STRUCTURAL MODIFICATION OF SOME FLAVONOIDS TO ENHANCE THEIR LARVICIDAL ACTIVITY

BY:

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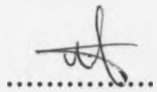
DECLARATION

This thesis is my original work and has not been presented for a degree in any University.



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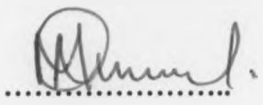

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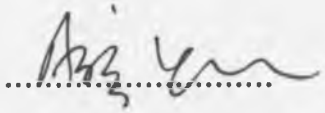

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This work is dedicated to my family.

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My sincere gratitude to my supervisors: Dr. Albert Ndakala, Dr. Martin Mbugua, and Prof. Abiy Yenesew, for introducing me to synthetic organic chemistry and also providing me with the support I needed during the research work. Lots of thanks to Dr. Ndakala (synthesis is his area of specialty) for his unending support and having him on my corner was satisfactory and enough guarantee that the research would come to an end. He was there for me at all times and his continuous encouragement made everything seem alright despite the many challenges. Lots of thanks to Dr. Mbugua for his moral support, finding time for me despite his tight schedule, his continual appraisal of my progress and all his doings during my time in this Department. I would also like to register my appreciation to Prof. Yenesew for his endless encouragement and diverse support that he accorded me.

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ABSTRACT

The aim of this study was to modify three naturally occurring flavonoids namely: abyssinone V-4'-methyl ether **(5)**, (+)-usararotenoid-A **(6)** and (+)-12α-*epi*milletosin **(7)** in a bid to enhance their larvicidal and antioxidant activities. Five modified analogues including abyssinone V-4'-methyl ether oxime **(59)**, tetrahydro-abyssinone V-4'-methyl ether **(60)**, 7-acetyl-abyssinone V-4'-methyl ether **(61)**, 5,7-diacetyl-abyssinone V-4'-methyl ether **(62)** and dehydromilletosin oxime **(63)** were prepared. The compounds were characterized by thin-layer chromatography (TLC) and nuclear magnetic resonance (NMR) spectroscopic techniques.

The reaction of abyssinone V-4'-methyl ether **(5)** with hydroxylamine hydrochloride in the presence of pyridine gave an oxime in 99% yield, while that with hydrogen gas in the presence of 5% palladium on carbon yielded 99% of the hydrogenated compound. The reaction of abyssinone V-4'-methyl ether **(5)** with acetic anhydride in the presence of pyridine yielded a mixture of 5% of the monoacetate and 45% of the diacetate.

The reaction of (+)-12α-*epi*milletosin **(7)** with hydroxylamine hydrochloride in the presence of pyridine gave 50% of dehydromilletosin oxime **(63)** whereas the hydrogenolysis of the 9-hydroxyhomoisoflavanone **(8)** on 5% palladium on carbon yielded 58% of the corresponding 9-deoxylhomoisoflavanone **(64)**. The reaction of (+)-usararotenoid-A **(6)** with hydroxylamine hydrochloride in the presence of pyridine gave 99% of a mixture of diastereoisomers.

Abyssinone V-4'-methyl ether **(5)**, (+)-12α-*epi*milletosin **(7)**, (+)-12α-usararotenoid-A **(6)**, and their analogues were tested for larvicidal activity against 2nd, 3rd and 4th instar larvae of *Aedes aegypti* and compared with the standard - Rotenone. Potent larvicidal activities were observed against the 2nd instar larvae of *Aedes aegypti* with LC_{50} values of 0.46, 4.08, 1.68 and 13.4 µg/ mL at 24 hours for rotenone **(9)**, abyssinone V-4'-methyl ether **(5)**, abyssinone V-4'-methyl ether oxime **(59)** and hydrogenated abyssinone V-4'-methyl ether **(60)** respectively. Compounds **9** and **59** showed 100% mortality at all concentrations tested whereas compounds **5** and **60** had LC_{50} values of 2.78 and 7.07 µg/ mL respectively at 48 hours.

Rotenone **(9)**, degeulin **(15)**, (+)-12α-*epi*milletosin **(7)**, (+)-12α-usararotenoid-A **(6)**, oximes **59**, and **63** showed larvicidal activity against the late third and early fourth instar larvae of *Aedes aegypti* with LC_{50} values of 1.31, 10.4, 38.4, 13.9, 18.5, and 20.8 µg/ mL respectively at 24 hours. Rotenone **(9)** showed 100% mortality at 48 hours while compounds **15**, **7**, **6**, **59** and **63** had LC_{50} values of 2.42, 27.6, 5.29, 9.63, and 10.8 µg/ mL at 48 hours and 1.43, 19.1 2.8, 4.52, and 7.99 µg/ mL respectively at 72 hours.

Rotenone **(9)** showed 100% mortality at 24 hours against the late third and early fourth instar larvae of *Anopheles gambiae*, whereas (+)-12α-usararotenoid-A **(6)** exhibited strong larvicidal activity with LC_{50} value of 10 µg/ mL at 24 hours and achieved 100% mortality at 72 hours.

No antioxidant activity was observed for abyssinone V-4'-methyl ether and its analogues towards 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) when compared with quercetin as a standard.

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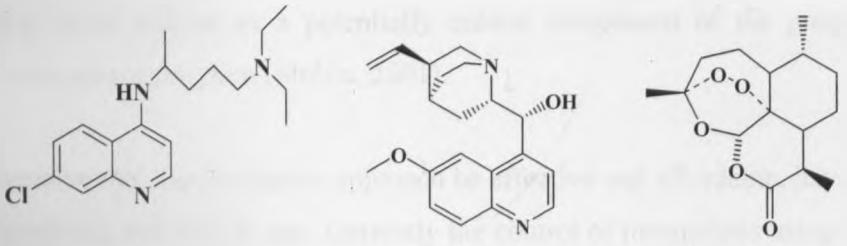
LIST OF ABBREVIATIONS AND SYMBOLS

δ	Chemical shift in delta values
µM	Micromolar
µg/ mL	Microgram per millilitre
brd	Broad doublet
d	Doublet
dd	Doublet of a doublet
dddd	Doublet of a doublet of a doublet of a doublet
m	Multiplet (multiplicity)
q	Quartet
s	Singlet
t	Triplet
AR	Analytical Reagent
^{13}C	Carbon-13 isotope
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane
DEPT	Distortionless Enhancement of Polarisation Transfer
DPPH	2, 2-Diphenyl-1-picrylhydrazyl radical
EC_{50}	Concentration of 50% effectiveness
^{1}H	Proton
Hz	Hertz
J	Coupling constant
KB	Human epidermoid carcinoma of oral cavity cell line
NCI-H187	Human small cell lung cancer cell line
LC_{50}	Concentration causing 50% lethality
NMR	Nuclear Magnetic Resonance
NMCP	National Malaria Control Program
PTLC	Preparative thin layer chromatography
TLC	Thin layer chromatography
UV	Ultra violet
WHO	World Health Organization

CHAPTER ONE

1.0 General Introduction

Malaria is the world's most parasitic disease, ranking high among the major health and developmental challenges in the poor countries of the world. This disease is one of the main causes of mortality among infants and young children (Achille et al., 2010); expectant women are also especially vulnerable. About 300-500 million clinical cases and 1.2–2.8 million deaths due to malaria occur each year (Chandel & Bagai, 2010). It also poses a risk to travellers and immigrants, with imported cases increasing in non-endemic areas (Vicente et al., 2008). The death rate is expected to double in the next 20 years. Precise statistics are unknown because many cases occur in rural areas where people do not have access to hospitals and/or means to affordable healthcare. Consequently, many cases are undocumented (Cragg et al., 1997). The situation has become even more complex over the last few years with the increase in resistance to the drugs [chloroquine **(1)**, quinine **(2)** and artemesinin **(3)**] normally used to combat *Plasmodium falciparum,* the parasite that causes the disease (Okochi & Okpuzor, 2005).



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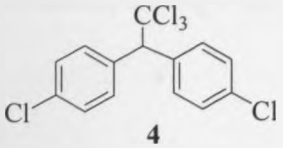
In sub-Saharan Africa where stable transmission is common, *Plasmodium falciparum* malaria is the main parasitic disease. Mosquitoes, the vector for transmitting the disease to humans, constitute a major public health menace in the region (Gopiesh & Kannabiran, 2007). Two main vectors are responsible for the disease transmission in Africa: *Anopheles gambiae* and *Anopheles funestus* (Achille et al., 2010).

In the year 1996, the World Health Organization (WHO), recommended the use of insecticide-treated nets and indoor spraying of insecticides at controlled levels in a bid to

reduce the intensity of human-vector contact. Unfortunately today, the resistance of vectors to insecticides has been observed and constitutes a barrier to their use (Achille et al., 2010). Hence, an alternative approach for mosquito control that uses plant extracts; identifies new natural insecticides, which do not have any ill effects on the non-target population and are easily biodegradable, remains to be one of the top priority strategies in the tropical countries (Gopiesh & Kannabiran, 2007).

Control of such mosquito-borne diseases is becoming more and more difficult because of increasing resistance to pesticides, lack of effective vaccines and drugs against disease-causing organisms (Gopiesh & Kannabiran, 2007). Nowadays, mosquito control is mostly directed against larvae, but only targets adults when necessary. This is because the fight against adult mosquitoes is temporary, unsatisfactory and polluting to the environment, while larval treatment is more localized in time and space resulting in less dangerous outcomes. Larval control can be an effective control tool due to the low mobility of larval mosquitoes, especially where the principal breeding habitats are man-made and can be easily identified. The National Malaria Control Program (NMCP), in redefining its long-term vector control strategies in the context of the country's Roll Back Malaria Program, has renewed interest in examining larval control as a potentially critical component of the program's integrated vector management program (Shililu, 2001).

It is imperative that the alternative approach be effective and affordable, have rapid action, be readily available and easy to use. Currently the control of mosquitoes using DDT **(4)** has not only attracted a lot of interest in some circles, but also caused a great uproar from environmentalists (Matovu & Olila, 2007). Since DDT is non-biodegradable, it is biomagnified in the food chain (bioaccumulates) and it kills non-target organisms. Such qualities commonly lead to pesticide resistance and have negative effects on the environment and non-target organisms including man (Bosire, 2010).



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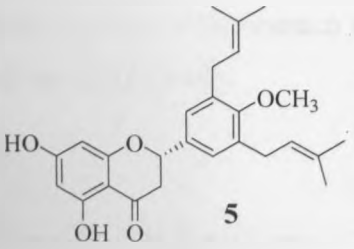
With the increasing awareness on environmental conservation and rapidly growing population, it is prudent to investigate biological control and botanical/natural insecticides in preference to the use of non-biodegradable synthetic insecticides (Matovu & Olila, 2007). Although many synthetic and botanical insecticides are available, there is still an increase in incidences of mosquito resistance. The use of natural products and biological insect control methods is gaining importance because of concerns about the environment, since they are more easily biodegradable; capable of being decomposed e.g. by bacteria (Kasturi et al, 2010). Not only might certain natural plant products be a source of new pesticides, but also botanical derivatives that may be more environmentally friendly than synthetic chemicals (Cantrell et al., 2010).

In light of this, several flavonoids known to possess larvicidal activity were structurally modified in a bid to enhance their activity. The compounds used in this study were prenylated flavanone abyssinone V-4'-methyl ether **(5)** identified from the root and stem bark of the plant *Erythrina abyssinica* (Yenesew et al., 2003b), (+)-12α-usararotenoid-A **(6)**, and (+)-12α-*epi*milletosin **(7)** that occur in *Milletia usaramensis* subspecies *usaramensis* (Yenesew et al., 1998).



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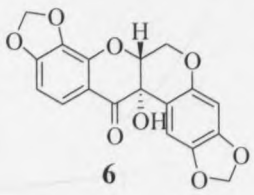

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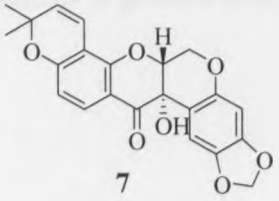

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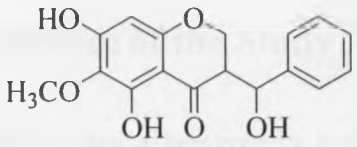
In addition to these modifications, the hydrogenolysis of two diastereoisomeric 9-hydroxyhomoisoflavanones **(8)** identified from *Polygonum senegalense* plant found in Kenya (Midiwo et al., 2006), and *Polygonum ferruginuem* plant grown in Argentina (Lopez et al., 2006) was carried out. This was done in a bid to establish which of the diastereoisomers is more reactive in the conversion to its corresponding 9-deoxyhomoisoflavanone.



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1.1 Problem Statement

For many years, the malaria vector has mainly been controlled with synthetic insecticides. However, these insecticides commonly suffer problems of pesticide resistance, non-biodegradability and detrimental effects on non-target organisms including man (Ghayal et al., 2010). To surmount these challenges, there is need for continuous development of new botanical insecticides that could also serve as lead compounds for developing more potent and biodegradable derivatives.

1.2 Objective of the Research

1.2.1 General Objective

The general objective of the research was to modify some flavonoids to generate analogues with enhanced larvicidal activity.

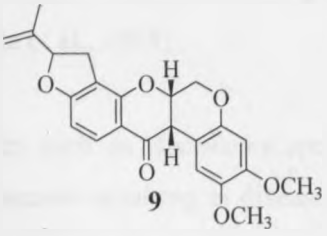
1.2.2 Specific Objectives

1. To perform structural modification of abyssinone V-4'-methyl ether, (+)-12α-usararotenoid-A and (+)-12α-*epi*milletosin;
2. To elucidate the structure of the derivatives of abyssinone V-4'-methyl ether, (+)-12α-usararotenoid-A and (+)-12α-*epi*milletosin;
3. To evaluate the larvicidal activity of abyssinone V-4'-methyl ether, (+)-12α-usararotenoid-A, (+)-12α-*epi*milletosin in comparison to their derivatives.
4. To perform hydrogenolysis of two diastereoisomeric 9-hydroxyhomoisoflavanones to its corresponding 9-deoxyhomoisoflavanone.

1.3 Rationale and Significance of the Study

Medicinal plants have continued to play a key role in world health. In spite of the great advances observed in modern medicine in recent decades, plants still make an important contribution to health-care. The use of conventional chemical pesticides has resulted in the development of pest resistance, undesirable effects on non-target organisms and fostered environmental and human health concerns. The use of herbal products is one of the best alternatives for mosquito control (Nandita et al., 2008).

Plant-derived (botanical) insecticides comprise of an array of chemical compounds which act in concert on both behavioural and physiological processes. Based on the combinatorial mode of action, the chances of pests developing resistance to such botanicals is less likely. In light of this, there is need to identify compounds with less bioaccumulation potential and biodegradability. The search for insecticides from plants can be guided by focusing on plants that have been known to be used traditionally. Botanical insecticides, such as rotenone (**9**) and other rotenoids offer this potential (Bosire, 2010).



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Bearing in mind that pest resistance exists, there is need to perform structural modification on these compounds to improve their larvicidal activity. Although there is scant literature on the chemical modification of flavonoids, the structural modifications done on several flavonoids to incorporate nitrogenous heterocycles have led to improved biological activities (Kul'magambetova et al., 2002).

CHAPTER TWO

2.0 Literature Review

2.1 Larval Control

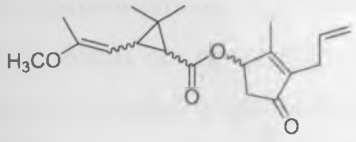
One of the approaches for control of mosquito borne diseases such as malaria is the interruption of disease transmission, either by killing or preventing mosquitoes from biting human beings or by causing larval mortality in a large scale at breeding centers of the vectors (Kasturi et al., 2010). Mosquitoes in the larval stage are attractive targets for pesticides because they breed in water and, thus, are easy to deal with in this habitat.

The use of conventional chemical pesticides has resulted in the development of resistance, undesirable effects on non-target organisms and fostered environmental and human health concerns. The use of herbal products is one of the best alternatives for mosquito control. The search for herbal preparations that do not produce adverse effects in the non-target organisms and are easily biodegradable remains a top research goal for scientists associated with alternative vector control (Nandita et al., 2008).

Fresh water breeding mosquitoes such as *Anopheles* species and *Aedes aegypti* are very difficult to control during rainy season resulting in diseases like malaria. The eradication or control of such mosquitoes is considered as the only option.

2.1.1 Synthetic Insecticides and Larvicides

Mosquitoes are a public health menace since they transmit diseases such as malaria that cause millions of deaths every year. Synthetic insecticides (pyrethroids) have been and are still being used to control this disease vector. Pyrethroids are synthetic (human-made) forms of pyrethrins. There are two types that differ in chemical structure and symptoms of exposure. Type I pyrethroids include allethrin **(10)** and resmethrin **(11)** whereas type II pyrethroids include cypermethrin **(12)** and cyfluthrin **(13)**. Both type I and II pyrethroids inhibit the nervous system of insects. This occurs at the sodium ion channels in the nerve cell membrane (NPTN., 1998).



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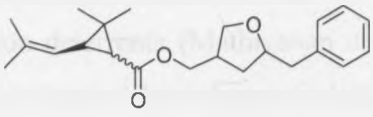

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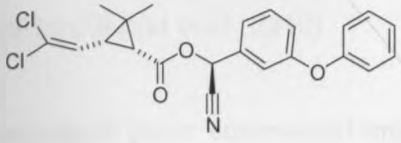

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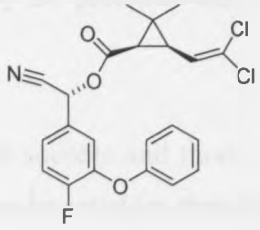

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One form of the synthetic larvicides, chlorinated hydrocarbons, of which DDT **(4)** is the best known are so called because they contain chlorine in combination with hydrogen and carbon. They vary widely in chemical structure and activity. Some are stable and long lasting, which accounts for their effectiveness as residual insecticides. Their ability to accumulate in the fatty tissues of humans, animals, fish and other living organisms makes their use as mosquito larvicides undesirable except under certain conditions. The ability of mosquitoes as well as other insects to develop resistance to these effective and economical insecticides has also reduced their usefulness in many areas.

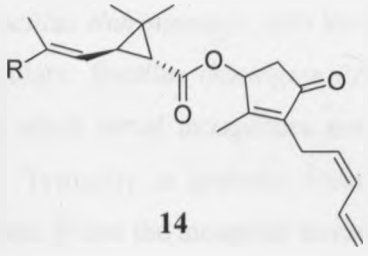
The greatest harm from synthetic insecticides is that once introduced into the eco-system, they persist for a very long duration. This persistence not only poses a threat to life, but also triggers insects to develop resistance against them. This is the reason for the desire to produce an insecticide which is powerful, with lesser side effects, biodegradable and with reduced chances of developing resistance against it. These problems have renewed interest in exploiting the pest-control potential of plants and their metabolites (Mathivanan et al., 2010).

2.1.2 Botanical Insecticides and Larvicides

Plants, being a natural source of various compounds including flavonoids, are known to contain larvicidal agents, which may act in combination or independently. Some phytochemicals act as general toxicants both against adult as well as larval stages of

mosquitoes, while others interfere with growth and development (growth inhibitors) or with reproduction (chemosterilent) or produce olfactory stimuli acting as repellent or attractant. Other phytochemicals act as ovicides or oviposition deterrents (Mathivanan et al., 2010). Natural products of plant origin with insecticidal properties have been tried in the recent years for control of a variety of insect pests and vectors; they may therefore be employed as an alternative source of mosquito control agents. They are preferred also due to their innate biodegradability (Ghayal et al., 2010).

Plant compounds of major commercial importance as sources and models of insect-control agents include pyrethrins and rotenoids. Pyrethrins are insecticides that are derived from the extract of *chrysanthemum* flowers (pyrethrum) that contains pyrethrin I **(14)** (R= CH_3) and pyrethrin II **(14)**, (R = CO_2CH_3). Pyrethrins affect the nervous system of insects by causing multiple action potentials in the nerve cells by delaying the closing of an ion channel (NPTN., 1998).



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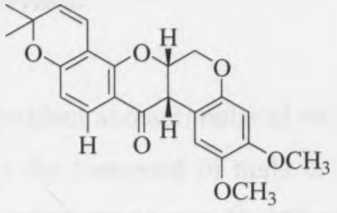
Rotenoids are a group of isoflavonoids obtained from the roots of certain tropical Leguminosae especially from *Derris* species. Rotenone **(9)** is the most important of such compounds; it is a selective, non-specific insecticide, used in home gardens for insect control of lice and tick control on pets, and for fish eradications as part of water body management. Moreover, it is both a contact and stomach poison to insects; it kills them slowly, but causes them to stop their feeding almost immediately. It exerts its toxic action by acting as a general inhibitor of cellular respiration (Bosire, 2010).

The presence, in different forms, of the isoprenoid chain attached to a flavonoid skeleton can lead to impressive changes in biological activity, mostly attributed to an increased affinity for biological membranes and to an improved interaction with proteins. The potent insecticidal

activity of rotenone **(9)**, which contains a 1,1-dimethyl chromanone ring on the basic rotenoid skeleton, may serve as a good example.

Several rotenoids including rotenone **(9)**, (+)-usararotenoid-A **(6)**, degeulin **(15)** and (+)-12α-*epi*milletosin **(7)**, have been known to be active against fourth-instar larvae of *Aedes aegypti* L. with LC_{50} values of 0.45, 9.3, 1.6 and 35 μg/ mL respectively (Yenesew et al., 2003a).



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2.1.3 Biological Agents

The biological control agent *Bacillus thuringiensis*, also known as *Bt*, is a bacterial disease specific to Lepidopteran caterpillars. *Bacillus thuringiensis israelensis*, also known as *Bti*, and *Bacillus sphaericus*, which affect larval mosquitoes and some midges, have come into increasing use in recent times. Typically in granular form, pellets are distributed on the surface of stagnant water locations. When the mosquito larvae ingest the bacteria, crystallized toxins are produced which destroy the digestion tract, resulting in death. These larvicides will last only a few weeks in water and pose no danger to humans, non-target animal species, or the environment when used according to directions (Weinzierl et al., 1997).

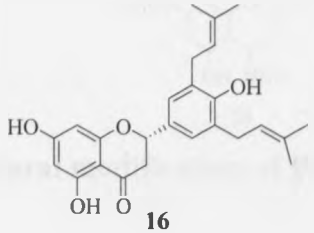
2.1.4 Mosquito Fish

The mosquitofish, *Gambusia affinis*, is native to southern and eastern United States. Originally introduced into California as early as 1922, their use has been one of the most effective non-insecticidal and non-chemical methods of controlling mosquitoes for over eighty years. Mosquito fish do not lay eggs, but rather give birth to live young. These fish, therefore, require no special environment, as most other fish do, for depositing and hatching their eggs. They breed throughout the summer and new broods are produced at intervals of about six weeks, with 50 to 100 young in a single brood. The young are approximately 1/4

inch in length when born and grow to a maximum size of about three inches. They are ready to begin the work of destroying mosquito larvae at once. Mosquito fish can eat mosquito larvae as fast as the larvae hatch from eggs, as many as 100 per day. The earliest brood of the season, born in April and May, become sexually mature and produce young when six to eight weeks old. Mosquito fish live 2-3 years and can tolerate a wide range of temperatures (Rajkumar, 1987).

2.2 Other Biological Activities

In an effort to explore other antioxidant and antimalarial natural products; an investigation of some *Erythrina* species, used for the treatment of malaria and microbial infections showed the presence of prenylated flavonoids abyssinone V **(16)** and abyssinone V-4'-methyl ether **(5)** from the roots and stem bark of *E. abyssinica* (Yenesew et al., 2003b; 2004).



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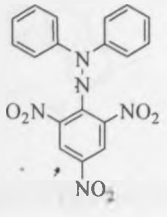
Flavanones **(5)** and **(16)** have also been isolated from the extract of the root bark and stem bark of *Erythrina burttii* (Yenesew et al., 1998, 2002, 2003c). On extraction, the flavanone **5** is obtained in large amounts compared to the flavanone **16**, though it is less biologically potent (Yenesew et al., 2004 and 2009). The radical scavenging activities of flavanone **16** towards 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) **(17)** had an EC_{50} of 30.1 μM compared to the standard; quercetin **(18)**, which had an EC_{50} of 5.4 μM (Yenesew et al., 2009).



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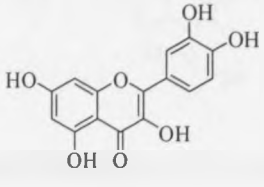


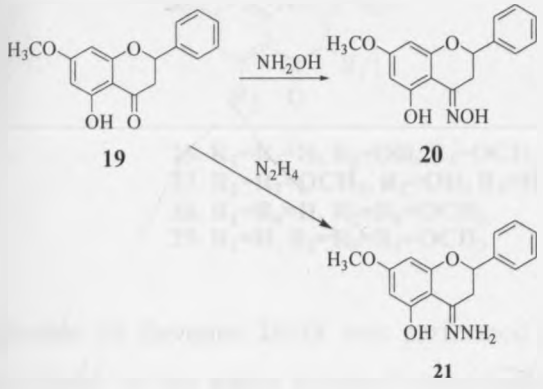
18

2.3 Effects of Chemical Modifications

There is scant literature on the chemical modification of bioactive flavonoids (Kul'magambetova et al., 2002). However, some of the structural modifications done have shown improved biological activities. Highlighted below are some of these cases.

2.3.1 Pinostrobin

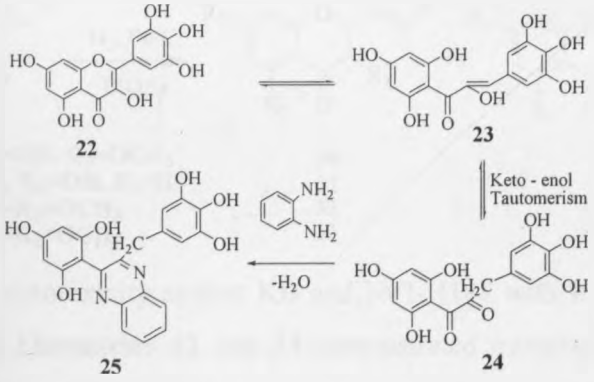
The structural modification of Pinostrobin **(19)** to its oxime **(20)** and hydrazone **(21)** enhanced its hepatoprotective activity (Kul'magambetova et al., 2002).



Scheme 1: Structural modification of Pinostrobin (19)

2.3.2 Myricetin

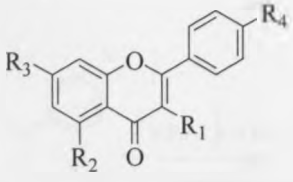
Chemical modification of the flavonol myricetin **(22)** through chalcone **(23)** and keto-tautomer **(24)** to quinoxaline **(25)** generated derivatives of interest with potential antitumoral, antimicrobial, antifungal, antioxidant, and antitubercular activities (Polyakov, 1999).



Scheme 2: Structural modification of Myricetin (22)

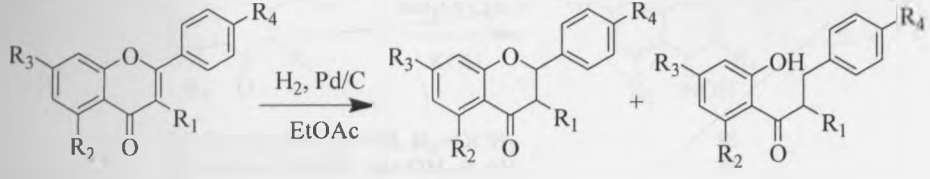
2.3.3 Flavonoids from *Kaempferia parviflora*

Flavones **26-29** isolated from *Kaempferia parviflora* were subjected to structural modification so as to improve their cytotoxicity. Sixteen flavonoid derivatives, including four new derivatives, were prepared from these flavones and evaluated for cytotoxicity against human epidermoid carcinoma of oral cavity (KB) and human small cell lung cancer (NCI-H187) cell lines (Yenjai & Wanich, 2010).



26. $R_1=R_4=H$, $R_2=OH$, $R_3=OCH_3$
27. $R_1=R_3=OCH_3$, $R_2=OH$, $R_4=H$
28. $R_1=R_4=H$, $R_2=R_3=OCH_3$
29. $R_1=H$, $R_2=R_3=R_4=OCH_3$

Catalytic hydrogenation of flavones **26-29** was performed using palladium on carbon to furnish flavanones **30-33** as the major products. Dihydrochalcones **34**, **35**, and **36** were detected as minor products which were obtained from benzylic cleavage of flavanones **30**, **32**, and **33**, respectively. However, there was no product from benzylic cleavage of **31** under the same conditions. This may be due to the methoxy group at C-3 position retarding the benzylic cleavage (Yenjai & Wanich, 2010).

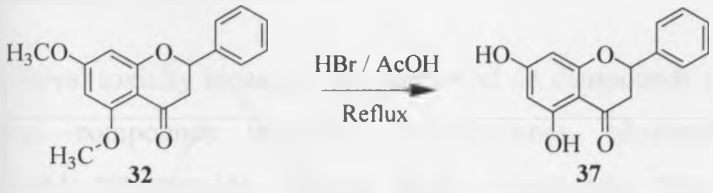


26. $R_1=R_4=H$, $R_2=OH$, $R_3=OCH_3$	**30**	**34**
27. $R_1=R_3=OCH_3$, $R_2=OH$, $R_4=H$	**31**	-
28. $R_1=R_4=H$, $R_2=R_3=OCH_3$	**32**	**35**
29. $R_1=H$, $R_2=R_3=R_4=OCH_3$	**33**	**36**

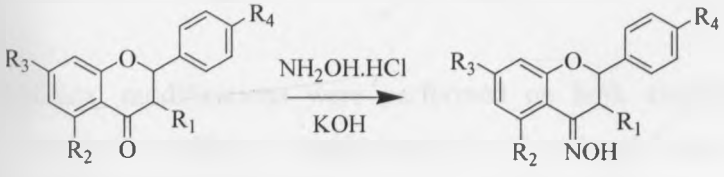
Flavanone **31** showed cytotoxicity against KB and NCI-H187 with IC_{50} values of 30.4 and 98.3 μM, respectively. Flavanones **32** and **33** demonstrated moderate cytotoxicity against NCI-H187 cells with IC_{50} values of 33.1 and 25.1 μM, respectively, while showing weak cytotoxicity against KB cells with IC_{50} values of 39.9 and 78.8 μM, respectively. However,

these three compounds were more cytotoxic than the parent flavones (Yenjai & Wanich, 2010).

The demethylation reaction of flavanone **32** was carried out using hydrobromic acid in acetic acid to obtain the corresponding 5,7-dihydroxyflavanone **37** in 48% yield. Demethylated flavanone **(37)** showed weak cytotoxicity against KB and NCI-H187 with IC_{50} values of 99.2 and 95.5 μM, respectively in comparison with the parent compound **(32)** (Yenjai & Wanich, 2010).



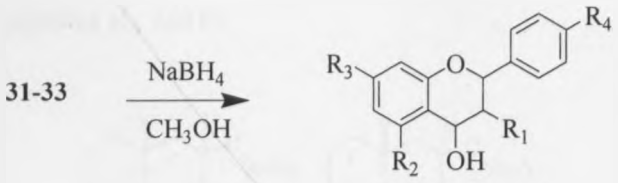
Treatment of flavanones **30-33, 37** with hydroxylamine hydrochloride in the presence of potassium hydroxide provided oximes **38-42** in high yield. Oxime **38** exhibited dramatically strong cytotoxicity against KB and NCI-H187 cell lines with IC_{50} values of 0.3 and 0.1 μM, respectively, while **39** showed strong cytotoxicity against NCI-H187 cell line with an IC_{50} value of 0.3 μM. Oximes **41** and **42** showed strong cytotoxicity against NCI-H187 cell line with IC_{50} values of 4.1 and 2.3 μM, respectively (Yenjai & Wanich, 2010).



30. R_1=R_4=H, R_2=OH, R_3=OCH_3 **38**
31. R_1=R_3=OCH_3, R_2=OH, R_4=H **39**
32. R_1=R_4=H, R_2=R_3=OCH_3 **40**
33. R_1=H, R_2=R_3=R_4=OCH_3 **41**
37. R_1=R_4=OH, R_2=R_3=OH **42**

Flavanones **31-33** were treated with sodium borohydride at room temperature to afford a single diastereomer of flavanols **43-45** in moderate to high yield. Flavanols **43-45** showed less potent cytotoxicity against KB and NCI-H187 cell lines in comparison with the parents'

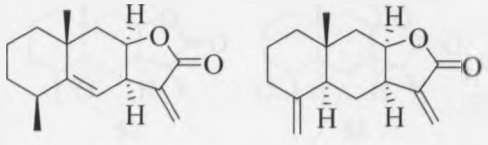
31-33. These results confirm that the 4-carbonyl groups or carbonyl analogs of flavanones are crucial for the activity (Yenjai & Wanich, 2010).



43-45

2.3.4 Eudesmanolides as Larvicides

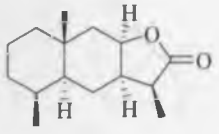
An *Aedes aegypti* larval toxicity bioassay was performed on compounds representing many classes of natural compounds including polyacetylenes, phytosterols, flavonoids, sesquiterpenoids, and triterpenoids. Among these compounds, two eudesmanolides, alantolactone **(46)** and isoalantolactone **(47)** showed larvicidal activities against *Aedes aegypti* and, therefore, were chosen for further structure–activity relationship studies (Cantrell et al., 2010).



46 **47**

In this study, structural modifications were performed on both alantolactone **(46)** and isoalantolactone **(47)** in an effort to understand the functional groups necessary for maintaining and/or enhancing their activity, and to possibly lead to more effective insect-control agents. All parent compounds and their analogues were evaluated for their larvicidal activities against *Aedes aegypti* larvae and adults. None of the synthetic isomers synthesized and screened against *Aedes aegypti* larvae were more active than isoalantolactone **(47)** itself which had an LC_{50} value of 10.0 µg/ mL. This was not the case for analogs of alantolactone **(46)** which had larvicidal activities ranging from 12.4 to 69.9 µg/ mL (Cantrell et al., 2010).

Compounds 11,13-dehydroalantolactone **(48)** and 11,13-dehydroisoalantolactone **(49)** were included in this study. Both compounds demonstrated LC_{50} values of >125 µg/ mL against larvae of *Aedes aegypti* indicating the importance of the α,β-unsaturated lactone moiety for larvicidal activity (Cantrell et al., 2010).



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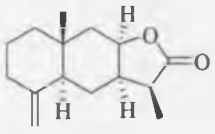
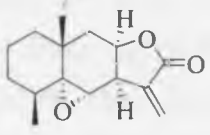


49

Initial synthetic modifications included the peracid epoxidation of compounds **46-49** to their corresponding epoxides **50-53**. All four epoxy synthetic isomers were also inactive up to the top testing concentration of 125 µg/ mL (Cantrell et al., 2010).



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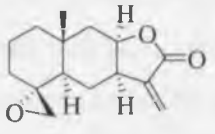

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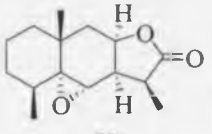

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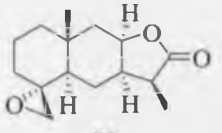
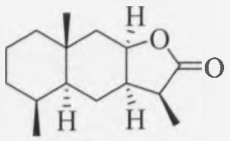


53

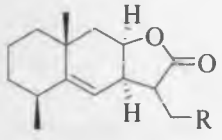
Catalytic hydrogenation of 11,13-dehydroalantolactone **(48)** provided product **54** which was inactive up to the top testing concentration of 125 µg/ mL (Cantrell et al., 2010).



54

Michael addition reactions using the nucleophilic amines piperidine and diethylamine (Et_2NH) were performed on both compounds alantolactone **(46)** and isoalantolactone **(47)**. Additions of Et_2NH to both alantolactone **(46)** and isoalantolactone **(47)** produced the desired

products **55** and **56; R = diethylamino**. *Aedes aegypti* larvicidal activity for the diethylamino analogs were 14.4 and >125 µg/ mL for **56** and **55**, respectively. Additions of piperidine to alantolactone **(46)** and isoalantolactone **(47)** provided the desired products **57** and **58** respectively; **R = piperidine-1-yl)** (Cantrell et al., 2010).



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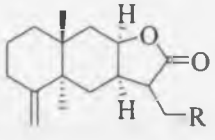
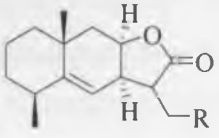


56



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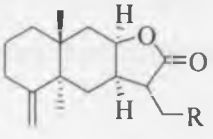


58

Compound **56** was only slightly less active than its parent compound **47**. Piperidine analogs **58** and **57** were active against *Aedes aegypti* larvae with LC_{50} values of 55.1 and 12.4 µg/ mL, respectively (Cantrell et al., 2010). These results lend support to the hypothesis that structural modification may provide analogues with enhanced larvicidal activity.

CHAPTER THREE

3.0 Experimental

3.1 Materials

Samples of abyssinone V-4'-methyl ether **(5)**, rotenone **(9)**, (+)-12α-usararotenoid-A **(6)**, degeulin **(15)** and (+)-12α-*epi*milletosin **(7)** were obtained from Professor Abiy Yenesew while. 9-hydroxyhomoisoflavanone **(8)** was obtained from Professor Midiwo, both from the Department of Chemistry, University of Nairobi.

Table 3.1: Source of the starting materials

Sample	Source	Reference
Abyssinone V-4'-methyl ether **(5)**	*Erythrina abyssinica*	(Yenesew et al., 2003b)
Rotenone **(9)**	*Derris* species	(Yenesew et al., 2003a)
(+)-12α-Usararotenoid-A **(6)** and (+)-12α-*epi*milletosin **(7)**	*Milletia usaramensis*	(Yenesew et al., 1998)
Degeulin **(15)**	*Milletia dura*	(Yenesew et al., 2003a)
9-Hydroxyhomoisoflavanone **(8)**	*Polygonum senegalense*	(Midiwo et al., 2006)

3.1.1 Reagents and Chemicals

Hydrogen gas, nitrogen gas, hydrobromic acid (48%), acetic anhydride, acetic acid, pyridine, hydrazine hydrate (98%), phenylhydrazine, ethanol 95%, absolute ethanol, methanol AR and triethylamine, sodium borohydride, hydroxylamine hydrochloride, hydrazinium chloride, 5% palladium on carbon, sodium sulphate, sodium bicarbonate, zinc metal, copper sulphate, diethyl ether were obtained from Sci-Lab chemicals, Fisher Scientific, Laborama, Kobian Limited and Pyrex Limited. These reagents were available in the University of Nairobi laboratories and store.

3.1.2 Chromatography

All the solvents used in chromatography were obtained from Kobian Kenya Limited and purified by distillation before use. Column chromatography was carried out using Merck silica gel 60 (70-230 mesh) and Sephadex LH 20. Analytical TLC and preparative thin layer chromatography (PTLC) were done using Merck pre-coated 60 F_{254} and Merck 60 PF_{254}, respectively.

Preparative TLC plates were prepared by adding 200 mL of water to 80 g of silica gel to form a slurry that was allowed to stand for 30 minutes. The slurry (40 mL) was then poured and spread evenly on six clean 20 cm x 20 cm glass plates and left to dry at room temperature. The silica gel was then activated in an oven for one hour at a temperature of 100 °C, removed and allowed to cool at room temperature before use. Chromatographic zones were detected under UV light at a wavelength of 254 nm.

3.1.3 Instrumentation

The NMR spectra were recorded on a Varian-Mercury 200 MHz instrument. Chemical shifts were recorded in ppm (δ) relative to the internal standard tetramethyl silane (TMS) with deuterated chloroform or acetone as the solvent wheareas coupling constants (*J*) values are given in Hertz (Hz). Signal multiplicities are represented by: *s* (singlet), *brs* (broad singlet), *brd* (broad doublet), *d* (doublet), *t* (triplet), *q* (quartet), *dd* (doublet of a doublet), *ddd* (doublet of a doublet of a doublet), *dddd* (doublet of a doublet of a doublet of a doublet) and *m* (multiplet).

3.1.4 Apparatus

All reusable glassware (test tubes, conical flasks, round bottomed flasks, measuring cylinders, vials, beakers, volumetric flasks, pasture pipettes, syringes) were soaked in warm water with liquid detergent before washing thoroughly and rinsing with tap water. They were then dried on the bench. Just before use, the apparatus were rinsed with a mixture of distilled solvents to remove any organic impurities.

3.1.5 Test Organisms

The eggs of *Aedes aegepti* were obtained from the Department of Zoology, University of Nairobi. The eggs were then transferred into trays containing a sodium chloride solution (pH 7.5) after which they were allowed to hatch awaiting the bioassay to be carried out (Kasturi et al., 2010).

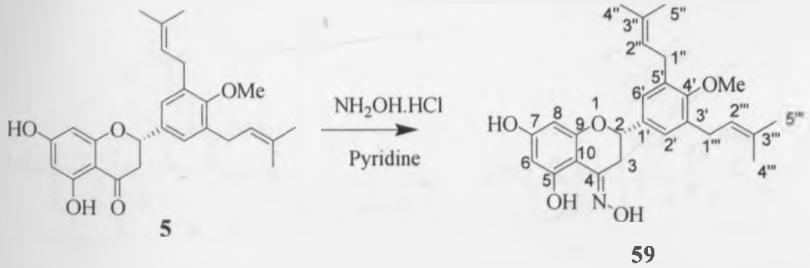
3.1.6 Thin Layer Chromatography

The progress of the reactions was monitored by TLC using either *n*-hexane: ethyl acetate (7:3 V/V), dichloromethane: ethyl acetate (8:2 V/V) or n-hexane: ethyl acetate (8:2 V/V). A few drops of triethylamine were then added into each solvent system to deactivate the TLC plates. After development, the spots were then visualized under an ultra violet lamp at a wavelength of 254 nm.

3.2 Preparation of the Analogues

3.2.1 Abyssinone V-4'-Methyl Ether (5) Analogues

3.2.1.1 Transformation of Abyssinone V-4'-Methyl Ether (5) to its Oxime (59)



5 59

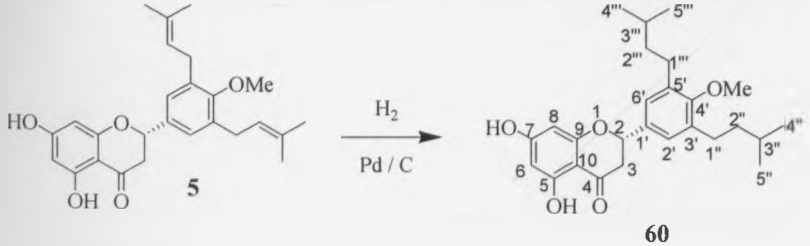
A 100 mL, round bottomed flask, equipped for mechanical stirring, was charged with 100 mg of flavanone 5 and 12 mL absolute ethanol. The solution was then treated with 169 mg of hydroxylamine hydrochloride and eight drops of pyridine and stirred for 5 days at room temperature for reaction to go to completion. Reaction progress was monitored by thin layer chromatography every 4 hours using hexane: ethylacetate (7:3 V/V) solvent system.

The resulting solution was diluted with 50 mL dichloromethane, filtered, dried over sodium sulphate, filtered and then concentrated by rotary evaporation; the residue was then allowed to dry. The resulting oil was then flushed with nitrogen gas. Thin layer chromatography was then redone to ascertain that no change to the product formed had occurred during the workup procedure and detected using a UV lamp at 254 nm. This afforded a green oil (103 mg, 99%) which was characterized as the oxime **59** by proton and carbon nuclear magnetic resonance.

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 7.31 (2H, *s*, H-2', H-6'), δ 6.17, 6.14 (2H, *s*, H-6, H-8), δ 5.03 (1H, *brd*, J = 11.2 Hz, H-2), δ 5.36 (2H, *t*, J = 6.0 Hz, H-2", H-2"'), δ 3.80 (3H, *s*, OCH_3-4'), δ 3.45 (4H, *d*, J = 6.8 Hz, H-1", H-1"'), δ 3.59 (1H, *brd*, J = 15.2, 16.6 Hz, H-3), δ 2.80 (1H, *dd*, J = 12.2, 17.6 Hz, H-3), δ 1.79 (12H, *s*, CH_3-4", CH_3-4"', CH_3-5", CH_3-5"').

^{13}C-NMR (50 MHz, $CDCl_3$): δ 171.2 (C-4), δ 159.8 (C-7), δ 160.5 (C-5), δ 158.8 (C-9), δ 156.6 (C-4'), δ 138.2 (C-3' and C-5'), δ 135.3 (C-1'), δ 133.0 (C-3" and C-3"'), δ 126.3 (C-2' and C-6'), δ 123.1 (C-2" and C-2"'), δ 98.1 (C-10), δ 97.6 (C-6), δ 96.4 (C-8), δ 76.8 (C-2), δ 61.3 (4'-OMe), δ 30.2 (C-3), δ 28.8 (C-1" and C-1"'), δ 26.1 (C-4" and C-4"'), δ 18.2 (C-5" and C-5"').

3.2.1.2 Hydrogenation of Abyssinone V-4'-Methyl Ether (5)



A 40 mL flat bottomed flask, charged with 49 mg of the flavanone **5** and 8 mL of absolute ethanol was warmed to allow dissolution. The solution was then cooled to room temperature, flushed with nitrogen gas, followed by the addition of 15 mg of 5% palladium on carbon. The flat bottomed flask was then equipped for hydrogen bubbling and the reaction process carried

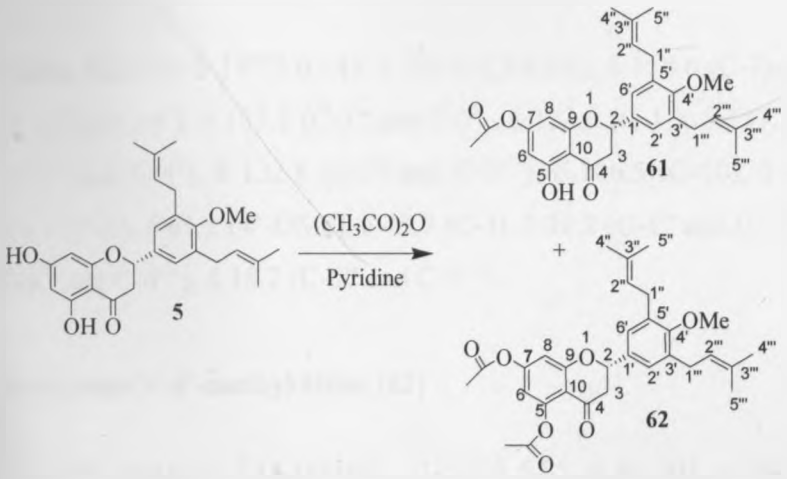
out for 17 hours at room temperature. The reaction progress was monitored by thin layer chromatography after 16 hours using hexane: ethylacetate (7:3 V/V) solvent system.

The resulting solution was diluted with 50 mL dichloromethane, filtered and concentrated by rotary evaporation; the residue was then allowed to dry. This afforded a yellow oil of compound **60** (47 mg, 99%) which was characterized by proton and carbon nuclear magnetic resonance.

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 12.13 (1H, *s*, OH-5), δ 7.17 (2H, *s*, H-2', H-6'), δ 6.08 (2H, *s*, H-6, H-8), δ 5.38 (1H, *brd*, *J* = 13.2 Hz, H-2), δ 3.83 (3H, *s*, OCH_3-4'), δ 3.17 (1H, *dd*, *J* = 13.2, 15.8 Hz, H-3), 2.83 (1H, *brd*, *J* = 17.6 Hz, H-3), δ 2.72 (4H, *t*, *J* = 5.8 Hz, H-1", H-1"'), δ 1.76 (2H, *m*, H-3", H-3"'), δ 1.58 (4H, *dd*, *J* = 7.8, 10.4 Hz, H-2", H-2"'), δ 1.04 (12H, *d*, *J* = 6.4 Hz, CH_3-4", CH_3-4"', CH_3-5", CH_3-5"').

^{13}C-NMR (50 MHz, $CDCl_3$): δ 196.4 (C-4), 165.7 (C-7), 164.6 (C-5), 163.6 (C-9), 157.1 (C-4'), 137.0 (C-3' and C-5'), 133.9 (C-1'), 125.9 (C-2' and C-6'), δ 103.2 (C-10), δ 97.0 (C-6), δ 95.9 (C-8), δ 79.7 (C-2), δ 61.5 (4'-OMe), δ 43.6 (C-3), δ 40.4 (C-1" and C-1"'), 28.6 (C-3" and C-3"'), δ 28.2 (C-2" and C-2"'), δ 22.9 (C-4", C-4"', C-5" and C-5"').

3.2.1.3 Acetylation of Abyssinone V-4'-Methyl Ether (5)



To a 100 mL, three necked, round bottomed flask, and fitted with a reflux condenser containing 198 mg of the flavanone **5** dissolved in 6 mL acetic anhydride, one drop of pyridine was added. The mixture was then refluxed at 60 °C for 5 hours. Reaction progress was monitored by thin layer chromatography every hour using hexane: ethylacetate (7:3 V/V) solvent system. The TLC revealed one spot.

The resulting solution was then transferred to a 50 mL beaker; ice cold water poured into it and the mixture stirred over an ice bath. After precipitation, the water was decanted off and 50 mL dichloromethane added to the precipitate. The resulting mixture was then dried over sodium sulphate, filtered and concentrated by rotary evaporation. The resulting oily residue was then flushed with nitrogen gas. Thin layer chromatography was then redone to ascertain that no change to the product formed had occurred during the workup. The TLC showed four spots which were separated using preparative TLC and detected using a UV lamp at 254 nm. This afforded yellow crystals (9 mg, 5%) of flavanone **61** and a yellow oil (89 mg, 45%) of flavanone **62** which were then characterized by proton and carbon nuclear magnetic resonance. The two remaining spots were the starting material and a mixture of the acetates.

7-Acetyl-abyssinone V-4'-methyl ether (61)

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 11.93 (1H, *s*, OH-5), δ 7.14 (2H, *s*, H-2', H-6'), δ 6.36, 6.35 (2H, *brs*2*, H-6, H-8), δ 5.43 (1H, *brd*, J = 13.8 Hz, H-2), δ 5.32 (2H, *t*, J = 5.2 Hz, H-2", H-2"'), δ 3.80 (3H, *s*, OCH_3-4'), δ 3.45 (4H, *d*, J = 6.8 Hz, H-1", H-1"'), δ 3.20 (1H, *dd*, J =

13.2, 17.2 Hz, H-3), δ 2.88 (1H, *brd*, *J* = 17.0 Hz, H-3), δ 2.34 (3H, *s*, $COCH_3$) δ 1.81 (12H, *s*, CH_3-4", CH_3-4"', CH_3-5", CH_3-5"').

^{13}C-NMR (50 MHz, $CDCl_3$): δ 197.7 (C-4), δ 168.6 ($COCH_3$), δ 158.6 (C-7), δ 163.6 (C-5), δ 162.7 (C-9), δ 157.0 (C-4'), δ 135.7 (C-3' and C-5'), δ 133.6 (C-1'), δ 133.3 (C-3" and C-3"'), δ 126.1 (C-2' and C-6'), δ 122.8 (C-2" and C-2"'), δ 106.5 (C-10), δ 103.5 (C-6), δ 102.1 (C-8), δ 79.7 (C-2), δ 61.2 (4'-OMe), δ 43.7 (C-3), δ 30.2 (C-1" and C-1"'), δ 26.7 ($CO\underline{C}H_3$), δ 20.6 (C-4" and C-4"'), δ 18.2 (C-5" and C-5"').

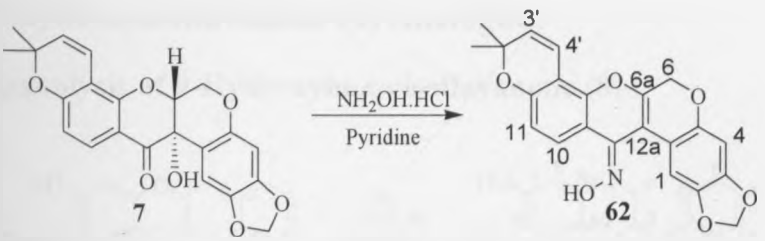
5,7-Diacetyl-abyssinone V-4'-methyl ether (62)

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 7.14 (*s*, H-2', H-6'), δ 6.85, 6.50 (2H, *s*, H-6, H-8), δ 5.46 (1H, *brd*, *J* = 13.8 Hz, H-2), δ 5.35 (2H, *t*, *J* = 6.4 Hz, H-2", H-2"'), δ 3.80 (3H, *s*, OCH_3-4'), δ 3.45 (4H, *d*, *J* = 7.2 Hz, H-1", H-1"'), δ 3.14 (1H, *dd*, *J* = 13.8, 16.6 Hz, H-3), δ 2.79 (1H, *brd*, *J* = 16.6 Hz, H-3), δ 2.36, 2.45 (6H, *s**2, $COCH_3$), δ 1.79 (12H, s, CH_3-4", CH_3-4"', CH_3-5", CH_3-5"').

^{13}C-NMR (50 MHz, $CDCl_3$): δ 189.9 (C-4), δ 169.6, 168.4 ($COCH_3$), δ 158.6 (C-7), δ 151.5 (C-5), δ 163.8 (C-9), δ 157.0 (C-4'), δ 135.7 (C-3' and C-5'), δ 133.7 (C-1'), δ 133.3 (C-3" and C-3"'), δ 126.1 (C-2' and C-6'), δ 122.8 (C-2" and C-2"'), δ 112.1 (C-10), δ 110.7 (C-6), δ 109.5 (C-8), δ 80.0 (C-2), δ 61.2 (4' - OMe), δ 45.3 (C-3), δ 28.6 (C-1" and C-1"'), δ 26.1 (C-4" and C-4"'), δ 21.5 ($CO\underline{C}H_3$), δ 18.2 (C-5" and C-5"').

3.2.2 (+)-12α-*epi*milletosin (7) Analogue

3.2.2.1 Transformation of (+)-12α-*epi*milletosin (7) to its Oxime (63)

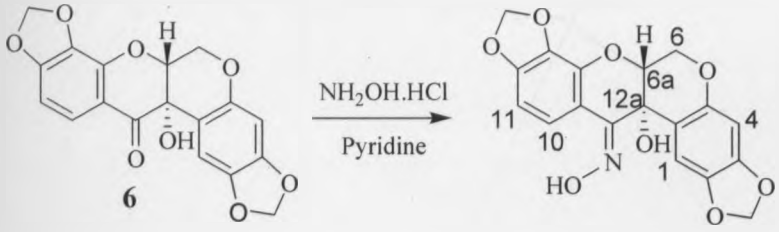


The reaction was carried out as in **3.2.1.1.** In this case, 20 mg of rotenoid **7** dissolved in 6 mL of absolute ethanol was treated with 10 mg of hydroxylamine hydrochloride and two drops of pyridine. The mixture was then stirred for 72 hours at room temperature. The solvent system used for TLC was hexane: ethylacetate (8:2 V/V). The TLC showed two spots which were separated using preparative TLC and detected using a UV lamp at 254 nm. This afforded a yellow oil (10 mg, 50%) of product **63** which was characterized by proton and carbon nuclear magnetic resonance though, the **^{13}C-NMR** was not pronounced. The other spot was the starting material.

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 8.66 (1H, *d*, *J* = 9.2 Hz, H-11), δ 7.76 (1H, *s*, H-1), δ 6.65 (1H. *d*, *J* = 3.4 Hz, H-4'), δ 6.61 (1H, *d*, *J* = 2.6 Hz, H-10), δ 6.46 (1H, *s*, H-4'), δ 5.86 *s* (2H, *s*, OCH_2O), δ 5.61 (1H, *d*, *J* = 9.6, H-3'), δ 4.70 (2H, *s*, H- 6α, 6β).

3.2.3 (+)-12α -Usararotenoid-A (6) Analogue

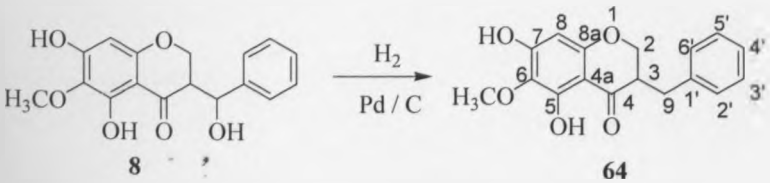
3.2.3.1 Transformation of (+)-12α -Usararotenoid-A (6) to its Oxime



Reaction process was similar to that of **3.2.2.1**. However, in this case, a mixture of diastereoisomers with the same polarity was afforded as a yellow oil (21 mg, 99%). This was evident from the proton nuclear magnetic resonance.

3.2.4 9-Hydroxyhomoisoflavanone (8) Analogue

3.2.4.1 Hydrogenolysis of 9-Hydroxyhomoisoflavanone (8)



Reaction process was similar to **3.2.1.2**. In this case, 79 mg of compound **8** was utilized; the hydrogenolysis was carried out for 48 hours. The reaction progress was monitored by TLC using hexane: ethylacetate (8:2 V/V) as the solvent system. The TLC showed three spots which were separated using preparative TLC and detected using a UV lamp at 254 nm. This afforded a yellow powder (46 mg, 58%) of compound **64** which was characterized by proton and carbon nuclear magnetic resonance. The other two spots were the overlapping diastereomers.

^{1}H-NMR ($CDCl_3$, 200 MHz): δ 12.27 (1H, *s*, OH–5), δ 7.21 - 7.30 (5H *m*, H 2'- 6'), δ 6.47 (brs, OH-7), δ 6.06 (1H, *s*, H-8), δ 4.28 (1H, *dd*, *J* = 4.0, 11.6 Hz, H-2), δ 4.10 (1H, *dd*, *J* = 2.8, 11.6 Hz, H-2), δ 3.93 (3H, *s*, OCH_3), δ 3.27 (1H, *dd*, *J* = 3.4, 12.6 Hz, H-9), δ 2.90 (1H, *dddd*, *J* = 3.4, 4.0, 7.0, 10.4, H-3), δ 2.76 (1H, *dd*, *J* = 10.0, 12.6 Hz, H-9).

^{13}C-NMR (50 MHz, $CDCl_3$): δ 198.8 (C-4), δ 158.8 (C-5), δ 157.5 (C-7), δ 154.9 (C-8a), δ 138.0 (C-6), δ 129.3 (C-3' and 5'), δ 128.9 (C-2' and C-6'), δ 128.5 (C-1'), δ 127.0 (C-4'), δ 102.7 (C-4a), δ 94.3 (C-8), δ 69.2 (C-2), δ 46.9 (C-3), δ 32.9 (C-9).

3.3 Bioassay

The larvicidal activity of abyssinone V-4'-methyl ether **(5)**, (+)-12α-*epi*milletosin **(7)**, (+)-usararotenoid-A **(6)** and their derivatives were investigated to establish if the various modifications carried out had any effect on their activities.

3.3.1 Larvicidal Test on the Larvae of *Aedes aegypti* Mosquito Colony

Mosquito eggs (*Aedes aegypti*) were obtained from the Zoology Department, University of Nairobi. The eggs were kept in a tray with clean water for hatching. The larvae were fed with yeast.

The dry test compounds including the standard (rotenone) were dissolved in dimethyl sulfoxide in different quantities to obtain 20 µg/ mL of the stock solution. Serial dilution was then carried out to prepare the other concentrations (0.75, 1.25, 2.5, 5, 10 µg/ mL) from the stock solutions which were then added to the respective narrow mouthed plastic cups (25 mL capacity) containing 20 mL of a 0.8 g/ L sodium chloride solution in water and left to stand overnight for stabilization. Twenty freshly molted second, late third and early fourth instar larvae (at different times) were then introduced into the plastic cups by means of a dropper. Control larvae received DMSO of the corresponding volumes. The cups were then covered with muslin. The treatments were replicated three times.

The test containers were held at 25 – 28 °C under a photoperiod of 12 hours light followed by 12 hours dark. The mortality rate was recorded at 24 hours interval and the LC_{50} values calculated using probit analysis for quantal data (Bosire, 2010).

3.3.2 Larvicidal Test on the Larvae of *Anopheles gambiae* Mosquito Colony

A stock solution of 5000 ppm was prepared by dissolving 5 mg of compounds **6** and **9** in 50 µL DMSO and the solution made to 1 ml with distilled water. Different concentrations (20, 10, 5 and 1 ppm) were prepared by serial dilution and the volume made up to 100 ml. Freshly molted late 3rd and early 4th instar larvae (20) of *Anopheles gambiae* were tested with control

running simultaneously. During the experiment, the larvae were fed on Tetramin® fish food (Terta GmbH, Germany) at about 1 mg per beaker every 24 h. The experiment room was kept at a temperature of 30±2°C and a photo period of 12 hours of light and 12 hours of darkness.

3.4 Antioxidant Assay

3.4.1 Preliminary Radical Scavenging Test

Solutions in dichloromethane of the compounds to be tested: abyssinone V-4'-methyl ether (5) and its generated analogues were deposited on the plate prior to development. TLC development was then carried out appropriately in a TLC tank using hexane: ethylacetate (7:3 V/V) as the solvent system. The TLC plate was then dried in open air and the compounds detected using a UV lamp at 254 nm. The dried plate was then sprayed with a 200 µg/ mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) that was prepared by dissolving 20 mg of DPPH in 100 mL of methanol. Lack of or very slight colour change indicated absence of or weak radical scavenging properties.

3.4.2 Estimation of Radical Scavenging Effects on DPPH

Stock solutions of 320 µg/ mL of quercetin (standard) and the structurally modified compounds respectively were prepared by dissolving 4 mg in 12 mL of anhydrous methanol. Serial dilution was then carried out by adding 2 mL methanol and 2 mL of DPPH (200 µg/ mL) to each of the stock solutions. This was done so as to have concentrations of 160, 80, 40, 20, 10, 5, 2.5, 1.25 µg/ mL. The solutions were then mixed to acquire uniformity and allowed to stand for 30 minutes.

The radical scavenging activities were then measured as the decrease in absorbance of DPPH radical expressed as a percentage of the control solution; consisting of 2 mL of methanol AR and 200 µg/mL DPPH solution at 517 nm. In all cases the mean values were used from triplicate experiments. EC_{50} values were calculated using Finney's probit analysis for quantal data [Induli, 2009].

CHAPTER FOUR

4.0 Results and Discussion

4.1 General

Today. the environmental safety of an insecticide is considered to be of paramount importance. An insecticide does not have to cause high mortality on target organisms in order to be acceptable but it should prevent the breeding. Phytochemicals may serve as suitable alternatives to synthetic insecticides in future as they are relatively safe, inexpensive, and are readily available.

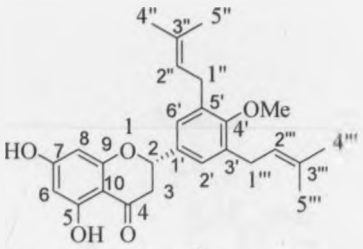
This study investigated the potential use of natural flavonoids and their analogues as larvicides. Six compounds namely: abyssinone V-4'-methyl ether oxime **(59)**, tetrahydro abyssinone V-4'-methyl ether **(60)**, 7-acetyl-abyssinone V-4'-methyl ether **(61)**, 5,7-diacetyl-abyssinone V-4'-methyl ether **(62)** and (+)-12α-dehydromilletosin oxime **(63)** were prepared from abyssinone V-4'-methyl ether **(5)** and (+)-12α-*epi*milletosin **(7)** respectively. The larvicidal activity of these compounds was assayed against *Aedes aegypti* and *Anopheles gambiae*. In addition to this, 9-deoxyhomoisoflavanone **(64)** was generated from 9-hydroxyhomoisoflavanone **(8)**; the identification of these products was based on NMR analyses whose spectra are in the appendix. The preparation, identification and biological evaluation of these compounds is discussed below.

4.2 Abyssinone V-4'-Methyl Ether Analogues

All the analogues of abyssinone V-4'-methyl ether prepared were identified by comparison of their NMR spectra against abyssinone V-4'-methyl ether, whose chemical shifts are listed in Table 4.1.

Table 4.1: ^{1}H-NMR (200 MHz, $CDCl_3$) and ^{13}C-NMR (50 MHz, $CDCl_3$) data of abyssinone V-4'-methyl ether (5)

Position	^{1}H (*J* in Hz)	^{13}C
2	5.55 *brd* (12.2)	79.3
3	2.83 *brd* (17.0)	43.0
	3.24 *dd* (12.8, 17.0)	
4		196.3
5		164.2
6	6.04 *s*	96.6
7		165.3
8	6.04 *s*	95.6
9		163.3
10		103.0
1'		133.7
2'	7.31 *s*	125.9
3'		135.4
4'		156.4
5'		135.4
6'	7.31 *s*	125.9
1''	3.48 *d* (7.2)	28.4
2''	5.41 *t* (6.8)	122.5
3''		133.0
4''	1.81 *s*	25.7
5''	1.81 *s*	17.9
1'''	3.48 *d* (7.2)	28.4
2'''	5.41 *t* (6.8)	122.5
3'''		133.0
4'''	1.81 *s*	25.7
5'''	1.81 *s*	17.9
4' – Ome	3.83 *s*	60.9
5 – OH	12.25 *s*	



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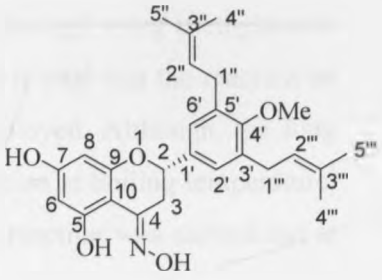
4.2.1 Transformation of Abyssinone V-4'-Methyl Ether (5) to its Oxime (59)

Oxime **59** was isolated as a green oil (103 mg) by reacting the flavanone **5** with hydroxylamine hydrochloride in the presence of pyridine. Evidence that oxime formation had occurred was apparent on the **^{1}H-NMR** and **^{13}C-NMR** spectra, whose chemical shifts are listed in Table 4.2.

Table 4.2: ^{1}H-NMR (200 MHz, $CDCl_3$) and ^{13}C-NMR (50 MHz, $CDCl_3$) data of abyssinone V-4'-methyl ether Oxime (59)

Position	^{1}H (*J* in Hz)	^{13}C
2	5.03 *brd* (11.2)	76.8
3	2.80 *dd* (12.2, 17.6)	30.2
	3.59 *brd* (16.6)	
4		171.2
5		160.5
6	6.17 *s*	97.6
7		159.8
8	6.14 *s*	96.4
9		158.8
10		98.1
1'		133.0
2'	7.18 *s*	126.3
3'		135.4
4'		156.6
5'		135.4
6'	7.18 *s*	126.3
1''	3.45 *d* (6.8)	28.8
2''	5.36 *t* (6.0)	123.1
3''		133.0
4''	1.79 *s*	26.1
5''	1.79 *s*	18.2
1'''	3.45 *d* (6.8)	28.8
2'''	5.36 *t* (6.0)	123.1
3'''	-	133.0
4'''	1.79 *s*	26.1
5'''	1.79 *s*	18.2
4' – Ome	3.80 s	61.3
5 – OH		



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^{1}H-NMR showed no chelation between the OH group at C-5 and N-OH group at C-4 in comparison to compound **5** that showed chelation between OH group at C-5 and C=O group at C-4 with a chemical shift of 12.25 ppm that appeared as a singlet. **^{13}C-NMR** showed a peak at 171 ppm compared to that of the carbonyl (196.3 ppm) in the starting material. The compound was thus identified as abyssinone V-4'-methyl ether oxime **(59)**.

Various challenges were experienced while attempting to isolate the oxime. It was observed that the oxime was sensitive to the presence of water. It readily underwent hydrolysis. This was evident when liquid-liquid extraction was employed using distilled water and diethyl ether in an attempt to separate the water-soluble pyridine from the diethyl ether soluble oxime. Secondly, chromatographic purification of the oxime was hampered by its hydrolysis on Sephadex LH 20 and silica gel. An attempt to deactivate the silica gel using triethylamine (0.5% V/V in the solvent systems used) was unsuccessful making it vital that the reaction be carried out to completion and hence the long reaction times employed. Although, the long reaction time would have been countered by carrying out the reaction at boiling temperature, this caused the decomposition of the product and as a result the reaction was carried out at room temperature.

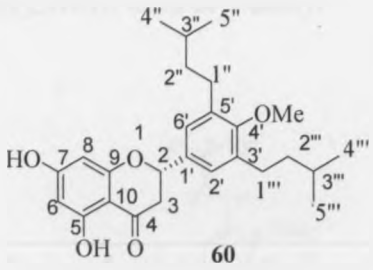
4.2.2 Hydrogenation of Abyssinone V-4'-Methyl Ether (5)

The hydrogenated flavanone product **60** was isolated as a yellow oil (47 mg) by reacting compound **5** with hydrogen gas using 5% palladium on carbon as a catalyst. Evidence that hydrogenation had occurred was apparent on the **^{1}H-NMR** that had δ 2.72 [4H] *t*, *J* = 5.8 Hz, H-1", H-1"', δ 1.76 [2H], *m*, H-3", H-3"', δ 1.58 [4H], *q*, *J* = 7.8, 10.4 Hz, H-2", H-2"', δ 1.04 [12H], *d*, *J* = 6.4 Hz, H-4", H-5", H-4"' and H-5"'. This is in comparison to the starting material ^{1}H-NMR spectra which had δ 3.48 [4H] *d*, *J* = 7.2 Hz, H-1", H-1"', δ 5.41 [2H], *t*, *J* = 6.8, Hz, H-2", H-2"', δ 1.81 [12H], *s*, H-4", H-5", H-4"' and H-5"'. The **^{13}C-NMR** showed chemical shifts of 40.4 (C-1" and C-1"'), 28.6 (C-3" and C-3"'), 28.2 (C-2" and C-2"'), 22.9 (C-4" and C-4"') and 22.9 (C-5" and C-5"'), in comparison to the starting material ^{13}C-NMR spectra which had chemicals shifts of 28.4 (C-1" and C-1"'), 133.0 (C-3" and C-3"'), 122.5 (C-2" and C-2"'), 25.7 (C-4" and C-4"') and 17.9 (C-5" and C-5"'). The compound was thus

identified as tetrahydro-abyssinone V-4'-methyl ether **(60)**. The NMR chemical shifts are listed in Table 4.3.

Table 4.3: ^{1}H-NMR (200 MHz, $CDCl_3$) and ^{13}C-NMR (50 MHz, $CDCl_3$) data of tetrahydro-abyssinone V-4'-methyl ether (60)

Position	^{1}H (*J* in Hz)	^{13}C
2	5.38 *brd* (13.2)	79.7
3	2.83 *brd* (17.6)	43.6
	3.17 *dd* (13.2, 15.8)	
4		196.4
5		164.6
6	6.08 *s*	97.0
7		165.7
8	6.08 *s*	95.9
9		163.6
10		103.2
1'		133.9
2'	7.17 *s*	125.9
3'		137.0
4'		157.1
5'		137.0
6'	7.17 *s*	125.9
1''	2.72 *t* (5.8)	40.4
2"	1.58 *ddd* (7.8,10.4,17.4)	28.2
3"	1.76 *m*	28.6
4"	1.04 *d* (6.4)	22.9
5"	1.04 *d* (6.4)	22.9
1'''	2.72 *t* (5.8)	40.4
2'''	1.58 *ddd* (7.8,10.4,17.4)	28.2
3'''	1.76 *m*	28.6
4'''	1.04 *d* (6.4)	22.9
5'''	1.04 *d* (6.4)	22.9
4' – Ome	3.83 *s*	61.5
5 – OH	12.13 *s*	



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Removal of 5% palladium on carbon from the product was the challenge during the purification of the hydrogenated product due to its fineness and inability to be held back upon filtering using filter paper or cotton wool. This limitation was overcome by use of glass wool. It can also be overcome by the use of a sintered filter that is embedded with clay to hold back the fine 5% palladium on carbon. However, if one intended to recycle it, it may be impossible since the 5% palladium on carbon will be contaminated with the clay.

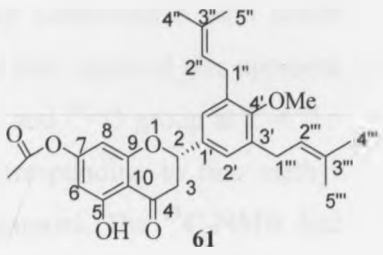
4.2.3 Acetylation of Abyssinone V-4'-Methyl Ether (5)

4.2.3.1 7-Acetyl abyssinone V-4'-methyl ether (61)

Flavanone **61** was isolated as yellow crystals (9 mg) as a secondary product during the work up procedure of the reaction of flavanone **5** with acetic anhydride in the presence of pyridine. The **^{1}H-NMR** and **^{13}C-NMR** chemical shifts are listed in Table 4.4.

Table 4.4: ^{1}H-NMR (200 MHz, $CDCl_3$) and ^{13}C-NMR (50 MHz, $CDCl_3$) data of 7-acetyl abyssinone V-4'-methyl ether (61)

Position	^{1}H (*J* in Hz)	^{13}C
2	5.43 *brd* (13.8)	79.7
3	2.88 *brd* (17.0)	43.7
	3.20 *dd* (13.2, 17.2)	
4		197.7
5		163.6
6	6.36 *brs*	103.5
7		158.6
8	6.35 *brs*	102.1
9		162.7
10		106.5
1'		133.6
2'	7.14 *s*	126.1
3'		135.7
4'		157.0
5'		135.7
6'	7.14 *s*	126.1
1''	3.45 *d* (6.8)	30.2
2'', 2'''	5.32 *t* (5.2)	122.8
3'', 3'''		133.3
4''	1.81 *s*	20.6
5''	1.81 *s*	18.2
1'''	3.45 *d* (6.8)	30.2
4'''	1.81 *s*	20.6
5'''	1.81 *s*	18.2
4' – OMe	3.80 *s*	61.2
5 – OH	11.93 *s*	
7 - COMe	2.34 *s*	26.7
7 - COMe		168.6



61

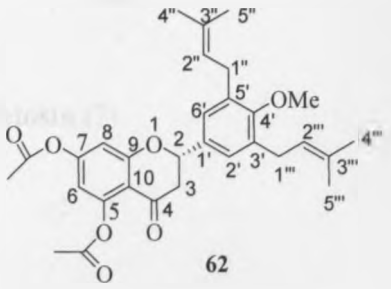
Evidence that mono acetylation had occurred was apparent on the **^{1}H-NMR** that showed chelation between OH group at C-5 and C=O group at C-4 with a chemical shift of 11.93 ppm that appeared as a singlet. Moreover, an additional singlet with chemical shift of 2.34 corresponding to a methyl group was observed (3H, s, $COCH_3$), which was not present in the starting material. The **^{13}C-NMR** had chemical shifts of 168.6 (COMe), 26.7 ($COCH_3$) confirming the presence of an acetate group. The compound was thus identified as 7-acetyl abyssinone V-4'-methyl ether **(61)**.

4.2.3.2 5,7-Diacetyl-abyssinone V-4'-methyl (62)

Flavanone **62** was isolated as a yellow oil (89 mg) by reacting compound **5** with acetic anhydride in the presence of pyridine. Evidence that diacetylation had occurred was apparent on **^{1}H-NMR** that showed no chelation between OH group at C-5 and C=O group at C-4. To add on, two singlets with chemical shifts of 2.34 and 2.45 corresponding to two methyl groups were observed which were not present in the starting material. The **^{13}C-NMR** had chemical shifts of 169.6 (COMe), δ 168.4 ($COCH_3$) and δ 21.5 ($COCH_3$) confirming the presence of two acetate groups. The compound was thus identified as 5,7-diacetyl abyssinone V-4'-methyl ether **(62)**. The NMR chemical shifts are listed in Table 4.5.

Table 4.5: ^{1}H-NMR (200 MHz, $CDCl_3$) and ^{13}C-NMR (50 MHz, $CDCl_3$) data of 5,7-diacetyl-abyssinone V-4'-methyl ether (62)

Position	^{1}H (*J* in Hz)	^{13}C
2	5.46 *brd* (13.8)	80.0
3	2.79 *brd* (16.6)	30.2
	3.14 *dd* (13.8, 16.6)	
4		189.9
5		151.5
6	6.85 *s*	110.7
7		156.2
8	6.59 *s*	109.5
9		163.8
10		112.1
1'		133.7
2'	7.14 *s*	126.1
3'		135.7
4'		157.0
5'		135.7
6'	7.14 *s*	126.1
1''	3.45 *d* (7.2)	28.6
2'', 2'''	5.35 *t* (6.4)	122.8
3'', 3'''		133.3
4''	1.81 *s*	26.1
5''	1.81 *s*	18.2
1'''	3.45 *d* (7.2)	28.6
4'''	1.81 *s*	26.1
5'''	1.81 *s*	18.2
4' – OMe	3.81 *s*	61.2
5 - COMe	2.36 *s*	21.5
7 – COMe	2.45 *s*	21.5
5 – COMe		168.4
7 - COMe		169.6



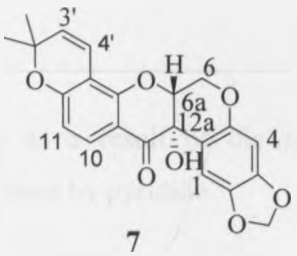
62

4.3 (+)-12α-*epi*milletosin Analogue

The generated analogue of (+)-12α-*epi*milletosin **(63)** was compared and contrasted with NMR spectra of (+)-12α-*epi*milletosin **(7)** shown in Table 4.6.

Table 4.6: ^{1}H-NMR (200 MHz, $CDCl_3$) data of (+)-12α-*epi*milletosin (7)

Position	^{1}H (*J* in Hz)
1	7.64 *s*
4	6.41 *s*
6α	4.42 *dd* (3.4, 7.0)
6β	4.39 *dd* (3.4, 5.0)
6a	4.63 *dd* (1.6, 3.6)
10	6.55 *d* (3.0)
11	6.65 *d* (3.4)
12a-OH	2.99 *s*
-Me	1.47 s 1.56 s
3'	5.68 *d* (3.4)
4'	7.75 *d* (2.8)
OCH_2O	5.91 *s*



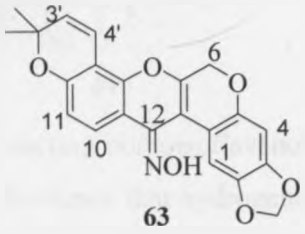
7

4.3.1 Dehydromilletosin Oxime (63)

The oxime **63** was isolated as a yellow oil (10 mg) by reacting rotenoid **7** with hydroxylamine hydrochloride in the presence of pyridine as a base. Evidence that oxime formation had occurred was apparent on **^{1}H-NMR** which showed that the OH group at B / C ring junction had been lost through dehydration. This was indicated by absence of the 12a-OH singlet at 3 Hz and loss of chirality at H–6a indicated by the singlet generated at 4.7 Hz corresponding to H-6α and H-6β. This was in comparison to the starting material that had a singlet at δ 2.75 and three doublet of a doublets corresponding to H-6a, H-6α and H-6β. The **^{13}C-NMR** was not that pronounced due to insufficient amount of product formed, however, it didn't show the presence of the deshielded carbonyl. This is in comparison to the starting material that has a deshielded carbonyl at δ 187.4. The NMR chemical shifts are listed in Table 4.7.

Table 4.7: ^{1}H-NMR (200 MHz, $CDCl_3$) data of dehydromilletosin oxime (63)

Position	^{1}H (*J* in Hz)
1	7.76 *s*
4	6.46 *s*
6α	4.70 *s*
6β	4.70 *s*
10	6.61 *d* (2.6 Hz)
11	6.65 *d* (3.4 Hz)
-Me	1.45 s 1.48 s
3'	5.61 *d* (9.6 Hz)
4'	8.66 *d* (9.2 Hz)
OCH_2O	5.86 *s*



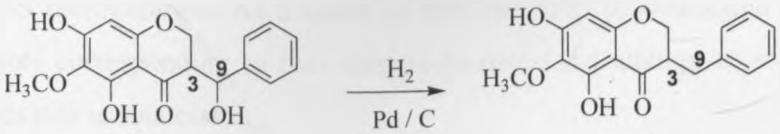
63

The dehydration at the B/C ring junction was as a result of the presence of excess hydrochloric acid in the reaction mixture that was freed by pyridine.

4.4 (+)-12α-Usararotenoid-A Analogue

A mixture of diastereoisomers was isolated as a yellow oil (21 mg) by reacting compound **6** with hydroxylamine hydrochloride in the presence of pyridine as a base. Evidence that a product had formed was apparent from the **^{13}C-NMR** which showed the absence of the carbonyl peak; however the C-NMR was not pronounced due to an insolubility problem. Moreover, the **^{1}H-NMR** spectra had additional peaks of which the multiplicity showed presence of more protons not corresponding to the parent compound; this was due to compound having two chiral centres and as a result of which it forms a mixture of two diastereoisomers which can only be determined if separation was possible. Separation can be carried out using a chiral column that separates such on the basis of chirality.

4.5 Hydrogenolysis of 9-Hydroxyhomoisoflavanone (8)

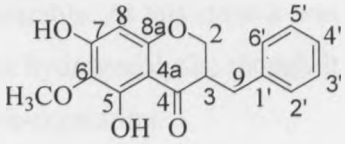


8 64

Compound **63** was isolated as yellow powder (46 mg) by reacting homoisoflavanone **8** with hydrogen gas using 5% palladium on carbon as a catalyst. Evidence that hydrogenolysis had occurred was noted on the **^{1}H-NMR** that showed peaks at δ 2.76 *dd* and δ 3.27 *dd* corresponding to protons at position 9. To add on this, the proton at position 3 showed evidence that it was being split by four protons at δ 2.90 *dddd* in comparison to the starting material. Evidence was also available from **^{13}C-NMR** that showed a shielded peak of δ 32.9 that corresponded to C-9. The NMR chemical shifts are listed in Table 4.8.

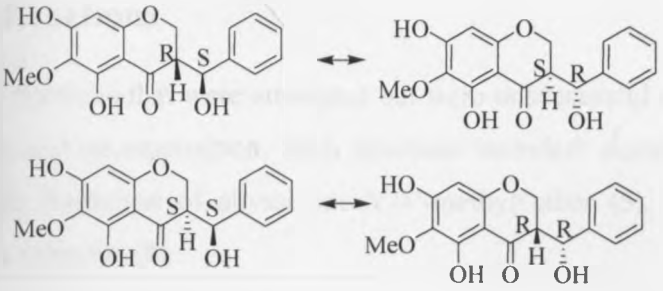
Table 4.8: ^{1}H-NMR (200 MHz, $CDCl_3$) and ^{13}C-NMR (50 MHz, $CDCl_3$) data of the 9-deoxyhomoisoflavanone (64) product

Position	^{1}H-NMR (*J* in Hz)	^{13}C-NMR
2	4.28 *dd* (4.0, 11.6) 4.10 *dd* (2.8, 11.6)	69.2
3	2.90 *dddd* (3.4, 4.0, 7.0, 10.4)	46.9
4		198.8
4a		102.7
5 - OH	12.27 *s*	158.8
6		138.0
6 - OCH_3	3.93 *s*	61.2
7 - OH	6.47 *brs*	157.5
8	6.06 *s*	94.3
8a		154.9
9	2.76 *dd* (10.0, 12.6) 3.27 *dd* (3.4, 12.6)	32.9
1'		128.5
2'	7.21 – 7.30 *m*	128.9
3'	7.21 – 7.30 *m*	129.3
4'	7.21 – 7.30 *m*	127.0
5'	7.21 – 7.30 *m*	129.3
6'	7.21 – 7.30 *m*	128.9



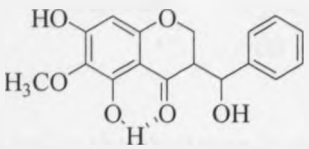
64

Compound **8** has two stereocentres giving room to four possible stereoisomers, of which the enantiomers with S-R and R-S configuration have the same polarity; the same case applies for R-R and S-S configuration. As a result of this, the TLC of compound **8** showed two overlapping spots corresponding to two diastereoisomers. An attempt to separate the two diastereoisomers was unsuccessful.



The diastereomeric mixture was subjected to hydrogenolysis to investigate if one of the diastereoisomers is more reactive to hydrogenolysis than the other. When the reaction was monitored by TLC, it became apparent that the reaction did not go to completion. A keen look at the TLC of the reaction mixture showed that the intensity of the less polar diastereoisomer had reduced while that of the slightly polar diastereoisomer persisted. When the reaction was stopped and PTLC carried out, the 9-deoxyhomoisoflavanone was successfully purified whereas the diastereoisomers were still inseparable. At this stage it was certain that one of the diastereoisomers predominantly underwent hydrogenolysis; though it was not possible to point out conclusively which one due to the non-separation.

The two diastereomers may arise from the enhanced epimerization due to the enolizable H-3. This was further facilitated by the H-3 being α to the carbonyl group at C-4 that is chelated and the hydrogen bonding further increases the exchanging ability of that proton. The acidity of palladium on carbon may further enhance the epimerization.



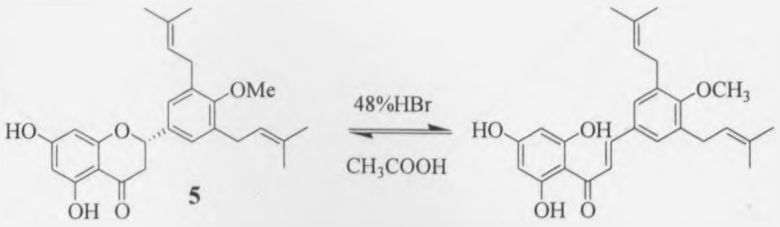
The mechanism of this reaction involves both hydrogenolysis and dehydration-hydrogenation, whereby hydrogenolysis constitutes the direct cleavage of the OH group while dehydration-hydrogenation involves the initial dehydration to form an alkene, followed by hydrogenation to an alkane.

4.6 Attempted Reactions

There were various reactions that were attempted but were unsuccessful as a result of use of non potent reagents and decomposition. Such reactions included: demethylation, carbonyl reduction, hydrazone formation of abyssinone V-4'-methyl ether **(5)**, hydrogenation and benzylic cleavage of rotenone **(9)**.

4.6.1 Demethylation of Abyssinone V-4'-methyl ether (5)

An attempt to demethylate flavanone **5** using 48% hydrobromic acid was unsuccessful. While these standard methods of dealkylation have been effective for a variety of simple ethers, the rate of dealkylation of more complicated ethers is often sufficiently slow that rather harsh reaction conditions or long reaction times are necessary to effect complete dealkylation, resulting in extensive decomposition and hence lower yield of the desired dealkylated product. In this case there was no dealkylation. Severe decomposition as a result of the high temperature employed (100 °C) was evident as indicated by the colour change in the reaction mixture (from slightly yellow to orange then red). The process also resulted in opening of ring C; a product that wasn't stable at room temperature; and was only evident using TLC.



The general structure of this molecule belies the challenge in executing a strategy that installs and maintains the configuration at the C-2 position. This stereocenter is sensitive since basic

conditions promote reversible ring opening to achiral 2'-hydroxy chalcones. Flavanones containing alkoxy- or hydroxy- substituents in the C4' position are particularly susceptible to racemization due to stabilized benzylic cation formation. As a result of this, abyssinone V (**15**) could not be accessed and hence the modifications were done on abyssinone V-4'-methyl ether (**5**).

4.6.2 Carbonyl reduction of Abyssinone V-4'-methyl ether (5)

An attempt to reduce flavanone **5** carbonyl with sodium borohydride at room temperature and at 80°C under reflux conditions was unsuccessful. Reaction progress monitored using TLC showed that no product had formed. This was as a result of the sodium borohydride not being potent enough to reduce the carbonyl group.

4.6.3 Hydrazone Formation of Abyssinone V-4'-methyl ether (5)

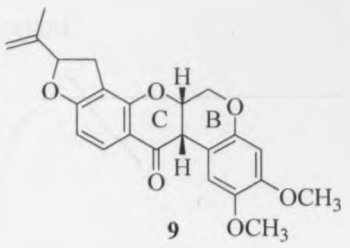
In a bid to form flavanone **5** hydrazone, flavanone **5** was subjected to various hydrazoning reagents including hydrazinium chloride, hydrazine hydrate (98%), and phenylhydrazine. None of these reactions was successful possibly as a result of the reaction being carried out in a non-inert atmosphere thus resulting in an increased rate of hydrolysis compared to that of oxime **58**.

4.6.4 Hydrogenation of Rotenone (9)

Hydrogenation of rotenoid **9** using palladium on carbon as catalyst was unsuccessful as a result of the presence of an impurity in the rotenone that could have been suppressing the reaction from taking place. Attempted purification of the rotenone using preparative TLC was unsuccessful as there was overlapping of products.

4.6.5 Transformation of Rotenone (9) to its Oxime

In an effort to form rotenone oxime, rotenone **(9)** was subjected to hydroxylamine hydrochloride in the presence of pyridine. This was not successful as result of the presence of an impurity in the rotenone that hindered the reaction from going to completion. Purification using preparative TLC was done to separate the products, though overlapping occurred. Moreover, rotenoid **9** has a *cis* configuration at the B/C ring junction that results in folding of the molecule which in turn results in hindering access of the hydroxylamine to the carbonyl.



9

4.6.6 Benzylic Cleavage of Rotenone (9)

In a bid to cleave rotenoid **9** at ring C various reaction conditions were employed without success. First, there was the use of zinc metal in glacial acetic acid, then in glacial acetic acid and distilled water (3:1) and finally in glacial acetic acid and 2.5 g/10 mL copper sulphate in distilled water (3:1). The copper sulphate was employed so as to act as a catalyst to free the zinc which could have oxidized to zinc oxide so that a reaction could take place.

4.7 Bioassay

The present study sought to determine whether structural modification of abyssinone V-4'-methyl ether **(5)**, (+)-12α-usararotenoid-A **(6)** and (+)-12α-*epi*milletosin **(7)** could lead to improvement of their larvicidal activity and be used for mosquito control. It was observed that abyssinone V-4'-methyl ether oxime **(59)**, tetrahydro-abyssinone V-4'-methyl ether **(60)**, showed improved larvicidal activities against second instar larvae of *Aedes aegypti*. Further analysis showed that (+)-12α-dehydromilletosin oxime **(63)** had good larvicidal activities to late third and early fourth instar larvae. These compounds independently produced morbidity and mortality effects in *Aedes aegypti*.

4.7.1 Larvicidal Activity

4.7.1.1 *Aedes aegypti*

The larvicidal activities of all the six compounds synthesized: abyssinone V-4'-methyl ether oxime **(59)**, tetrahydro-abyssinone V-4'-methyl ether **(60)**, 7-acetyl-abyssinone V-4'-methyl ether **(61)** and 5,7-diacetyl-abyssinone V-4'-methyl ether **(62)**, dehydromilletosin oxime **(63)** and the starting materials: abyssinone V-4'-methyl ether **(5)**, and (+)-12α-*epi*milletosin **(7)** were tested against the second instar larvae of *Aedes aegypti*.

Rotenone **(9)**, compounds **5**, **59** and **60** showed dose dependent activity of 0.46, 4.08, 1.68 and 13.4 µg/ mL respectively at 24 hours, Rotenone **(9)** and oxime **59** showed 100% mortality whereas compounds **5** and **60** had LC_{50} values of 2.78 and 7.07 µg/ mL respectively at 48 hours as shown in Table 4.9.

Table 4.9: Larvicidal test results for rotenone and compounds 5, 59 and 60 against second instar larvae.

Compound	LC_{50} in µg/ mL ± SD At 24 hours	LC_{50} in µg/ mL ± SD At 48 hours
Standard (rotenone)	0.46 ± 0.09	100% mortality*
Abyssinone V-4'-methyl ether **(5)**	4.08 ± 0.92	2.78 ± 1.20
Abyssinone V-4'-methyl ether oxime **(59)**	1.68 ± 0.13	100% Mortality*
Tetrahydro-abyssinone V-4'-methyl ether **(60)**	13.4 ± 0.7	7.07 ± 0
* Lowest concentration was 1.25 µg/ mL; more than 50% of the larvae were dead.		

The mortality was observed to increase with increasing exposure as well as the concentrations of the compounds. 100% mortality was observed as a result of employing the abyssinone V-4'-methyl ether oxime derivative at 48 hours. The moribund and dead larvae in three replicates were combined and expressed as a percentage of larval mortality for each concentration. Moribund larvae were those incapable of rising to the surface (within reasonable period of time) or showing the characteristic diving reaction when the water was disturbed. Dead larvae were identified when they failed to move after probing with a needle in the siphon or cervical region.

Acetylated products **61** and **62** showed no activity against second instar larvae of *Aedes aegypti* compared to flavanone **5** due to the structure of this compound containing one and two acetate groups respectively. After four days, over 50% of the larvae had changed into pupae which matured into adult mosquitoes.

In an attempt to access compounds with enhanced larvicidal activity, various functional groups were targeted for modification. From the activity of tetrahydro-abyssinone V-4'-methyl ether **(60)**, it was decided to change the carbonyl group to oxime and investigate the activity. Abyssinone V-4'-methyl ether oxime **(59)** showed strong activity against second instar larvae of *Aedes aegypti* with an LC_{50} of 1.68 µg/ mL. The results show convincingly that the oxime group at C-4 of flavanone plays an important role in larvicidal activity. This

substance is likely to be useful as a promising compound for the development of a novel class of larvicidal agents.

With these results and curiosity of why rotenoids are the best larvicidal compounds, larvicidal activity of the well known larvicidal rotenoids was carried out to yield the following results: rotenone **(9)**, degeulin **(15)**, (+)-12α-*epi*milletosin **(7)** and (+)-12α-usararotenoid-A **(6)** showed larvicidal activity against the late third and early fourth instar larvae of *Aedes aegypti* with LC_{50} values of 1.47, 10.4, 38.4 and 13.4 µg/ mL respectively at 24 hours. Rotenone **(9)** showed 100% mortality at 48 hours, whereas the other compounds showed LC_{50} values of 2.51, 29.4 and 4.83 µg/ mL respectively. At 72 hours degeulin **(15)**, (+)-12α-*epi*milletosin **(7)** and (+)-12α-usararotenoid-A **(6)** showed LC_{50} values of 1.34, 25.4 and 2.24 µg/ mL respectively. At 96 hours the larvae that were not dead had changed into pupae which matured into adult mosquitoes.

These results proved interesting as rotenoids are known to be highly larvicidal. As a result, the assay was repeated at the top concentration of 120 µg/ mL for oximes **59** and **63**. Rotenone **(9)**, degeulin **(15)**, (+)-12α-*epi*milletosin **(7)**, (+)-12α-usararotenoid-A **(6)**, oximes **59** and **63** showed larvicidal activity against the late third and early fourth instar larvae of *Aedes aegypti* with LC_{50} values of 1.31, 10.4, 38.3, 13.9, 18.5, and 20.8 µg/ mL respectively at 24 hours. Rotenone **(9)** showed 100% mortality at 48 hours, while the other compounds showed LC_{50} values of 2.42, 27.6, 5.29, 9.63 and 10.8 µg/ mL at 48 hours and 1.43, 19.1, 2.80, 4.52 and 7.99 µg/ mL respectively at 72 hours. At 96 hours the larvae that were not dead had changed into pupae which matured into adult mosquitoes.

Total mortality (100%) for all the compounds tested was observed between 40 and 120 µg/ mL. Rotenone **(9)** was the most active at 20 µg/ mL in 24 hours with 100% mortality, whereas dehydromilletosin oxime **(63)** showed 95% mortality at 20 µg/ mL in 48 hours. At 96 hours the larvae that were not dead had changed into pupae which matured into adult mosquitoes. The LC_{50} values of the compounds tested are shown in the Table 4.10.

Table 4.10: Larvicidal activity of compounds 6, 7, 15, 59 and 63 against late third and early fourth instar larvae.

Compound	LC_{50} in µg/ mL ± SD At 24 hours	LC_{50} in µg/ mL ± SD At 48 hours
Standard (Rotenone)	1.31 ± 0.09	100% mortality*
Deguelin **(15)**	10.4 ± 0.7	2.42 ± 0.16
Abyssinone V-4'-methyl ether oxime **(59)**	18.5 ± 1.3	9.64 ± 0.62
Usararotenoid A **(6)**	13.9 ± 2.3	5.29 ± 0.30
(+)-12α-*epi*milletosin **(7)**	38.4 ± 2.5	27.6 ± 1.1
Dehydromilletosin oxime **(63)**	20.8 ± 0.7	10.8 ± 0.7
* Lowest concentration was 1.25 µg/ mL; 100% of the larvae were dead.		

4.7.1.2 *Anopheles gambiae*

The larvicidal activities of rotenone **(9)** and (+)-12α-usararotenoid-A **(6)** were tested against the late third and early fourth instar larvae of *Anopheles gambiae*. Rotenone **(9)** showed 100% mortality at 24 hours, whereas (+)-12α-usararotenoid-A **(6)** exhibited strong larvicidal activity with an LC_{50} of 10 µg/ mL at 24 hours and achieved 100% mortality at 72 hours.

4.8 Antioxidant Assay

Preliminary tests for radical scavenging activities (RSA) using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical as a spray reagent on TLC plates spotted with the generated analogues including flavanone **5** did not reveal any active compounds. Flavanones **5** and **60** showed only a slight change in colour in the DPPH **(16)** solution in methanol. The radical scavenging activities of compounds **5**, **59-62** were assessed and compared with that of the standard (quercetin) and detected using UV at a wavelength of 517 nm. The scavenging activity was measured as the decrease in absorbance of the DPPH radical expressed as a percentage of the absorbance of the control solution. All the compounds were inactive as shown in Table 4.11.

Table 4.11: Antioxidant activity EC_{50} values of compounds 5, 59 - 62 using quercetin as standard

Flavanones	EC_{50} Values (µg/ mL)
Quercetin	8
Abyssinone V-4'-methyl ether **(5)**	N/A*
Abyssinone V-4'-methyl ether oxime **(59)**	180
Tetrahydro-abyssinone V-4'-methyl ether **(60)**	135
7-Acetyl-abyssinone V-4'-methyl ether **(61)**	N/A*
5,7-diacetyl-abyssinone V-4'-methyl ether **(62)**	N/A*
*The highest concentration used was 320 µg/ mL	

Previous radical scavenging activity test results on some of the different sub-classes of flavonoids and phenolics clearly indicate the importance of the free phenolic group in the B-ring of the flavonoids for radical scavenging activity (Induli, 2009). Furthermore several investigations have determined correlations between flavonoid structural features and anti-oxidative and free radical scavenging activities. The hydroxyl group at carbon position three, which is shared by flavonoid **18** seems to play a part in the inhibition of lipid oxidation. Other characteristics include the double bond between carbon positions two and three, the carbonyl group in carbon position four, and the catechol functional groups on the aromatic ring B (Tseng, 2003).

In summary, a series of modified flavonoids were prepared using simple reactions and their larvicidal and antioxidant activities evaluated. It was found that abyssinone V-4'-methyl ether oxime **(59)** and dehydromilletosin oxime **(63)** possess high larvicidal activity against *Aedes aegypti* larvae compared to their respective parent compounds. In addition, tetrahydro-abyssinone V-4'-methyl ether **(60)** showed moderate larvicidal activity affecting the morbidity of the second instar larvae of *Aedes aegypti* and may thus serve as promising leads for the development of botanical insecticides. These results encourage the isolation of flavonoid analogues for improving larvicidal activity.

CHAPTER FIVE

5.1 Conclusion and Recommendations

5.1.1 Conclusion

In this study six analogues of natural products were prepared. These were: abyssinone V-4'-methyl ether oxime **(59)**, tetrahydro-abyssinone V-4'-methyl ether **(60)**, 7-acetyl-abyssinone V-4'-methyl ether **(61)**, 5,7-diacetyl-abyssinone V-4'-methyl ether **(62)**, dehydromilletosin oxime **(63)** and 9-deoxyhomoisoflavanone **(64)**. All these compounds were generated from their respective parent materials and their biological activities investigated except for compound **(64)**.

The reactions of abyssinone V-4'-methyl ether **(5)** with hydroxylamine hydrochloride and hydrogen resulted in the formation of their respective compounds **59** and **60** in high yields (99%) whereas that with acetic anhydride yielded **61** (5%) and **62** (45%). When the effect of temperature was investigated, it was evident that a temperature of 60°C gave the highest yields of compound **62** (100%).

The reaction of (+)-12α-*epi*milletosin **(7)** with hydroxylamine hydrochloride in the presence of pyridine gave 50% of dehydromilletosin **(59)** whereas the reaction of 9-hydroxyhomoisoflavanone **(8)** with hydrogen gas in the presence of 5% palladium on carbon yielded 53% of 9-deoxyhomoisoflavanone **(64)**. The reaction of (+)-12α -usararotenoid-A **(6)** with hydroxylamine hydrochloride in the presence of pyridine gave 99% of a mixture of diastereoisomers.

Oxime **59** showed strong larvicidal activity against the second instar larvae of *Aedes aegypti* and also at 40 μg/ mL against the late third and early fourth instar larvae of *Aedes aegypti*. Hydrogenation and acetylation of the flavanone **5** resulted in the reduction of larvicidal activities, though compound **60** showed moderate activity. Oxime **63** showed an improved

larvicidal activity compared to its parent compound. These compounds did not reveal any significant antioxidant activity.

5.1.2 Recommendations

1. Cytotoxicity of abyssinone V-4'-methyl ether oxime **(59)** be done to establish its efficacy and safety.
2. More modification reactions on isolated compounds with low larvicidal and antioxidant activities should be done to improve these activities.

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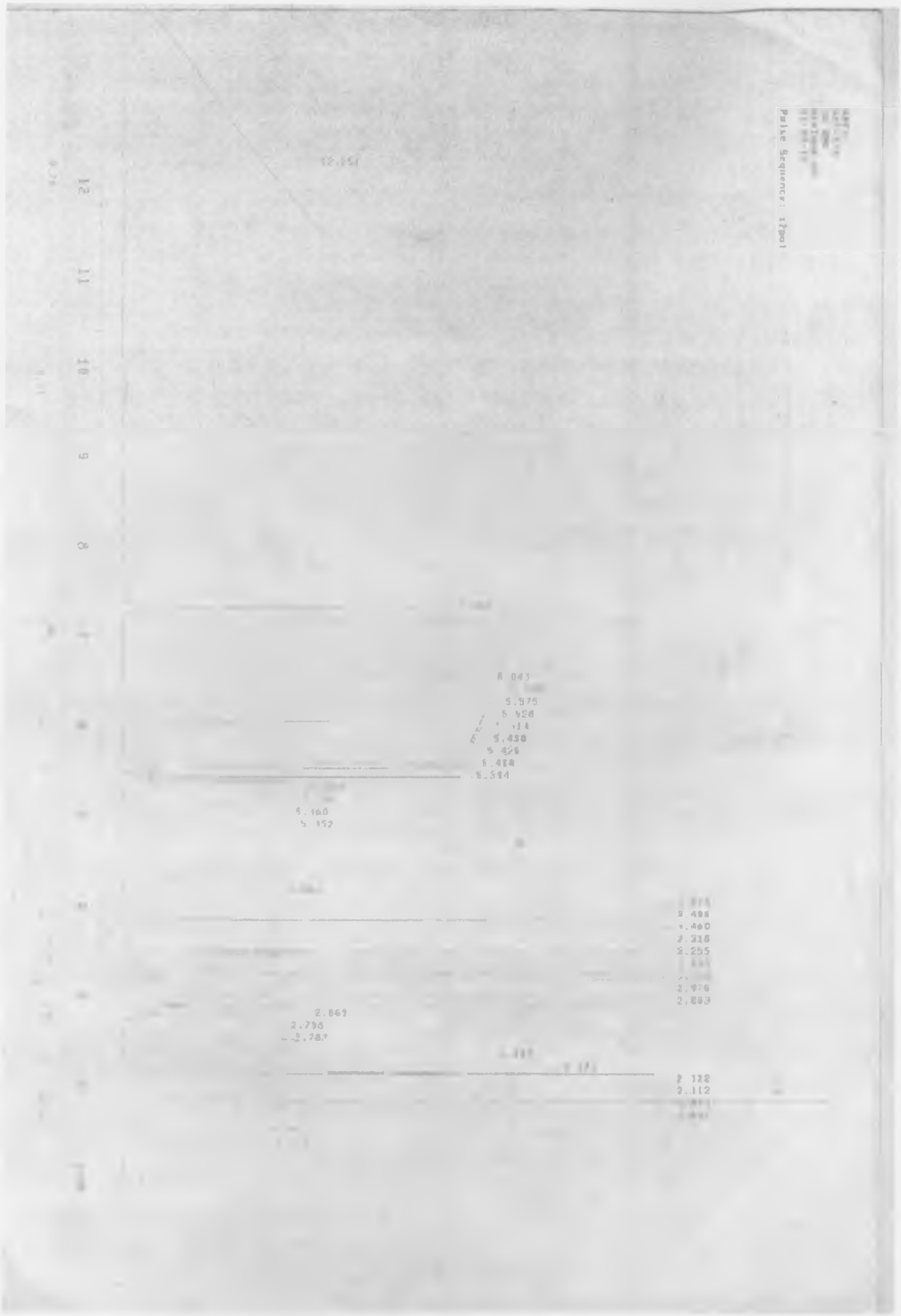
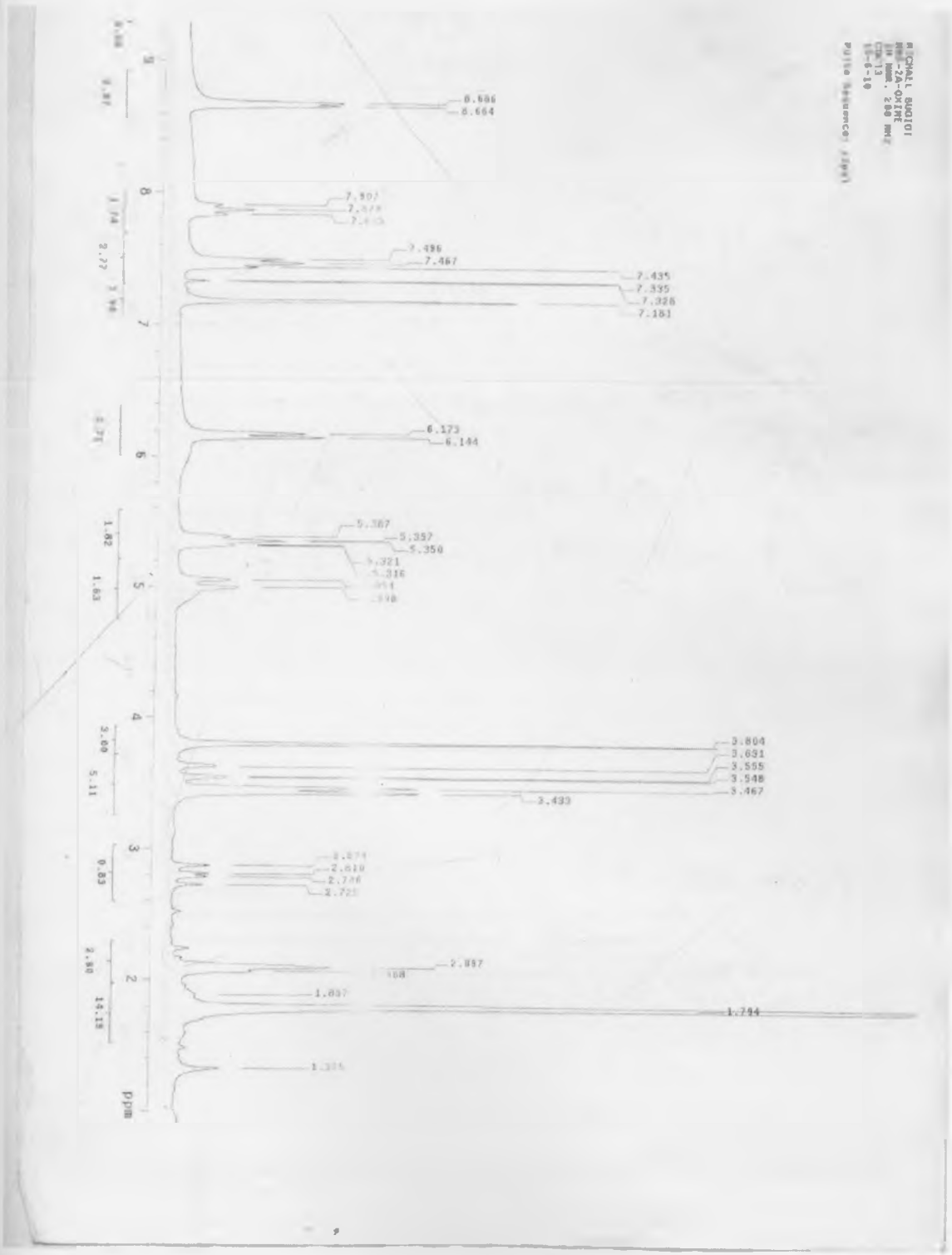
APPENDIX

^{1}H-NMR (200 MHz, $CDCl_3$), ^{13}C-NMR (50 MHz, $CDCl_3$) and DEPT

Abyssinone V-4'-Methyl Ether (5) ^{1}H-NMR Spectra

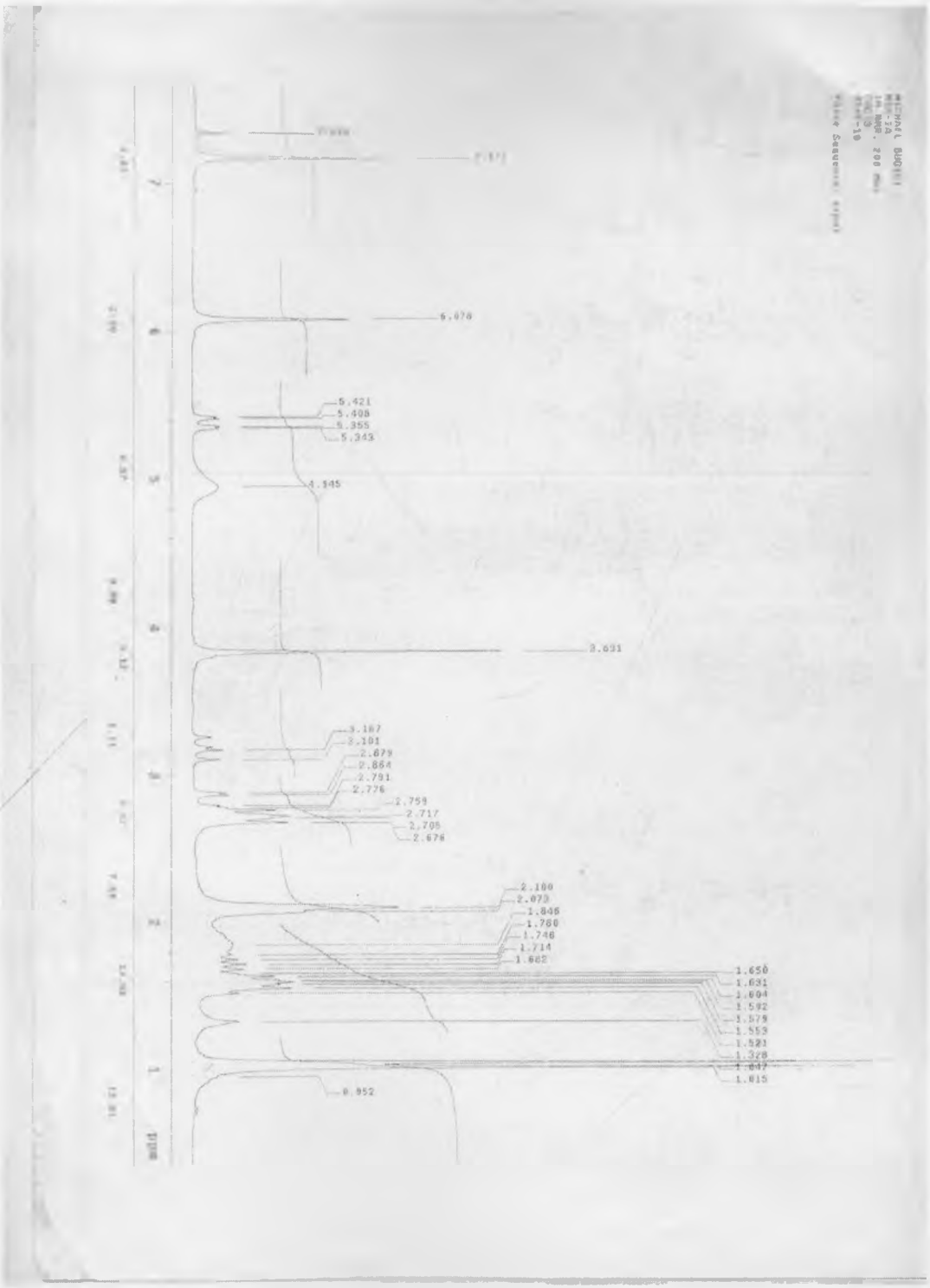


Abyssinone V-4'-Methyl Ether Oxime (58) ^{1}H-NMR Spectra



Abyssinone V-4'-Methyl Ether Oxime (58) ^{1}C-NMR Spectra

Tetrahydro-Abyssinone V-4'-Methyl Ether (59) ^{1}H-NMR Spectra



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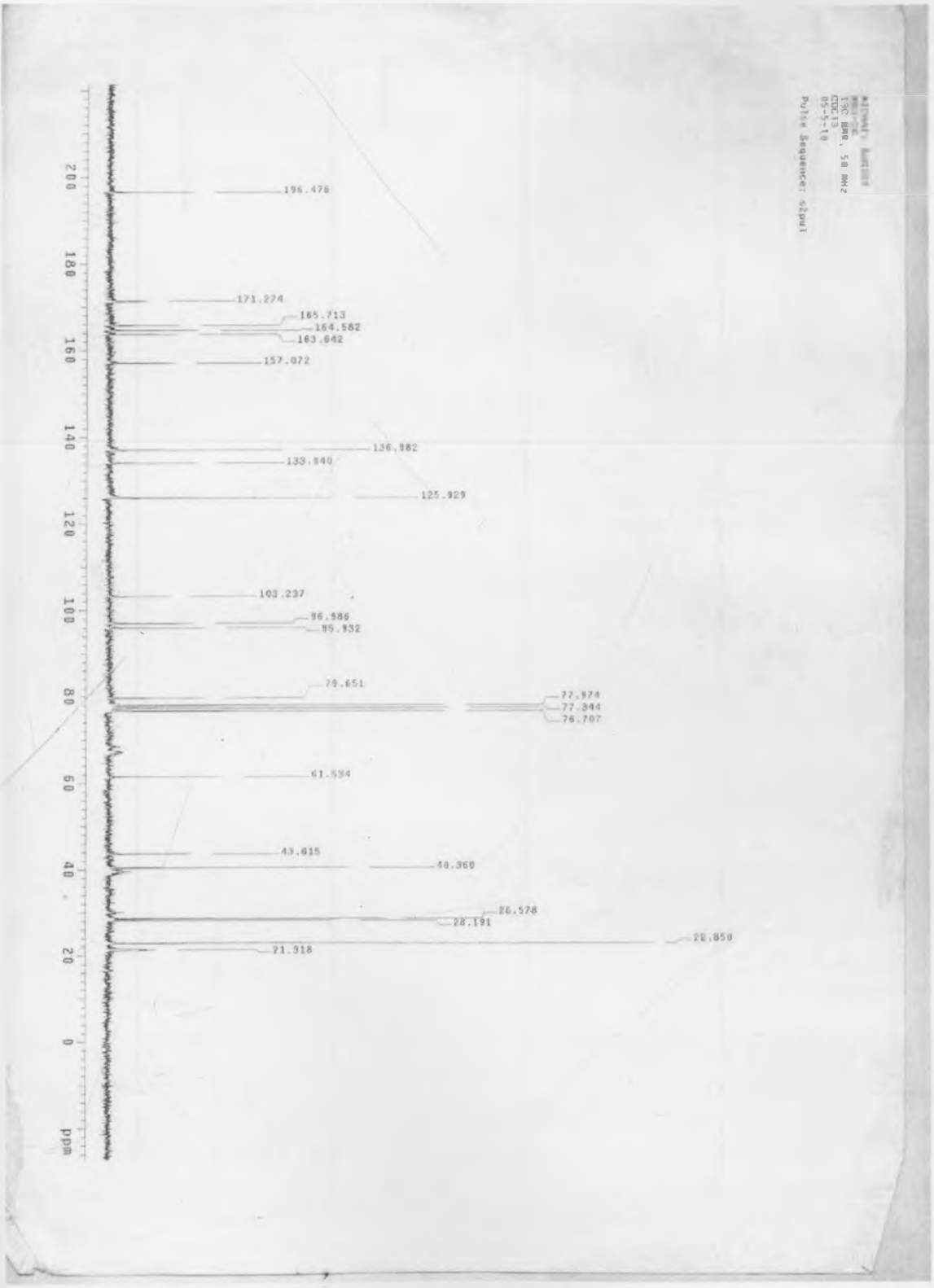
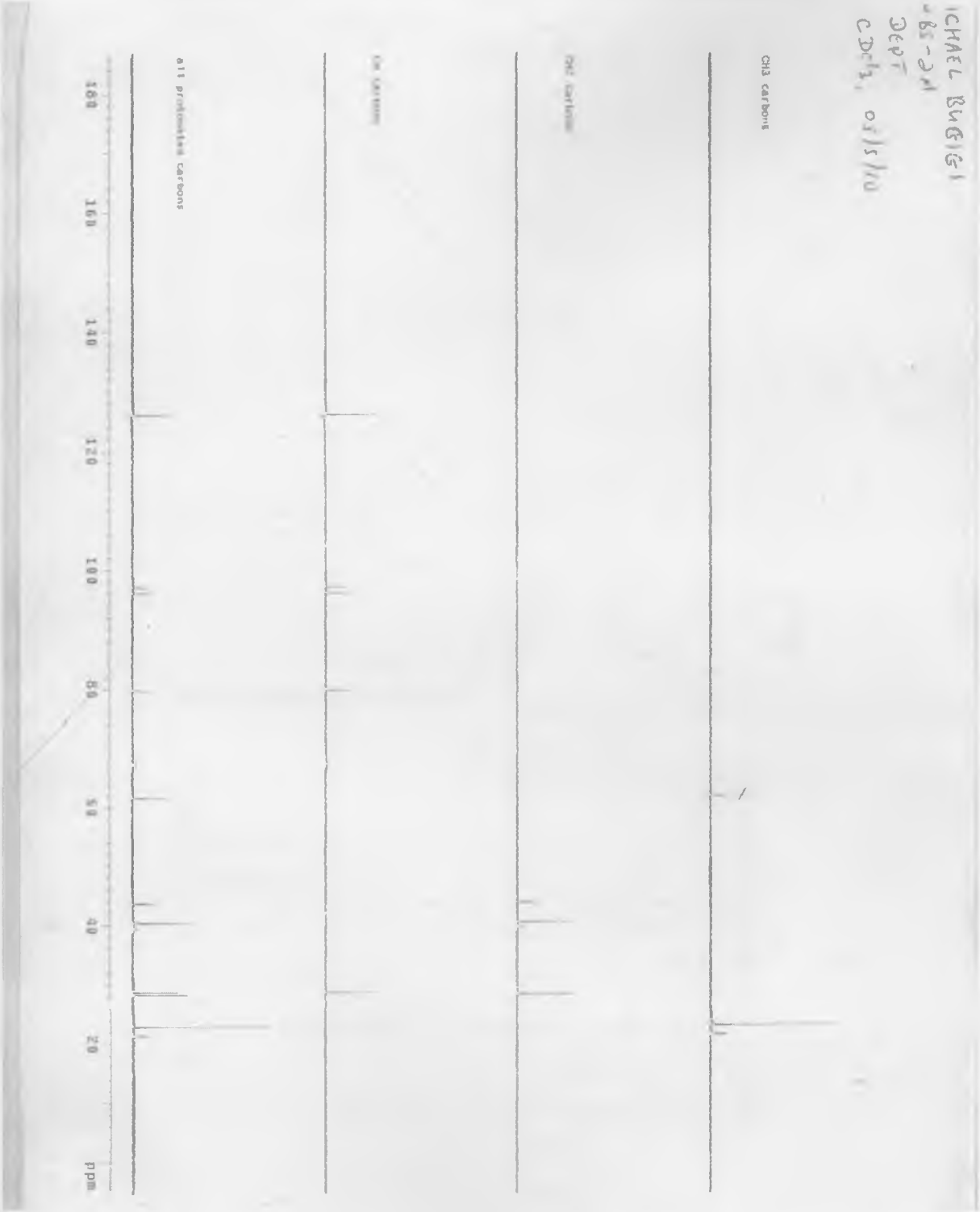
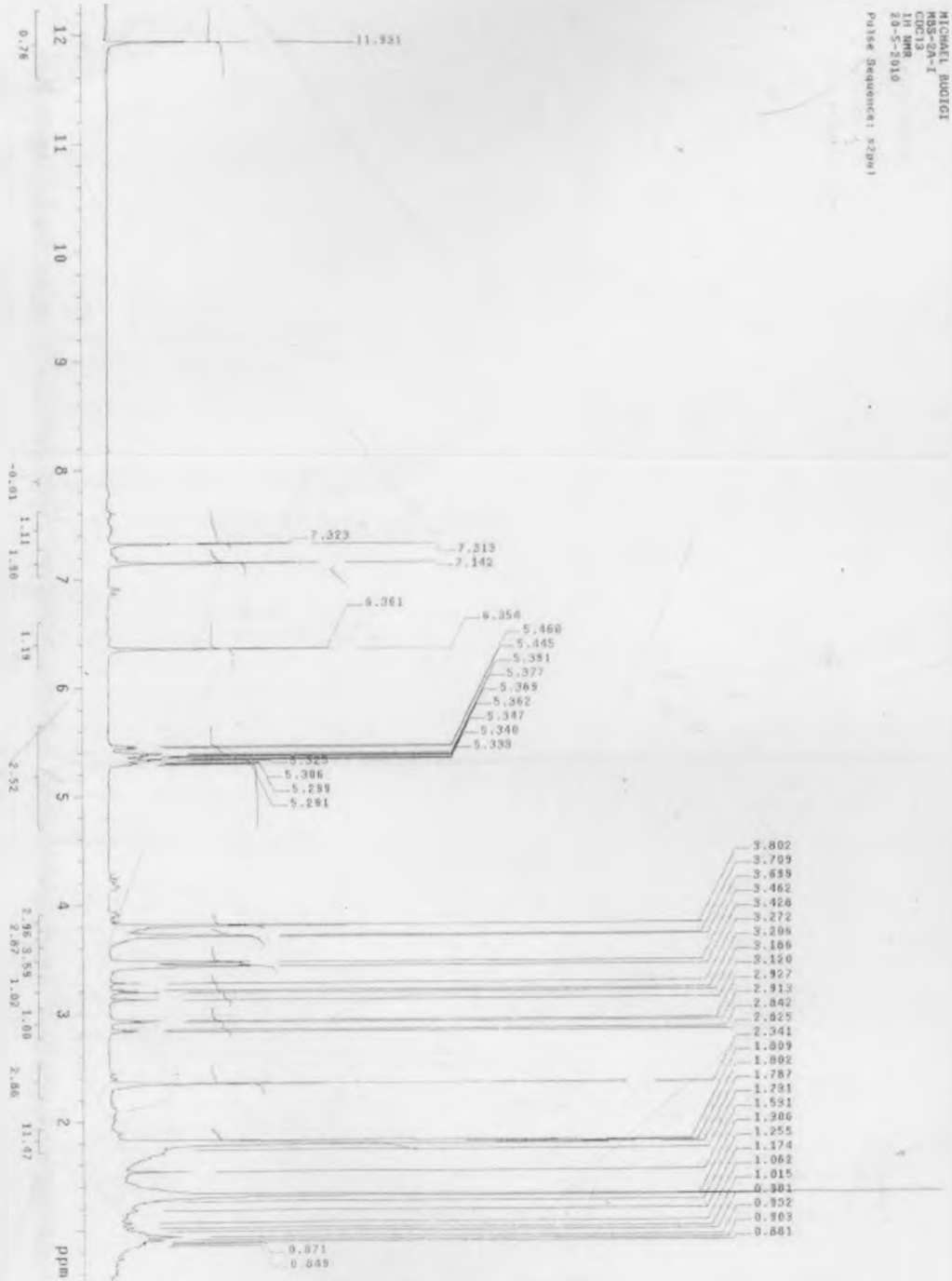


Tetrahydro-Abyssinone V-4'-Methyl Ether (59) DEPT



7-Acetyl-Abyssinone V-4'-Methyl Ether (60) ^{1}H-NMR Spectra



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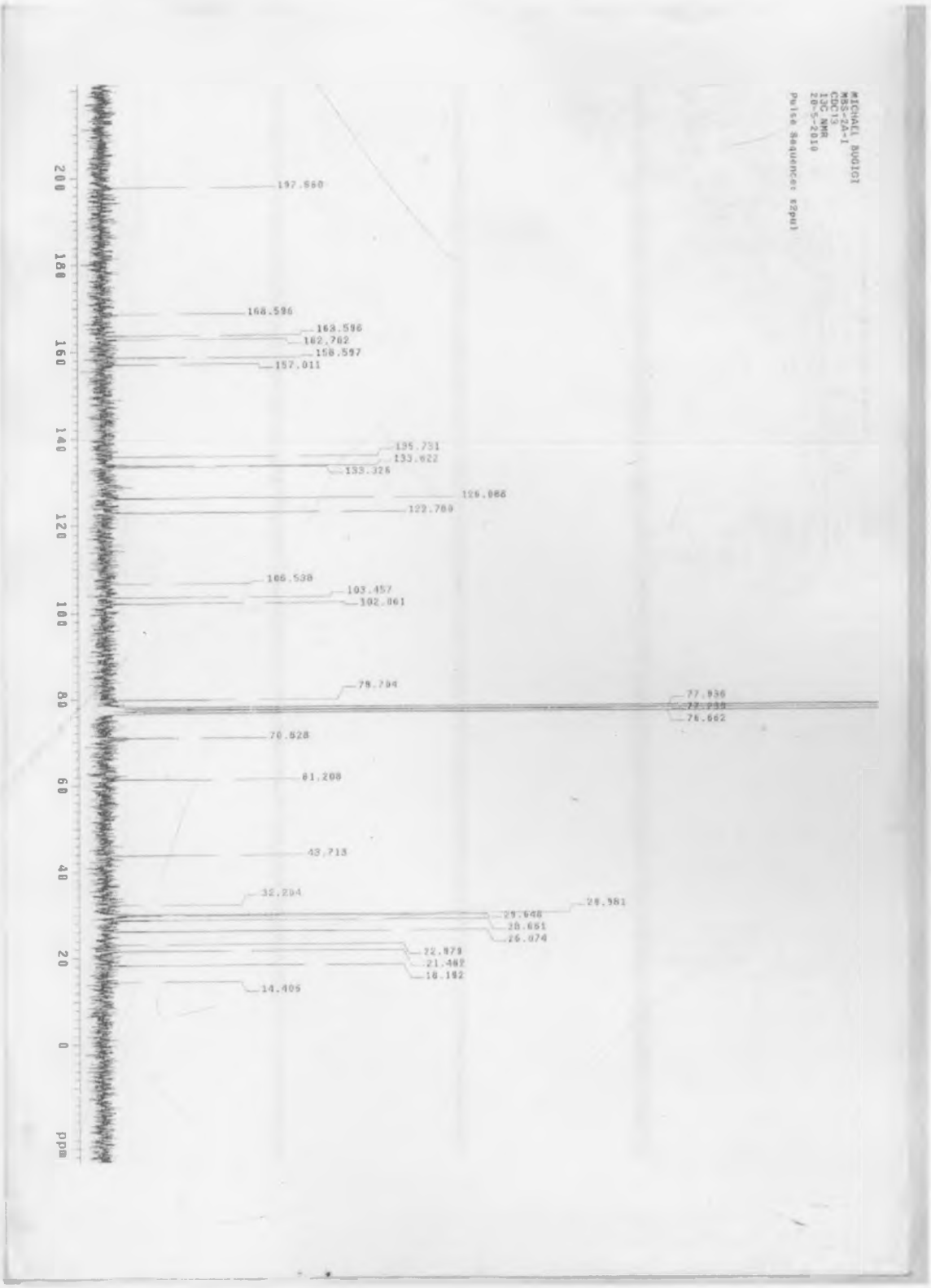

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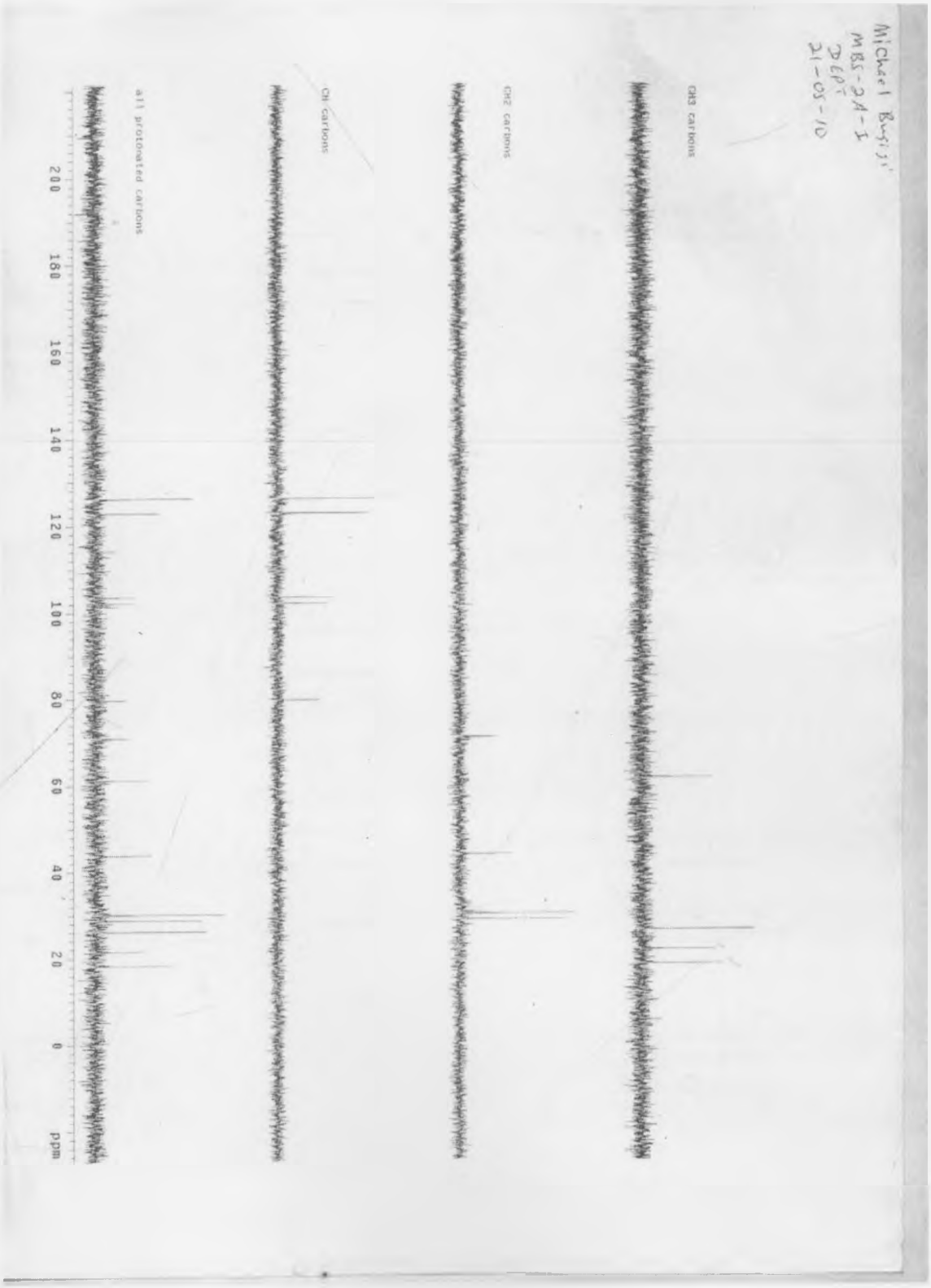
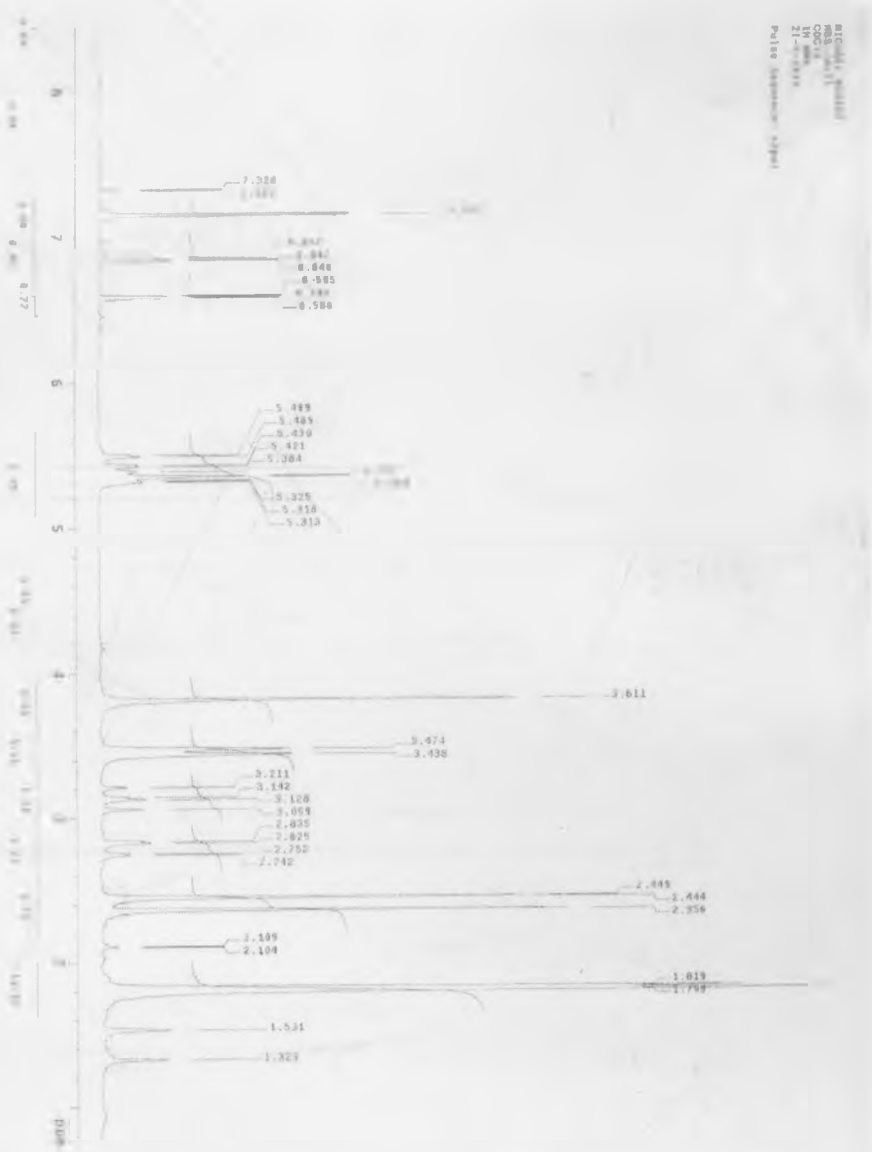


5,7-Diacetyl-Abyssinone V-4'-Methyl Ether (61) ^{1}H-NMR Spectra



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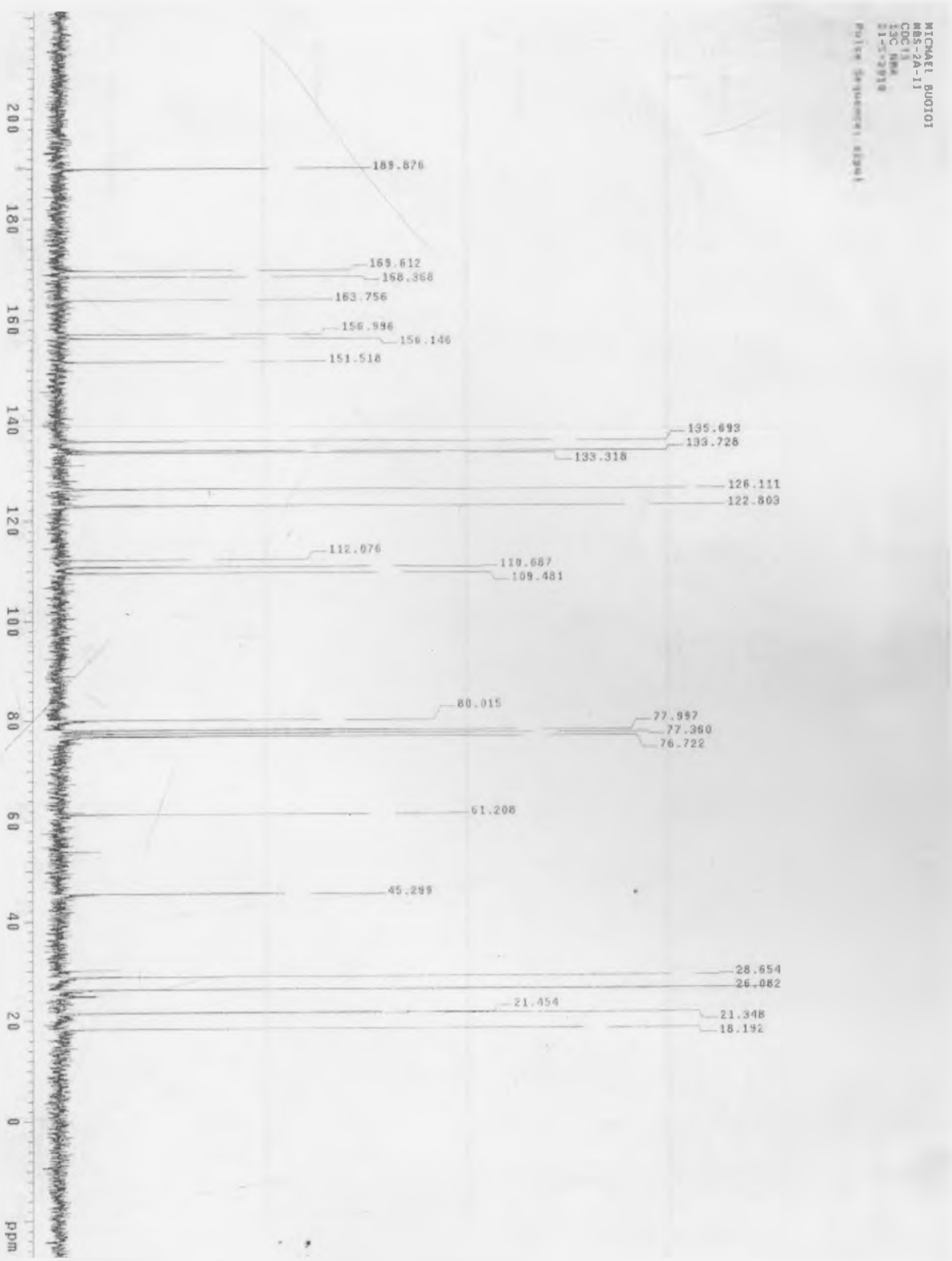
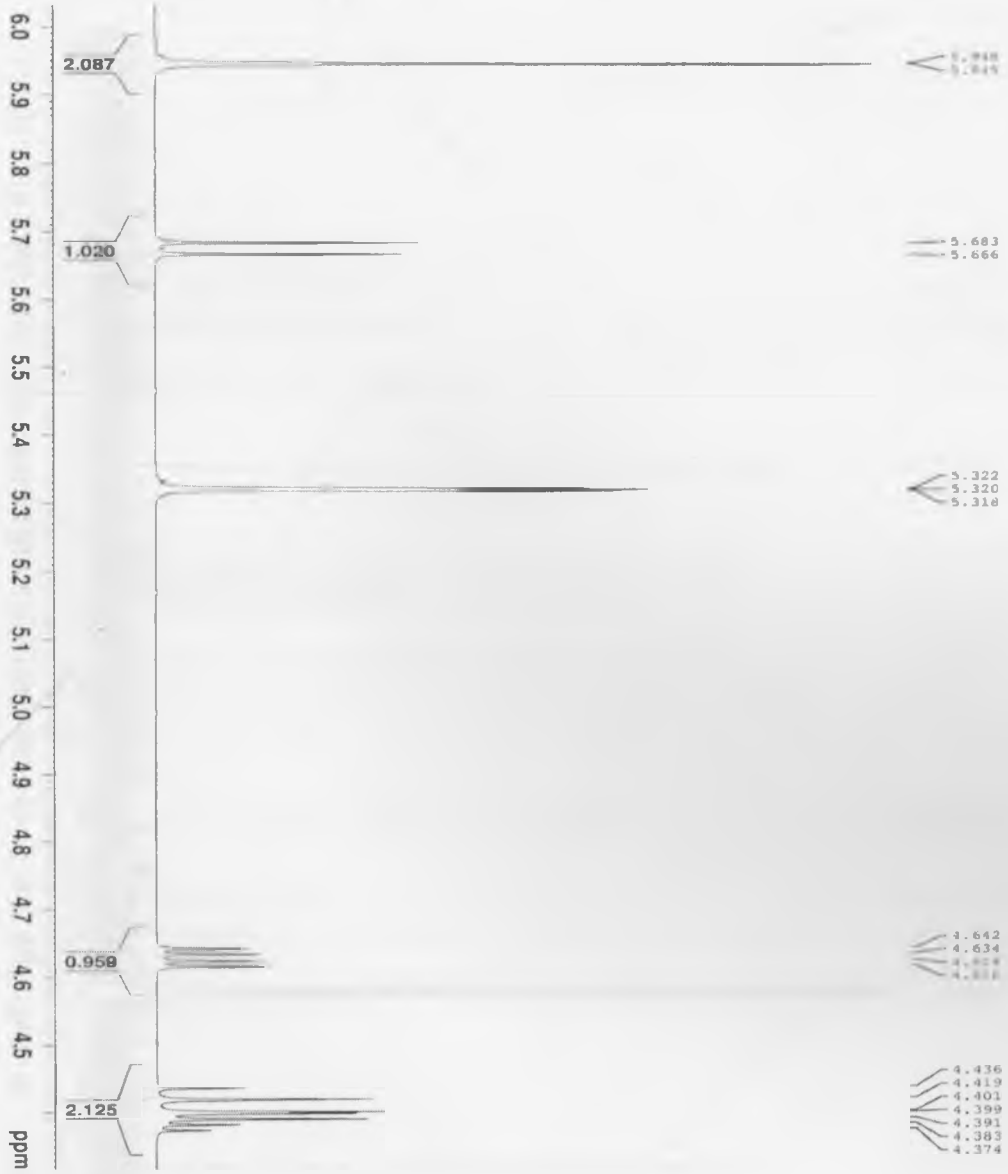


5,7-Diacetyl-Abyssinone V-4'-Methyl Ether (61) DEPT

12α-*epi*milletosin (7) ^{1}H-NMR Spectra

12α-*epi*milletosin (7) 1H-NMR Spectra



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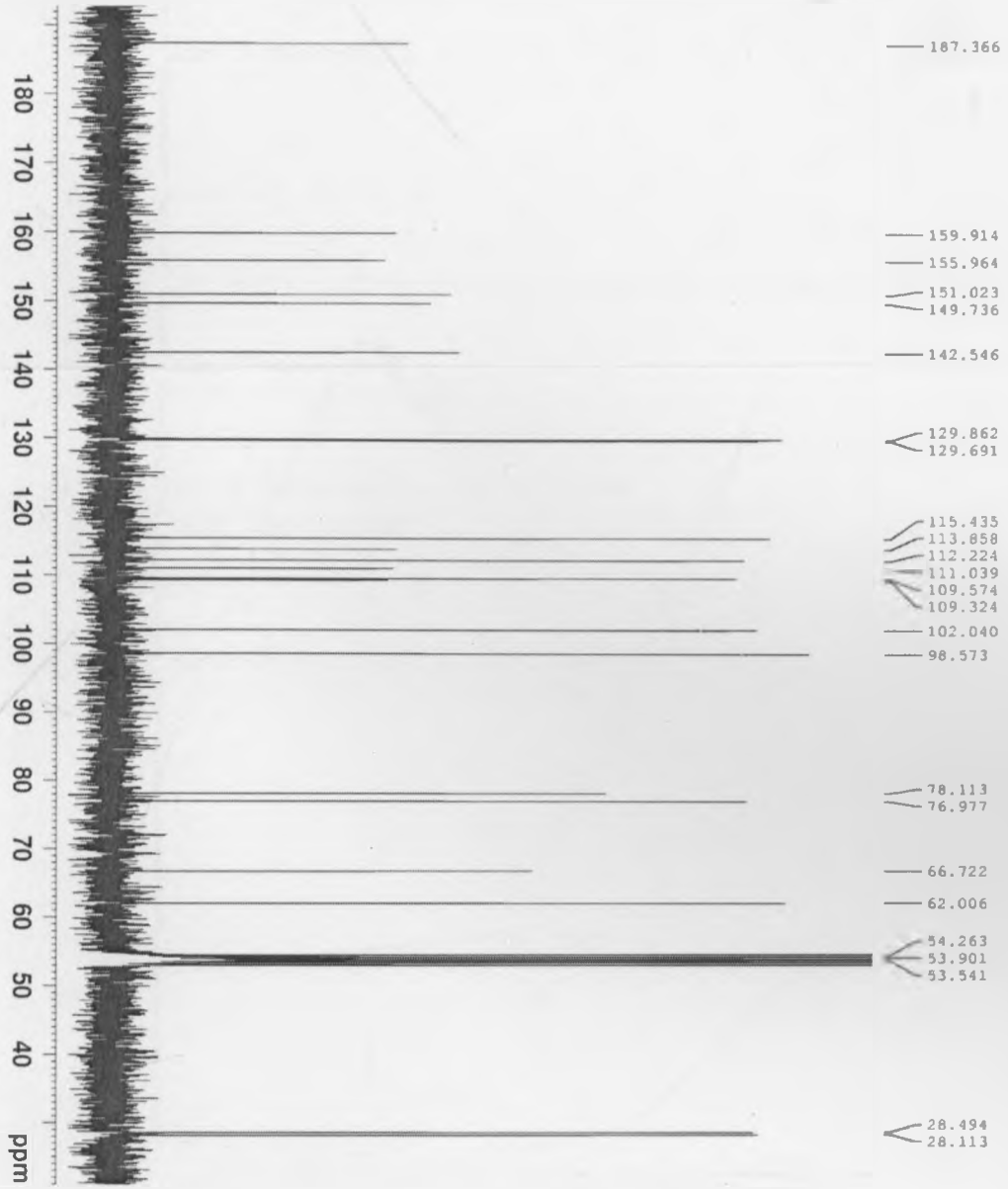
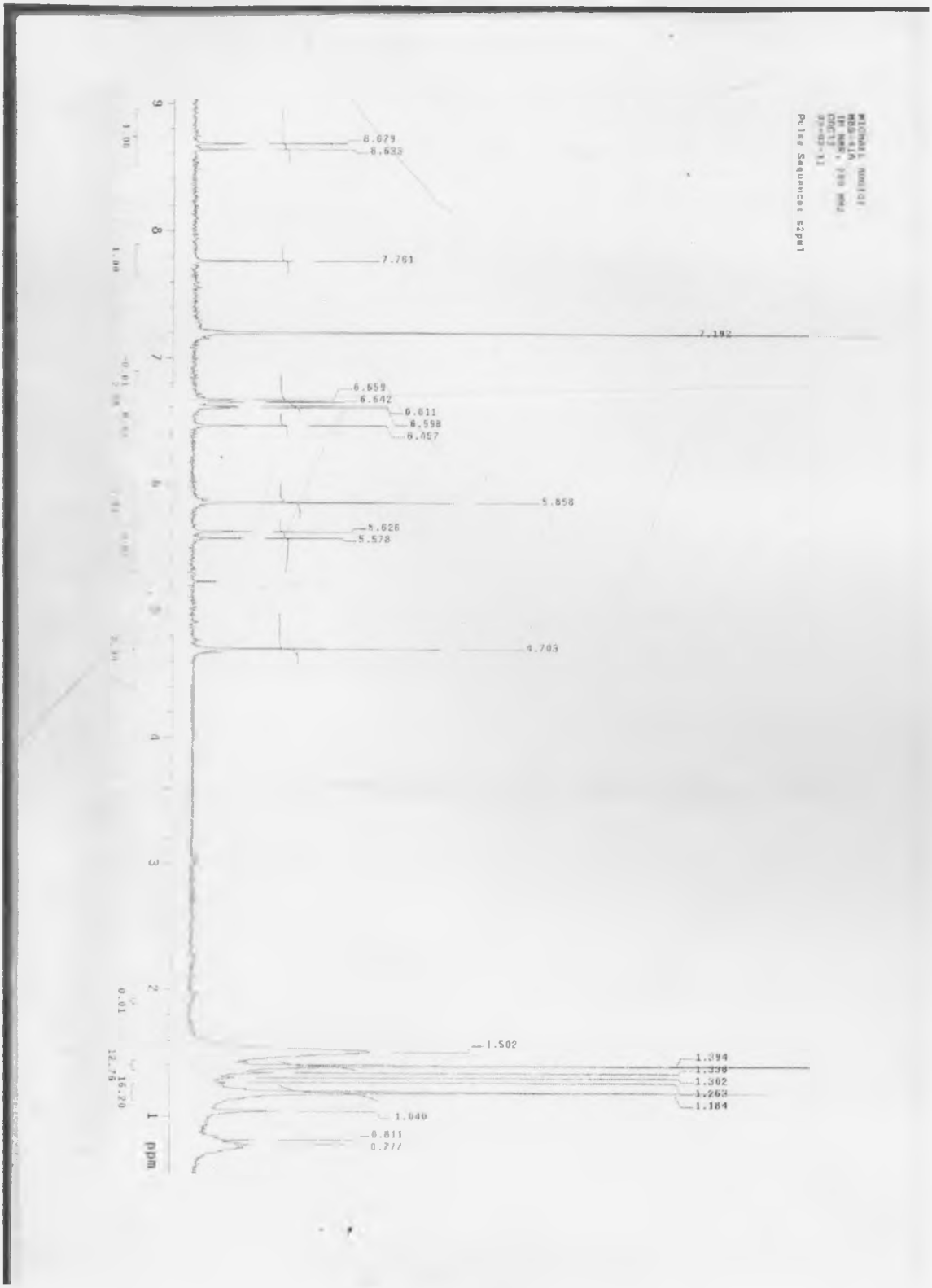
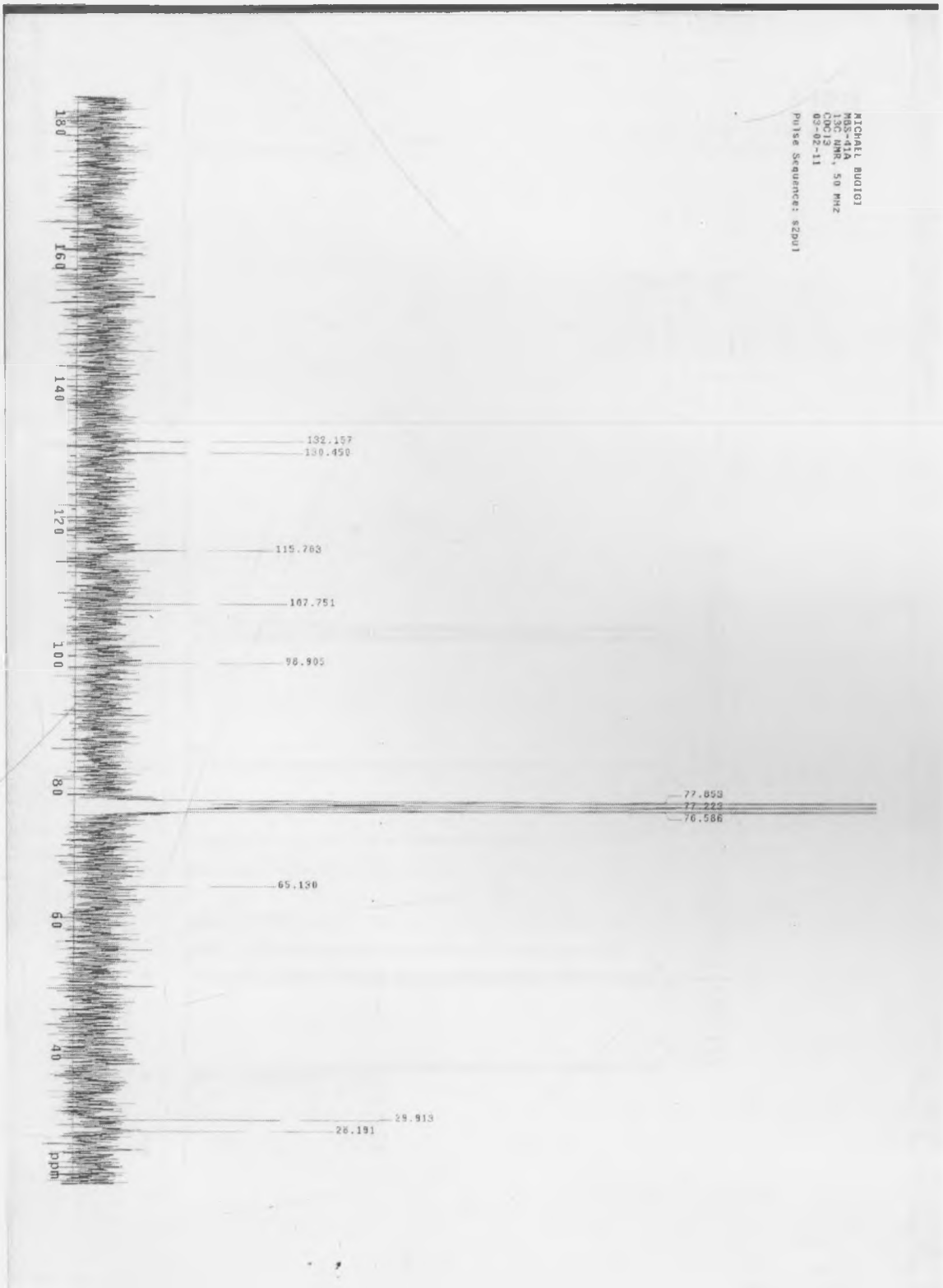


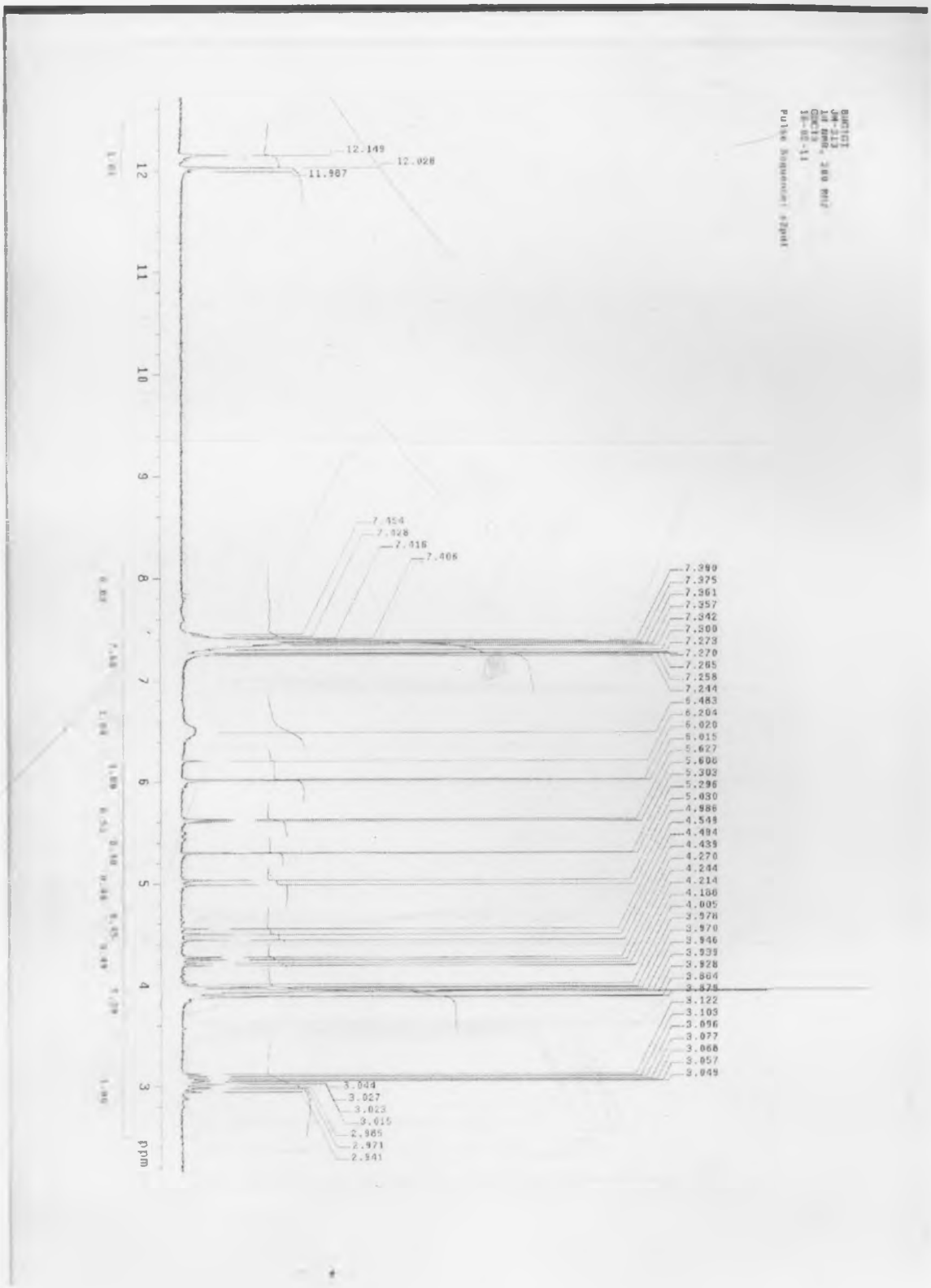
(+)-12α-Dehydromilletosin Oxime (62) ^{1}H-NMR Spectra



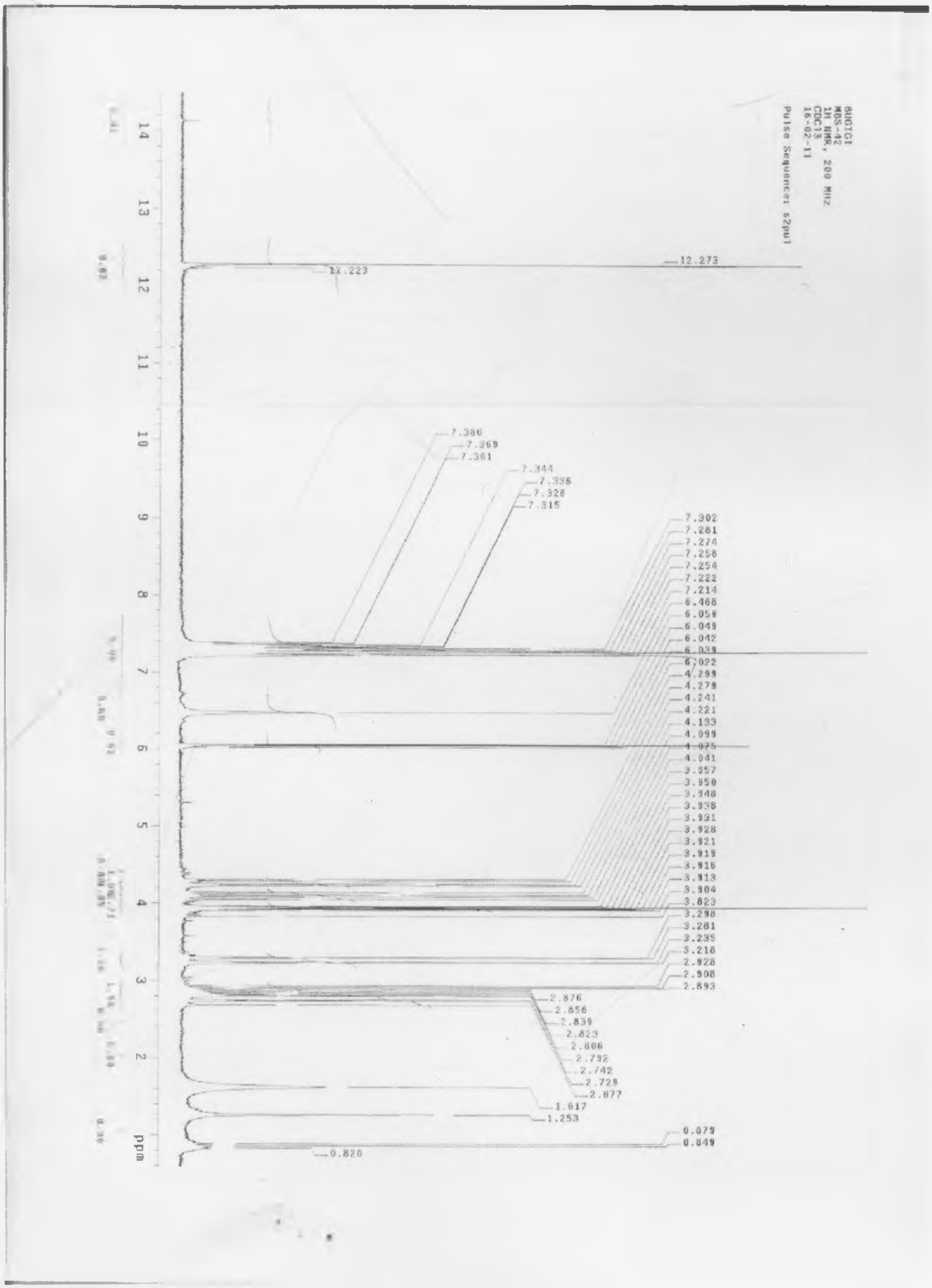
(+)-12α-Dehydromilletosin Oxime (62) ^{13}C-NMR Spectra



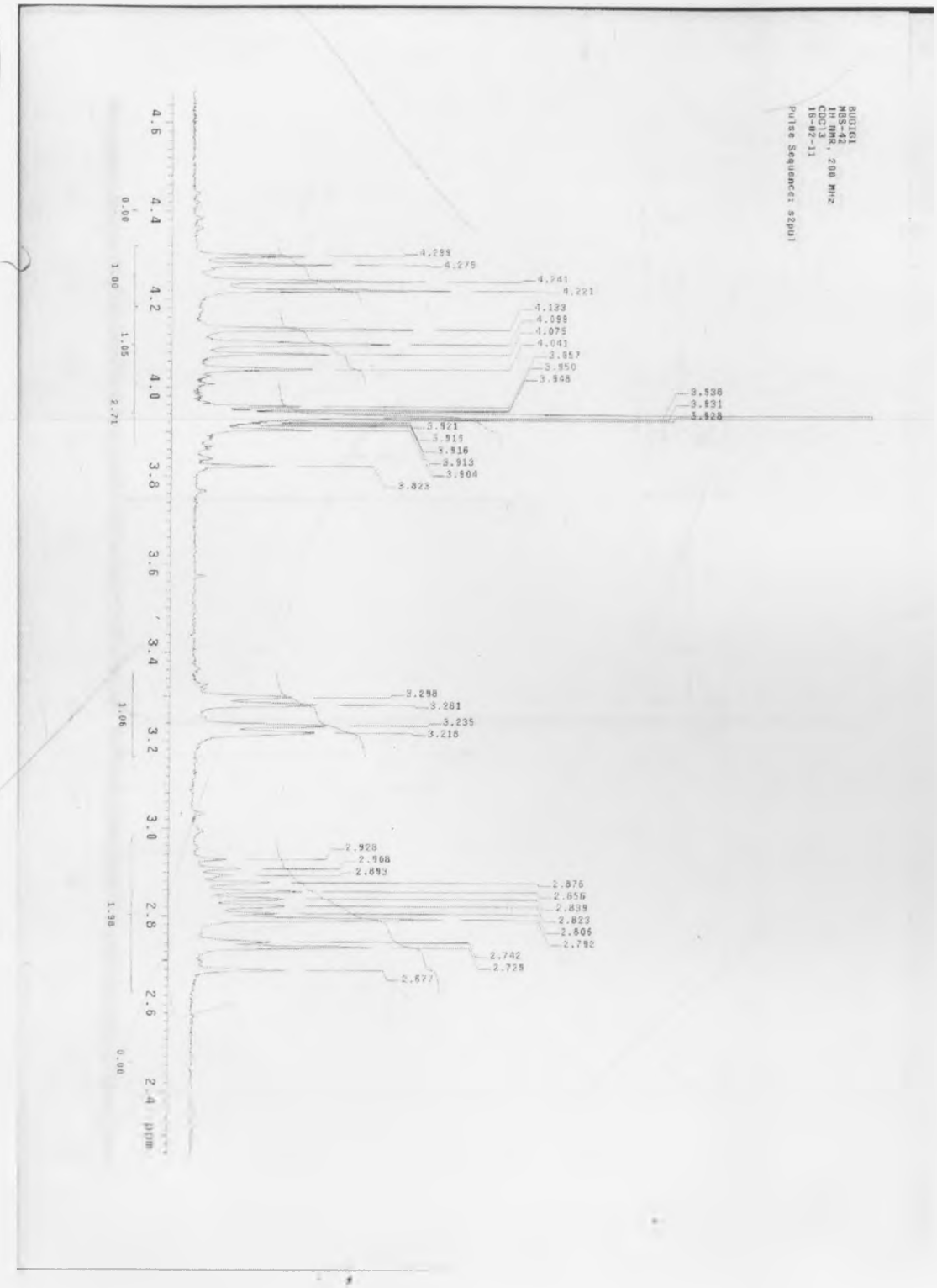
9-hydroxyhomoisoflavanone (diastereoisomers) ^{1}H-NMR Spectra



9-deoxyhomoisoflavanone (63) ^{1}H-NMR Spectra



9-deoxyhomoisoflavanone (63) ^{1}H-NMR Spectra



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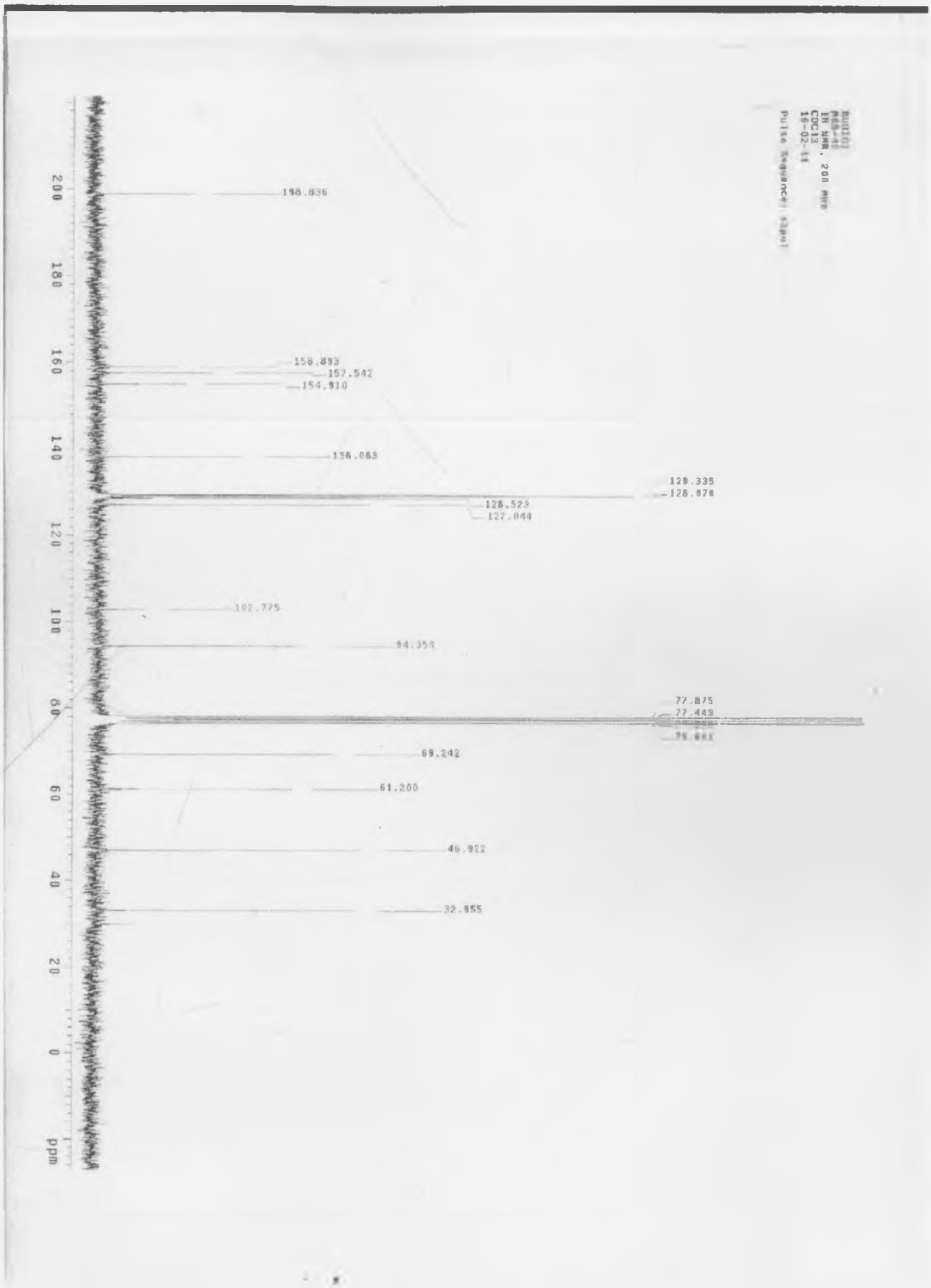

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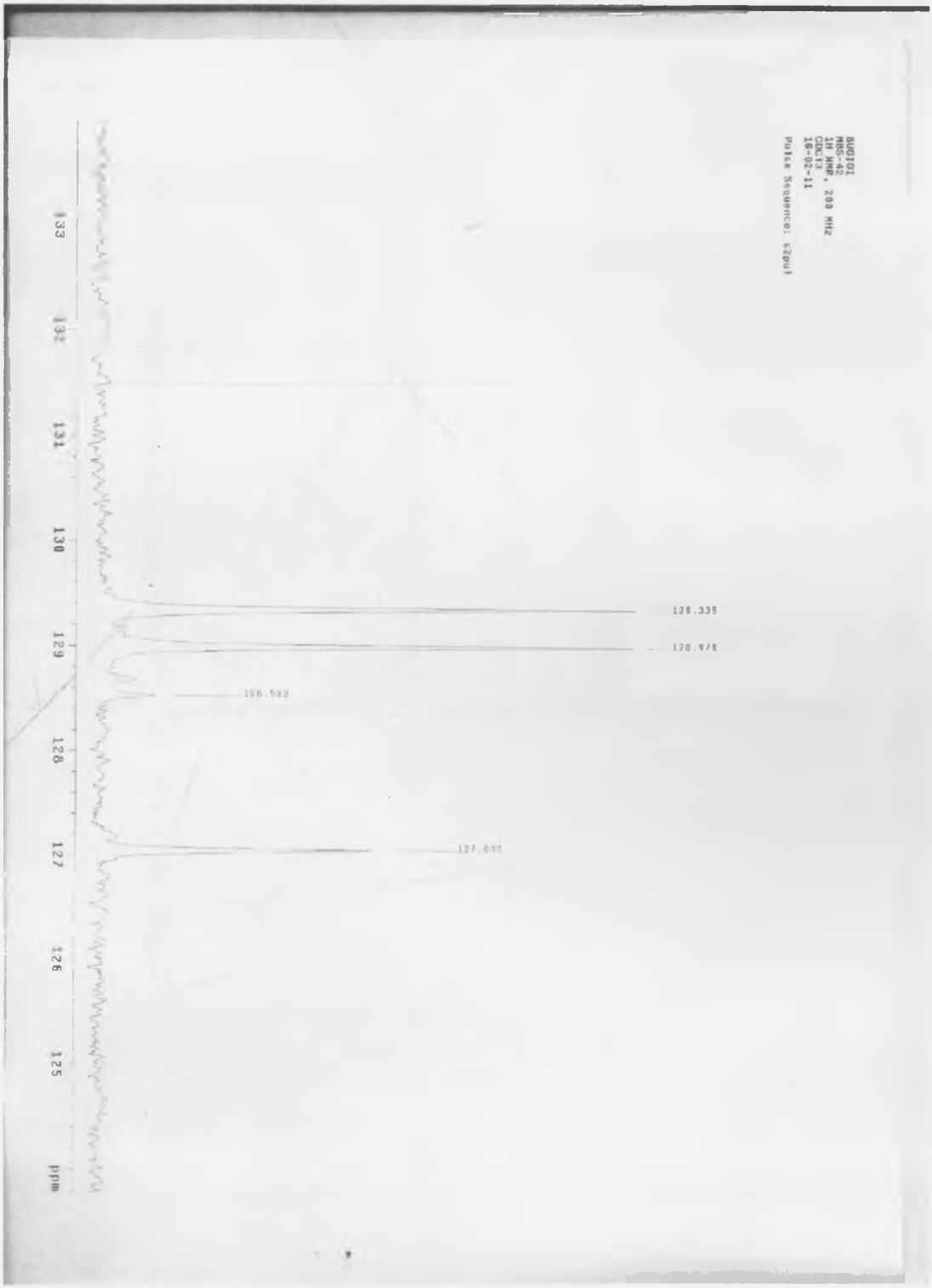

(+)-12α-Usararotenoid Analogues ^{1}H-NMR Spectra