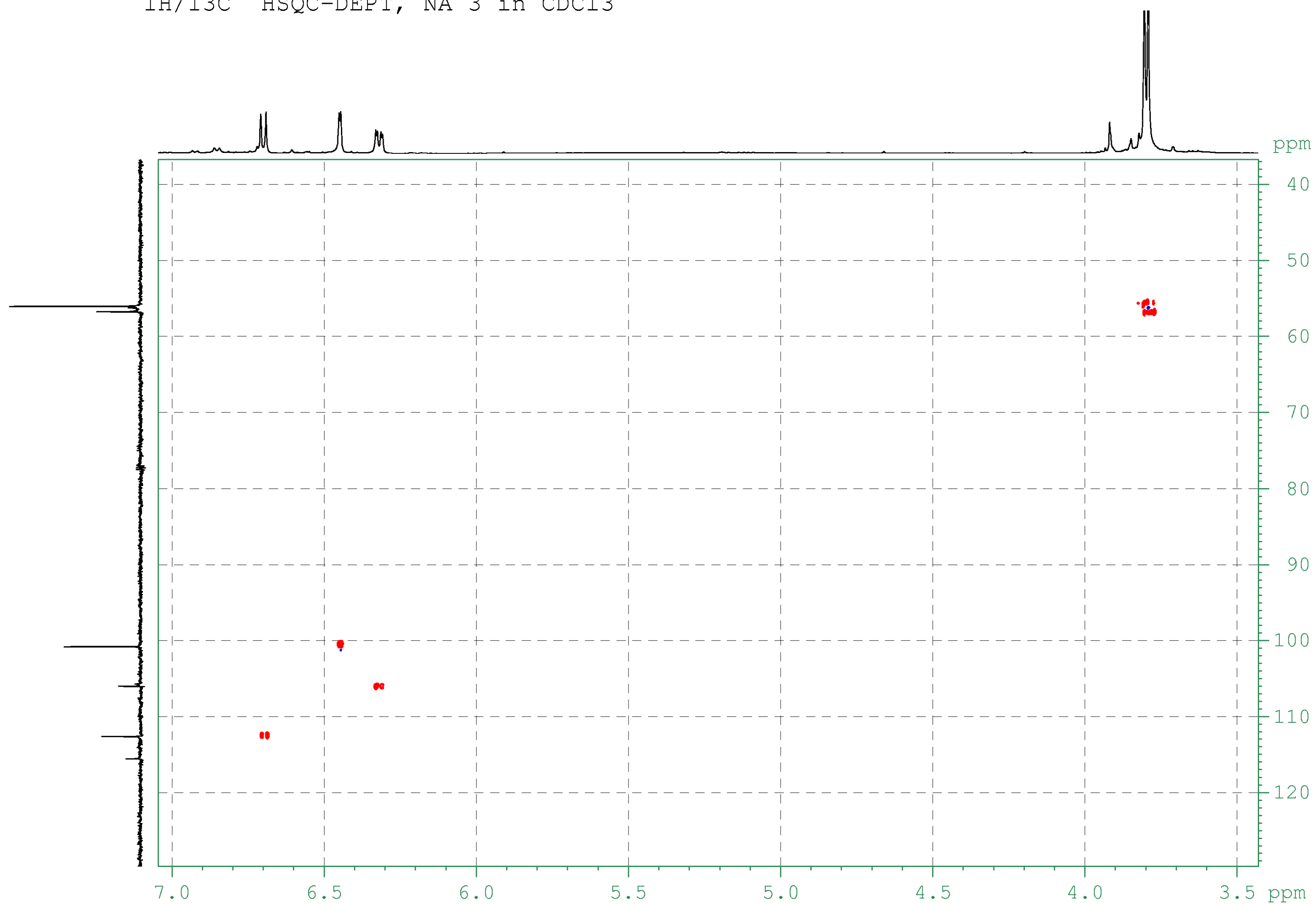
APPENDIX 7: $^{1}H/^{1}H$ COSY NMR Spectrum for NA3

APPENDIX 8: DEPT Spectrum for NA3

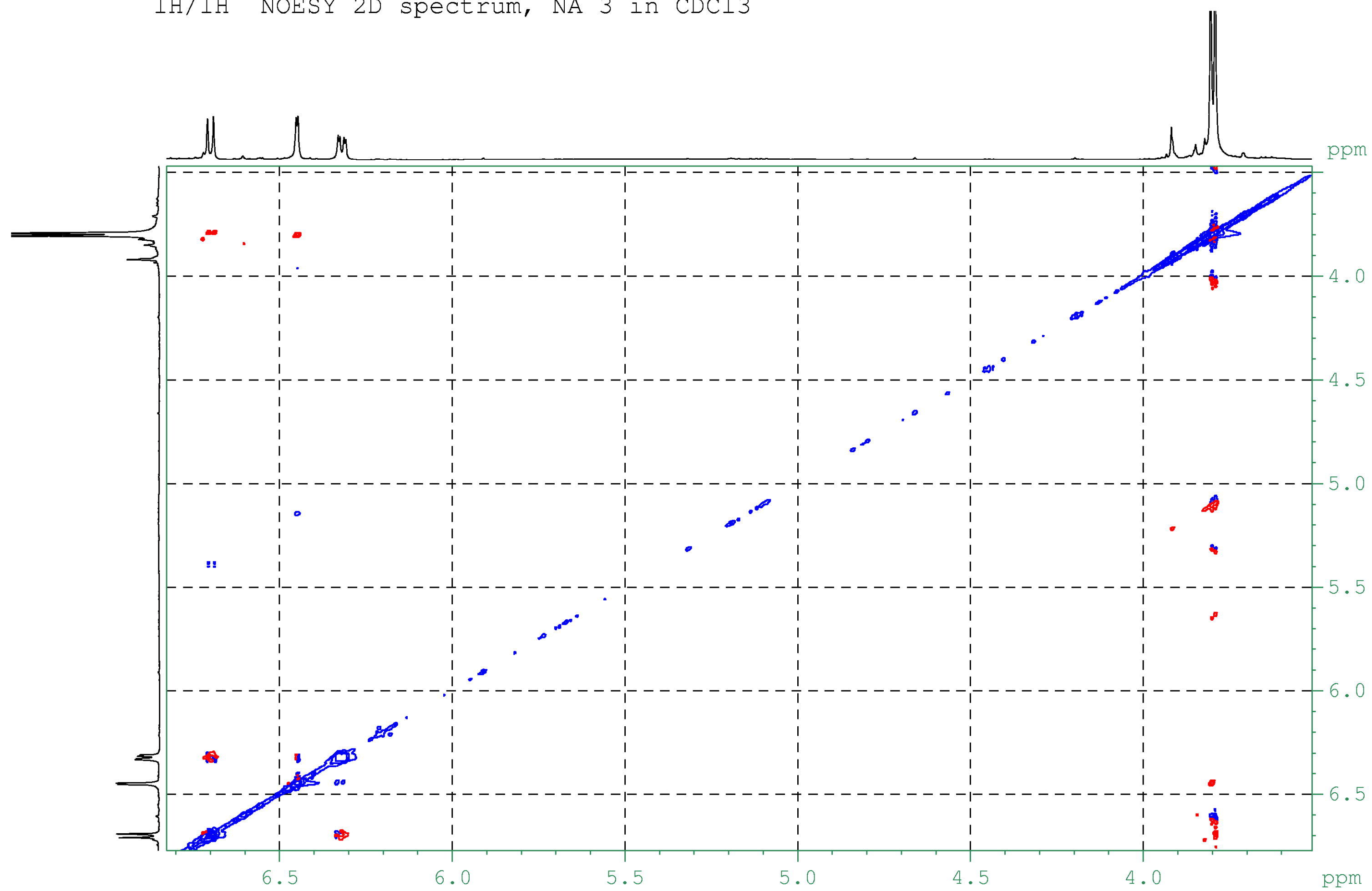


APPENDIX 9: $^{1}H/^{13}C$ HMBC Spectrun for NA3

APPENDIX10: $^{1}H/^{13}C$ HSQC-DEPTSpectrum for NA3



APPENDIX 11: $^{1}H/^{1}H$ NOESY 2D Spectrum for NA 3



APPENDIX 12: ^{1}H NMR Spectrum for NA6

1H NA 6 in CDCl3 MKL 300MHz proton spectrum

7.259, 7.021, 7.017, 6.970, 6.965, 6.439, 6.431, 6.379, 6.371, 5.706, 5.694, 5.671, 5.657, 5.643, 5.620, 5.008, 4.988, 4.970, 2.923, 2.884, 2.876, 2.862, 2.854, 2.821, 2.813, 2.655, 2.641, 2.625, 2.615, 2.600, 2.584, 2.373, 2.347, 2.338, 2.328, 2.231, 2.224, 2.184, 2.169, 2.150, 2.140, 2.108, 2.097, 1.805, 1.786, 1.774, 1.755, 1.733, 1.722, 1.715, 1.658, 1.635, 1.612, 1.592, 1.584, 1.554, 1.545, 1.530, 1.512, 1.493, 1.455, 1.444, 1.428, 1.365, 1.345, 1.315, 1.303, 1.242, 0.876, 0.867

Current Data Parameters
NAME apr01365.mkl
EXPNO 1
PROCNO 1

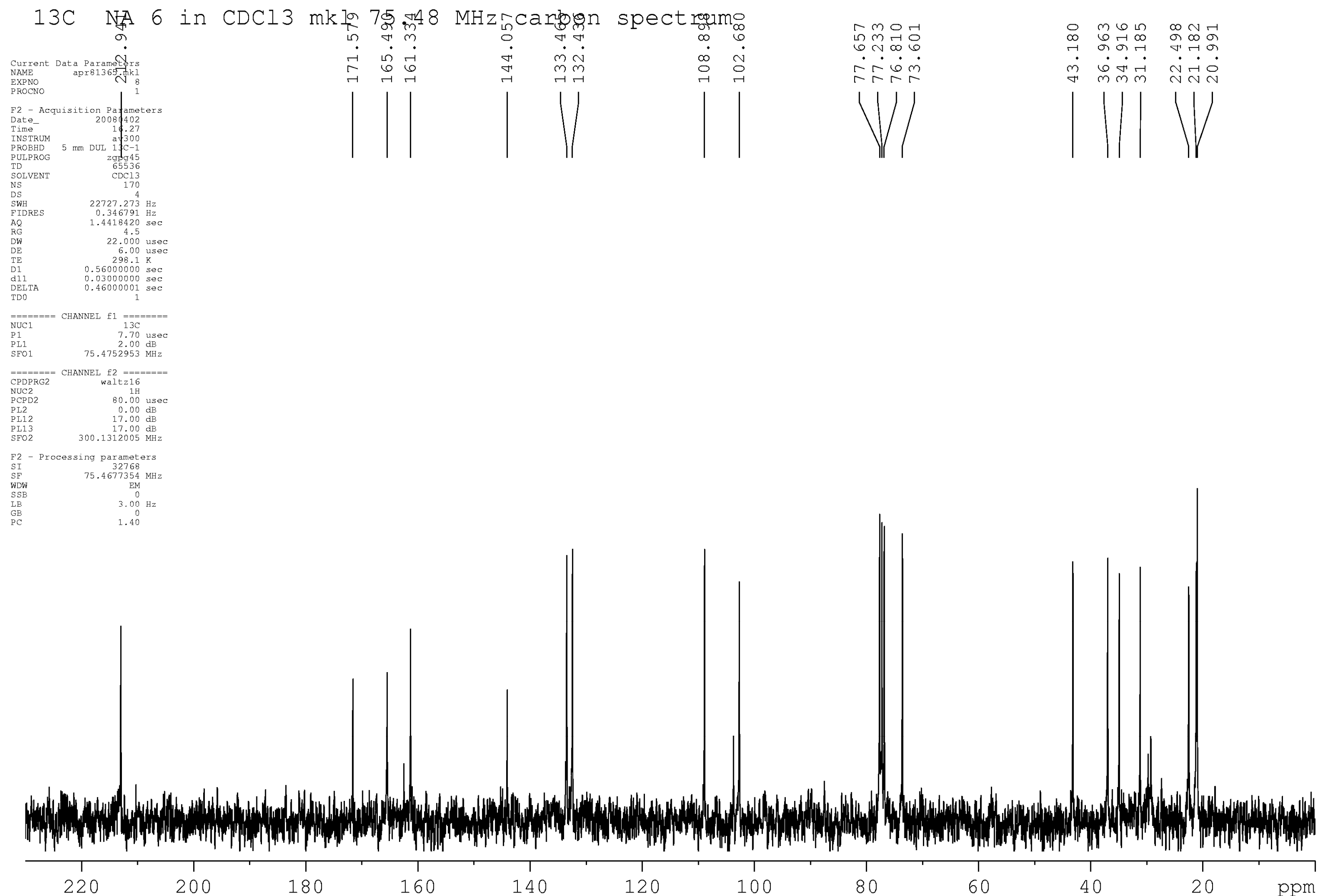
F2 - Acquisition Parameters
Date_ 20080402
Time 16.23
INSTRUM av300
PROBHD 5 mm DUL 13C-1
PULPROG zg30
TD 40840
SOLVENT CDCl3
NS 16
DS 2
SWH 6188.119 Hz
FIDRES 0.151521 Hz
AQ 3.2999220 sec
RG 90.5
DW 80.800 usec
DE 6.00 usec
TE 298.0 K
D1 0.10000000 sec
TD0 1

======== CHANNEL f1 ========
NUC1 1H
P1 11.20 usec
PL1 0.00 dB
SFO1 300.1324010 MHz

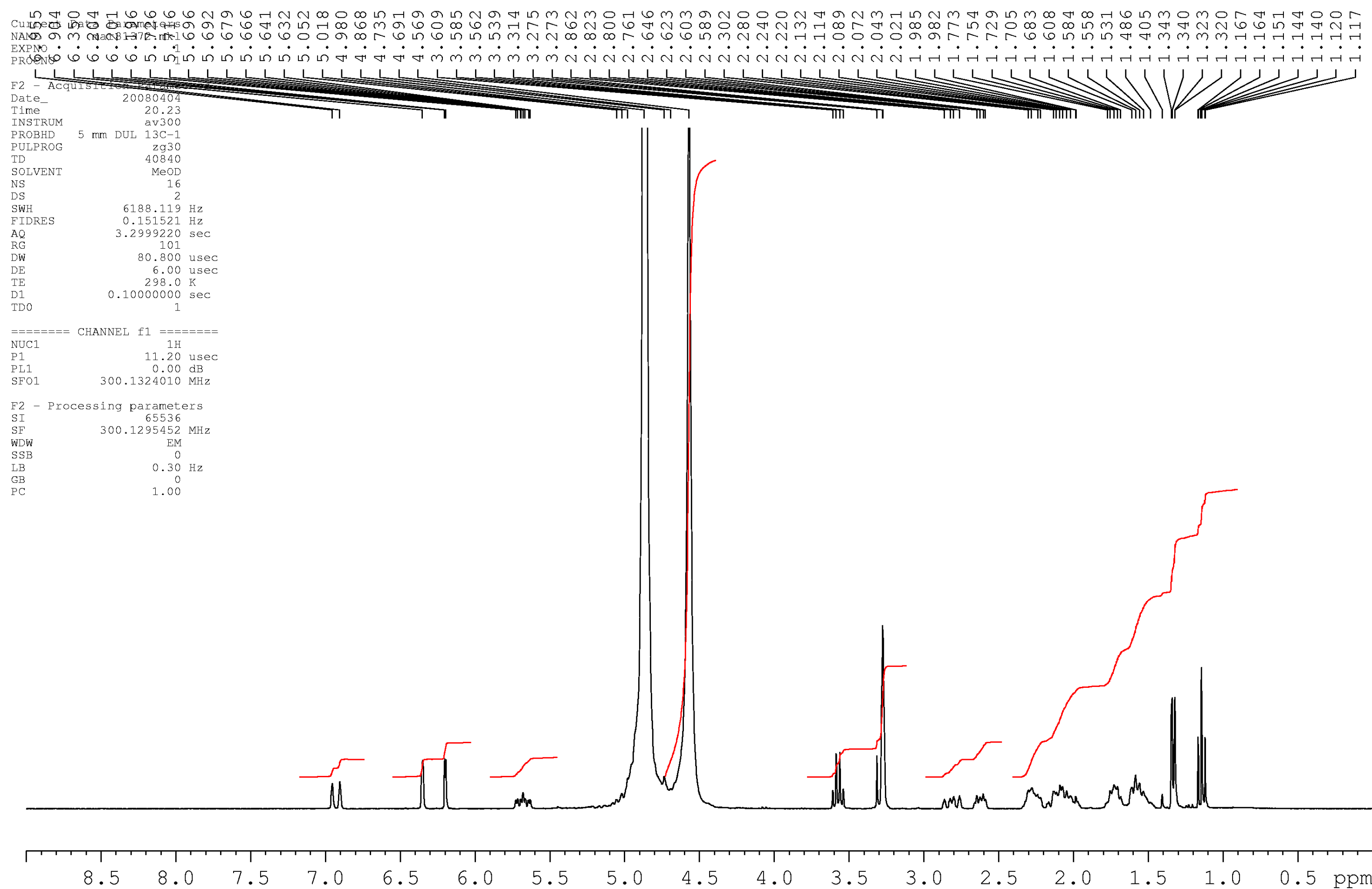
F2 - Processing parameters
SI 65536
SF 300.1300062 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

12 11 10 9 8 7 6 5 4 3 2 1 ppm

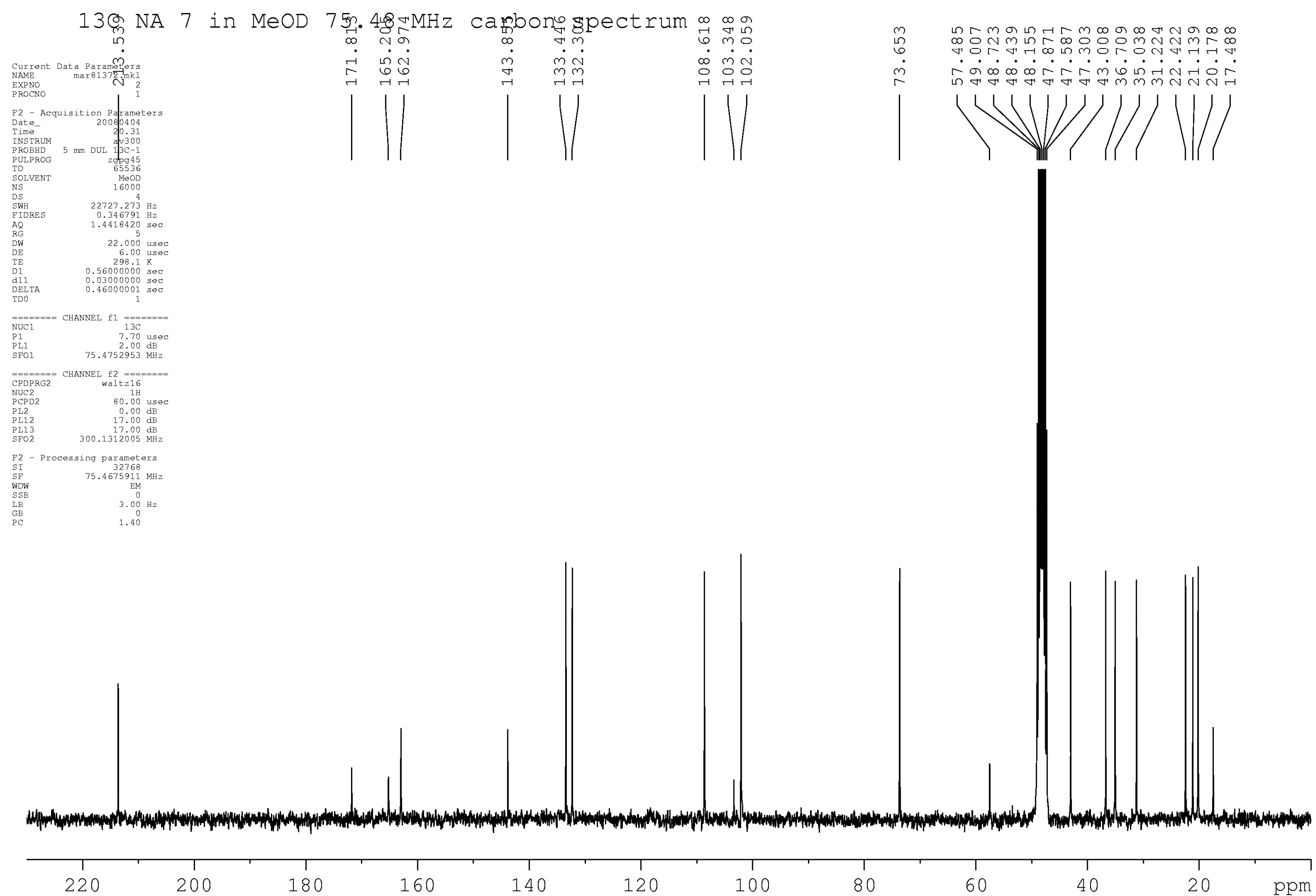
APPENDIX 13: ^{13}C NMR Spectrum for NA6



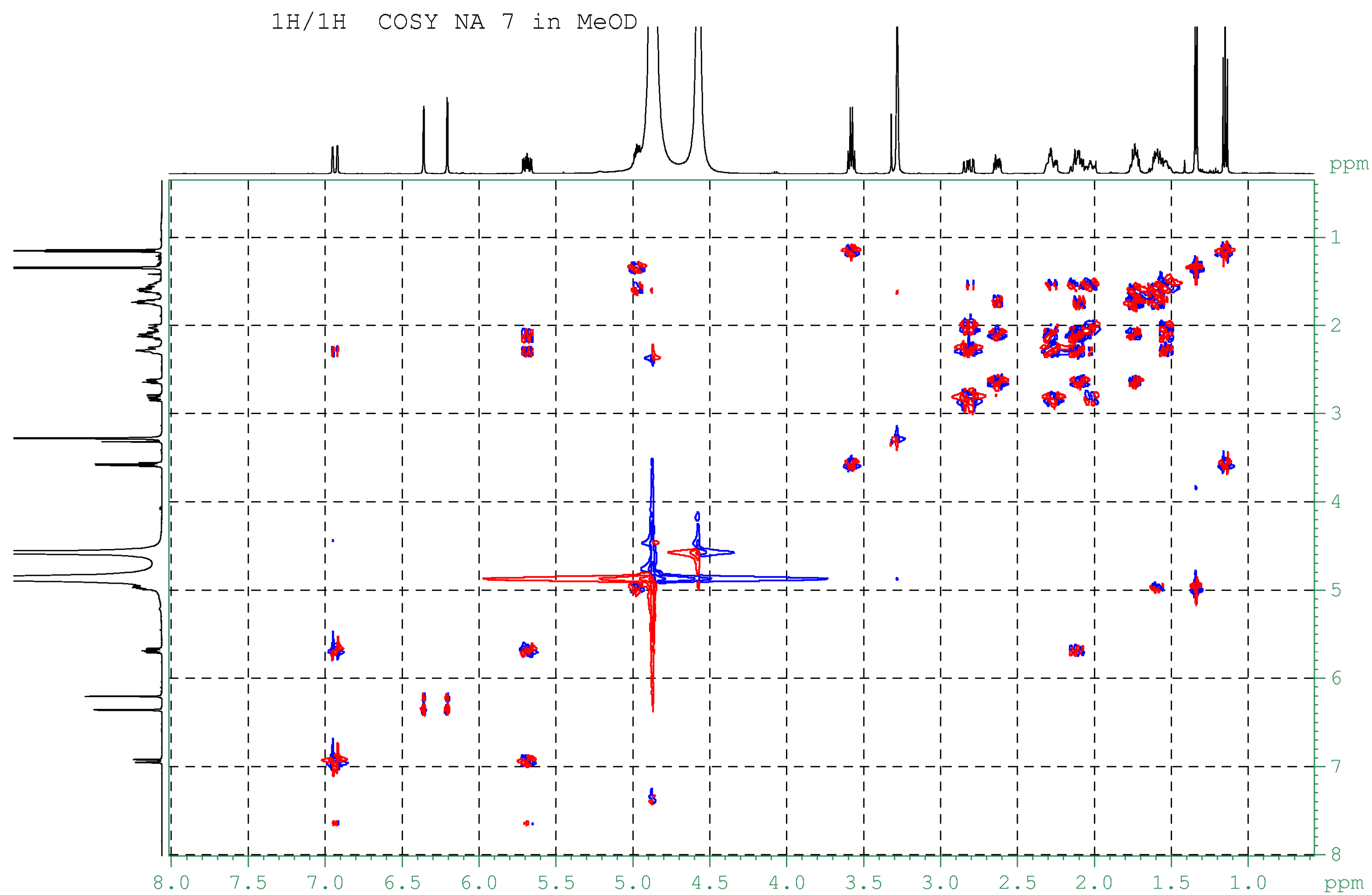
APPENDIX 14: ^{1}H NMR Spectrum for NA7



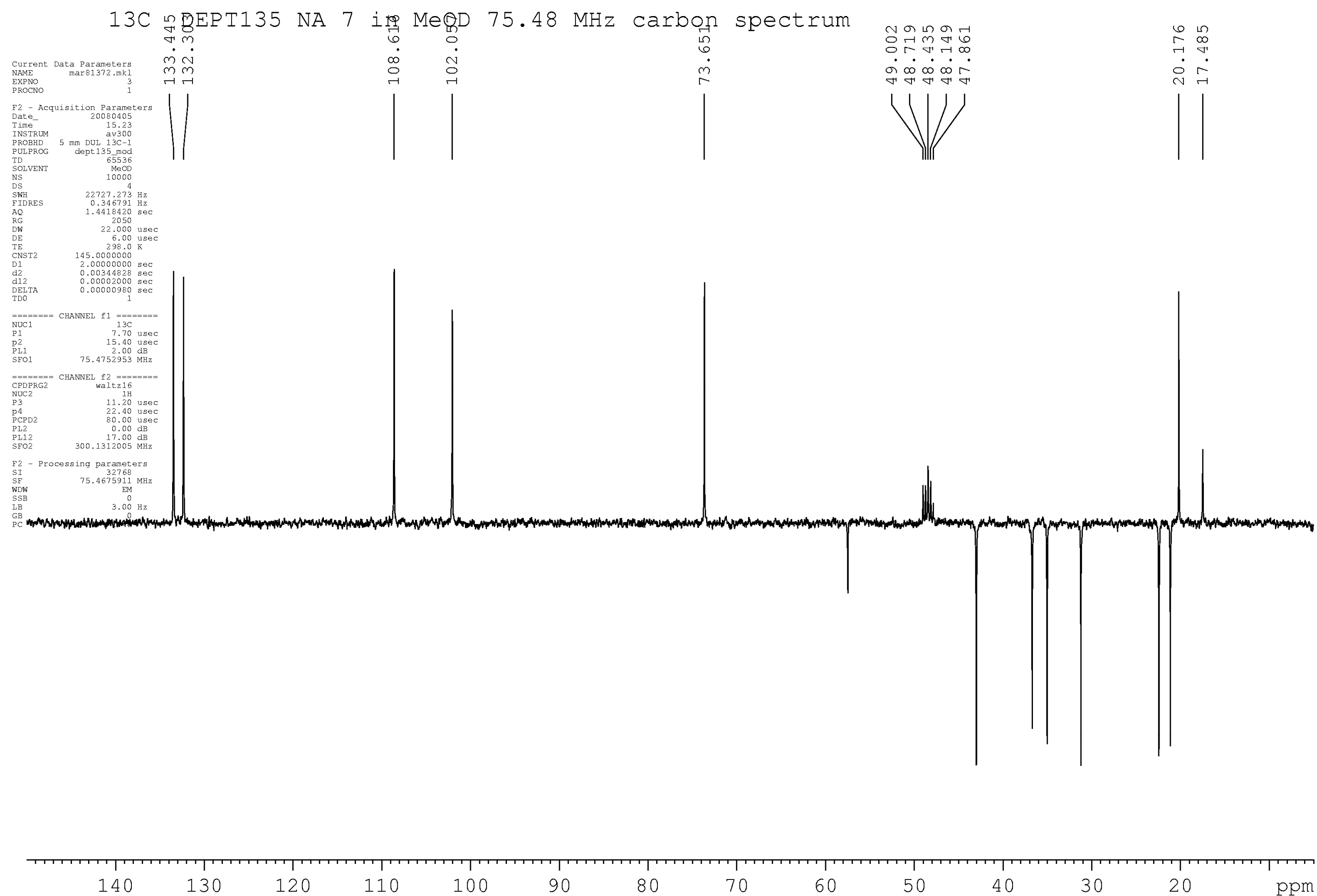
APPENDIX 15: ^{13}C NMR spectrum for NA7



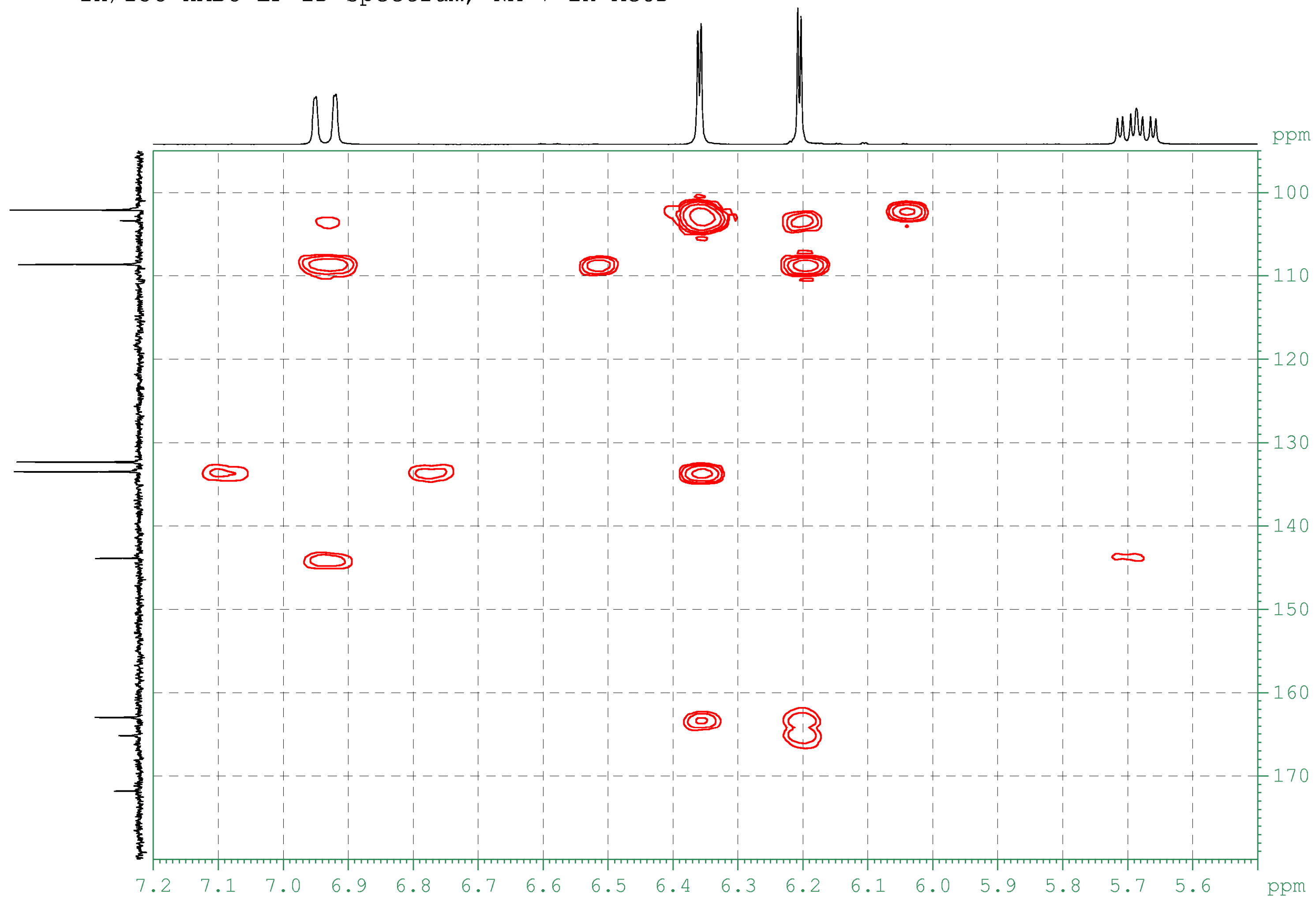
APPENDIX 16: $^{1}H/^{1}H$ COSY Spectrum for NA7



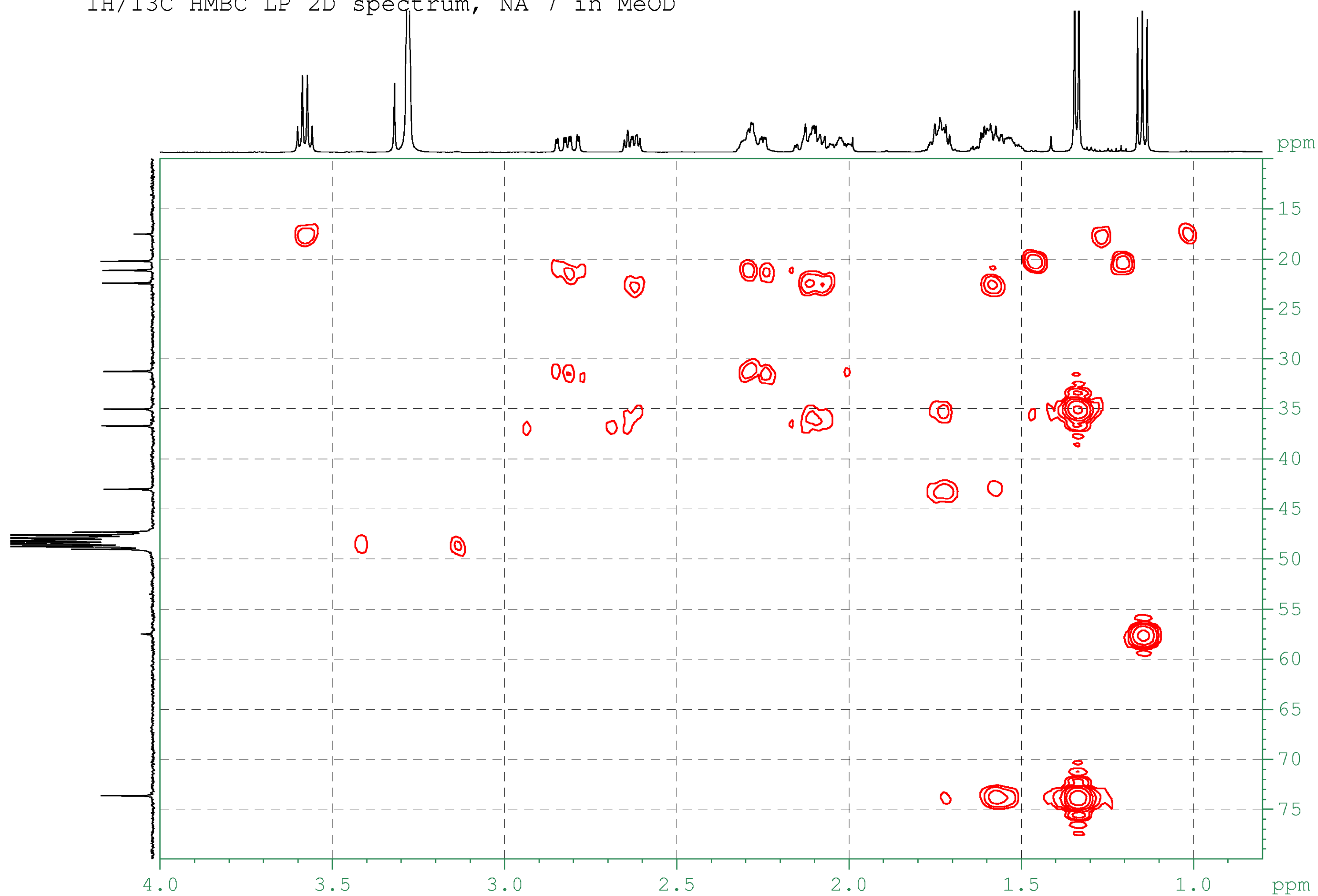
APPENDIX 17: DEPT Spectrum for NA7



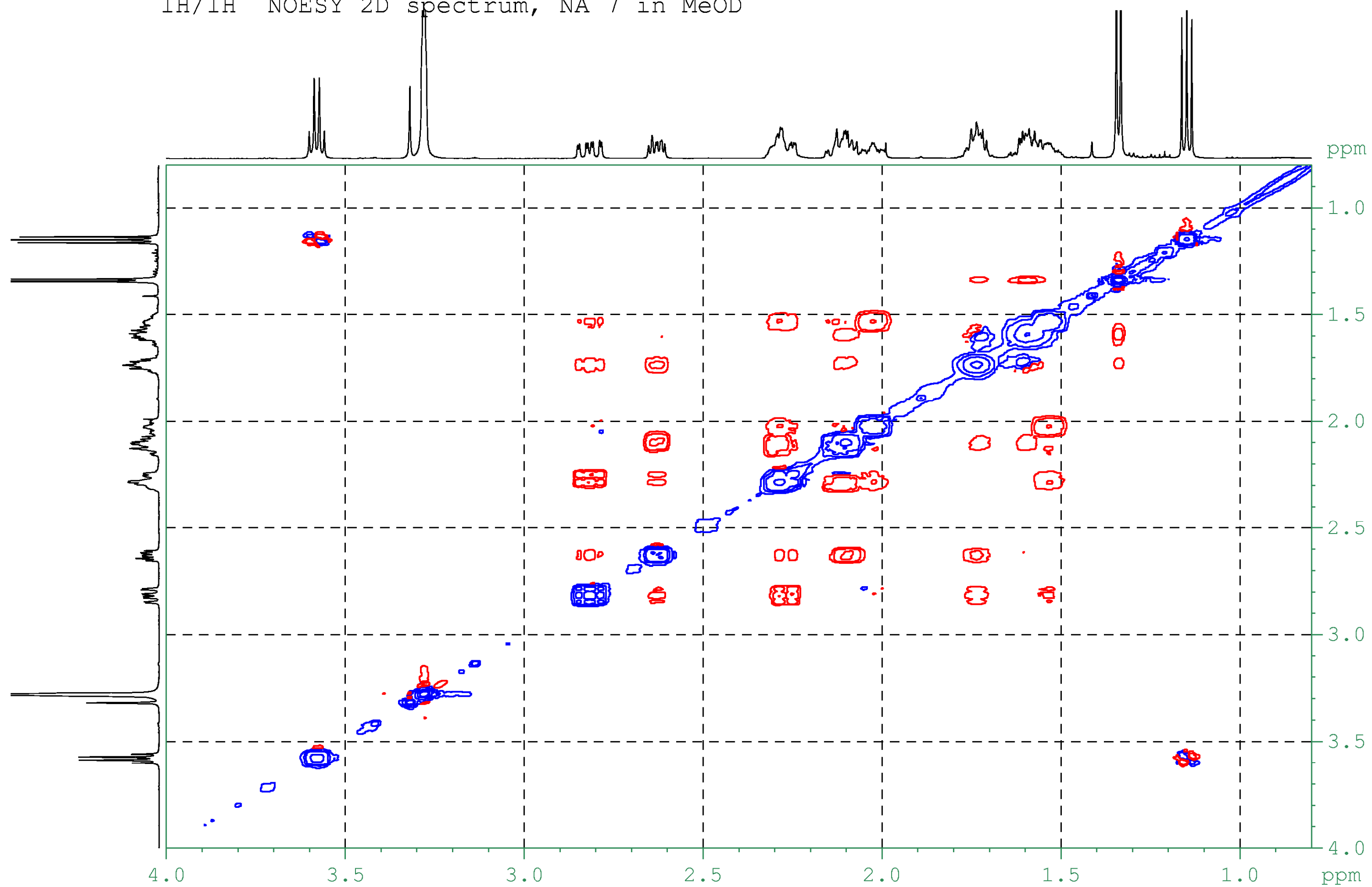
APPENDIX 18: $^{1}H/^{13}C$ HMBC Spectrum for NA7



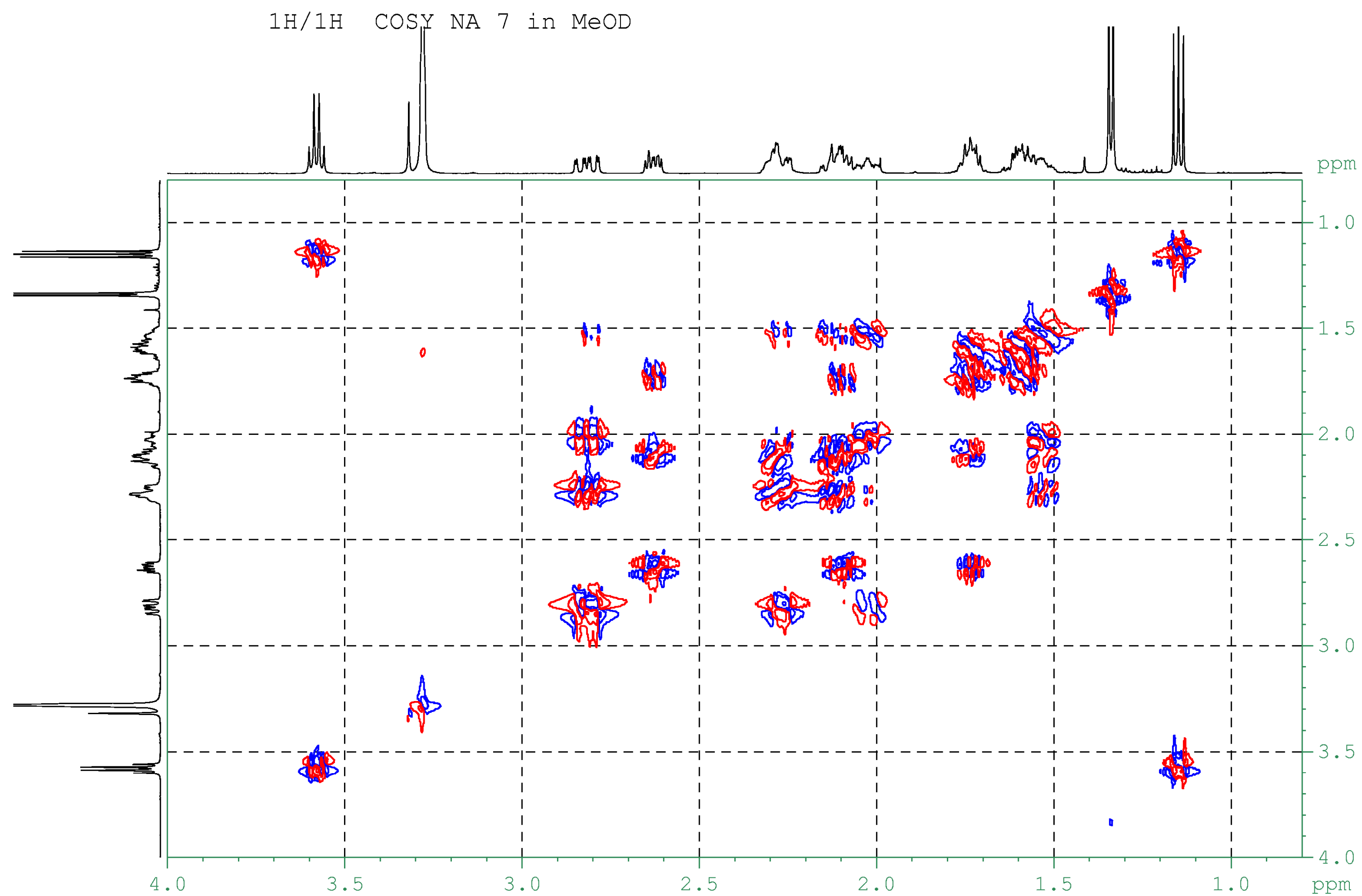
APPENDIX 19: $^{1}H/^{13}C$ HMBC Spectum for NA7



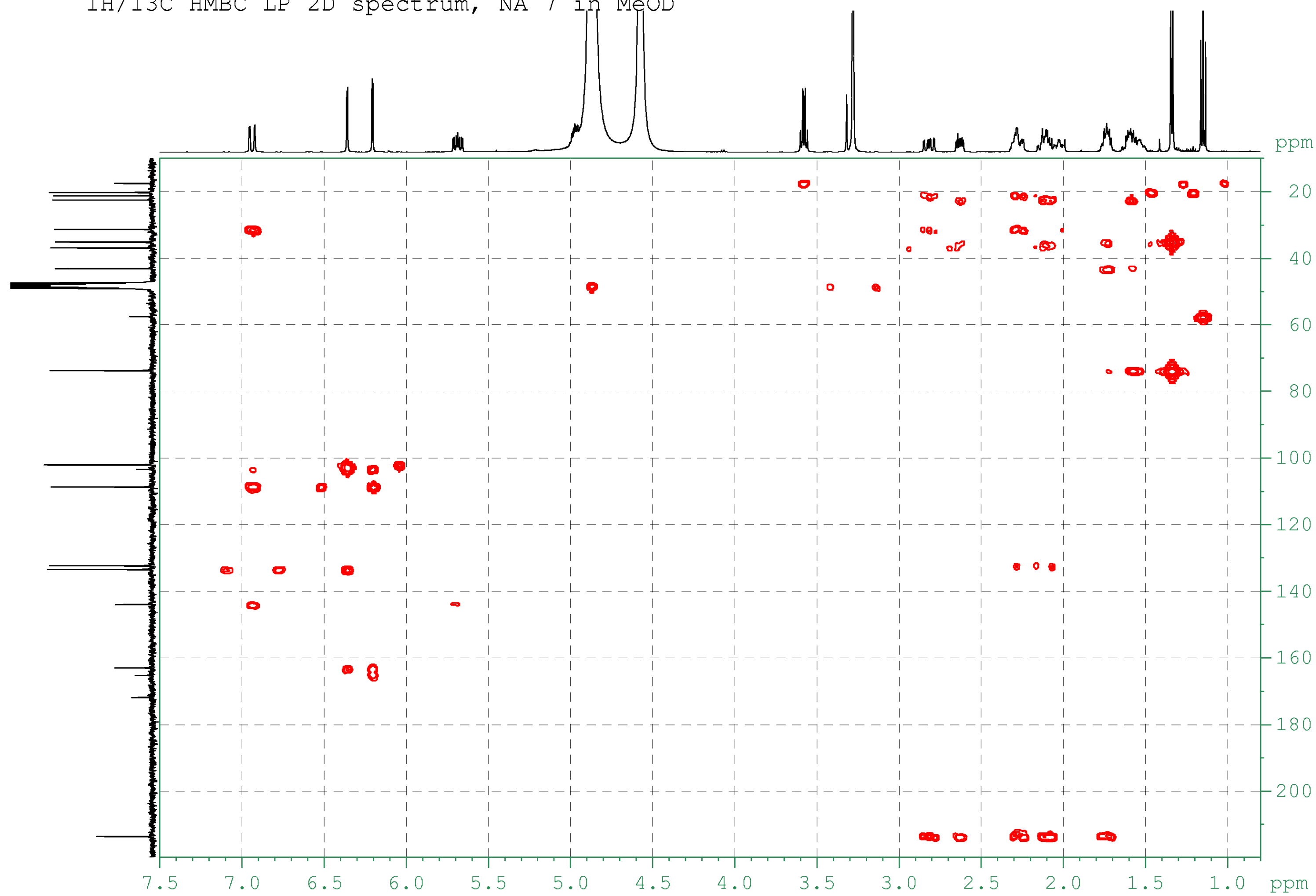
APPENDIX 20: $^{1}H/^{1}H$ NOESY Spectrum for NA7



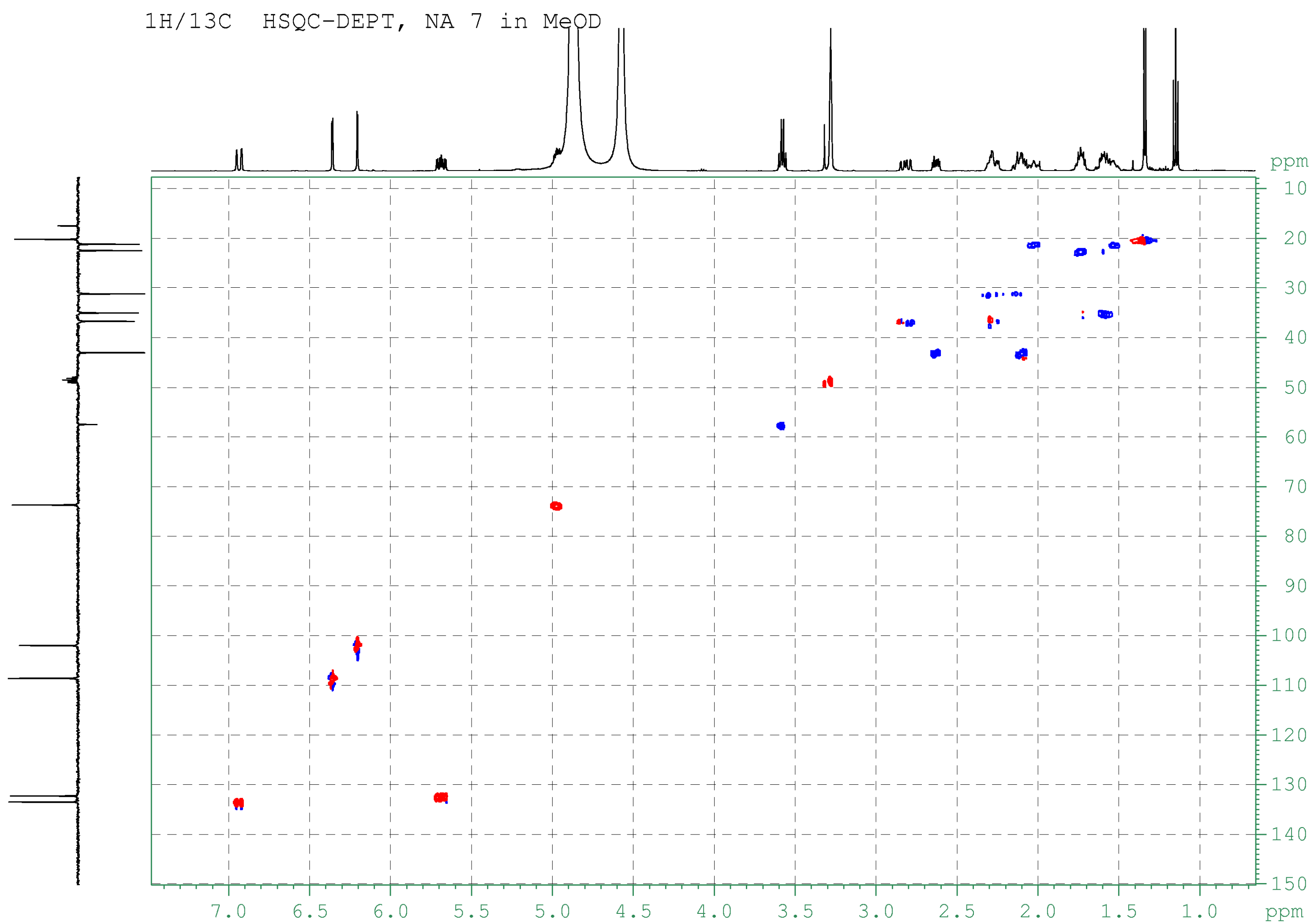
APPENDIX 21: $^{1}H/^{1}H$ COSY Spectrum for NA 7



APPENDIX 22: $^{1}H/^{13}C$ HMBC Spectrum for NA7



APPENDIX 23: $^{1}H/^{13}C$ HSQC-DEPT Spectrum for NA7



APPENDIX 24: $^{1}H/^{1}H$ NOESY Spectrum for NA7