CHEMICAL PROSPECTING OF MEDICINAL PLANTS FOR DRUG DISCOVERY

By

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Submitted in fulfillment of the academic requirements for the degree of Doctor of Philosophy in the School of Chemistry, University of KwaZulu-Natal, Westville Campus, Durban, South Africa.

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DECLARATION

I, **Opeoluwa Oyehan Oyedeji** declare that

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2. This thesis has not been submitted for any degree or examination at any other university.

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O O Oyedeji

I hereby certify that the above statement is correct.

…….

Prof. Francis O. Shode

EPILOGUE

. . . He who built the house has more honor than the house

For every house is built by someone

But He who built all things is God… Heb. 3:3b-4.

DEDICATION

This thesis is dedicated to the memories of my late mother -

Mrs. Rebecca Adeola Oyedeji

Who struggled among all odds to get me educated

ACKNOWLEDGEMENT

There is no island that can exist without the support of other living and non living things therefore I am most grateful to all for making this study a success:

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ABSTRACT

African traditional medicine relies largely on the abundant African flora estimated at several tens of thousands of species. These plants, like other living organisms, produce natural products which are organic molecules exhibiting a remarkable wide range of chemical diversity and a multiplicity of biological properties.

Over the past 20 years, interest in drugs of plants origin has been reviving and growing steadily. Among the broad spectrum of natural products that are showing promise as possible leads to useful therapeutic agents are the terpenoids. In the present study, selected African medicinal plants were investigated for the presence of extractable and exploitable terpenoids as leads or raw materials for producing more potent bioactive compounds for pre-clinical drugs discovery programme for chemoprotective agents against cancer, HIV/AIDS, diabetes, hypertension, malaria and other chronic diseases.

The plants investigated in this study included *Callistemon salignus*, *C. viminalis*, *Melaleuca bracteata* var. *revolution* gold, *M. bracteata* var. *revolution* green, *M. trichostachya* var. *compata*, *Syzygium aromaticum* and *Tectona grandis*.

These plants were subjected to two separate regimes of phytochemical extraction protocols namely volatile and non-volatile-extraction protocol. The *Callistemon* species and *Melaleuca* species upon hydrodistillation afforded essential oils. The gas chromatographic and mass spectrometric (GC-MS) analysis of these essential oils reveals that 1,8-cineole was the major constituent of the *Callistemon* oils. Similarly, 1,8-cineole was the major constituents of the essential oil of *M. trichostachya* var. *compata*, while methyl eugenol was the predominant constituent of the oils of *Melaleuca bracteata* var. *revolution gold*, and *M. bracteata* var. *revolution green*.

Antibacterial investigation of the essential oils showed that they possess strong to moderate inhibitory effect against selected bacteria.

In the non-volatile extraction protocol, various parts of the plants were sequentially extracted with organic solvents to obtain crude extracts which were subjected to fractionation and purification protocols (chromatographic techniques and re-crystallization). The crude extracts

from the leaves of the *Callistemon* and *Melaleuca* species gave a crystalline mixture of betulinic acid and oleanolic acid in an appreciable yield. The crude extract from the cloves of *Syzygium aromaticum* yielded oleanolic acid as the major extractive and maslinic acid as minor extractive. The crude extracts from *Tectona grandis* afforded betulinic acid in an appreciable yield. The elucidation of the structures of the pure extractives was achieved by extensive 1D and 2D nuclear magnetic resonance (NMR) spectroscopy as well as infra-red spectroscopy (FT-IR) and mass spectrometry (MS).

Betulinic acid and oleanolic acid were chosen as seed molecules for making known and unknown derivatives for lead optimization study. The semi-synthesized compounds were 3-acetoxyoleanolic acid, 3-acetoxyoleanolic hydrazide, 3-acetoxyloleanolic hydrazone, 3-succinyl oleanolic acid, 3-acetoxybetulinic acid, 3-succinylbetulinic acid and maslinic acid di-acetate.

Table of Content

List of Tables

Table of Figures

Appendix 6

Table of Scheme

List of Abbreviations

Acetyl CoA	Acetyl coenzyme A
ADP	Adenosine diphosphate
AIDS	Acquired immune deficiency syndrome
amu	Atomic mass unit
ATM	African traditional medicine
ATP	Adenosine triphosphate
BA	Betulinic acid
d	doublet
dd	doublet of doublet
ddd	doublet of doublet of doublet
DMSO	Dimethylsulfoxide
DMPP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
FPP	Farnesyl diphosphate
GPP	Geranyl diphosphate
HIV	Human Immunovirus
IPP	Isopentenyl diphosphate
MM	Methyl maslinate
m	Multiplet
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
OA	Oleanolic acid
ppm	Parts per million
R_f	Retention factor
s	singlet
t	triplet
UA	Ursolic acid
US	United States
UV	Uvaol

WHO	World Health Organisation

CHAPTER ONE

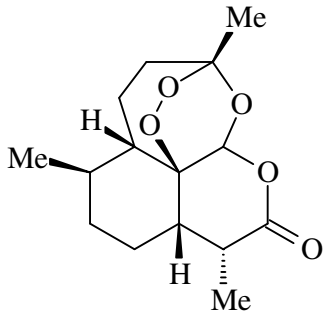
GENERAL INTRODUCTION

1.1 Introduction

Plants are the oldest source of pharmacologically-active compounds, and have provided humankind with medically useful compounds for centuries.[1] Today, it is estimated that more than two thirds of the world's population rely on drugs from plants.[2] Africa is known to have rich biodiversity of flora and this has made African Traditional Medicine (ATM) the oldest in the world.[3] ATM involves a holistic approach method in which the body and mind are subjected to treatment. In Africa, medicinal plants are used to treat diseases such as HIV/AIDS, malaria, sickle-cell anaemia, diabetes, hypertension to mention a few.[4] Many Africans believe that herbal medicines made from medicinal plants are more beneficial than synthetic drugs. Apart from the fact that they are cheaper and easier to get, herbal medicines are generally acceptable and suitable for chronic treatments.[5] Today, the largest users of traditional medicines are the Chinese, with more than 5,000 plants and plant products in their pharmacopoeia.[6] The Chinese medicine has contributed greatly to the growth and popularity of herbal medicine around the world. *Angelica polymorpha* var. *sinensis* (dang gui), *Artemisia annua* (qing hao), *Ephedra sinica* (ma huang), *Paeonia lactiflora* (bai shao yao), *Panax ginseng* (ren shen), and *Rheum palmatum* (da huang) are among the famous Chinese medicinal herbs.[7] It is believed that traditional medicine has been the focus for wider coverage of primary health care around the world. Examples abound of natural-product use, especially in small native populations in a myriad of remote locations on earth.[8] According to Iwu *et al* [9], medicinal plants were first used in their crude form; this formed the first generational usage of plants used for medicinal purposes. Researches on these medicinal plants led to isolation of pure compounds in their original form. This is referred to as the second stage of medicinal plants usage and it sees medicinal plants as phytopharmaceutical agents. This second stage also enhanced the synthesis of therapeutic agents. Recent uses of medicinal plants have led to the formulation of drugs which are based on well-controlled double-blind clinical and toxicological studies with an aim to improve the quality, efficacy, stability and safety of the preparation. This had led to the study of phytomedicine in various forms. Phytomedicine is commonly defined as herbal preparation produced by subjecting plant material to extraction, fractionation, purification or other physical or biological processes.[4, 10-12]

Natural products are defined as chemical compounds or substances produced by living organisms or found in nature the majorty of which are known to have pharmacological or biological activity and could find use in pharmaceutical drug discovery and drug design.[3] Many chemists refer to natural products as secondary metabolites produced by plants, animals, and micro-organisms for ecological reasons and have limited distribution.[3,13-15] Thus, natural products can be obtained from the tissues of terrestrial plants, marine organisms or micro-organism fermented broths. In phytomedicine, natural products that are responsible for significant biological activities are known as the "active principles" and search for such compounds is known as *bioprospecting*.[16]

Natural products and their derivatives represent over 50% of all drugs used clinically with higher plants accounting for over 25% of this amount.[4, 17] Plants have formed the basis for traditional medicine system and these are well documented across various cultures.[4] In man's search for food, the poisonous and healing properties of many plants were discovered.[18] Large number of natural products that are now used as drugs were derived from plants (See Table 1.1). These include artemisinin **1.1**, diosgenin **1.2**, digoxin **1.3**, vincristine **1.4**, vinblastine **1.5**, d-tubocurarine **1.6**, quinine **1.7**, pilocarpine **1.8**, etoposide **1.9**, reserpine **1.10**, and taxol **1.11**, to mention a few.



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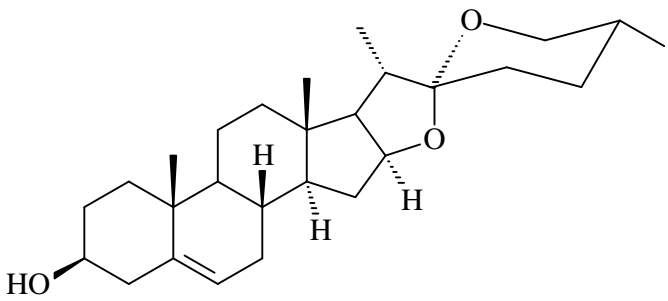

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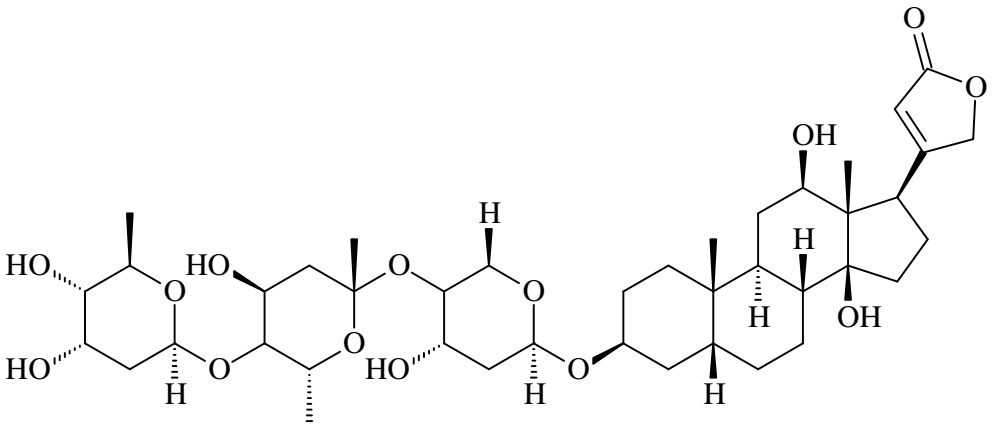

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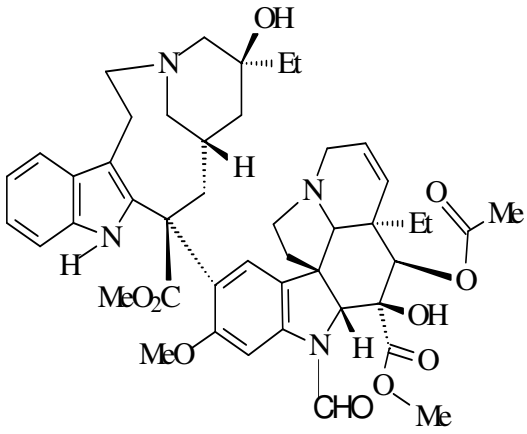

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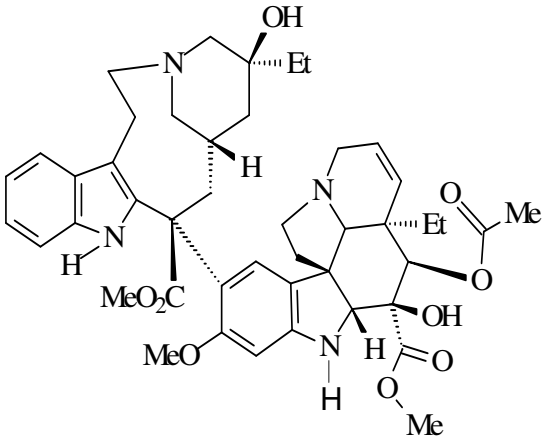

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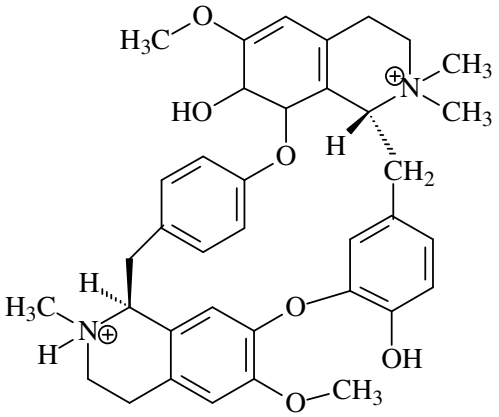

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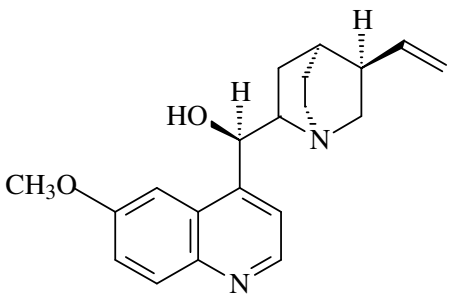

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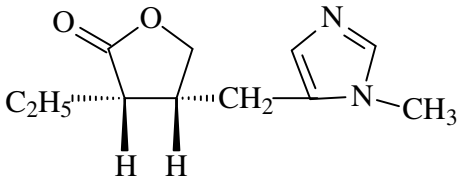

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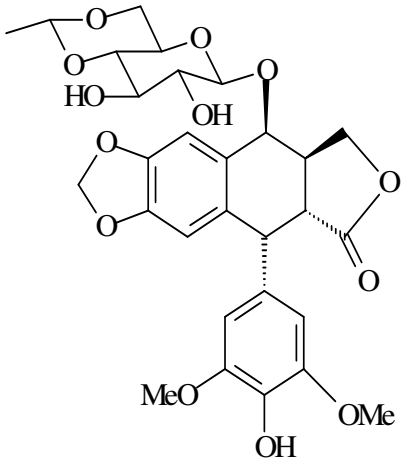

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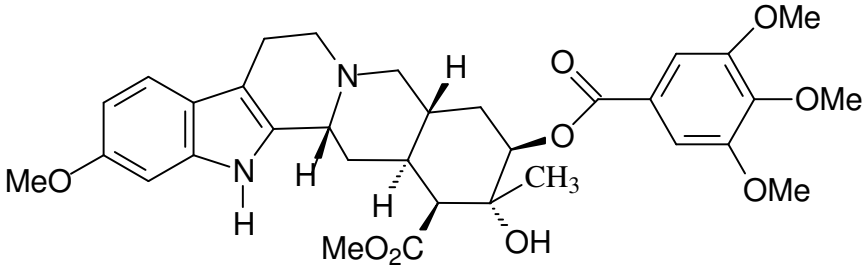

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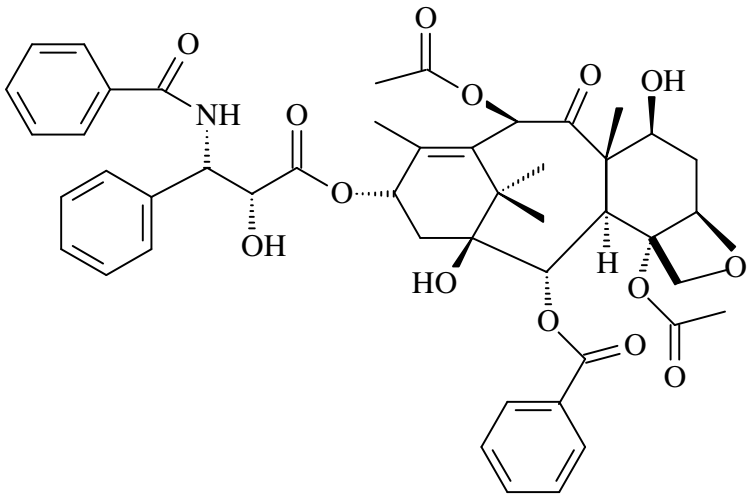

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Table 1.1 Examples of natural products from plants and their uses

Plant name / Species	Natural Product	Uses	Reference
Artemisia annua	Artemisinin (**1.1**)	Antimalarial	19
Dioscrea villosa	Diosgenin (**1.2**)	Contraceptive	20
Digitalis purpurea	Digoxin (**1.3**)	Cardiotonic	20
Catharanthus roseus	Vincristine (**1.4**)	Anticancer and Leukemia	21
Chondrodendron tomentosum	d-tubocurarine (**1.6**)	Muscles relaxant in surgery	17
Cinchona officinalis	Quinine (**1.7**)	Antimalarial	22
Pilocarpus jaborandi	Pilocarpine (**1.8**)	Glaucoma	22
Podophyllum peltatum	Etoposide (**1.9**)	Leukaemia, lymphoma lung and Testicular cancer	23
***Rauwolfia* species**	Reserpine (**1.10**)	Antihypertensive	23
Taxus brevifolia	Taxol (**1.11**)	Breast and Ovarian cancer	23

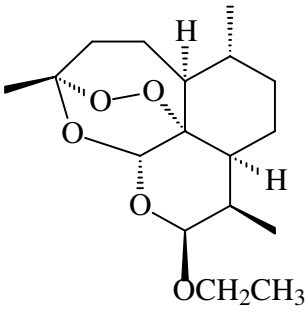
Medicinal plants play important role in health care delivery systems in many parts of the world. WHO estimates that over 80% of the world inhabitants rely and continue to rely on traditional medicine.[3] According to research conducted in the US from 1959-1980, over 25% of dispensed prescription-drugs were plant extracts or the active principles derived from higher plants.[3] About 119 chemical substances were derived from 90 plant species of which 74% were discovered from plants used in traditional medicine.[24] Hence, the discovery of drug can be linked to traditional medicine.[3, 17, 24] Between 1983 and 1994, 41% of newly approved drugs (medicines) have natural

product as their source.[5] Table 1.2 summaries few examples of medicines (drugs) derived or semi-synthesised from natural products.

Table 1.2 Examples of drugs derived or semi-synthesized from natural products

S/N	Drug (Generic name)	Therapeutic Class	Reference
1	Artemotil (arteether) § (**1.12**)	Antimalarial	25
2	Valstar (valrubicin) § (**1.13**)	Anticancer	26
3	Invanz (ertapenem) § (**1.14**)	Antibacterial	27
4	Cancidas (caspofungin) §§ (**1.15**)	Antifungal	28
5	Ketek (telithromycin) §§ (**1.16**)	Antibacterial	29
6	Myfortic (mycophenolate sodium)§§§(**1.17**)	Immunosuppressant	30

Key: § Natural product derived; §§ Semi-synthetic natural product; §§§ Natural product



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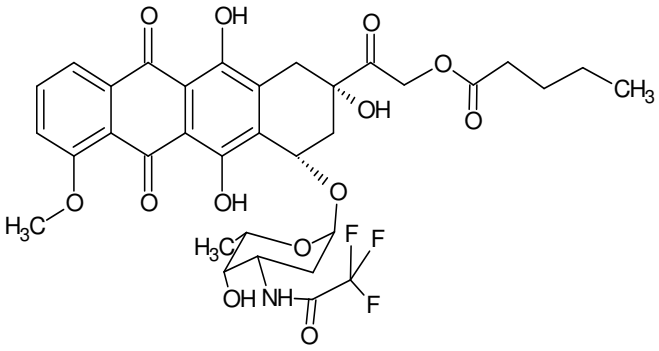

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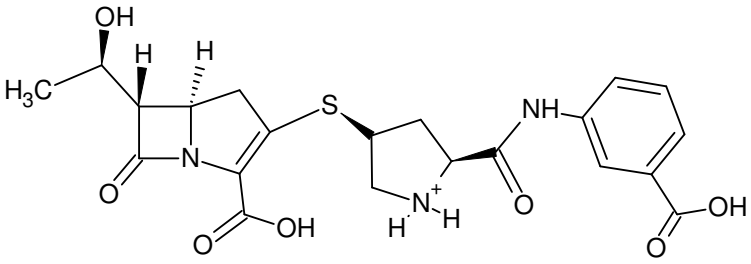

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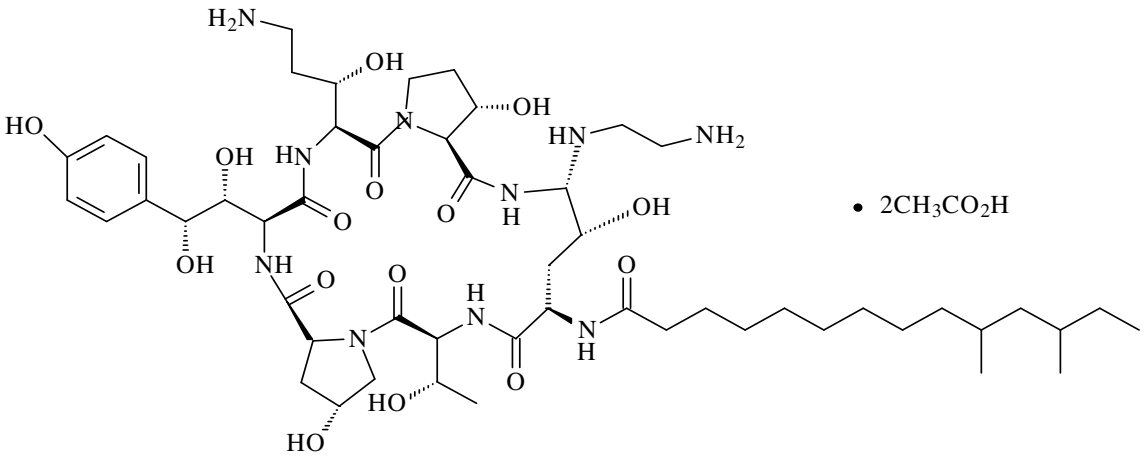

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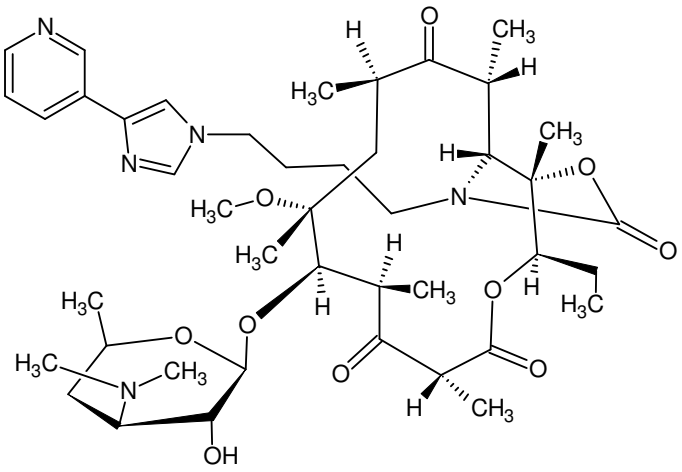

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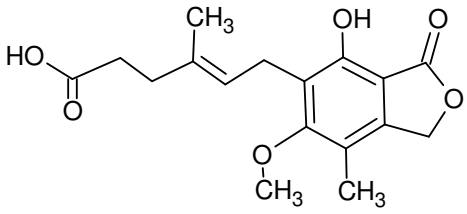

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1.2 Motivation for the Present Study

Natural products have the potential of providing medicine with a source of novel structure that cannot be obtained through combinatorial synthesis[14, 31]. This is because nature has the ability to produce complex molecules with multiple chiral centres that are designed to interact with biological systems. Living organisms use these compounds as self defence mechanism.[3] Most biologically active natural products are secondary metabolites with complex structures.[14, 31]

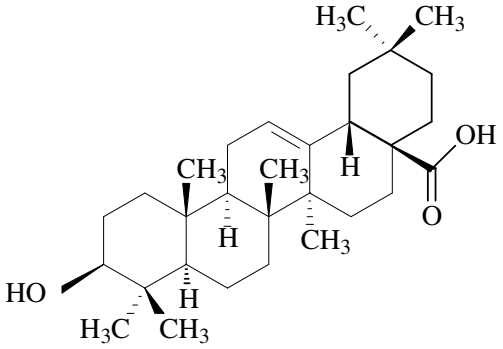
Natural products have chemical diversity which determines their end usage. The biodiversity of nature has lead to novel natural products and greater amount are yet to be discovered. It is estimated that from about 250,000 plants species available in the world only about 5-15% has been studied for bioactive compounds.[17] The biodiversity of the marine ecosystem remains a grave yard which has not been exploited to the fullest.[32] Marine organisms tend to produce a wealth of natural products with over 3,000 new compounds isolated.[15] The microbial world is richer than the two aforementioned ecological systems with only about 1% of its biodiversity explored.[33, 34]

Africa is plagued with many diseases which include malaria, diabetes, hypertension, filariasis, HIV/AIDS, and sickle cell anaemia to mention just a few.[3, 4] Many ethnobotanical surveys show that many African medicinal plants are used by traditional healers to treat some of these diseases.[4] Figure 1 shows a typical traditional herbal medicinal market in Durban, South Africa.

Figure 1.1 A typical traditional medicine market in Durban, South Africa.[35]

Furthermore, most of the phytomedicines derived from the medicinal plants have not been subjected to any scientific study to validate their efficacy and safety and also identify their biologically active ingredients.[3] The ubiquity of triterpenes in many medicinal plants used in Asian

and European phytomedicines encouraged the present focus on triterpenes as lead compounds for drug discovery purposes. Previously, Somova *et al.*[36] investigated the anti-hypertensive activity of triterpenoids from the leaves of *Olea europaea* subspecies *africana*. The study showed that oleanolic acid (OA) **1.18**, and ursolic acid (UA) **1.19** are the active constituents in the leaf extract.



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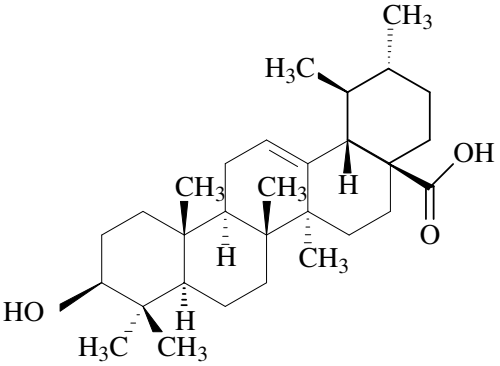

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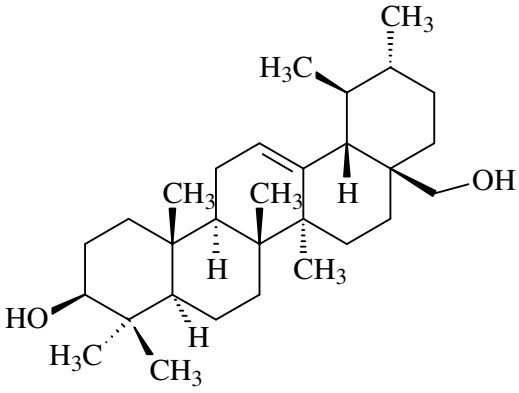
Furthermore, Somova *et al.*[37] showed that OA, UA, and uvaol (UV) **1.20** as well as methyl maslinate (MM) **1.21**, a derivative of OA, isolated from the leaves of *Olea europaea* (Cape cultivar) have cardiotonic and antidysrhythmic effects.



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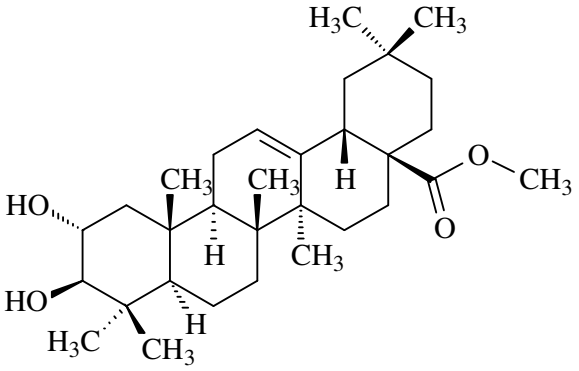

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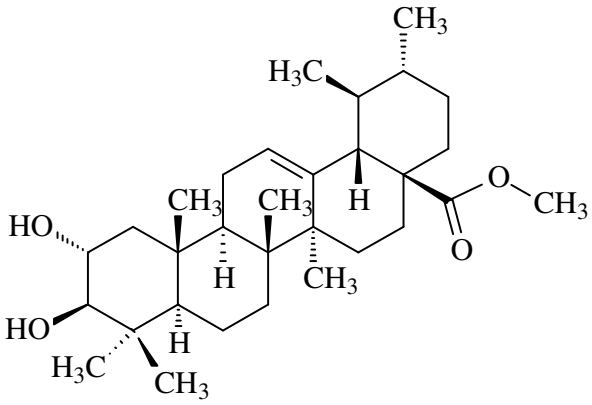
In a separate study, Musabayane *et al.*[38] implicated OA, UA, and their derivatives, methyl maslinate **1.21** and methyl corosolate **1.22**, in the observed anti-diabetic activity of the leaf extract of *Syzygium cordatum* in streptozotocin-induced diabetic rats. Furthermore, of recent, many reports on the anti-cancer and anti-HIV activities of natural pentacyclic compounds such as betulinic acid **1.23**, platanic acid **1.24**, OA **1.18**, pomolic acid **1.25**, glycyrrhetic acid **1.26**, UA **1.19**, morolic acid **1.27**, and other structurally related triterpenoids as potent and selective inhibitors of HIV type 1 replication have appeared in the chemical and biological literature.[39]



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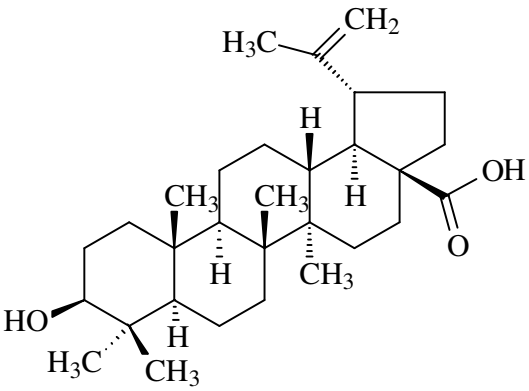

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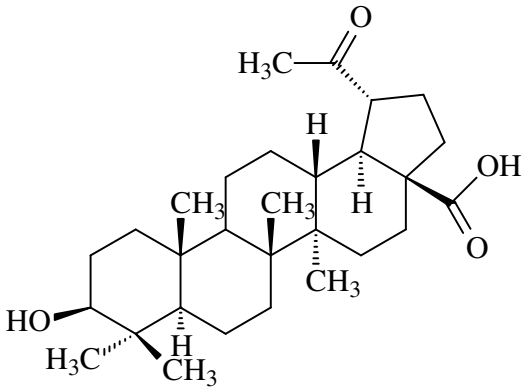

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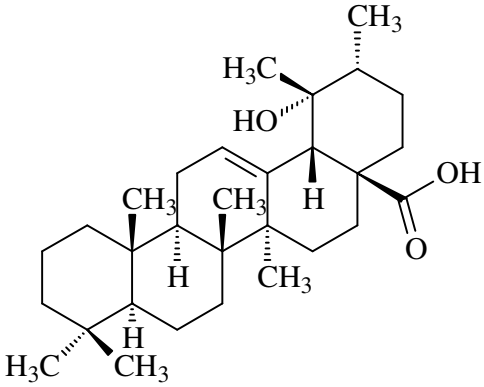

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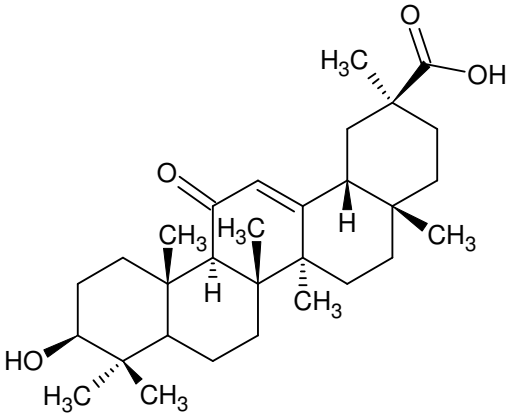

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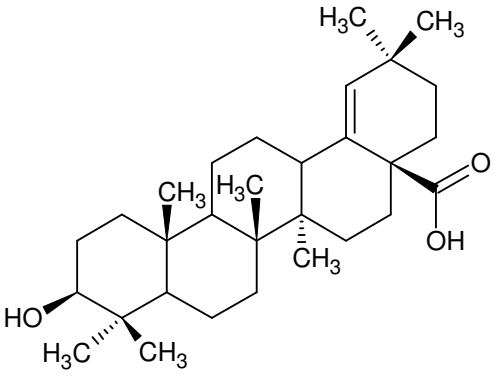

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In recognition of the broad-based biological activities of triterpenoids [40] and the ubiquity of triterpenic acids in nature as well as the latent functionalities present in the molecules, the following hypotheses were proposed.

1.3 Hypotheses

- Bulk quantities of triterpenoids such as OA, BA, UA and other classes of natural products can be extracted from local and non-local economic and horticultural plants after qualitative and quantitative phytochemical screening. These extractives can be used as active pharmaceutical ingredients (APIs) and/or precursors for lead compound optimisation programme which can lead to potent chemotherapeutic agents.
- The isolated natural products (triterpenoids and others) which are bioactive and have latent or obvious functional groups can be decorated with pharmacophoric groups for enhanced biological activities for drug discovery purposes.

1.4 Aims and Objectives of Research

The principal aims of the present research were:

(i) To isolate and characterize the chemical constituents of *Callistemon species*, *Melaleuca species*, *Syzygium aromaticum* and *Tectona grandis* for chemical and biological evaluation.

(ii) To produce (potent biologically-active) derivatives of pentacyclic triterpenoids from the study plants.

1.4.1 Specific Research Objectives

(i) To review scientific literature of previous work done on plants containing triterpenoids, especially OA and BA.

(ii) To isolate and characterize the essential oils and pentacyclic triterpenoids of *Callistemon viminalis* and *Callistemon salignus*.

(iii) To characterise the essential oils of *Melaleuca bracteata* var. *revolution gold*, *Melaleuca bracteata* var. green, and *Melaleuca trichostachya* var. *compacta*.

(iv) To isolate and characterise the pentacyclic triterpenoids of *Melaleuca bracteata* var. *revolution gold*, *Melaleuca bracteata* var. *revolution green*, and *Melaleuca trichostachya* var. *compacta*.

(v) To isolate and characterise the pentacyclic triterpenoids of *Syzygium aromaticum*.

(vi) To isolate and characterise the pentacyclic triterpenes of *Tectona grandis*.

(vii) To semi-synthesise some known and unknown derivatives of OA and BA.

(viii) To study the anti-diabetic activity and anti-ulcer activity of OA, BA and their derivatives.

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CHAPTER TWO

LITERATURE REVIEW

2.0 Phytochemicals as Leads in Drug Discovery

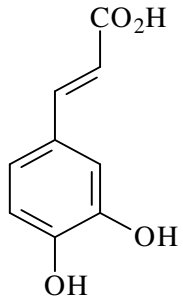
2.1 Introduction

Secondary plant metabolites (also referred to as phytochemicals) are low molecular weight compounds that do not play a role in the primary plant metabolism[1-2] They are compounds derived biosynthetically from primary metabolites in specialized cell types and at distinct developmental stages thereby making their extraction and purification challenging to even, experienced chemists.[3] These secondary plant metabolites constitute the active ingredients of medicinal plants. Plant secondary metabolites are unique to each plant and do occur in low concentration. Their function or importance is mainly related to ecological aspects as they are used for defence against predators, parasites and disease, interspecies competition and facilitation of reproductive processes.[1-5] Secondary metabolites could be classified according to their basic skeletal structure.

2.2 Classification of Phytochemicals

2.2.1 Simple Phenols and Phenolic Acids

Simple phenols and phenolic acids are secondary metabolites with only one single substituted phenol ring. Caffeic acids (**2.1**) found in herbs, thyme and tarragon belong to this class of compound and known to be strong antifungal and antiviral agent. Eugenol (**2.2**) found in cloves and alfalfa possesses good antibacterial and antifungal activity which accounts for its uses in tooth and mouth infection. [2, 6-9]



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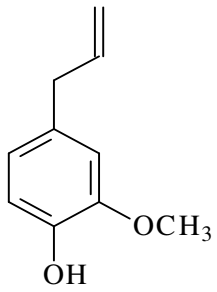

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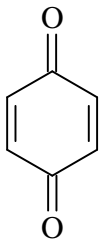
2.2.2 Quinones

These are group of aromatic compounds with two ketone substitutions for example benzoquinone (**2.3**). They differ from the simple phenol class of compounds by the oxygen-carbon group as against hydroxyl group in the former.



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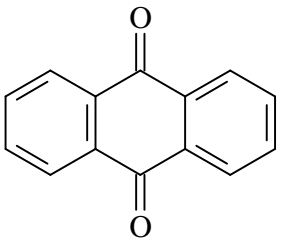
Quinones are very reactive class of compounds occurring in various forms in nature. They are responsible for the brown colouration that occurs when a plant is cut or injured. They have been proven to be good antimicrobial and antidepressant agents.[2] Some examples of naturally occurring quinones are naphthoquinone (**2.4**), hydroquinone **2.5**) and ubiquinone (**2.6**)[8-9].



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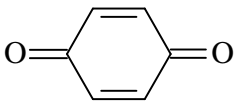

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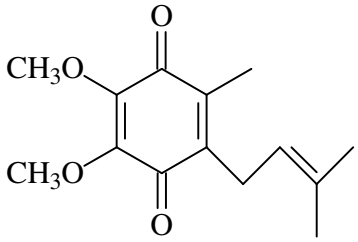

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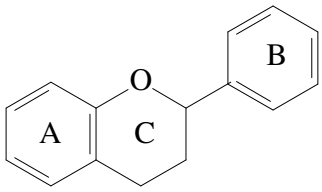
2.2.3 Flavonoids

Flavonoids are hydroxylated phenolic compounds with a flavan nucleus consisting of 15 carbon atoms in a C_6-C_3-C_6 arrangement which is labelled A, B, C in carbon frame (**2.7**).[9]



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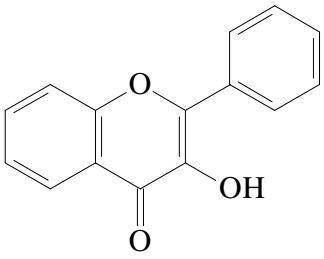
Flavonoids have been further classified as follows: flavonols (**2.8**), flavones (**2.9**), isoflavones (**2.10**), flavanones (**2.11**), coumarins (**2.12**) and anthocyanidins (**2.13**).



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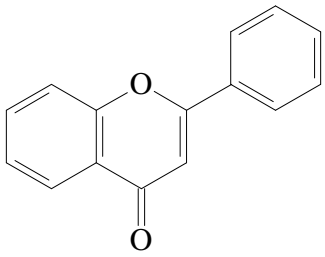

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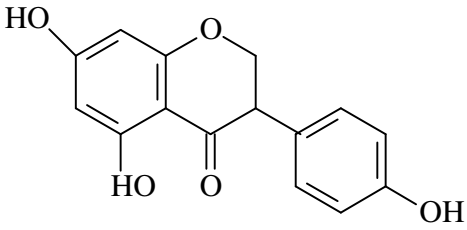

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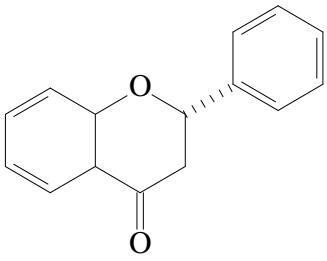

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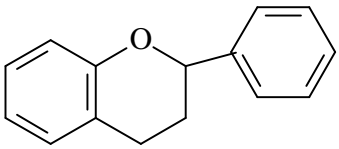

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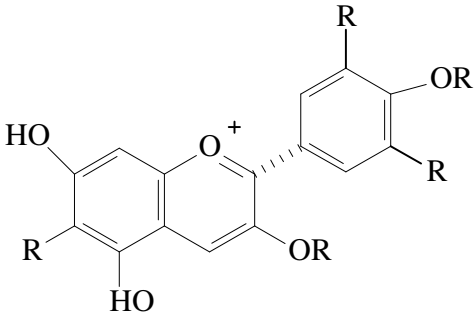
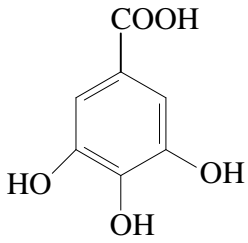


2.13

Flavonoids are the most studied group of secondary plant metabolites found in vegetables, fruits, nuts and beverages.[10] They are known to have anti-inflammatory, anti-allergic, antiviral, antispasmodic and diuretic effects. Flavones are class of compounds that are generally water-soluble pigments in plants and are responsible for the colour of flowers and fruits. Some flavonoids are synthesized by plants in response to microbial invasion and hence they are referred to as phytoalexins but not all flavonoids are phytoalexins.[11] Flavonoids have been found to be good *in vitro* antimicrobial agents.[8-14]

2.2.4 Tannins

These are polymeric phenolic compounds that can precipitate as gel from a solution.[2] They are classified into hydrolysable- and condensed-tannins according to their structure and origin. [9] Hydrolysable tannins are esters of a sugar and a phenolic acid (**2.14**) while condensed tannins are polymeric flavonoids (proanthocyanidins), for example (**2.15**) that decompose into anthocyanidins at high temperature when treated with acids.[2,8,9]



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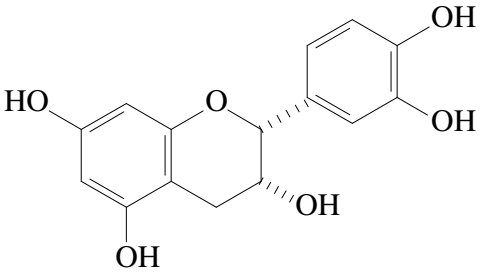
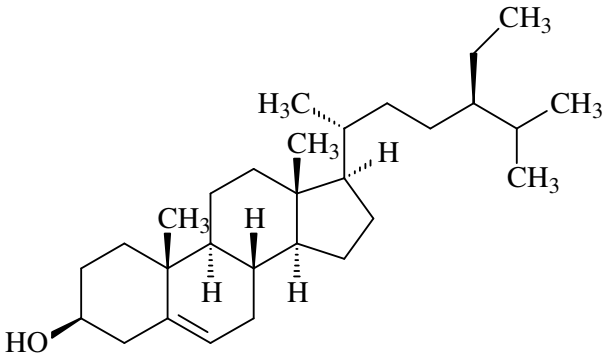


2.15

Tannins are good antimicrobial agents which precipitate protein thereby providing waterproof layer on the skin when used externally or protect the underlying layers of the skin and limit the loss of fluid. They are also known to be good antiviral agents.[6,10]

2.2.5 Saponins

Saponins are secondary metabolites that occurs in many plants with a characteristic skeletal structure derived from a 30-carbon precursor oxidosqualene to which glycosyl residue are attached.[15-16] Saponins are non-volatile, surface active compounds with soap-like behaviour in aqueous solutions.[8, 9, 15, 16] They have wide application in beverages, confectioneries as sweeteners, cosmetics and pharmaceutical products.[15,16] They are subdivided into triterpenoid and steroidal saponins based on their biosynthesis.[17] The difference between the two classes is that steroidal saponins have three methyl groups removed (**2.16**) thus having 29 carbon atoms in their molecule while triterpenoids retain their 30 carbon atoms (**2.17**). However, recent review has classified saponins into three subgroups namely triterpenoid saponins, spirostanol saponins, and furostanol saponins which is based on structural elements due to secondary biotransformations.[18]



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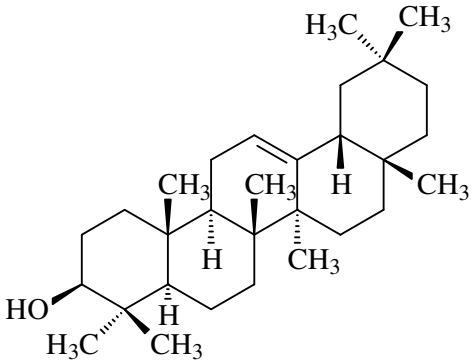

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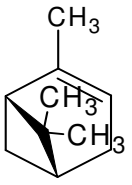
2.2.6 Terpenes

Terpenes are a wide group of natural compounds with significant biological importance.[19] They are class of secondary metabolites that are made up of isoprene units ($C_5H_{10} = C_5$). The linking up of isoprene units produces sub-classes of terpenes named as follows: monoterpenes (C_5 x 2 = C_{10}), sesquiterpenes (C_5 x 3 = C_{15}), slightly different from here diterpenes (2 x C_{10}= C_{20}), triterpenes (2 x C_{15} = C_{30}), and tetraterpenes (2 xC_{20} 8= C_{40}).

(i) Monoterpenes

Monoterpenes are the simplest members in the terpenes series and are C_{10} compounds. They are obtained by the head to tail coupling of two isoprene units (see Section 2.6.2), and are widely distributed in nature with more than 400 naturally occurring monoterpenes identified. They are commonly found in essential oils.[8] Examples of monoterpenes include α-pinene (**2.18**), camphor (**2.19**), limonene (**2.20**) and 1,8-cineole (**2.21**).



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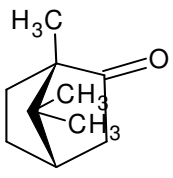

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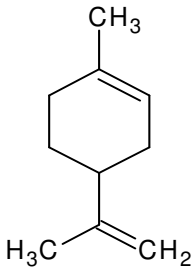

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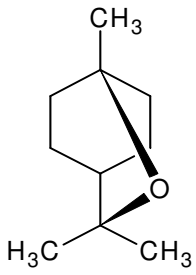

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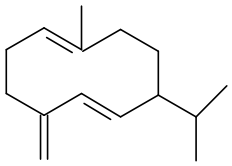
(ii) Sesquiterpenes

Sesquiterpenes are C_{15} compounds which are constituents of many essential oils plants. Examples of sesquiterpenes include germacrene D (**2.22**), humulene (**2.23**), δ-cadinene (**2.24**), and caryophyllene (**2.25**). They constitute a very large group of secondary metabolites, some having been shown to be stress compounds formed as a result of disease or injury.[19] They occur in plant families like the *Asteraceae.* Sesquiterpenes lactones (**2.26** and **2.27**) are of interest from chemical and chemotaxonomic viewpoints, but also possess antitumor, anti-leukaemic, cytotoxic and antimicrobial activities.[8,20] They can be responsible for skin allergies in humans and they can also act as insect feeding deterrents.[8,20]



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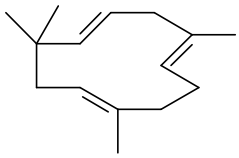

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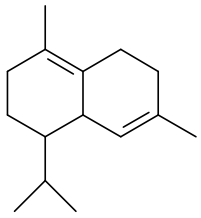

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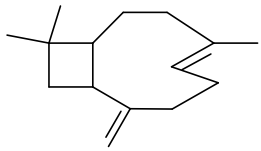

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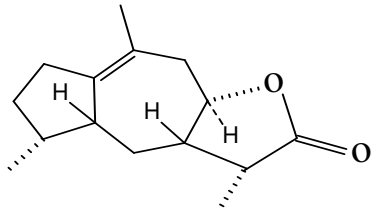

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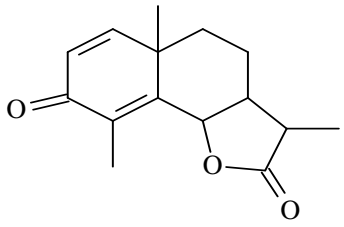
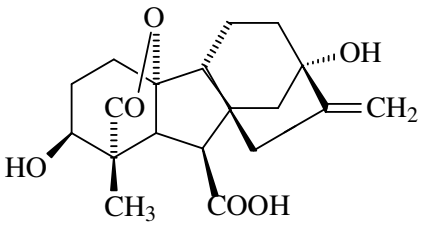


2.27

(iii) Diterpenes

Diterpenes constitute a vast group of C_{20} compounds arising from the metabolism of 2*E*, 6*E*, 10E-geranylgeranyl pyrophosphate. They are present in animals and plants and there are about 2500 known diterpenes that belong to 20 major structural types.[8] Plant hormones, for example gibberellins e.g. GA1 (**2.28**) and phytol (**2.29**) occurring as a side chain on chlorophyll are diterpenic derivatives.[8, 9] Some diterpenes have therapeutic applications.[20]



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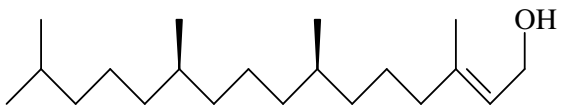
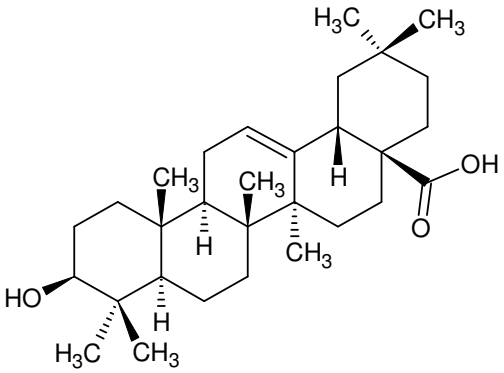


2.29

(iv) Triterpenes

Triterpenes are C_{30} compounds arising from the cyclization of 3*S*-2,3-epoxy-2,3-squalene.[8] Pentacyclic triterpenes and steroids have similar structures (A-B-C- rings) but have different biosynthetic pathway. The pharmaceutical applications of triterpenes and steroids are of great interest. The most important triterpenoids structures are the oleanane, ursane, lupane and dammarane-eupane. Triterpenoids are studied for their anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory and tonic effects.[21] Oleanolic acid (**2.31**) and betulinic acid (**2.32**) are triterpenoids known to have inhibitory effect on the HIV pathogen.[22, 23]



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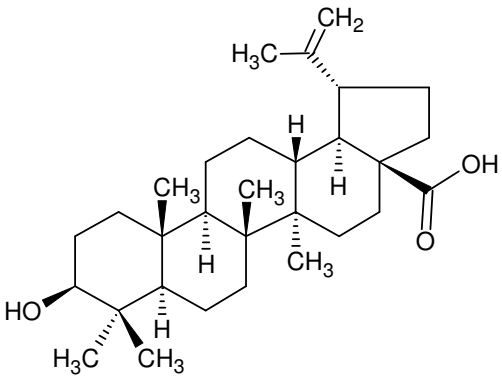
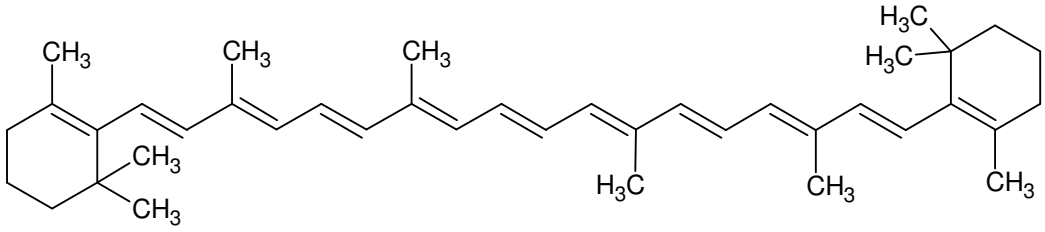


2.32

(v) Tetraterpenes

Tetraterpenes contain eight isoprene units. Important among these are the C_{40} yellow or orange-red carotenoid pigments of which about 180 have been reported. Carotenes, e.g. β-carotene (**2.33**) in association with chlorophyll participate in photosynthesis, but also occur in other plants' organs as the carrot (β-carotene 30). Among these compounds, the hydrocarbons are collectively referred to

as carotenes and the hydroxylated derivatives as xanthophylls e.g. crypoxanthen (**2.34**). Xanthophylls are the yellow pigment of leaves and they are from the carotene family.[8-9]



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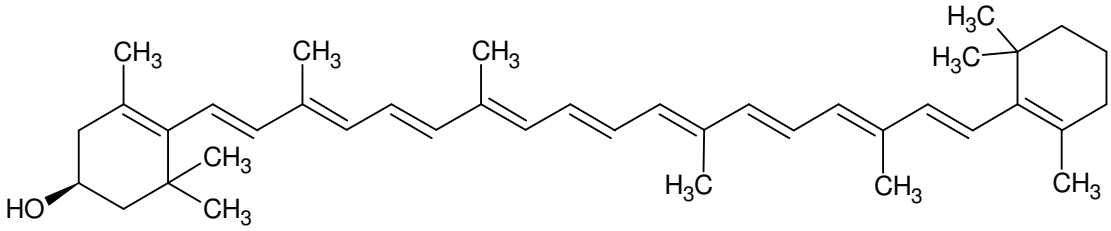
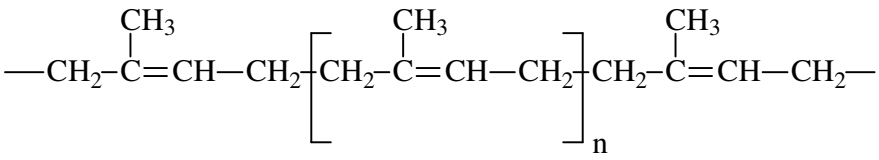


2.34

(vi) Polyterpenes

Polyterpenes, for example, rubber (**2.35**), consist of long chains of many isoprene units, an example of rubber having macromolecules of molecular weight over 100,000, is found in India rubber and *gutta-percha.* [6-8, 11]



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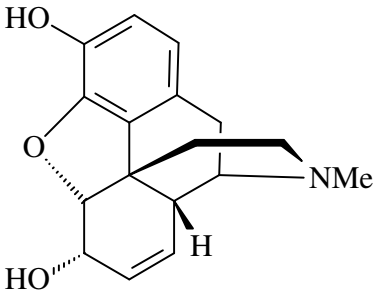
2.2.7 Alkaloids

Alkaloids are a large group of cyclic compounds that contain at least one nitrogen atom in the ring system.[8, 9] They are further sub-grouped according to their skeletal structure. The presence of the nitrogen atom in alkaloids makes them alkaline in nature. They are known to have good pharmacological properties.[9, 24] Examples of pharmacologically active alkaloids include, morphine

(**2.36**) as antidepressant; caffeine (**2.37**) as stimulant; cocaine (**2.38**) as anaesthetic; vinblastine (**2.39**) as antitumor; quinine (**2.40**) as antimalaria; berberine (**2.41**) as antibacterial; and emetine (**2.42**) as amoebicide.[1, 8, 23, 25]



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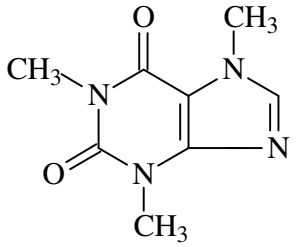

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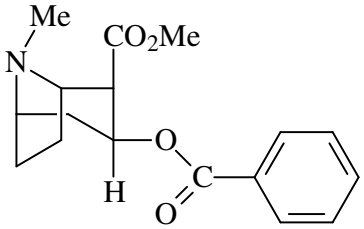

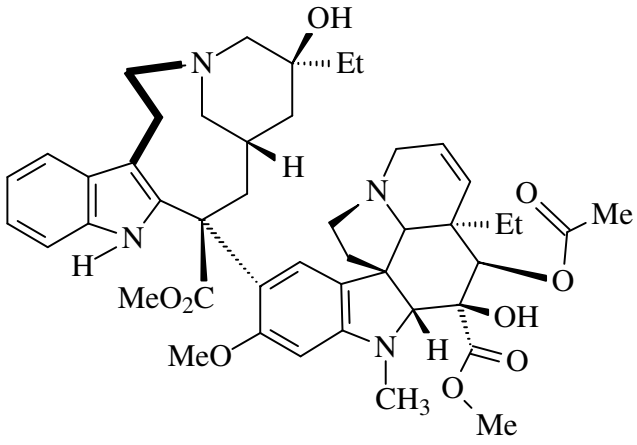

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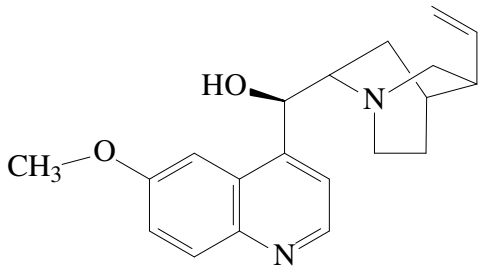

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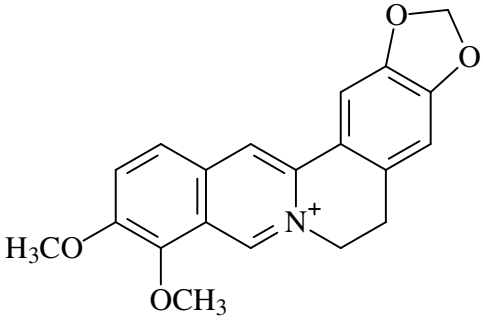

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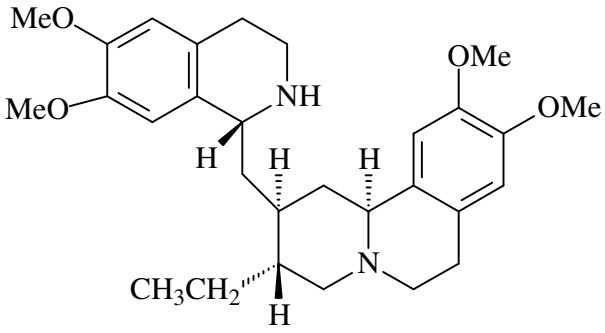
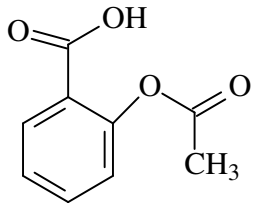


2.42

2.3 Examples of Drugs Developed from Phytochemicals

2.3.1 Aspirin

Aspirin (**2.43**) also known as acetylsalicylic acid is often used as an analgesic to relieve minor aches and pains.[25] It is also used as an antipyretic to reduce fever and as an anti-inflammatory medication. Aspirin is probably the most widely used pain killer in the world. It has also been used to treat toothaches, headaches, arthritis and other painful maladies for 100 years.



2.43

Aspirin also has an antiplatelet, or "anti-clotting" effect and is used in long-term, low doses to prevent heart attacks, strokes, and blood clot formation in people at high risk for developing blood clots. Aspirin was the first member of the class of drugs known as non-steroidal anti-inflammatory drugs (NSAIDs) to be discovered. The NSAIDs are not all salicylates like aspirin, but they all have similar effects and mechanism of action.[25-26]

It is relevant at this point of this thesis to give a short historic view of the discovery and development of Aspirin. In mid 18th century, the bark of *Salix alba* (Salicaceae) (Figure 2.1) known as (willow plant) was found to be rich in salicylate-compounds which were effective for treating fever, pain and inflammation. By the 19th century pharmacists were experimenting with

and prescribing a variety of chemicals related to salicylic acid, the active component of willow extract. A French chemist, Charles Frederic Gerhardt, was the first to prepare acetylsalicylic acid in 1853 (patented under the name aspirin on March 6, 1899). He synthesized salicylic-acetic anhydride by reacting acetyl chloride with a sodium salt of salicylic acid (sodium salicylate). This preparation of aspirin ("salicylic-acetic anhydride") was one of the many reactions of Gerhardt.[25, 27, 28] Six years later, von Gilm obtained analytically pure acetylsalicylic acid by a reaction of salicylic acid and acetyl chloride. In 1869 Schröder, Prinzhorn and Kraut repeated both Gerhardt's (from sodium salicylate) and von Gilm's (from salicylic acid) syntheses and concluded that both reactions gave the same compound—acetylsalicylic acid. They were the first chemists to assign the correct structure to Aspirin. By 1897, scientists at the drug and dye firm Bayer, began investigating acetylsalicylic acid as a less-irritating replacement for the standard common salicylate medicines at that time and by 1899; Bayer branded the acetylsalicylic acid as *Aspirin* and was selling it around the world.[25]

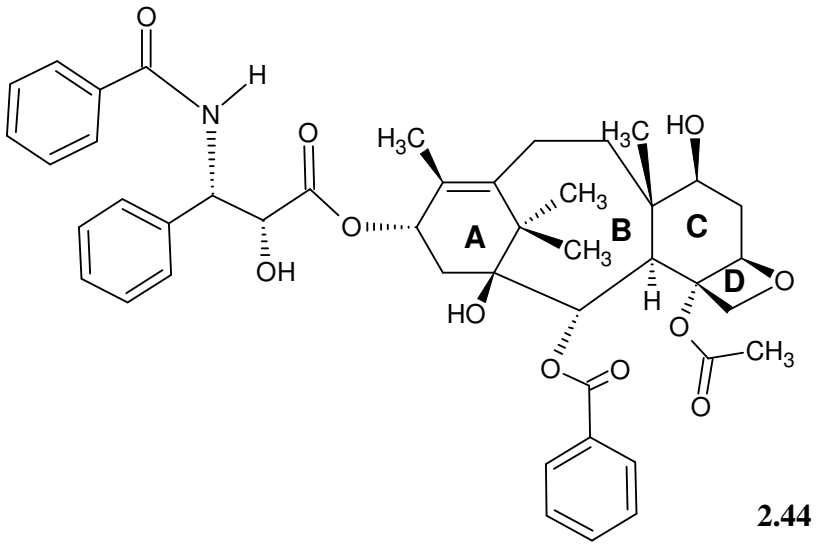
Figure 2.1: *Salix alba* Tree

2.3.2 Taxol

Taxol (**2.44**) also known as Paclitaxel is a natural product extracted from the stem bark of *Taxus brevifolia* (Taxaceae) (figure 2.2) to fight cancer. It was first collected in August 21, 1962 by a team of botanists led by Arthur Barclay from Pacific Northwest forests, Packwood, Washington. Taxol is now used to treat patients with lung, ovarian, breast cancer, head and neck cancer, and advanced forms of Kaposi's sarcoma.[28-31] Taxol has been well established and approved by Food and Drug Administration (FDA) as a very important effective chemotherapeutic agent against wide range of tumors since 1992.[32]

Figure 2.2 *Taxus brevifolia* Nutt (Specimen presentation and Stem Bark)

Taxol is a complex and highly oxygenated diterpene molecule commonly found in most *Taxus* species, although in small yields. All species and subspecies of the genus *Taxus* produce taxol-like compounds termed taxoids and over 350 distinct taxoid compounds have been isolated.[33] Cancer is a disease that is known to exhibit un-controlled cell division and taxol has the ability to inhibit the malignant tumour cell from growing. This is achieved when taxol inhibits the tubulin molecules of the chromosomes which are responsible for cell division, thus interfering with the cancerous growth.[8] Furthermore, the slow release of a cytostatic dose of taxol over an extended period, thereby reducing the neointimal growth after stent deployment make the demand for taxol is high.[33]



2.44

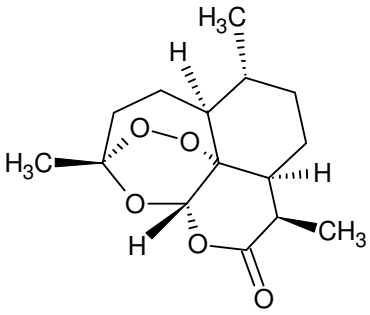
Figure 2.3 Structure of Taxol

The supply of taxol has been the major challenge facing the development and availability of taxol as an anti-cancer drug. The laboratory synthesis of taxol posed serious technical challenges. The

structure of taxol consists of rings A, B, and C (Figure 2.3) along with an oxetane ring, D, N-benzophenylisoserine side chain and the benzoate group. The total synthesis of taxol by Holton *et al.*,[34] Nicolaou *et al.*,[35] and Danishefsky *et al.*,[36] gave a yield of about 2.4, 0.7, and 2% taxol respectively. Today, taxol is produced commercially by plant tissue culture technology.[37-39]

2.3.3 Artemisinin

Artemisinin (**2.45**) is a sesquiterpenes lactone obtained from the dried leaves or flower clusters of *Artemisia annua* (Asteraceae) plant (Figure 2.4) It is used as an anti-malarial drug to treat multi-drug resistant strains of *Plasmodium falciparum* malaria. It can be synthesized from artemisinic acid (**2.46**).[3]



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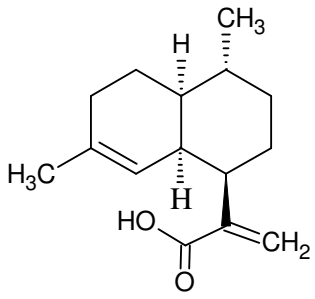


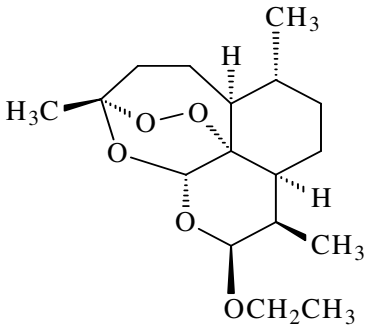
2.46

Figure 2.4: *Artemisia annua*

The antipyretic (fever-reducing) properties of *Artemisia annua* were first recognized in the 4th century by Chinese physicians, who called the plant *qinghao* and recommended a natural remedy in the form of *qinghao* tea. In the centuries that followed, *quinghao tea*, was commonly prescribed for haemorrhoids and malaria. The active agent, called *qinghaosu*, was isolated from the plant in the 1970s; this compound became widely known as artemisinin.[40, 41]

2.3.4 Arteether

Arteether (**2.47**) is a sesquiterpenes lactone isolated from *Artemisia annua* L. It is a potent anti-malarial drug which can also be derived from artemisinin (**2.45**).[44]



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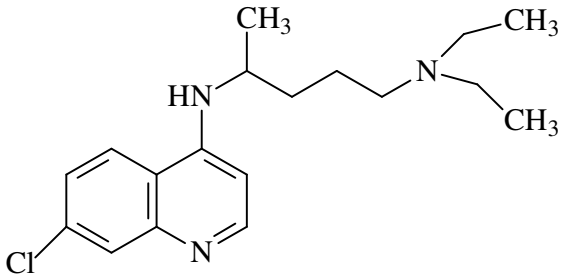
2.3.5 Chloroquine

Chloroqunine (**2.48**), *N*'-(7-chloroquinolin-4-yl)-*N*,*N*-diethylpentane-1,4-diamine, is a 4-aminoquinoline drug. It is used for the treatment and prevention of malaria.[45] Chloroquine has also been clinically tested and found to have some antiretroviral activity in humans with HIV-1/AIDS and as a potential antiviral agent against chikungunya fever.[45-47] One of the earliest natural compounds that highlighted the use of natural product to combat malaria was quinine (**2.40**) which was isolated from the bark of *Cinchona* (Rubiaceae) tree bark and serves as templates, for the development of structurally simpler analogues such as chloroqunine (**2.48**).



2.48

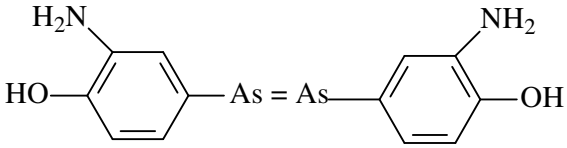
Many examples of drugs developed from natural products are discussed in a review by Butler, M.S.[48]

2.4 Chemotherapeutic Agents

Chemotherapeutic agents are substances that are specifically toxic to parasitic organism invading man's body. They are usually given over a period of time. These agents have the ability to attack and reduce or kill the infected cells causing the illnesses. Due to the large number of illnesses, chemotherapeutic agents are classified as follows:

2.4.1 Antibacterial Agents

Bacteria are single-cell microorganisms which were first identified in the 1670's by van Leeuwenhoek. However, the link of bacteria to diseases was only known in 19th century when a scientist name Koch was able to identify microorganisms responsible for tuberculosis, cholera and typhoid diseases.[49] This discovery led to the study of vaccines and antibiotics in the middle 19th century. In 1910 Ehrlich first synthesised an antimicrobial drug called salvarsan (**2.49**).[49] Salvarsan was found not to have a broad inhibitory effect on a wide range of bacteria but was more effective in inhibiting protozoal borne sleeping sickness diseases. By 1934, proflavin (**2.50**)[49] was introduced into the market and was used during the Second World War against bacteria infected wounds. Proflavin was short lived because of its toxicity.



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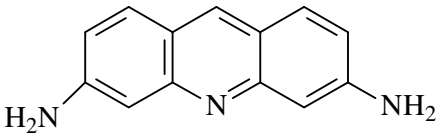
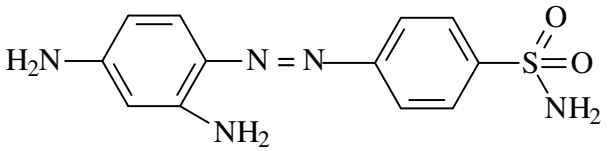


2.50

Prontosil (**2.51**) a sulphur drug was discovered in 1935 which is effective against streptococcal infection. Penicillin (**2.52**) was successfully isolated from *Penicillium notatum* and came into use in the 1940s.



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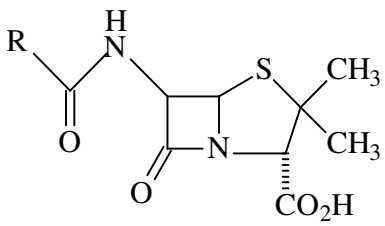
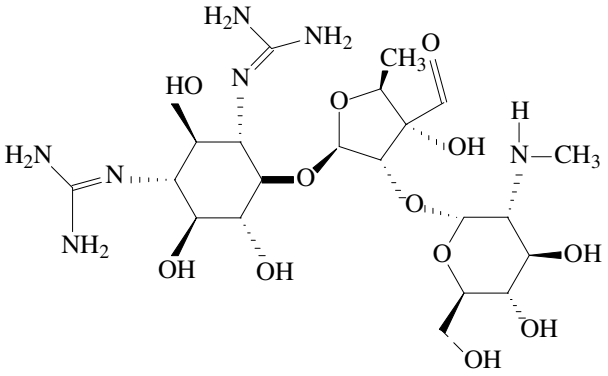


2.52 (R =Ph)

This was a breakthrough in fighting bacteria borne-diseases. Streptomycin (**2.53**), chloraphenicol (**2.54**), tetracycline (**2.55**), erythromycin A (**2.56**), cephalosporin C (**2.57**) and their derivatives were either isolated or synthesized between 1945 and late 1960s.[49,50]



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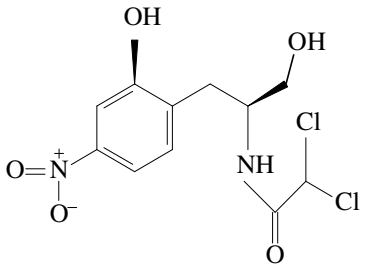

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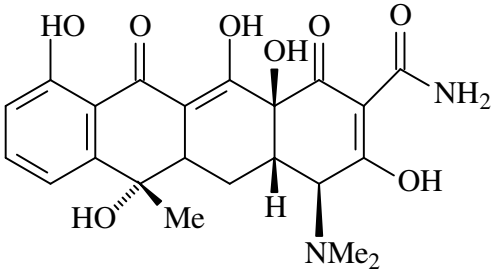

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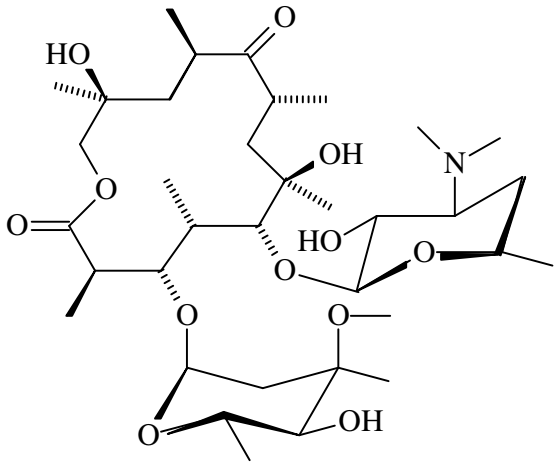

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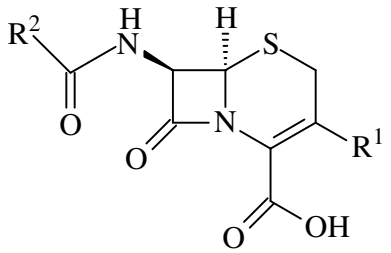


2.57

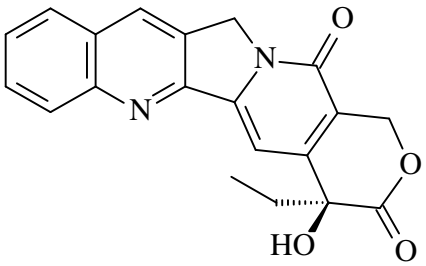
($R^1 = CH_2OCOCH_3$, R^2= $HOOCCH(NH_2)CH_2CH_2CH_2$)

The resistance of most of these drugs to bacterial infection helped in the discovery of more antibacterial agents from either natural source or synthesized drugs.[49]

2.4.2 Anticancer Agents

Cancer is one of the most feared diseases in the world; it is the second largest cause of death after heart disease.[49] Cancer cells are formed when normal cells lose the normal regulatory mechanism that controls growth and multiplication. Cancer can be localized or could be malignant. If the cells are localized they are known as tumour but when the cancer cells are malignant they invade other parts of the body and set up secondary tumour. The malignant cancer cells are life threatening. Proto-oncogenes [49] and DNA helps to control the cell division in the body and repair damaged DNA cells. If the proto-oncogenes are seriously affected such that normal function of the cells are disrupted then the cell becomes cancerous. The inability of the DNA to repair the damaged DNA leads to tumour. However, there are three traditional treatments of cancer namely surgery, radiotherapy and chemotherapy.[49-51]

Most anticancer drugs work by disrupting the function of DNA, these drugs are classed as cytotoxic, others act on DNA directly, while some act directly by inhibiting the enzymes involved in DNA synthesis. Other side effect of most traditional anticancer drugs are impaired wound healing, loss of hair, damaged to the epithelium of the gastrointestinal tract, depression of growth in children, sterility, nausea and kidney damaged. Many natural products such as camptothecin (**2.58**), podophyllotoxin (**2.59**), genistein (**2.60**) and amsacrine (**2.61**) to mention a few have been found useful at various stages of the cell growth.[49]



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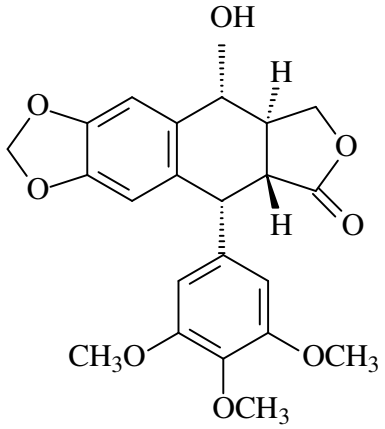

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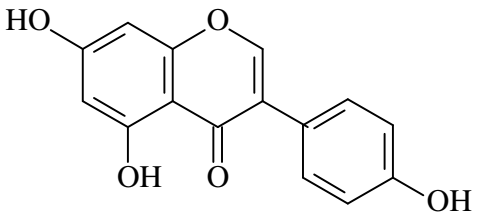

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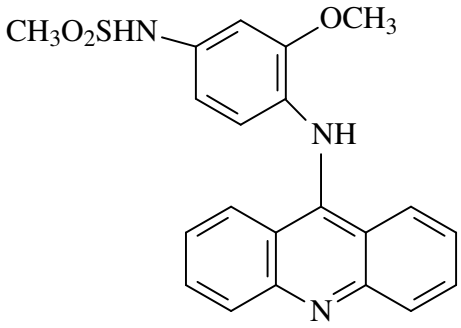

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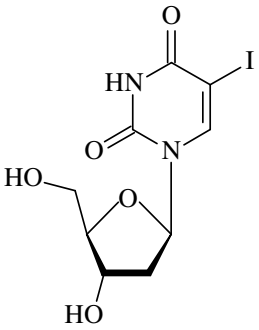
2.4.3 Antiviral Agents

Viruses are non-cellular infections pathogen which takes over a host cell in order to survive and multiply. Over 400 different known viruses infect man.[49] Influenza, chickenpox, measles, mumps, viral pneumonia, rubella and smallpox are air-borne viruses transmitted from an infected host through coughing or sneezing. Viruses can be transmitted by arthropods or ticks thereby causing Colorado tick fever and yellow fever. Some viruses cannot survive outside the host and are therefore transmitted through physical contact leading to for example HIV-AIDs, cold sores, common cold, genital herpes and certain leukaemia and rabies. Food-borne and water-borne viruses lead to diseases known as hepatitis A and E, poliomyelitis and viral gastroenteritis. Vaccination is the preferred method of protection against viral diseases. This is achieved by introducing the body to foreign materials which bears molecular similarity to some component of the virus but lacks the infectious nature or toxic effect. The body recognizing the fingerprints of the virus and builds an immunity against such virus.[49]

Antiviral drugs come handy in treating viral diseases when vaccines are ineffective. The first generation of antiviral drugs include idoxuridine (**2.62**) and vidarabine (**2.63**) for herpes infection and amantadine (**2.64**) for influenza A infection was introduced into the market in 1960s. By 1981, aciclovir (**2.65**) a relatively safe and non-toxic drug was used in the treatment of herpes simplex I and II infections.[49]



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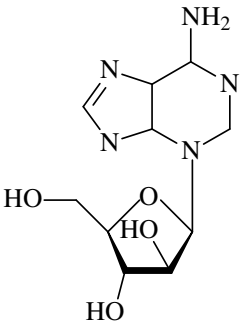

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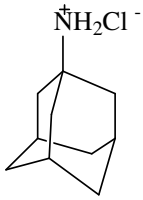

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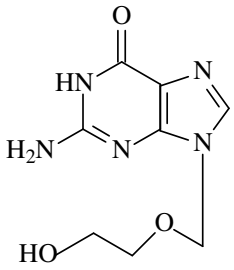

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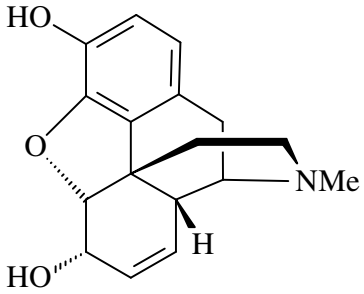
2.5 Drug Discovery, Design and Development[49, 52-55]

Man has always been afflicted by disease and illness. Herbs, berries and other substances from his environment has been used by man to treat illness. Evidences of these activities are documented as early as 2100 BC on Sumerian clay table where a number of recipes on the usage of some plants were written. By 1500 BC, the Egyptians compiled a list of remedies with over 800 recipes and a number of rituals incantations to help control diseases in man. However, 2000 – 3000 years ago, China and India made significant progress in combating diseases using natural source alone. In China till date, documentation of over 1892 entries of which 57% are from plants, 23 from animals and 14 from minerals are still in used and are rich source of interesting lead for therapeutic active agents of which artemisinin (**2.45**), an antimalaria agent, is one. Among the early natural substance to be investigated was the Opium plant in which Serturners in 1805, isolated morphine (**2.66**) although not in the pure form from opium. He holds the record for the first isolation of a pharmacological active substance from natural product. In 17th century, Jesuit missionaries introduced herbal remedies to Europe through the extract of the *Cinchona officinalis* bark from South America Indians for the treatment of chills and fever. This herbal remedy soon became the favoured medicine for fever, chills and malaria. By 1820, active compound quinine (**2.40**) was isolated by Runge. By 18th century in England, Withering introduced the use of an extract from foxglove plant (*Digitalis purpurea*) for the treatment of dropsy, a heart condition characterised by accumulation of excess liquid in the lower limbs of the patient.



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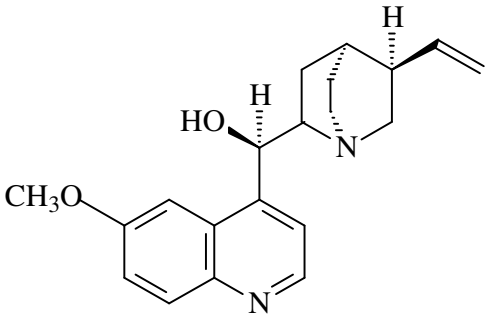

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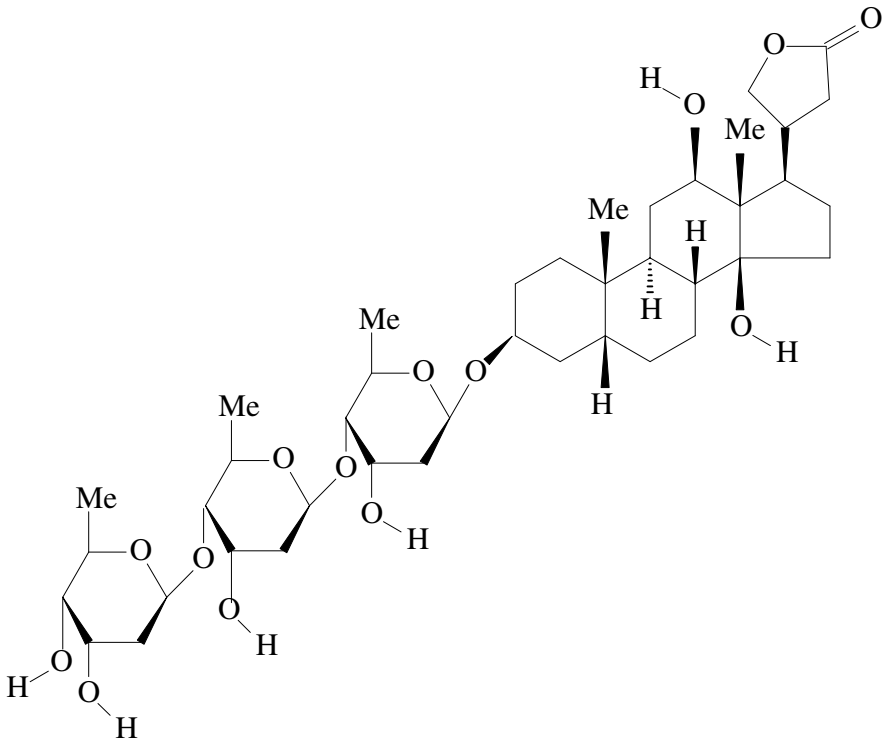
The active ingredient digoxin (**2.67**) was isolated by Smith in 1930 used for controlling threaten heart failure. The most beneficial innovation came in 19th century with the introduction of sedative drugs for use during surgical operations.



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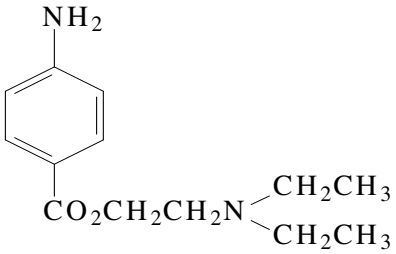
Today the risks and rigours of surgery are lessened with the array of pain-killers, anaesthetics, muscles relaxants and antiinfectives agents use. By the end of 19th century, the most widespread modern drug aspirin (**2.43**) was introduced into the market. In 1903 Eishom synthesized the first local anaesthetic procaine (**2.68**) while Ehrlich patented arsphenamine (**2.69**) the first man-made chemotherapeutic agent in 1909. The 1930s and 1940s witnessed the introduction of antibiotics

e.g. pencillin (**2.70)** and prontosil (**2.51**), which helped overcome the life threaten bacterial infections. The 1950s and 1960s saw the adverts of mental disorder drugs e.g. chlorpromazine (**2.71)**, imipramine (**2.72)** and tranylcycnopromine (**2.73**).



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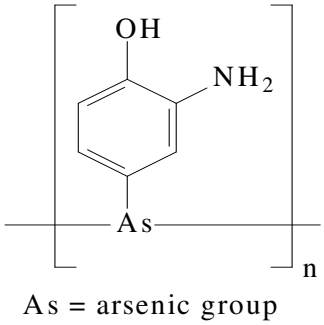

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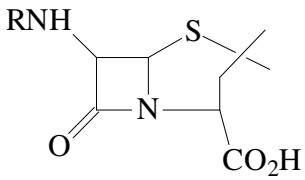

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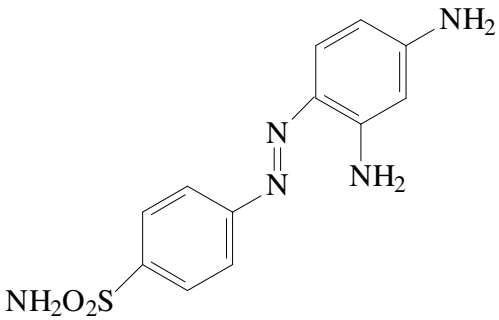

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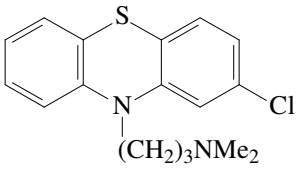

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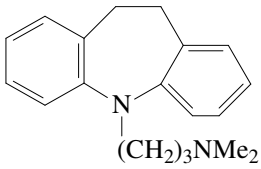

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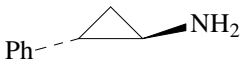

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Drug design is a multidisciplinary activity which involves the expertise of a chemist, biochemist, biologist, pharmacologist and many others in related fields. The role of the chemist in the discovery of a therapeutic agent commences at the discovery process. The process of drug discovery consists of two important processes:

i) The discovery of a new molecule as a lead structure which shows some elements of desired biological activity

ii) Modification of the lead structure until the desired pharmacological profile for the required therapeutic agent is obtained.

A lead compound is a prototype compound that exhibits some useful biological activity. This lead compound does not get to the market but serves as a starting material or point for the design of a new target molecule with

i) Optimized activity

ii) Reduced toxic or other undesirable side-effects

iii) Reduced problems of delivery to the site of action.

In rational drug design, there is a net work as shown in Figure 2.5 of four major activities as shown below before the lead compound is available to the public. A series of bioassay is carried out on the lead compound the result of data analysis is then used in assisting the molecular modelling of the lead compound.[52,53]

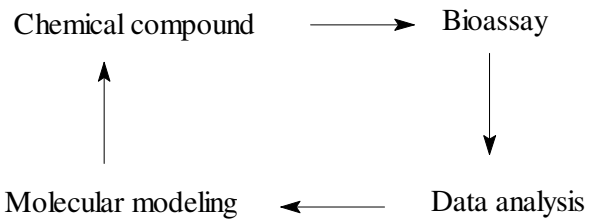


Figure 2.5: Drug design network [52]

2.6 Classification of Drugs

In the early days, drugs were classified and discussed by their chemical structure irrespective of their therapeutic property, but with the isolation of over 5000 compounds that have been characterised and investigated as potential therapeutic agents, effort have been made to classify them in a systematic manner. This classification is based on their therapeutic effect with subsections for specific disease. There are four major classes of drugs each with various sub-sections.[53]

2.6.1 CNS or Psychopharmaceutical Agents

i) Antipsychotic agents

ii) Anticonvulsants

iii) Analgesic

iv) Anti Parkinsonian agents

v) Antidepressants

vi) Anxiolytics
vii) Sedative-hypnotics
viii) Drugs for cognitive disorders
ix) Stimulants

The central nervous system of man comprises of the brain and spinal cord which controls the thoughts, emotions, senses and motor functions. Antipsychotic agents are drugs used in alleviating severe mental disorders. Anticonvulsants are used to control the involuntary convulsions of epilepsy while, analgesic are used as pain relieving drugs. Anti Parkinsonian agents are used for the treatment most motor functional illness such as tremors and gait disorder, Parkinson's disease. Antidepressants are used for people with severe depression. Alzheimer's disease which is known to be the illness of the elderly occurs as a severe and distressing loss of memory is treated using cognitive disorder drug. Sedative-hypnotics are used to promote sleep while stimulants are used to fight fatigue.[53]

2.6.2 Pharmacodynamic Agents

i) Antihypertensive agents
ii) Calcium modulators
iii) Antiischaemic (antistroke) drugs
iv) Antiulcer-gastrointestinal agents
v) Antithrombotic
vi) Pulmonary and antiallergic agents
vii) Antiarrythmics
viii) Antiglaucoma
ix) Vasodilators
x) Antianginals

Pharmacodynamic agents are used for the treatment of malfunctioning units of the body; they mostly act on the heart, respiratory system and gastrointestinal tract. Antihypertensive agents, calcium modulators, antiischaemic, antithrombotic, antiarrhythmic, antianginal and vasolidating agents all act on different parts of the heart and vasculatory system to help reduce the blood

pressure, heart rate or force and to improve blood flow. Pulmonary and antiallergic agents are used for lung and respiratory system disorder. Eye malfunctioning and glaucoma is treated with antiglaucoma agents. Antiulcer and gastrointestinal drugs are used to heal and prevent the recurrence of stomach ulcers and increase gastric motility.[53]

2.6.3 Chemotherapeutic Agents

i) Antimicrobial
ii) Antifungal
iii) Anticancer
iv) Antiparasitics
v) Antiviral

These are drugs used to fight the parasitic organism that invades the body. Antimicrobial agents act on bacterial infection, antifungal on fungal infections, antiviral on viruses and antiparasitics on parasitic infections such as worms, protozoa, for example, malaria parasite. Anticancer agents are used to attack, eliminate or reduce some rapid growing cancer cells and its spread.[53]

2.6.4 Metabolic Diseases

i) Contraceptives
ii) Antiandrogens
iii) Antiinflammatories
iv) Dermatologicals
v) Antiatherosclerotics
vi) Antriheumatics and autoimmune disease drugs
vii) Antiobesity agents

These are sometimes referred to as endocrine drugs to correct overproduction or underproduction of the body's natural hormone. Contraceptive agents are often used by women to interfere biologically with the conception process while antiandrogens are used by men in prostate cancer treatment. Antiinflammatories are used as palliatives for mild pain, joint disorders and rheumatism. Dermatological agents are often applied topically to alleviate skin disorder while antiobesity

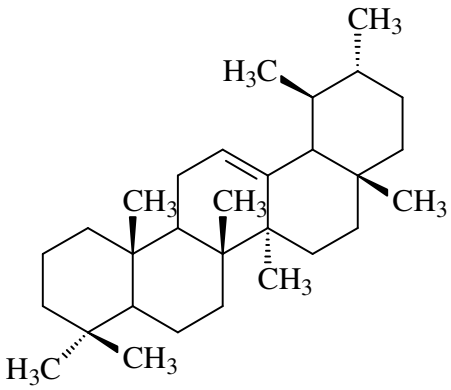
agents are used to correct eating disorder. True disease-remitting antirheumatic agents help modulating the underlying immune dysfunction.[53]

2.7 Bioactive Triterpenoids

2.7.1 Introduction

In view of present attention given to triterpenoids by researchers and the central position of triterpenes in this thesis, it is relevant to review some aspects of the chemistry of bioactive triterpenoids.

Triterpenoids are a diverse ubiquitous group of C_{30} compounds.[56] Triterpenoids, triterpenes, triterpenes glycosides (saponins), and triterpenic acids represent one of the numerous classes of secondary metabolites with mixed biosynthesis but with wide spectrum of biological activities.[19] They are bactericidal, antiviral, fungicidal, cytotoxic, analgesic, anticancer, spermicidal, cardiovascular and antiallergic.[19] Triterpenoids and their glycosides are represented by tetracyclic and pentacyclic skeleta and are derived from ursane (**2.74**), gammacerane (**2.75**), oleanane (**2.76**), betulane (**2.77**), lupane (**2.78**) and hopane (**2.79**).[19]



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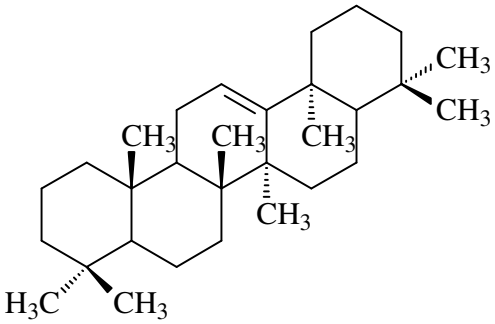

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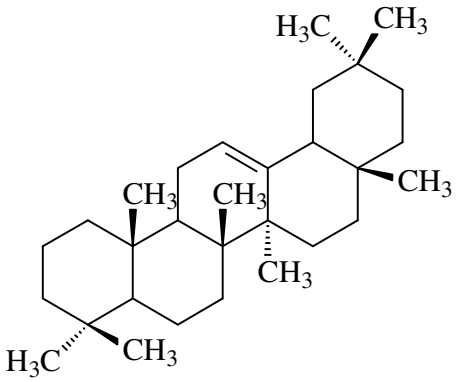

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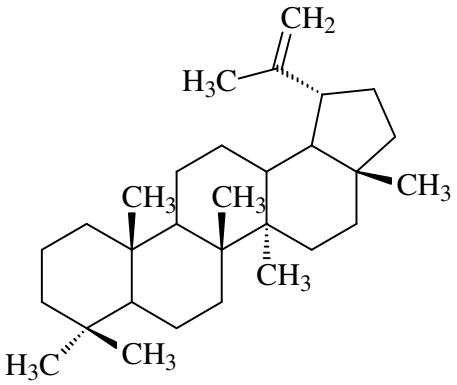

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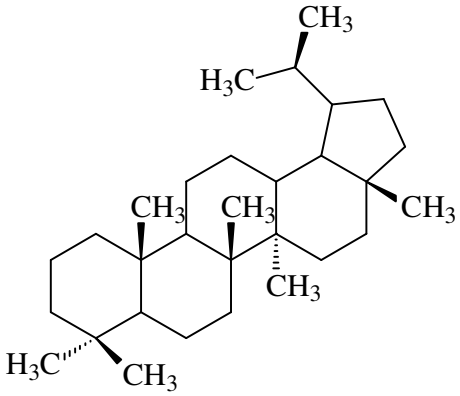

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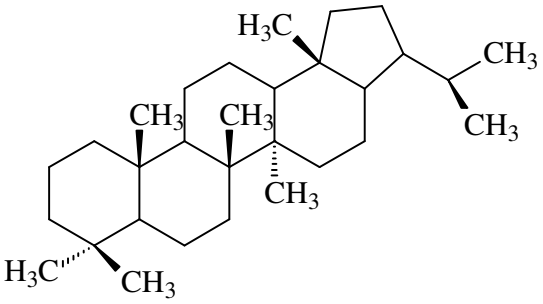

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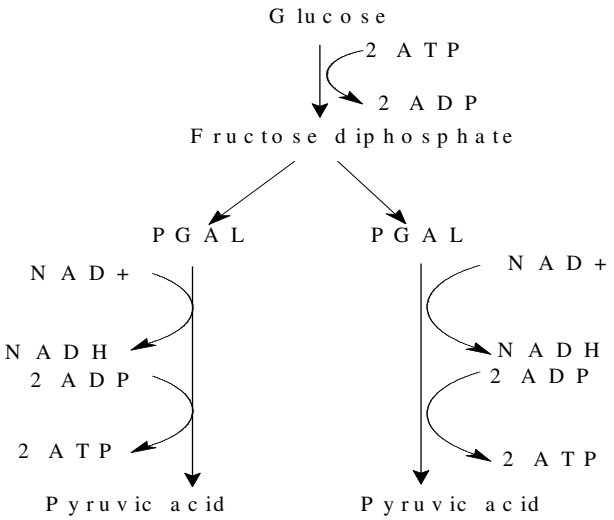
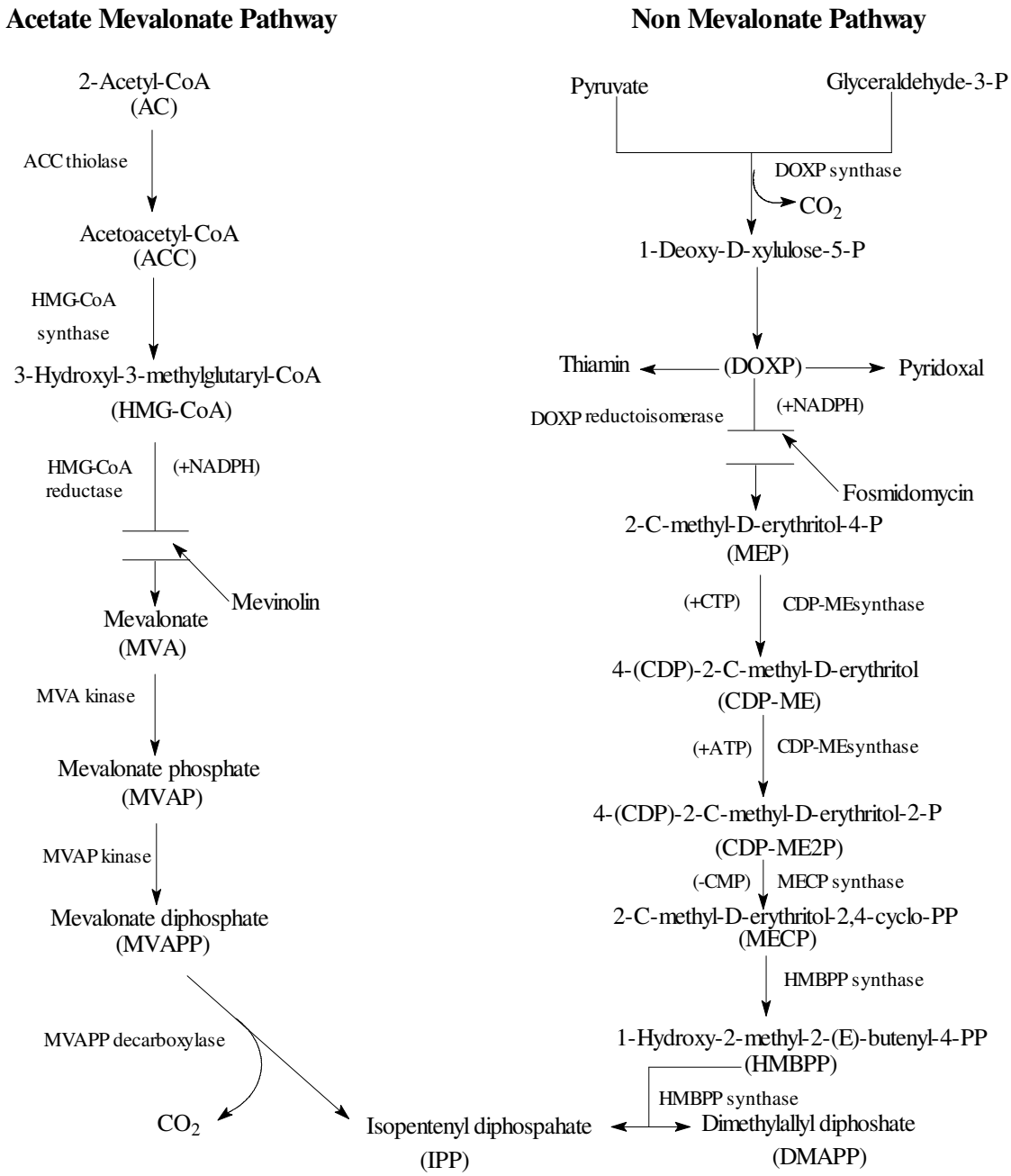
2.7.2 Biosynthesis of Triterpenoids

Biosynthesis in plants consists of two parts which are primary metabolism and secondary metabolism. In primary metabolism, carbohydrates, proteins, fats and nucleic acids are synthesized via a series of reactions, generally referred to as glycolysis. Glycolysis is the metabolic pathway that converts glucose ($C_6H_{12}O_6$) into pyruvate ($C_3H_5O_3$) (Figure 2.6). The energy released during this conversion is used to form the high energy compounds, ATP and NADH.[57] Some of these primary metabolites are further used by some specific living organisms for the secondary metabolism of secondary metabolites such as polyphenols, phenylpropanoids, triterpenoids and alkaloids. These secondary metabolites are derived from the intermediates acetyl coenzyme A (acetyl CoA), shikimic acid and mevalonic acid pathways.[9,57]



Scheme 2.1 Simplified glycolysis metabolic pathway

Terpenoids constitute one of the largest families of natural products with over 30,000 members. Terpenoids are known to be of great biological and physiological potential to man.[58,59] Terpenoid biosynthesis occurs mostly through the head-to-tail additions of isopentyl diphosphate (IPP) and DMAPP. Two pathways have been postulated for the synthesis of IPP and DMAPP in plants which are the acetate-mevalonate pathway which occurs in cytoplasm and the non-mevalonate pathway in plastids (Scheme 2.2). Triterpenoids (C_{30}) are formed when IPP is added to DMAPP in a head to tail addition giving rise to geranyl diphosphate (GPP, C_{10}). Condensation reaction of the enzyme-bound geranyl diphosphate to a isopentyl diphosphate results in a larger pentyl diphosphate known as farnesyl diphosphate (FPP C_{15}). Combination of two farnasyl diphosphate (Figure 2.8) gives squalene which is the precursor for triterpenes.[60]



Scheme 2.2 Two independent pathways for biosynthesis of IPP and DMAPP in plants [Adapted from Dubey *et al*. [60]]

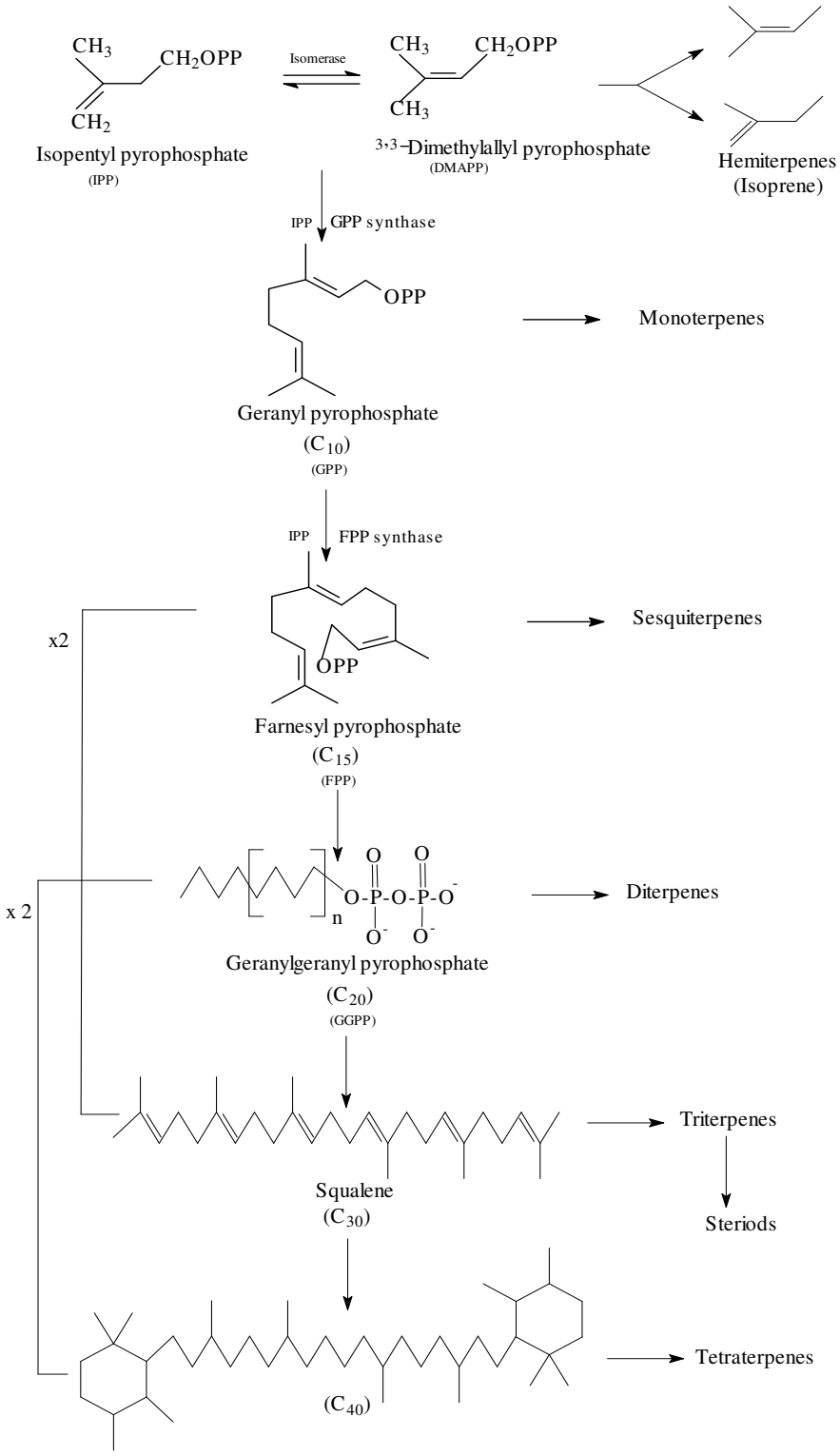


Figure 2.6: Biosynthesis of various classes of terpenoids in plants [Adapted from Dubey *et al.*[60]]

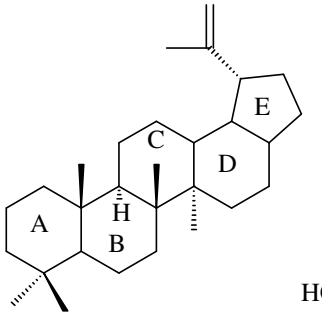
2.8 An Overview of Pentacyclic Triterpenoids

2.8.1 Introduction

Triterpenoids are the most ubiquitous class of natural secondary metabolites with about 400 compounds in the terrestrial and marine flora.[61] Triterpenoids can be divided into broad groups according to the number of rings in their chemical structure namely, (i) the tetracyclic group and (ii) pentacyclic group.[19] The pentacyclic triterpenes are further classified as follows : those comprising of 5 six-membered rings(6-6-6-6-6-6) (ursane, and olanane) or 4 six-membered rings and one five-membered ring (6-6-6-6-5) (lupane, and hopane).[19]

2.8.2 Lupane –Type Triterpenoids

Lupane triterpenoids are compounds with four six membered ring (A, B, C and D) while the E ring is a five membered rings (6-6-6-6-5) (**2.78**).[62] Betulin (**2.82**), betulinlic acid (**2.32**) and their derivatives are members with the lupane skeletal structure.



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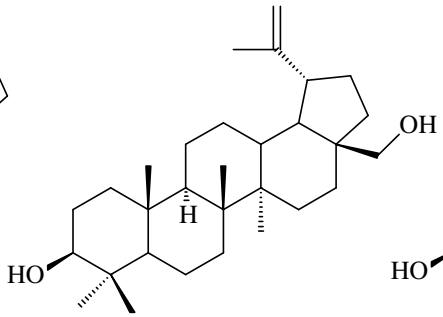

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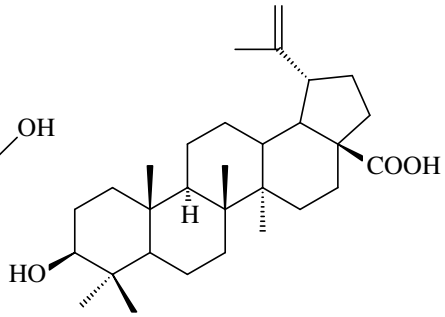

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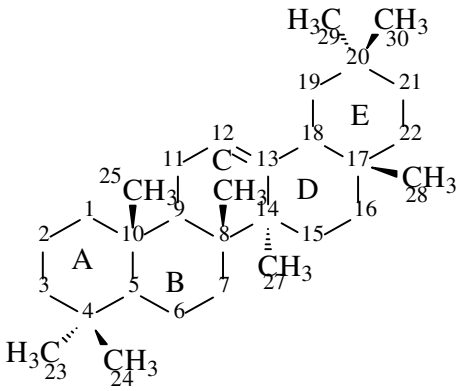
2.8.3 Oleanane and Ursane –Type Triterpenoids

Oleanane (**2.77**) and ursane (**2.75**) are biosynthesize by the cyclization of squalene.[63] They consist of five (A, B, C, D, E) rings which are six membered. In oleanane compounds the two methyl groups (29 and 30) are on carbon 20 while in ursane the two methyl group groups are on different carbons (19 and 20). Most common of these groups are the oleanolic acid commonly referred to as OA and ursolic acid referred to as UA. These groups of pentacyclic compounds are known for their anti-inflammatory activity, suppression of tumor promotion, protection of the liver against toxic injury, induction of collagen synthesis, and differentiation in leukemia or tertocarcinoma cells.[63]



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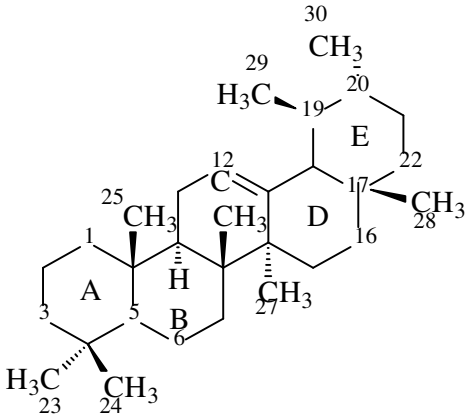

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2.9 Extraction, Isolation and Structural Elucidation

Extractive techniques with spectroscopic method of identification are often employed in the isolation and elucidation of natural products. Once the plant material is collected either by ethnobotanical or random method, the plant material is dried quickly to avoid degradation of components by air or microbes. The drying must be to a constant weight. Once this is achieved any of the solvent extraction techniques is used to extract various components in the plant material.[64]

2.9.1 Extraction, Purification and Isolation

The plant material is extracted in solvent of differing polarity at room temperature. Solvents used includes, hexane, methanol, ethanol, ethyl acetate, chloroform, dichloromethane, and water. This method of extraction allows maximum extraction of various components into like solvent. Sequential extraction (Figure 2.7) is one of the methods used for isolation of compounds from plant material. Maceration, percolation, accelerated solvent extraction, soxhlet extraction, supercritical fluid extraction (SFE), pressurized-liquid extraction (PLE), microwave-assisted extraction (MAE), solid-phase extraction (SPE) and solid-phase microwave extraction are other extraction techniques used in extracting compounds.

The extract obtained from the solvent extraction method is subjected to chromatographic methods for the isolation and purification of compounds. The most versatile laboratory technique for

separating and isolation of a vast variety of chemical compounds in micrograms to kilos is chromatography.

In chromatographic separation, the solute (extract) is distributed between the stationary phase and the mobile phase. Components of extract are separated according to how strongly they adsorb to the stationary phase against how much they dissolve in the mobile phase. Thin-layer chromatography (TLC) is the most commonly used technique for identifying component of an extract and purification of isolated compounds. It is faster and requires small quantities of sample, between $1\text{-}10^{-6}$g. It consists of a thin layer of stationary phase (usually silica) spread on an inert support which can either be glass, plastic or aluminium.[64-66]

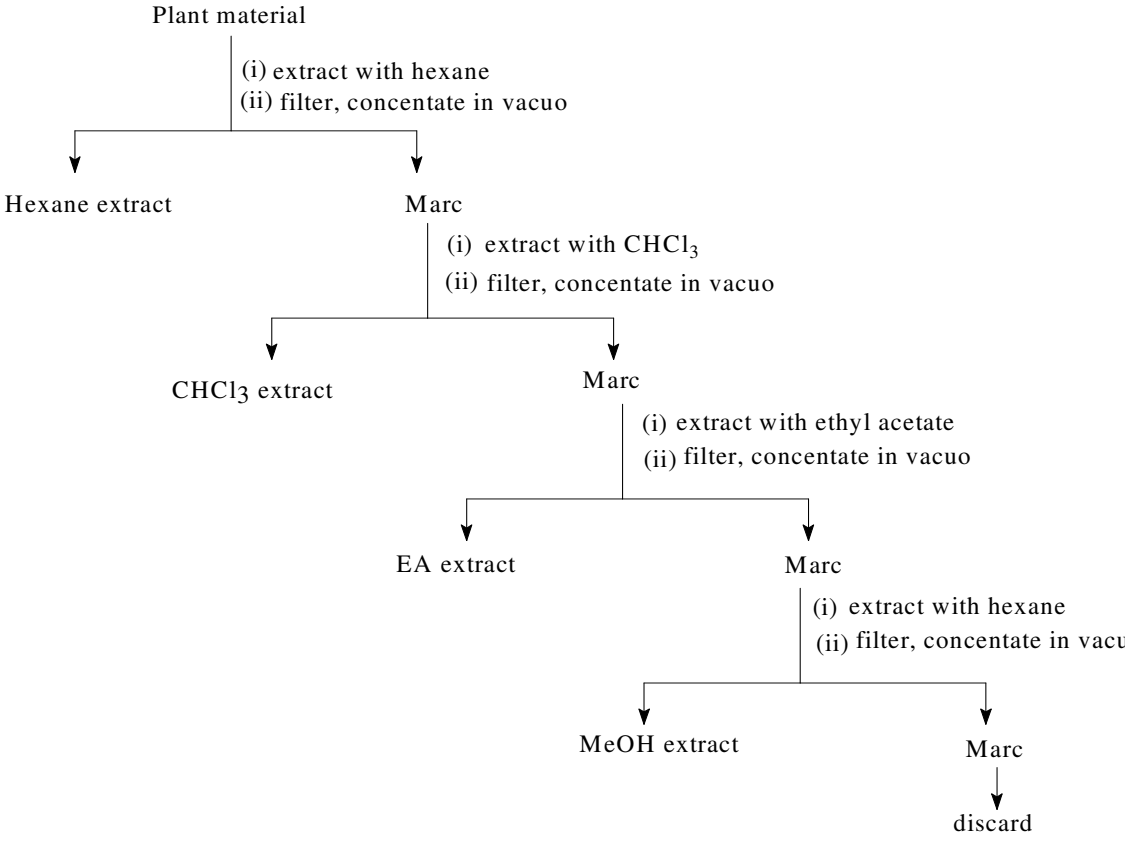

Figure 2.7: Sequential extraction of plant material

A. Column Chromatography (CC)

This method is employed when the extract consists of many components. The stationary phase which is a solid adsorbent, (for example, silica or alumina), is placed in a vertical glass column and

mounted on a retort stand. The sample to be analyzed is applied to the top of the column. The mobile phase, the eluent (solvent or mixture of solvents), is added to the top which flows down the column. Components of the sample separate from each other by partitioning between the stationary phase and the mobile phase. The eluent is collected in fractions and analyzed by TLC. When the solvent flows down the column through gravity or percolation, this method of column chromatography is known as gravity column chromatography. If pressurized gas or air is used to enhance the solvent movement down the column, it is referred to as flash column chromatography or medium pressure chromatography. Fine silica gel particles are used in flash column chromatography. It is rapid and gives high resolution.[65]

B. Vacuum Liquid Chromatography (VLC)[54]

This technique is similar to CC with a vacuum employ at the collection end of the column to speed up eluent flow rate. It differs from flash chromatography in that the column is allowed to dry after each fraction is collected.

C. High Performance Liquid Chromatography (HPLC)[66]

HPLC is the most powerful chromatographic technique that is currently employed for isolation of plant material. Applications of HPLC include separation, identification, purification, and quantification of compounds from mixtures. The stationary phase is a spherical shaped particles or a porous monolithic layer. Other chromatographic methods are ion-exchange chromatography, supercritical fluid chromatography, counter current chromatography and gel filtration chromatography.

2.9.2 Structure Elucidation of Compounds

Structure elucidation of pure isolates (compounds) can be done in many different ways. The common techniques are Nuclear Magnetic Resonance (NMR) spectroscopy, Mass Spectrometry (MS), Infrared (IR) Spectroscopy, and Ultraviolet (UV) Spectroscopy.

2.10 Essential oils

Essential oils are odoriferous oily products of plant materials extracted by distillation either by water or steam or by mechanical process in highly concentrated form. Essential oils are highly aromatic (odoferous) and volatile in nature and are derived mainly from the flowers, fruits, leaves and roots of many plants. However, essential oils are often found in the glands or intercellular spaces in the plant tissues. Essential oils are mostly concentrated in the seeds or flowers.[68-70] The oil usually bears the name of the plant from which it is derived, for example rose oil (genus *Rosa*, for example, *Rosa bracteata*) or peppermint oil (genus *Menta*, for example *Menta piperita*). Essential oils are stored as micro-droplets in the glands of the plant and the odour diffuses through the walls of the glands to the surface of the plant part before evaporating and filling the air with its aroma.[68] Most of the essential oils are insoluble in water but are soluble in organic solvents. Although, essential oils are immiscible with water but they are sufficiently soluble to impact their odour in water. Essential oils bear some characteristic odour, distinct high refractive indices and are optically active. [70, 71]

Plants, which contain essential oil, are widely spread in the remote geographical regions of the world including the tropical zone. Although plants growing in areas other than those in which they are indigenous often yield essential oils which vary slightly in chemical composition from the indigenous plants.[68] Temperate areas of the world produce about 150 important commercial essential oils from over 1,500 plants.[68]

The species and variety of a plant have direct relationship with the chemical composition of the essential oil. This includes the genetical background and its agronomic conditions such as harvest time and the type of processing used. Environmental and ecological characteristics of the growing region play important factors on the oil, by giving each aromatic plant of a particular species and variety its specific fingerprints, and reflects distinct organoleptic properties in relation to its composition. [72]

Essential oils are mainly mixtures of hydrocarbon and oxygenated compounds. Although the terpenoids constitute the major flavour and odour characteristics of the essential oils, the oxygenated compounds act as the principal odour carriers.[69] Essential oils are generally complex

mixtures of naturally occurring compounds or secondary metabolites and their utilisation in various industries is influenced by the nature of their constituents. Most of the groups of organic compounds found in essential oils include hydrocarbons, alcohols, ketones, aldehydes, oxides, esters with only a few essential oils contain a single component in high percentage.[69, 70]

The principal constituents of essential oils are the terpenes (which are the most abundant constituent), benzenoid and aliphatic compounds. The chemical composition of the essential oils are typically dominated by the occurrence of a wide variety of monoterpenoids and sesquiterpenoids with minor amounts of phenyl propanoids, diterpenoids and miscellaneous steam volatile organic compounds.[73] Most of the molecular structure of these terpene components has the basic skeleton isoprene units C_5H_8.[69,74] Several processes such as distillation, extraction with volatile solvent, expression, effleurage and maceration methods are employed in isolating essential oils. Expression, effleurage and maceration also utilised where high temperatures would have an undesirable effect on the essential oil. [70-71, 75]

2.10.1 Extraction, Analysis and Identification of Essential Oils

A. Extraction of Essential Oils [75-76]

The isolation procedure of essential oils can have a profound influence on their composition. Majority of essential oils is isolated from the plant through distillation.

(i) Distillation

This is the most widely used procedure for isolating plant volatile from non-volatile materials irrespective of whether the plants are fresh or dried. The technique permits raising the temperature of the distillates above 100^{o}C without the risk of decomposing the essential oils thermally. The major advantage of this method over solvent extraction method is that the isolates do not include non-volatile matter, which can interfere with the chromatographic analysis of the oil. The distillation method could either be direct distillation (hydrodistillation) in which the plant material is in direct contact with the boiling water or the indirect distillation (steam distillation) where wet or dry steam is introduced into the plant material in order to extract the volatile oils.

Hydrodistillation is a mild but slow process, which yields a superior product. In hydrodistillation, the plant sample is immersed in water in a quick-fit flask and heated to boil. The essential oil is thereby extracted from the glands of the plant during the boiling process and carried along with hot steam into a condenser where cooling takes place. The essential oil is automatically separated from the water phase.

In wet steam distillation, the plant sample is placed on a grid in a still and water is then positioned below the grid or it accumulates during the process. Steam is introduced into the flask from an external source directly onto the plant sample to extract the volatile components. Steam distillation is a rapid method of extraction, which could be used, for most essential oil extractions. In dry steam distillation on the other hand, the ground plant material is placed on the grid in the still. Direct steam is applied to the sample while the heat outside the flask supplied by a steam jacket at a temperature, which prevents any water condensation.
Clevenger-type distillation unit is widely accepted for hydrodistillation method. Other distillation methods used are vacuum distillation and vacuum degassing.

(ii) Solvent Extraction

This simple extraction method is based on the distribution coefficient of the volatiles between the solvent and the plant material. Boiling point determines the choice of solvent to be used in relation to the temperature of the volatiles to be isolated. In addition, solvents chosen must not react chemically with the essential oil extract and must be free of odour and impurities. Diethyl ether, petroleum ether, ethanol, pentane, dichloromethane are the common solvents used. The major defects of solvent extraction are the presence of contaminants such as colorants, tannin, pigment, partial loss of extracted volatile and the removal of excess solvent.

Combined steam distillation-extraction method proposed by Likens and Nickerson[75-76] is now being utilized. The advantages of this combined method include the high concentration of the volatile from a dilute solution, small amount of solvent used and reduction of thermal degradation using reduced pressure. The Likens and Nickerson apparatus, which consist of two separate flasks for the plant slurry and the solvent are connected through a condenser.[75-76]

(iii) Extraction with Fat

The process of absorption of essential oils into purified fat was developed for the treatment of flowers which do not yield appreciable quantity of oil through distillation. Fats possess high power of absorption such that it absorbs the fragrance of the flower along with the essential oil.[70] Alcohol is used to remove the essential oils from the fat.

(a) Effleurage

This involves the absorption of the volatile oils with cold fat in a special pressing equipment. This method extracts essential oils from flowers whose odour change when in contact with boiling water and steam. A mixture of tallow and lard are spread over the flower for a period of 24-72 hours in which most flower oils must have been absorbed by the fat.[70] The petals are removed and the process is repeated until the fat is saturated with oil and the final product is called 'Pomade'. The pomade is rarely used directly, rather, it is washed with ethyl alcohol so as to remove the essential oil and the alcoholic extract is known as 'Extant'. Its odour resembles that of the natural flower. The solution is frozen to remove traces of fat while the alcohol is removed by distillation in vacuum to yield the concentrated flower oil called 'Pomade Absolute'.[71]

(b) Maceration

This process is similar to effleurage. The difference is in the use of hot fat instead of cold fat. In addition, the process time of 1-2 hours is employed with the hot fat of about 45 ^{0}C to 80 ^{0}C.[70] The time differences which is a shorter period than in effleurage makes maceration process advantageous.

(iv) Extraction with Carbon dioxide

The use of carbon dioxide as an extraction solvent was first investigated in the 1960's. In 1980, extraction of essential oils with carbon dioxide (CO_2) became commercially accepted. Carbon dioxide (CO_2) penetrates plants quickly because of its low viscosity and low latent heat of evaporation making the carbon dioxide easily removable from the essential oil without any residue.[70,75] However, this method of extraction is expensive and the unusual balance of extracted essential oil component result is a drawback.

(v) Extraction through Expression

This procedure is used only for citrus oils. The outer coloured peels of the citrus fruit are squeezed; the essential oil is decanted and centrifuged to separate water from the cell debris.[70]

2.11 Analysis of Essential Oils

GC-MS (Gas Chromatography-Mass Spectrometer) is the method used for the analysis of the essential oil extracts. The GC-MS combines both functions of GC and MS. The sample mixture is injected into the column of the GC and is volatilized in the heated chamber. The volatilized mixture travels through the length of the column carried by the carrier gas sample, where it is separated according to the chemical properties of the compounds in the mixture. The separated compounds are eluted at different retention time, passed through the transfer line and enter the MS where they are ionized into fragments by either electron ionization (EI) or chemical ionization (CI). The ionized fragments are then detected using their mass-charge ratio. This is similar to MS procedure described in Section 2.6.4B above. GC-MS technique allows a finer degree of substance identification than either unit used separately.

2.11.1 Identification of Essential Oil Components [75-76]

A. Retention Time (t_R)

Retention time (t_R) is the time which elapses between injected sample and recording of the peak maximum, provided operational conditions are constant and is given as:

$$t_R = \frac{(1+k)L}{u} \qquad (2.1)$$

where k = capacity factor, L = column length, and u = true linear gas velocity in the column.

Adjusted retention time (t'_R) is the time from the point of injection to the peak maximum, minus the time required for unretained component to pass through the column t_M:

$$t'_R = t_R - t_M \qquad (2.2)$$

Retention volume (V_R) is the volume of gas passing through the column between injection of the sample and the maximum peak. Retention time is related to retention volume as follows:

$$t_R = \frac{V_R}{U_{av}} \qquad (2.3)$$

where U_{av} is the average flow rate through the column.

Earlier studies on essential oil analyses were performed by means of retention volume but since the introduction of capillary gas chromatography, retention time is frequently use.

B. Kovat Index

The retention time of a compound varies with temperature and flow rate, and it is impossible to maintain constant conditions throughout an experiment. Thus, reproducibility of retention times is not always possible. However, it is essentially important in the separation of components, that the retention time of a substance be measured relative to that of a reference substance on the same column and under the same conditions. The reference substance must be a known component of a mixture and could be added to the mixture prior to analysis. A homologous series of *n*-paraffin hydrocarbon is routinely used as the reference and the retention index system obtained is called Kovats retention index system. The kovat index is:

$$KI = 100N + 100n\ (\log t'_x - \log t_N / \log t_{R(N-n)} - \log t_{R(N)}) \qquad (2.4)$$

where $t_{R(N)}$ and $t_{R(N+n)}$ are the adjusted retention times of *n*-paraffin hydrocarbon of carbon number N and (N+n), respectively eluting before and after solute X with the adjusted retention times t.
Kovats indices for most known compounds on polar column (Carbowax 20M), nonpolar column (OV101) and medium polar column (DB-1) are now available.[78] Identification of some essential oil compounds is achieved through correlation with standard references regardless of the column. Table 2.1 displays Kovats index for some of these compounds. The Kovats index system is widely employed in essential oil analyses. Although the GC-MS spectrum gives information on the molecular mass and fragmentation pattern of the compound, better identification of essential oil compounds needs the combination of GC-MS information with Kovats retention index for accurate identification of the compound.[75-76]

Table 2.1 Kovats indices of some common essential oil constituents[76]

Compound	Carbowax 20M	OV-101	DB-1
α-Pinene	1020	940	941
α-Thujene	1036	938	934
β-pinene	1111	980	978
Sabinene	1118	975	975
Myrcene	1174	986	985
1,8-Cineol	1200	1028	1028
Limonene	1206	1030	1031
cis-Ocimene	1228	1025	1025
Trans-Ocimene	1250	1038	1039
γ-Terpinene	1257	1257	1255
p-Cymene	1257	1015	1015
Terpinolene	1273	1083	1082
n-Hexanol	1316	858	858
Linalool	1506	1092	1092
Camphor	1518	1136	1135
α-Terpineol	1661	1185	1182
α-Copaene	1519	1398	1398
β-Bourbonene	1546	1406	1406
α-Cedrene	1600	1436	1437
Caryophyllene	1617	1428	1427
β-Copaene	1626	1445	1444
Borneol	1682	1465	1465
α-Humulene	1698	1164	1164
α-Muurolene	1730	1500	1500
δ-Cadinene	1761	1524	1524
α-Ionene	1833	1416	1416
β-Ionene	1918	1474	1474
α-Nerolidol	1961	1524	1521
β-Neroidol	2000	1553	1553
Thymol	2001	1287	1286
Eugenol	2013	1351	1351
Vanillin	2100	1287	1287

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CHAPTER THREE

PHYTOCHEMICAL AND BIOLOGICAL STUDIES ON *CALLISTEMON SALIG*NUS AND *C. VIMINALIS*

3.1 Introduction

The genus *Callistemon* R. Br. (commonly known as bottlebrush) belongs to the family Myrtaceae and comprises over 300 species. They are woody aromatic trees or shrubs (*ca*. 0.5 m to 7 m tall) widely distributed in the wet tropics, notably Australia, South America and tropical Asia, but are now spread all over the world. *Callistemon* species has attractive narrow foliage and white papery bark. The leaves of *Callistemon* species are lanceolate (*ca.* 3-6 mm wide and 40-70 mm long) in arrangement and very aromatic. The flowers are borne in spikes of about 40-150 mm long with prominent red stamens. Petals are greenish or pale coloured, tiny, inconspicuous and in some cases deciduous.[1,2] The majority of *Callistemon* species are endemic to the more temperate regions of Australia, but are now spread all over the world. *Callistemon* species like *Melaleuca* and *Leptospermum* species are known for forestry, essential oil production, farm tree/windbreak plantings, degraded-land reclamation and ornamental horticulture among other applications.[1]

Callistemon species are also used as weed control and ornamental horticulture [3,4]and as bio-indicators for environmental management.[5] Previous phytochemical investigations of members of this genus resulted in the isolations and identification of C-methyl flavonoids, triterpenoids, betulinic acid, and phloroglucinol derivatives. [6-11] Furthermore, isolation of piceatannol and scirpusin B from the stem bark of *C. rigidus* from Japan, showed inhibitory effects on mouse α-amylase activity.[12] In addition, antimicrobial, anti-staphylococcal, antithrombin, repellent and nematicidal activities as well as larvicidal and pupicidal activity have been reported for the genus *Callistemon*.[13-19] Furthermore, an *in vitro* investigation of the crude water and methanol extracts from the leaves of some species of this genus revealed potential antimicrobial activity against a broad spectrum of multidrug-resistant human pathogens.[20] In the flora of South Africa, *Callistemon* species are grown as garden, street tree or ornamental plants due to their decorative flowers. *C. citrinus* (Curtis) Skeels (syn: *Metrosideros citrina* Curtis; commonly known as crimson or lemon Bottlebrush) is a handy medium shrub to large tree (ca. 5 – 7 m tall). *C. citrinus* is the most widely

cultivated member of the genus *Callistemon*. The bright red flower spikes of *C. citrinus* are very rich in nectar and attract many birds.[1-6]

Chemical studies of the oil compositions of some species of this genus have been previously reported.[17-18, 20-36] 1,8-Cineole (60.0-82.0%) is the predominant constituent of the oils of the species grown in Australia, Brazil**,** Egypt, India, Pakistan and Reunion. Although, other components such as α-pinene, β-pinene, myrcene and limonene have been reported to be in substantial amount. In the oil of *Callistemon rigidus* from Cameroon, linalool was the major component. It is interesting to know that linalool was not detected in the oils of species from other countries.[33] Some reports on the biological activities of *C. citrinus* and *C. viminalis* essential oils reveal anthelmintic and anti-quorum sensing activities.[37-39] There is no literature report on the essential oil of *C. salignus*. This was the first study of the essential oil content of this plant. Furthermore, the essential oil composition of *C. viminalis* from other countries has been reported. Consequentially, the oils from these two species were investigated as a part of an ongoing study on aromatic plants that also have OA or BA or both in large amounts. This was the first time this investigation was undertaken for both plants growing wildly all over South Africa.

3.2 *Callistemon salignus*

Callistemon salignus 'Rubra' (Figure 3.1) better known as the willow or white bottlebrush is one of the 34 species of the genus *Callistemon* (Myrtaceae).[3,40] *C. salignus* has attractive narrow foliage and white papery bark. The flower spikes are generally white or greenish, but pink, red and mauve forms are also found. In South Africa, *C. salignus* is planted as a garden, ornamental or street tree growing up to 5 – 12 m.[1]

Figure 3.1 *Callistemon salignus* [41]

3.3 *Callistemon viminalis*

C. viminalis (Sol. ex Gaertner) G. Don ex Loudon (Figure 3.2) commonly known as weeping Bottlebrush is a small tree or shrub with pendulous foliage although some forms are more pendulous than others. It reaches a height of about 4 m in its natural habitat but is usually smaller in cultivation, particularly in temperate areas. It is the most widely cultivated Callistemon. *C. viminalis* is known in Traditional Chinese Medicine pills for the treatment of haemorrhoids.[1, 3, 42]

Figure 3.2 *Callistemon viminalis* [41]

3.4 Experimental

3.4.1 Plant collection

Fresh leaf samples of *C. salignus* were collected July 2007 from University of Zululand, KwaDlangezwa campus, Empangeni (both in KwaZulu-Natal Province) and Johannesburg (Gauteng Province), South Africa. While, the plant materials of *C. viminalis* were collected from Durban and Johannesburg both in the Province of KwaZulu-Natal and Gauteng respectively, in South Africa. Dr. S.J. Siebert a senior plant taxonomist, of the Department of Botany, University of Zululand, identified the plants. Voucher specimens were deposited at the University of Zululand Herbarium as [OOO 10-12 (ZULU)] for *C. salignus* and [OOO 13 (ZULU)] for *C. viminalis*.

3.4.2 Extractives of *C. salignus* and *C. viminalis*

3.4.3 Extraction of Essential oil

Fresh matured leaves (300 g) of each plant sample were subjected to hydrodistillation in an all glassed Clevenger apparatus (Figure 3.3) for 3 h in accordance with the British Pharmacopoeia.[43-45] The resulting oils were collected, preserved in a dark vial bottles and stored under refrigeration at 4 $^{\circ}$C until analysis.

Figure 3.3 Clevenger apparatus for hydrodistillation of aromatic plants

3.4.4 Analysis and Identification of Essential Oil Components

(a) GC/MS analysis

GC/MS analyses of the oils were performed on a Hewlett Packard Gas Chromatography HP 6890 interfaced with Hewlett Packard 5973 mass spectrometer system operating in EI mode at 70 eV, equipped with a HP-5 MS capillary column (30 m x 0.25 mm, film thickness 0.25 µm). The initial temperature of the column was 70 ^{o}C and was heated to 240 ^{o}C at a rate of 5 ^{o}C/min. Helium was used as the carrier gas at a flow rate of 1 ml/min. The split ratio was 1:25. Scan time was 50 min with a scanning range of 35 to 450 amu. 1µl of the diluted oil was injection for analysis. *n*-Alkane of C_8-C_{30} were run under the same condition for Kovat indices determination.

(b) Identification of compounds

The components of the oils were identified by matching their spectra and retention indices (Kovat Index of calculated) with those of the authentic samples and literature. [46-48]

3.4.5 Extraction of OA

Literature has it that BA has been isolated from *C. viminalis*,[11] the study was therefore aimed at isolating OA and BA from *C. salignus*. The sequential extraction of the plant material was employed as described in Chapter 1. The dichloromethane and chloroform extracts were defatted using hexane. Column and flash chromatographic methods were used in an attempt to separate OA and BA which were eluted as a mixture but this was not successful.

3.4.6 Antibacterial Assay

The essential oils were tested against 12 reference bacterial strains obtained from the Department of Biochemistry & Microbiology, University of Fort Hare, Alice. Gram-positive bacteria: *Bacillus cereus* (ATCC 10702), *Bacillus pumilus* (ATCC 14884), *Staphylococcus aureus* (ATCC 3983), *Staphylococcus aureus* (ATCC 6538) and *Streptococcus faecalis* (ATCC 29212). Gram-negative strains: *Enterobacter cloacae* (ATCC 13047), *Escherichia coli* (ATCC 4983), *Klebsiella pneumoniae* (ATCC 2983), *Proteus vulgaris* (ATCC 6830), *Proteus vulgaris* (CSIR 0030), *Pseudomonas aeruginosa* (ATCC 19582) and *Serratia marcescens* (ATCC 9986). The stock cultures were maintained at 4 ^{o}C in Mueller-Hinton agar (MHA) (Oxoid).

A. Agar disk diffusion

The essential oils were tested for antibacterial activity by the agar disc diffusion method according to Kiehlbauch *et al*,.[49] The microorganisms were grown overnight at 37 ^{o}C in 20 ml of Mueller-Hinton broth (Oxoid). The cultures were adjusted with sterile saline solution to obtain turbidity comparable to that of McFarland no. 5 standard (1.0 x 10^{8}) CFU/mL. 90 mm Petri dishes (Merck, South Africa) containing 12 ml of sterilized Mueller-Hinton agar (Oxoid) were inoculated with these microbial suspensions. Sterile Whatman No. 1 (6 mm) discs papers were individually placed on the surface of the seeded agar plates and 10 µl of essential oil in DMSO was applied to the filter paper disk. The plates were incubated at 37 ^{o}C for 24 h and the diameter of the resulting zones of inhibition (mm) of growth was measured. All tests were performed in triplicates. Gentamycin and tetracycline were used as positive controls, while hexane and DMSO served as negative controls

B. Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the essential oils was determined using 96-well microtitre dilution method as described by Oyedeji & Afolayan, and Eloff. [50-51]

Bacterial cultures were incubated in Müller-Hinton (MH) broth overnight at 37 ^{o}C and a 1:1 dilution of each culture in fresh MH broth was prepared prior to use in the micro dilution assay. Sterile water (100 µL) was pipetted into all wells of the microtitre plate, before transferring 100 µL of essential oil in DMSO. Serial dilutions were made to obtain concentrations ranging from 10 mg/ml to 0.078 mg/mL. 100 µL of bacterial culture of approximate inoculums size of 1.0 x 10^{8} CFU/mL was added to all well and incubated at 37 ^{0}C for 24 h. After incubation, 40 µL of 0.2 mg/mL *p*-iodonitrotetrazolium violet (INT) solution was added to each well and incubated at 37 ^{o}C. Plates were examined after about 30-60 min. of incubation. Microbial growth is indicated by the presence of a reddish colour which is produced when *p*-iodonitrotetrazolium violet (INT), a dehydrogenase activity detecting reagent, is reduced by metabolically active micro-organisms to the corresponding intensely coloured formation. MIC is defined as the lowest concentration that produces an almost complete inhibition of visible micro-organism growth in liquid medium. Solvent controls (DMSO and Hexane) and the standard antibiotics gentamycin and tetracycline were included in the assay.

3.5 Results and Discussion

3.5.1. Essential oils

A. *Callistemon salignus*

Table 3.1 Chemical composition of the essential oils from three *C. salignus* leaf extracts from different locations.

		(%) Composition		
Compound	RI[a]	Sample A	Sample B	Sample C
α-thujene	928	0.3	0.3	-
α-pinene	936	17.8	6.2	27.3
camphene	951	-	-	1.0
sabinene	969	tr	-	-
β-pinene	974	1.5	1.4	2.7
myrcene	980	-	tr	-
α-phellandrene	1003	0.2	-	1.0
δ-3-carene	1009	0.6	-	-
α-terpinene	1015	-	-	0.1
1,8-cineole	1028	63.4	85.4	44.4
E-β-ocimene	1036	6.7	0.1	-
γ-terpinene	1058	0.6	0.4	0.7
cis-linalool oxide (furanoid)	1074	tr	tr	0.2
terpinolene	1084	0.6	0.6	0.1
linalool	1096	1.6	1.1	1.1
α-fenchol	1109	tr	tr	0.4
α-thujone	1116	0.1	-	-
trans-pinocarveol	1136	-	0.5	0.7
β-terpineol	1153	-	-	0.1
pinocarvone	1158	tr	0.3	0.2
borneol	1161	-	-	0.6
terpin-4-ol	1174	1.4	1.5	1.0
α-terpineol	1186	2.1	-	6.8
trans-carveol	1212	-	-	0.3
citronellol	1227	-	-	0.7
geraniol	1251	tr	-	0.8
citronellyl formate	1269	-	-	0.3
bornyl acetate	1284	-	-	0.1

Table 3.1 Continued.

β-caryophyllene	1424	0.8	-	-
geranyl acetone	1458	0.2	-	-
caryophyllene oxide	1581	0.6	-	-
globulol	1628	-	0.2	-
Monoterpene hydrocarbon		28.3	8.4	33.4
Oxygenated monoterpenes		68.8	89.4	57.7
Sesquiterpene hydrocarbon		0.8	-	-
Oxygenated sesquiterpenes		0.6	0.2	-
Total identified		98.5	98.0	91.1

RI[a] = Retention Indices relative to C_9-C_{24} *n*-alkanes on the DB-5 column, tr = trace amount ($\leq 0.05\%$), * = correct isomer not identified, Sample A = sample collected from University of Zululand, KwaDlangezwa, Sample B = sample collected from Empangeni, Sample C = sample collected from Johannesburg

Hydrodistillation of the leaf samples gave 1.12%, 1.04% and 1.71% yield of oils for University of Zululand, KwaDlangezwa campus (Sample A), Empangeni (Sample B) and Johannesburg (Sample C), respectively. The GC/MS chromatogram of the essential oils from three different locations is displayed in Figure 3.1.1-3.1.3 (Appendix 1). Comprehensive analyses of the oils indicated the presence of more than 33 compounds in general, out of which a range of 16 – 22 were identified, accounting for 90.1 – 98.5% of the oil samples that are predominantly monoterpenoids with oxygenated monoterpene dominating the oil compositions (57.7-89.4%).

1,8-Cineole (63.4%), α-pinene (17.8%) and *E*-(β)-ocimene (6.7%) were the major constituents identified in the KwaDlangezwa sample (Sample A). The Empangeni sample (Sample B) contained only 1,8-cineole (85.4%) and α- pinene (6.2%) as the main compounds present in the oil. While 1,8-cineole (44.4%), α-pinene (27.8%) and α-terpineol (6.8%) were the major components of the Johannesburg sample (Sample C). Although, 1,8-cineole was the major component of the oils from the two provinces, the percentage composition of the Johannesburg sample (44.4%) was relatively low, when compared to 85.4% from Empangeni sample or 63.4% from University of Zululand sample. Similarly, α-pinene from the Johannesburg sample (27.8%) was highly substantial when compared to University of Zululand (17.8%) and Empangeni (6.2%) samples from the same province. Also, the lack of detection of sesquiterpenoids in the Johannesburg sample was noticeable. The results obtained in this study showed that the chemical pattern of the

essential oils of *Callistemon salignus* from two provinces of South Africa is very similar to other *Callistemon* species from different countries.

B. *Callistemon viminalis*

Table 3.2 Chemical Constituents of the essential oil from *C. viminalis* leaves

		Percentage composition
Compound	RI[a]	*C. viminalis*
α-pinene	937	6.4
β-pinene	976	0.9
myrcene	989	tr
α-terpinene	1016	0.4
1,8-cineole	1027	83.2
linalool	1096	0.5
trans-pinocarveol	1138	0.9
pinocarvone	1154	tr
terpin-4-ol	1168	o.6
α-terpineol	1187	4.9
trans-carveol	1220	tr
geraniol	1244	0.5
Monoterpene hydrocarbon		7.7
Oxygenated monoterpenes		90.1
Sesquiterpene hydrocarbon		-
Oxygenated sesquiterpene		-
Total		98.3

RI[a] = Kovat index relative to C_9-C_{24} *n*-alkanes on DB-5 column. tr = trace (< 0.05%)

The yield of the oils obtained from the hydrodistillation of the leaves of *C. viminalis* were 0.9% (w/w), respectively. Table 3.2 lists the components identified in the essential oils with their percentage composition and relative retention indices. Twelve constituents were identified from the GC-MS oil analysis of *C. viminalis*, (Figure 3.2.1 – Appendix 1) accounting for (98.3%) of the oil composition. The major compounds were 1,8-cineole (83.2%), α -pinene (6.4%) and α -terpineol (4.9%). The most abundant constituents were the oxygenated monoterpenes (90.1%) with 1,8-cineole constituting the bulk of the oil when compared to monoterpene hydrocarbons (7.7%). α-Pinene was the prominent monoterpene found in the oil and no sesquiterpenoid was present in the oil. The abundance of 1,8-cineole in the essential oil *C. viminalis* makes the oil similar to those reported in previous studies from India, Australia, Egypt and Reunion. However, the key difference in the oils lies in the relative abundant of α-pinene, β-pinene, limonene, linalool and α-

terpineol. In contrast to the report concerning the analysis of the essential oil of *C. viminalis* growing in Egypt, our study (South African species) showed quantitative difference. 1,8-cineole represents 47.9% of the total oil of *C.viminalis* from Egypt, while, the South African species, constitute 83.2%. In addition, linalool (13.0%) and limonene (10.9%) which appear as major constituents in the Egyptian species were present in low concentration, linalool (0.5%) and limonene was absent from the South Africa species.[18, 31-32] Figure 3.4 shows the graphical comparison of the major components of the essential oils of *C. salignus and C. viminalis*.

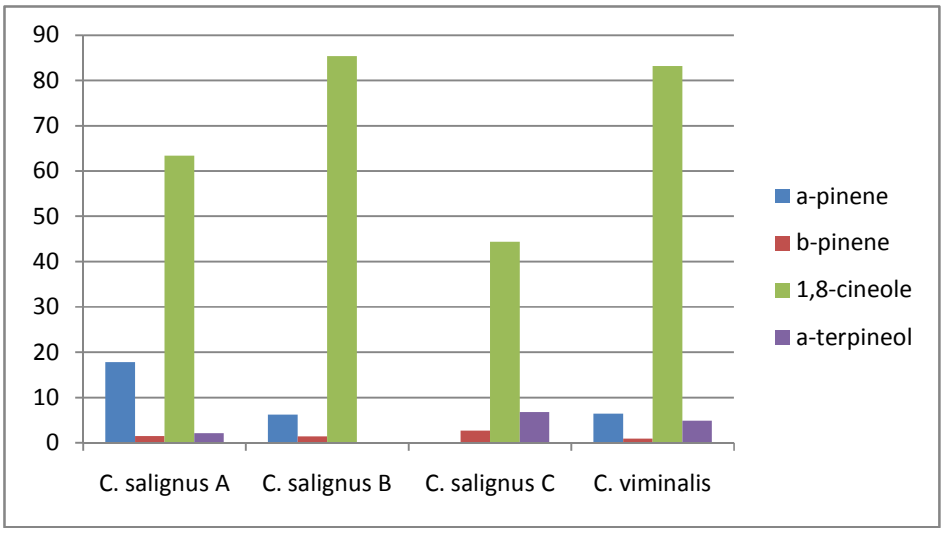

Figure 3.4: Comparison of major components of *C. salignus* and *C. viminalis* essential oils

3.5.2. Antibacterial Activities of the Essential Oils of *C. salignus* and *C. viminalis*

The inhibition zone of the disc diameters (IZ) and minimum inhibitory concentrations (MICs) of the essential oils of *C. salignus* and *C. viminalis* against the microorganisms tested are showed in Table 3.3. The results obtained from the agar disc diffusion method for the essential oils revealed *S. aureus* (ATCC 3983) to be the most sensitive microorganism with the largest inhibition zone of (26.5 and 23.3) mm for both oils. While the smallest inhibition zones were exhibited by *S. marcescens* and *E. coli*. Furthermore, the MIC values showed *S. aureus* (ATCC 3983) having the lowest MIC value (0.08 mg/mL) for *C. salignus* (sample B) and *C. viminalis*, while oil showed moderate to low concentrations. All the oils showed moderate to good inhibitory effect on *P. aeruginosa, B. cereus* and *B. pumilus* bacteria. When compared with standard antibiotics

(gentamycin and tetracycline), the essential oils showed inhibition zones from weak to moderate strong inhibition effect (10.3 to 26.3 mm) than the standard antibiotics (6.0 ± 0.0 to 23.7 ± 1.5 mm).

Generally, the essential oils exhibited notable antibacterial activity against all the bacteria species tested. However, Gram-positive bacteria were most susceptibility than Gram-negative bacteria. The antibacterial activity showed by the essential oils of *C. salignus* and *C. viminalis* could be attributed to the presence of some major components such as 1.8-cineole, α-pinene and α-terpineol, along with other components in lower amount such as, β-pinene and linalool, which were already known to exhibit antimicrobial and bacteriostatic activities.[37-39]

Table 3.3 Antibacterial activities of the essential oils of *C. salignus* and *C. viminalis*

Microorganism	*C. salignu* Sample A		*C. salignu* Sample B		*C. salignu* Sample C		*C. viminalis*		Gentamycin		Tetracycline	
	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC
B. cereus (ATCC 10702)	19.8± 2.0	0.63	20.4± 1.7	0.31	21.3± 1.5	0.63	19.0± 1.7	0.63	14.0± 2.0	0.63	13.3± 2.0	1.25
B. pumilus (ATCC 14884)	18.5± 1.2	2.50	20.5± 1.0	1.25	17.7± 0.6	2.50	15.3± 1.2	1.25	13.3± 2.1	1.25	14.0± 1.5	1.25
S. aureus (ATCC 3983)	23.3± 1.3	0.31	26.5± 0.6	0.08	25.6± 1.0	0.63	24.7± 1.2	0.08	17.3± 1.2	0.31	18.7± 2.6	0.31
S. aureus (ATCC 6538)	25.5± 1.0	0.31	22.3± 1.5	0.63	23.3± 1.3	0.63	23.0± 1.2	0.63	14.4± 1.5	0.63	ND	0.31
S. faecalis (ATCC 29212)	19.9± 1.8	0.31	21.1± 1.4	0.31	19.7± 1.6	0.31	20.3± 2.0	0.63	16.0± 2.0	1.25	ND	ND
E. cloacae (ATCC 13047)	20.3± 1.5	0.31	17.3± 2.1	0.63	18.7± 0.6	0.63	17.7± 2.5	0.63	12.6± 0.6	2.50	13.0± 0.6	2.50
E. coli (ATCC 4983)	13.3± 1.8	2.50	12.3± 0.9	1.25	13.3± 2.0	1.25	14.3± 1.5	2.50	21.3± 1.5	0.16	23.0± 1.7	0.31
K. pneumoniae (ATCC 2982)	19.9± 1.0	0.16	21.3± 2.0	0.16	24.8± 1.7	0.08	14.3± 0.6	2.50	23.7± 1.5	0.08	17.6± 1.5	0.63
P. vulgaris (ATCC 6830)	13.3± 1.7	2.50	15.7± 2.0	1.25	17.8± 0.0	1.25	16.0± 0.0	2.50	21.3± 1.2	0.31	6.0 ± 0.0	ND
P. vulgaris (CSIR 0030)	18.5± 1.3	1.25	19.2± 1.0	1.25	19.6± 0.0	1.25	18.3± 1.5	1.25	6.0 ± 0.0	5.00	6.0 ± 0.0	5.00
P. aeruginosa (ATCC 7700)	19.9± 1.3	0.31	21.3± 1.4	0.16	19.3± 1.9	0.31	10.3± 0.6	5.00	20.7± 1.2	0.63	14.7± 0.6	0.63
S. marcescens (ATCC 9986)	13.3± 1.3	2.50	10.3± 1.5	2.50	12.3± 0.0	2.50	11.3± 1.2	5.00	7.3 ± 0.0	2.50	15.7± 1.5	0.63

IZ = Zone of inhibition; MIC = minimum inhibitory concentrations; Dose: 5 mg/mL; Disc diameter; 6 mm. Values are the mean ± S.D of the mean; ATCC = American Type Culture Collection; ND = Not Determined

3.5.3 Triterpenoids

Extraction of OA and BA from *Callistemon* plant material did not yield good result as expected. Instead a mixture of OA and BA was obtained which was very difficult to separate due to their close R_f values. This was therefore left for future studies.

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CHAPTER FOUR

PHYTOCHEMICAL AND BIOLOGICAL STUDIES ON *MELALEUCA* SPECIES

4.1 Introduction

Melaleuca species are of the Myrtaceae family. The genus *Melaleuca* is entirely Australian, and has more than 100 species of trees and shrubs with leathery leaves and spicate inflorescences of red, white or yellow flowers. The trees and shrubs are mostly cultivated for the beauty of the flowers due to its conspicuous stamens, which are united in bundles. The leaves and the volatile oils are used in enthnomedicine of West Africa as a remedy for numerous diseases.[1-5] *M. bracteata var. revolution gold and green* are hybrid of *M. bracteata*. They are evergreen shrubs that grow up to 6 m in height having white flowers in spring. *Melaleuca bracteata* is a variable species. Some populations are rounded shrubs about two metres high, whilst others develop into trees about 15 metres tall. The leaves are dark green, oval in shape and scattered along the branches. Each leaf has a sharp point. Flowers are white, carried in clusters on or near the ends of branches. Flowering occurs between August and November. *Melaleuca bracteata* usually grows along water courses and may dominate stream-side vegetation. A number of cultivars are available commercially. "Revolution Gold" and "Revolution Green" are both bushy shrubs.[3-5]

4.1.1 *M. bracteata* var. *revolution gold*

M. bracteata var. *revolution gold* is a fast growing fine leafed shrub ±6 m. It is often referred to as Johannesburg gold (Figure 4.1). The essential oil obtained from the fresh leaves and twigs are antiseptic in nature and do have germicidal and insecticidal potential.[6, 7]

Figure 4.1: Leaves of *M. bracteata* var. *revolution gold* being harvested by Mr. Oyedeji

4.1.2 *M. bracteata var. revolution green*

M. bracteata var. *revolution green* is a tidy erect bush admired for brilliant fiery purple red new foliage throughout the year ±2.5 x 1m with small white flowers. Leaves are evergreen and hardy (Figure 4.2).[7]

Figure 4.2 *M. bracteata* var. *revolution green*

4.1.3 *M. trichostachya var. compacta*

M. trichostachya is synonymous with *M. linariifolia.* The leaves *M. trichostachya* are small, of about 2.6 cm in length, while the tree is between 9-12 m in height. The tree possesses long terminal branchlets and calyxes, the bark is hard, compact, and furrowed which distinguish it from other "Tea Trees" bark. The species was first described by Baron von Mueller.[8-10] *M. trichostachya* var. *compacta* (Figure 4.3) is a variety of *M. trichostachya* indigenous to South Africa.

Figure 4.3 *M. trichostachya* var. *compacta*

4.2 Ethnobotanical Review of *Melaleuca* Species

The 'cajuput oil', which is obtained from the leaves of some species, is of high germicidal value containing 40-60% cajuputol or eucalyptol (1,8-cineole). Cajuput oil is used in perfumes as a toner blender and in pharmacy as a flavouring agent.[11-13] Cajuput oil is used for the treatment of aches and pain, inhaled aromatic vapour from crushed leaves to reduced nasal and bronchial congestion.[14] *Melaleuca* plants are also known to be good reactive oxygen species (antioxidant) and metal accumulators (such as aluminium, copper, nitrogen) and thus often used to cleanse industrial sites of heavy metals.[15-16] Furthermore, it has been reported that some *Melaleuca* species have unique ability to withstand environmental stress. Their stress tolerance is attributed to their ability to accumulate large quantities of an organic compound known as proline (betaine). Proline

is extracted from *Melaleuca* species and used in seed treatment and foliar application to increase the stress tolerance of economic crops.[17-18]

There are numerous reports on the essential oil composition and biological activities of *Melaleuca* species from Australia [14, 17-25], Nigeria [26-27], France [28-29], Egypt [30-31], Brazil [33], Tokyo [34], Hungary [35], Japan [36], New Zealand [37]. 1, 8-cineole, α-terpineol, α-pinene, limonene are main components of the oils. The cineole content is often used to group the oil-type into chemotypes. There is no literature reference to the essential oils of *M. trichostachya* var *compacta*, however, there are two literature articles which reports the essential oil composition of *M. linariifolia* Smith which is syn. to *M trichostachya*. Terpinen-4-ol and γ-terpinene are reported to be the major constituents of the oil while, 1,8-cineole, *p*-cymene, α-terpinene, terpinolene and sabinene were found in moderate concentration.[38, 39] Methyl eugenol (97%) was the major compound found in the Egyptian *M. bracteata* essential oil[30-31] while the Thailand oil[40] had methyl eugenol ether (29%), eleminicn (21.6%) and methyl eugenol (14%) as the major components identified in the oil.

4.3 Extractives of *Melaleuca* Species

4.3.1 Experimental

A. Plant Collection

Fresh leaf samples of the three *Melaleuca* species were collected in September 2007 from Ethekwini Municipality Parks, Leisure and Cementeries Department Nursery in Durban, South Africa. Mr Pravin Poorun a senior plant taxonomist, of the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Westville Campus identified the plants. Voucher specimens were prepared, allocated voucher numbers, and deposited at the Ward Herbarium (UKZN, School of Bioloical & Conservation Sciences): *M. bracteata* var. *revolution gold* (Glow OO1), *M. bracteata* var. *revolution green* (Glow OO2), and *M. trichostachya* var. *compacta* (Glow OO3).

B. Extraction and Isolation

(i) Essential oil

Fresh matured leaves of *M. trichostachya* var. *compacta* (350 g), *M. bracteata* var. *revolution green* (345 g), and *M. bracteata* var. *revolution gold* (350 g) were subjected to hydrodistillation in an all glassed Clevenger apparatus for 3 h in accordance with the British Pharmacopoeia.[41-43] The resulting oils were collected, preserved in a dark vial bottles and labelled as MAG1, MG1 and MYG1. After about 7 hours of resting, the hydrodistilled plant in the flask was further distilled and gave oils which were labelled as MAG2, MG2 and MYG2. All these oils were stored under refrigeration at $4^{O}C$ until when required for analysis.

(ii) Pentacyclic Triterpenes

Fresh leaves of the three *Melaleuca* species (450 g, each) were extracted separately overnight with dichloromethane (4.5 L) and the solvent removed under reduced pressure to yield a dark green residue (21.18 g) (4.71%). The remaining plant material content (marc) was extracted with ethyl acetate and the solvent was removed under reduced pressure to give a light green residue (18 g) (4%).

Isolation of Compounds (BA/OA Mixture)

A portion of the ethyl acetate crude extract (3 g) was subjected to chromatographic separation on silica gel (60 – 120 mesh, 90 g) column (20 x 5.0 cm). Gradient elution with hexane-ethyl acetate solvent system (8:2 → 7:3), gave 64 fractions (20 ml each). The fractions were analysed by TLC and similar fractions were combined into eight sub-fractions, allowed to evaporate to dryness before weighing: F1 (290 mg), F2 (430 mg), F3 (490 mg), F4 (340 mg), F5 (190 mg), F6 (50 mg), F7 (10 mg) and F8 (40 mg). Further TLC analysis was carried out on all the sub-fractions which showed the presence of chlorophylls and a mixture of two compounds. All the sub-fractions were combined, redisolved in methanol. Some grams 2mg of charcoal was added to the hot merhanol and filtered while hot to give a white solid (1.8 g). Spectroscopic analysis confirmed that the white solid was a mixture of BA and OA. Repeated attempts to separate this mixture chromatographically were unsuccessful.

C. Antibacterial assay

The essential oils were tested against 12 reference bacterial strains obtained from the Department of Biochemistry & Microbiology, University of Fort Hare, Alice. Gram-positive bacteria: *Bacillus cereus* (ATCC 10702), *Bacillus pumilus* (ATCC 14884), *Staphylococcus aureus* (ATCC 3983), *Staphylococcus aureus* (ATCC 6538), and *Streptococcus faecalis* (ATCC 29212). Gram-negative strains: *Enterobacter cloacae* (ATCC 13047), *Escherichia coli* (ATCC 4983), *Klebsiella pneumoniae* (ATCC 2983), *Proteus vulgaris* (ATCC 6830), *Proteus vulgaris* (CSIR 0030), *Pseudomonas aeruginosa* (ATCC 19582) and *Serratia marcescens* (ATCC 9986). The stock cultures were maintained at 4 ^{o}C in Mueller-Hinton agar (MHA) (Oxoid).

(i) Agar disk diffusion

The essential oils were tested for antibacterial activity by the agar disc diffusion method according to Kiehlbauch *et al*,[44] The microorganisms were grown overnight at 37 ^{o}C in 20 ml of Müller-Hinton broth (Oxoid). The cultures were adjusted with sterile saline solution to obtain turbidity comparable to that of McFarland no. 5 standard (1.0 x 10^{8}) CFU/mL. 90 mm Petri dishes (Merck, South Africa) containing 12 ml of sterilized Mueller-Hinton agar (Oxoid) were inoculated with these microbial suspensions. Sterile Whatman No.1 (6 mm) discs papers were individually placed on the surface of the seeded agar plates and 10 µl of essential oil in DMSO was applied to the filter paper disk. The plates were incubated at 37 ^{o}C for 24 h and the diameter of the resulting zones of inhibition (mm) of growth was measured. All tests were performed in triplicates. Gentamycin and tetracycline were used as positive controls, while hexane and DMSO served as negative controls

(ii) Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the essential oils was determined using 96-well microtitre dilution method as described by Oyedeji & Afolayan, and Eloff. [45-46]

Bacterial cultures were incubated in Müller-Hinton (MH) broth overnight at 37 ^{o}C and a 1:1 dilution of each culture in fresh MH broth was prepared prior to use in the micro dilution assay. Sterile water (100 µL) was pipetted into all wells of the microtitre plate, before transferring 100 µL of essential oil in DMSO. Serial dilutions were made obtain concentrations ranging from 10 mg/ml to 0.078 mg/mL. 100 µL of bacterial culture of approximate inoculums size of 1.0 x 10^{8} CFU/mL

was added to all well and incubated at 37 ^{0}C for 24 h. After incubation, 40 µL of 0.2 mg/mL *p*-iodonitrotetrazolium violet (INT) solution was added to each well and incubated at 37 ^{o}C. Plates were examined after about 30-60 min. of incubation. Microbial growth is indicated by the presence of a reddish colour which is produced when *p*-iodonitrotetrazolium violet (INT), a dehydrogenase activity detecting reagent, is reduced by metabolically active micro-organisms to the corresponding intensely coloured formazan. MIC is defined as the lowest concentration that produces an almost complete inhibition of visible micro-organism growth in liquid medium. Solvent controls (DMSO and Hexane) and the standard antibiotics gentamycin and tetracycline were included in the assay.

D. Analysis and Identification of Essential Oil Components

(i) GC/MS analysis

GC/MS analyses of the oils were performed on a Hewlett Packard Gas Chromatography HP 6890 interfaced with Hewlett Packard 5973 mass spectrometer system operating in EI mode at 70 eV, equipped with a HP-5 MS capillary column (30 m x 0.25 mm, film thickness 0.25 µm). The initial temperature of the column was 70 ^{o}C and was heated to 240 ^{o}C at a rate of 5 ^{o}C/min. Helium was used as the carrier gas at a flow rate of 1 ml/min. The split ratio was 1:25. Scan time was 50 min with a scanning range of 35 to 450 amu. 1ul of the diluted oil was injection for analysis. *n*-Alkane of C_8-C_{30} were run under the same condition for Kovat indices determination.

(ii) Identification of compounds

The components of the oils were identified by matching their spectra and retention indices (Kovat Index) with those of the authentic samples and literature values.[47-49]

4.4 Results

4.4.1. Essential oils

Table 4.1 Physicochemical parameters of the three *Melaleuca* essential oils

	MAG1	MAG2	MG1	MG2	MYG1	MYG2
Weight of Plant Sample (gm)	350	350	345	345	350	350
% (w/w) yield	2.53	2.28	0.11	0.19	0.75	0.16
Physical Appearance	Cloudy	Colourless	Colourless	Colourless	Cloudy	Cloudy
Odour	Aromatic and minty	Aromatic and minty	Aromatic	Aromatic and minty	Aromatic	Aromatic

MAG1 = first isolates of *M. trichostachya* var. *compacta*, MAG2 = second isolates of *M. trichostachya* var. *compacta*, MYG1 = first isolates of *M. bracteata* var. *revolution gold*, MYG2 = second isolates of *M. bracteata* var. *revolution gold*, MG1 = first isolates of *M. bracteata* var. *revolution green*, MG2 =second isolates of *M. bracteata* var. *revolution green.*

Table 4.2 Chemical composition of the *essential o*ils *of* two *M. trichostachya var. compacta*

		(%) Composition	
COMPOUND	RI	MAG1	MAG2
m-xylene	867	0.3	-
α-thujene	940	tr	-
α-pinene	943	9.9	2.6
β-pinene	979	2.1	-
Myrcene	991	3.7	1.9
1,8-cineole	1036	64.8	69.3
γ-terpinene	1064	0.3	0.4
cis-linalool oxide	1078	0.1	-
α-terpinolene	1088	0.4	-
Linalool	1101	1.8	-
Fenchol	1112	0.1	-
δ-terpineol	1150	0.3	0.6
terpinen-4-ol	1178	0.6	0.9
α-terpineol	1190	3.9	12.4
Geraniol	1260	0.1	-
geranial (α-citral)	1278	0.1	-
Undecanone	1298	0.1	-
eugenol	1364	0.1	-
(-)-isoledene	1370	0.1	-

Table 4.2 Continued.

α-copaene	1379	0.1	-
β-elemene	1391	0.1	-
methyl eugenol	1423	0.1	-
α-gurjunene	1436	0.1	-
β-caryophyllene	1442	1.1	2.5
Aromadendrene	1470	0.7	0.4
germacrene D	1480	tr	-
α-humulene & β-selinene	1485	0.6	0.4
Alloaromadendrene	1494	0.1	0.7
α-amorphene	1500	0.2	-
epi-α-selinene	1517	0.7	-
δ-cadinene	1523	0.4	0.8
cadina-1,4-diene	1528	0.1	-
α–cadinene	1538	0.3	0.3
α–calacorene	1539	0.3	0.6
caryophyllene oxide	1581	0.1	-
Spathulenol	1589	0.7	-
Viridiflorol	1592	0.6	1.5
Isophytol	1994	-	-
Phytol	2109	0.1	-

RI[a] = Retention Indices relative to C_9-C_{24} n-alkanes on the DB-5 column. tr = trace amount (≤ 0.05%) MAG1 = first isolates of *M. trichostachya* var. *compacta*, MAG2 = second isolates of *M. trichostachya* var. *compacta*.

Table 4.3 Chemical composition of the essential oils *two M. bracteata var. revolution green*

		(%) Composition	
COMPOUND	RI	MG1	MG2
p-xylene	851	3.7	-
p-cymene	1025	1.0	-
Limonene	1034	1.8	-
1,8-cineole	1036	-	19.2
Linalool	1101	3.7	-
methyl eugenol	1423	89.7	72.5
methyl eugenol ether (isoeugenol ether)	1490	-	8.3

RI[a] = Retention Indices relative to C_9-C_{24} n-alkanes on the DB-5 column. MG1 = first isolates of *M. bracteata* var. *revolution green*, MG2 =second isolates of *M. bracteata* var. *revolution green.*

Table 4.4 Chemical composition of the *essential* oils *two M. bracteata* var. *revolution gold*

		(%) Composition	
COMPOUND	RI	MYG1	MYG2
cis-3-Hexenol	858	0.8	0.8
trans-3-Hexen-1-ol	860	1.1	0.9
α-thujene	940	0.1	-
Myrcene	991	0.3	0.3
α-phellandrene	1005	0.7	0.6
p-cymene	1025	2.8	1.9
Limonene	1034	-	0.5
trans-β-ocimene	1044	0.1	0.1
γ-terpinene	1064	0.1	-
Terpinolene	1088	0.9	0.6
Linalool	1101	5.0	3.8
terpinene-4-ol	1178	0.1	0.2
α-terpineol	1190	0.3	0.8
estragole (methyl chavicol)	1212	0.3	0.4
α-copaene	1379	0.2	0.2
methyl eugenol	1423	82.5	84.6
germacrene D	1478	1.7	1.6
Bicyclogermacrene	1495	0.7	0.9
cis-calamenene	1515	0.1	0.7
δ-cadinene	1523	0.6	0.1
cadina-1,4-diene	1528	0.1	0.1
caryophyllene oxide	1581	0.2	0.2
spathulenol	1589	0.3	0.2
t-cadinol	1640	0.2	-

RI[a] = Retention Indices relative to C_9-C_{24} n-alkanes on the DB-5 column. MYG1 = first isolates of *M. bracteata* var. *revolution gold*, MYG2 = second isolates of *M. bracteata* var. *revolution gold.*

Table 4.5 Antibacterial activities of the essential oils of three *Melaleuca* species

Microorganism	MAG1		MAG2		MG1		MG2		MYG1		MYG2		Gentamycin		Tetracycline	
	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC
B. cereus (ATCC 10702)	21.5 ± 1.5	0.06	22.3 ± 0.3	0.03	8.5 ± 0.5	2.50	10.8 ± 0.8	2.50	11.0 ± 1.5	1.25	11.6 ± 0.6	1.25	14.0 ± 2.0	0.62	13.3 ± 2.0	1.25
B. pumilus (ATCC 14884)	28.0 ± 0.5	0.06	22.0 ± 0.5	0.03	11.3 ± 0.8	1.25	9.0 ± 0.0	1.25	10.0 ± 1.5	2.50	10.0 ± 1.5	2.50	13.3 ± 2.1	1.25	14.0 ± 1.5	1.25
S. aureus (ATCC 3983)	19.5 ± 0.5	0.31	17.0 ± 1.0	0.31	10.3 ± 0.8	1.25	12.5 ± 1.0	0.62	12.5 ± 1.5	2.50	15.3 ± 0.3	0.62	17.3 ± 1.2	0.31	18.7 ± 2.6	0.31
S aureus (ATCC 6538)	20.5 ± 3.8	0.31	21.8 ± 1.75	0.31	10.5 ± 0.5	0.62	10.0 ± 0.0	0.62	17.3 ± 1.3	1.25	17.0 ± 1.5	0.62	14.4 ± 1.5	0.62	ND	0.31
S. feacalis (ATCC 29212)	20.3 ± 1.8	0.03	18.0 ± 1.0	0.06	9.0 ± 0.0	2.50	10.2 ± 2.0	1.25	13.8 ± 1.5	2.50	15.5 ± 0.5	1.25	16.0 ± 2.0	1.25	ND	ND
E. cloacae (ATCC 13047)	17.0 ± 2.0	0.03	17.8 ± 0.3	0.03	8.3 ± 0.8	2.50	11.0 ± 0.5	2.50	10.3 ± 2.5	2.50	16.5 ± 4.5	0.62	12.6 ± 0.6	2.50	13.0 ± 0.6	2.50
E. coli (ATCC 4983)	17.0 ± 0.5	0.62	16.5 ± 1.0	1.25	11.8 ± 0.3	1.25	12.3 ± 1.25	2.50	8.6 ± 0.5	ND	14.5 ± 0.5	1.25	21.3 ± 1.5	0.16	23.0 ± 1.7	0.31
K. pneumoniae (ATCC 2982)	18.3 ± 0.3	0.62	19.3 ± 0.3	0.62	11.5 ± 0.5	1.25	13.0 ± 1.0	1.25	18.0 ± 0.0	0.62	14.5 ± 1.5	1.25	23.7 ± 1.5	0.08	17.6 ± 1.5	0.62
P. vulgaris (ATCC 6830)	15.3 ± 0.3	1.25	17.3 ± 0.8	1.25	10.0 ± 0.0	1.25	11.5 ± 2.0	1.25	15.5 ± 0.5	0.62	13.6 ± 0.3	1.25	21.3 ± 1.2	1.31	6.0 ± 0.0	ND
P. vulgaris (CSIR 0030)	19.8 ± 0.8	0.62	21.5 ± 1.5	0.31	11.5 ± 1.5	1.25	12.8 ± 0.8	1.25	15.5 ± 1.5	1.25	18.5 ± 0.5	0.62	6.0 ± 0.0	5.00	6.0 ± 0.0	5.00
P. aeruginosa (ATCC 7700)	7.5 ± 1.0	2.50	17.8 ± 0.3	0.62	8.5 ± 1.5	2.50	12.0 ± 2.0	1.25	12.5 ± 2.0	1.25	9.0 ± 1.0	2.50	20.7 ± 1.2	0.62	14.7 ± 0.6	0.62
S. marcescens (ATCC 9986)	22.0 ± 0.3	0.31	30.5 ± 0.5	0.06	7.5 ± 0.5	5.00	10.8 ± 0.2	2.50	18.5 ± 1.5	0.62	20.3 ± 0.3	0.31	7.3 ± 0.0	2.50	15.7 ± 1.5	0.62

IZ = Zone of inhibition; MIC = minimum inhibitory concentrations, Dose: 5 mg/mL; Disc diameter; 6 mm. Values are the mean ± S.D of the mean, ATCC = American Type Culture Collection, ND = Not Determined, MAG1 = first isolates of *M. trichostachya* var. *compacta*, MAG2 = second isolates of *M. trichostachya* var. *compacta*, MYG1 = first isolates of *M. bracteata* var. *revolution gold*, MYG2 = second isolates of *M. bracteata* var. *revolution gold*, MG1 = first isolates of *M. bracteata* var. *revolution green*, MG2 =second isolates of *M. bracteata* var. *revolution green.*

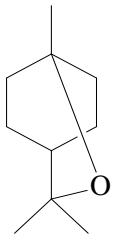
4.5 Discussion

4.5.1 Chemical Composition of the Three *Melaleuca* Species

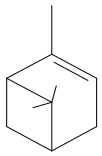
The oils of *M. trichostachya* var. *compacta* (MAG1 and MAG2) gave more oil yield when compared to the other two *M. bracteata* species (Table 4.1). The colour and odour of the oils were almost similar. MAG1, MG1 and MYG1 were the first distillates obtained after 3 hours of distillation from the respective plant materials. The distilled plant samples were cooled and

redistilled after 7 hours for another 3 hours to yield the second distillates tagged MAG 2, MG2 and MYG2 for *M. trichostachya* var. *compacta, M. bracteata* var. *revolution green*, and *M. bracteata* var. *revolution gold*, respectively. The percentage yields were almost similar for both sets except for MYG1 and MYG2 with a percentage difference was 0.59. It was also noticed that the percentage yield of the second distillate of MG followed a different pattern when compared to MAG and MYG.

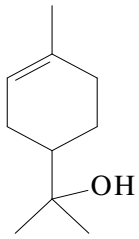
The GC-MS analysis of the leaf essential oils of *M. trichostachya* var. *compacta* (MAG1 and MAG2) (Figure 4.3.1 and 4.2.2 – Appendix 2) afforded the identification of 36 and 15 compounds, respectively which account for about 95% of the total oil compositions (Table 4.2). Oxygenated monoterpenes were identified to be the dominating group (72.1% and 83.2%, respectively), this was followed by monoterpenes (16.7% and 4.5%), sesquiterpenes (4.9% and 5.7%), while the same concentration of oxygenated sesquiterpenes (1.5%) was noticed for both oils. 1,8-cineole (**4.1**) was the main component of both oils (64.8% and 69.3%, respectively). Another prominent compound was α-pinene (**4.2**) and α-terpineol (**4.3**). The concentration of α-pinene in MAG1 was 9.9% compared to 2.6 % in MAG2 while a reverse trend was noticed in the case of α –terpineol with 12.4% in MAG2 oil and 3.9% in MAG1.



(**4.1**)

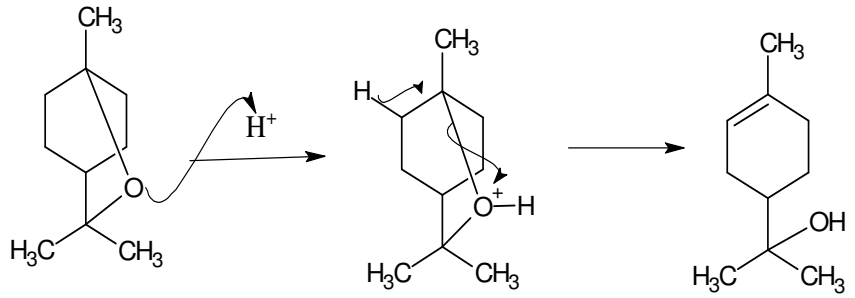


(**4.2**)



(**4.3**)

It is interesting to note that the compositional variation of these compounds (**4.1**: 4.4%, **4.2**: 7.3% and **4.3**: 8.5%) in the two oils isolated from the same samples is surprising. One can postulate that a rearrangement transformation of (**4.1**) could lead to (**4.3**) due to the long stay of MAG2 in water via enzymatic reaction. This, therefore, suggests that certain rearrangement might have occurred during and after distillation. This hypothesis has to be tested by simulating the conversion of **4.1** to **4.3** in the laboratory. A plausible mechanism is shown in Scheme 4.1.



4.1 **4.3**

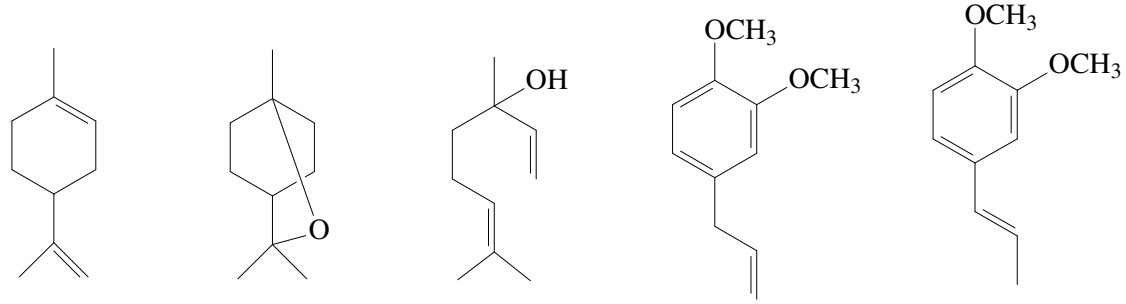
Scheme 4.1 Plausible mechanism for the conversion of **4.1** to **4.3**

It also interesting to note that some compounds like β-pinene (2.1%), linalool (1.8%), that were in significant amount in the MAG1 oil sample were completely absent in the MAG2 sample. Furthermore, more compounds were identified in the essential of MAG1 as compared to MAG2 while most of these compounds were completely absent in MAG2. The percentage yields of sesquiterpenoids are between 1.5 - 5.7% in total. Individual sesquiterpenoid component was at very low concentration except for β-caryophyllene (1.1% and 2.5%) in both oils. Comparing these results with those of Southwell and Stiff [38] and Shellie *et al* [39] it was very obvious that these results are in variance with the literature report as the major compound in this study was 1,8-cineole while terpinolene was very low (0.4%). This suggests possibly a chemotype different from those in literature.

Five compounds were identified in the oil sample of MG1 (Figure 4.2.1 - Appendix 2) while only three for MG2 (Figure 4.2.2 – Appendix 2) accounting for 99.9% and 100% of the total oil compositions, respectively (Table 4.3). Both oils were characterised by terpenoids with oxygenated monoterpene accounting for 93.4% and 100% of MG1 and MG2 oil composition. The major component of both oil extracts was methyl eugenol (89.7% and 72.5%, respectively) apart from this similarity the other oil constituents are at variance to each other. In MG1, monoterpene constitute about 6.5% with *p*-xylene (3.7%), *p*-cymene (1.0%) and limonene (1.8%). Linalool (3.7%) was the other oxygenated monoterpene identified in the GCMS analysis of the oil. Only three major compounds were detected in the MG2 oil which is a redistilled plant material from which MG1 oil was extracted. Except for methyl eugenol, the other two major compounds, 1,8-cineole and methyl eugenol ether, were in high percentage and were completely absent in MG1

chemical profile. The presence of these three compounds in MG2 makes it of better medicinal valuable than MG1. This also confirms the minty odour (Table 4.1) detected in the MG2 oil sample.

It is very difficult to account for the variance in the chemical profile of the two oils, especially with limonene (**4.4**) and linalool (**4.5**) in the MG1 which were not identified in MG2. This is because geranyl pyrophosphate is the precursor for acyclic monoterpenes while neryl pyrophosphate is for cyclic monoterpenes. However, a rearrangement can be postulated to have taken place during the cooling or redistillation process. Furthermore, isomerization can be said to have taken place in methyl eugenol (**4.6**) during the heating and/or redistillation process of MG2 to give methyl eugenol ether also known as isoeugenol methyl ether (**4.7**)



(**4.4**) (**4.1**) (**4.5**) (**4.6**) (**4.7**)

Analysis of the GC-MS spectrum of MYG1 (Figure 4.1.1 - Appendix 2) and MYG2 (Figure 4.1.2 – Appendix 2) afforded the identification of 23 and 21 compounds, respectively (Table 4.4). There was no much noticeable variance in the chemical profile when compared to the oil extracts of the other two *Melaleuca* species. Like the other *M. bracteata* var. *revolution green* discussed above, methyl eugenol was the major component of both oils. Methyl eugenol has been reported as the major constitute of the oil of *M. bracteata* from Egypt [30, 31], thus making the South African *M. bracteata var. revolution* similar to those in the chemical literature.

A graphical representation of the major compounds in the three *Melaleuca* species studied is presented in Figure 4.7. It is obvious from the graph that the major compound in the two *M. bracteata* species is methyl eugenol while 1, 8-cineole dominates *M. trichostachya* var. *compacta*. It could also be seen clearly that MG2 has significant amount of 1,8-cineole and this was

completely absent in MG1. The chemical profile of MYG1 and MYG2 oils had almost similar constitutes with only percentages differences when compared to MG1 and MG2 or MAG1 and MAG2 in which the variance chemical compoistion was very much pronounced.

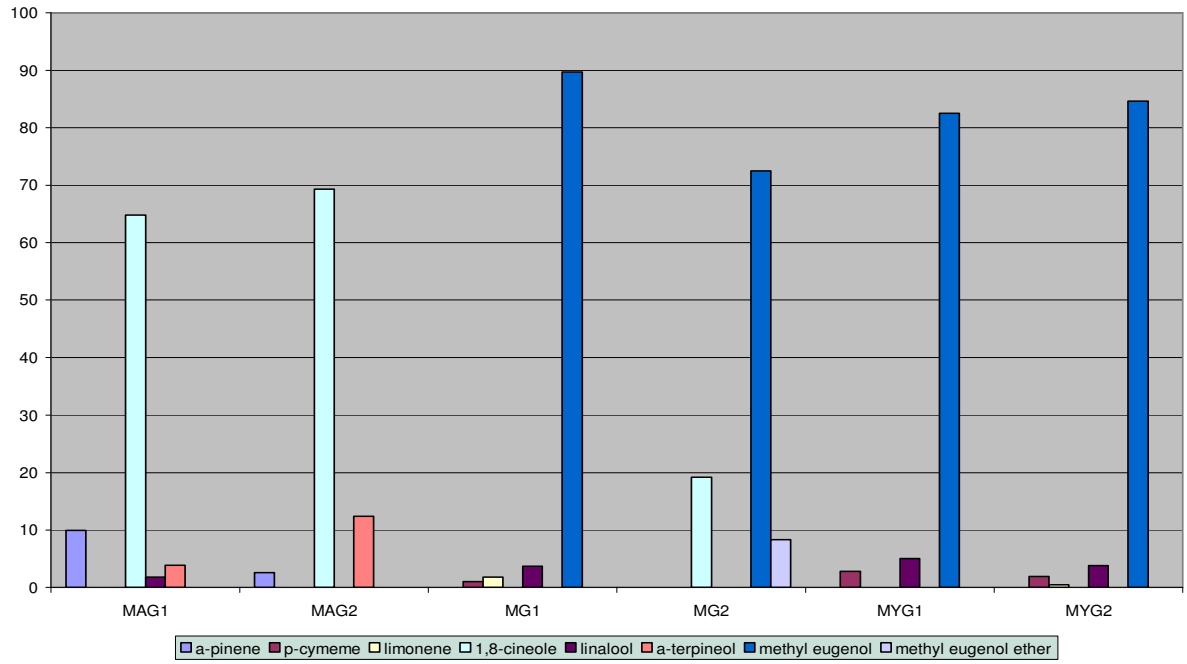


Figure 4.4: Comparison of major components of *three Melaleuca* essential oils

4.5.2 Antibacterial Activities of the Essential Oils of the Three *Melaleuca* Species

The result of the inhibition assay and minimum inhibition concentration (MIC) are presented in Table 4.5. Figure 4.5 - 4.7 are the 96 microwell plates of the essential oils against the 12 bacteria strained mention above (Section 4.3.1.B)

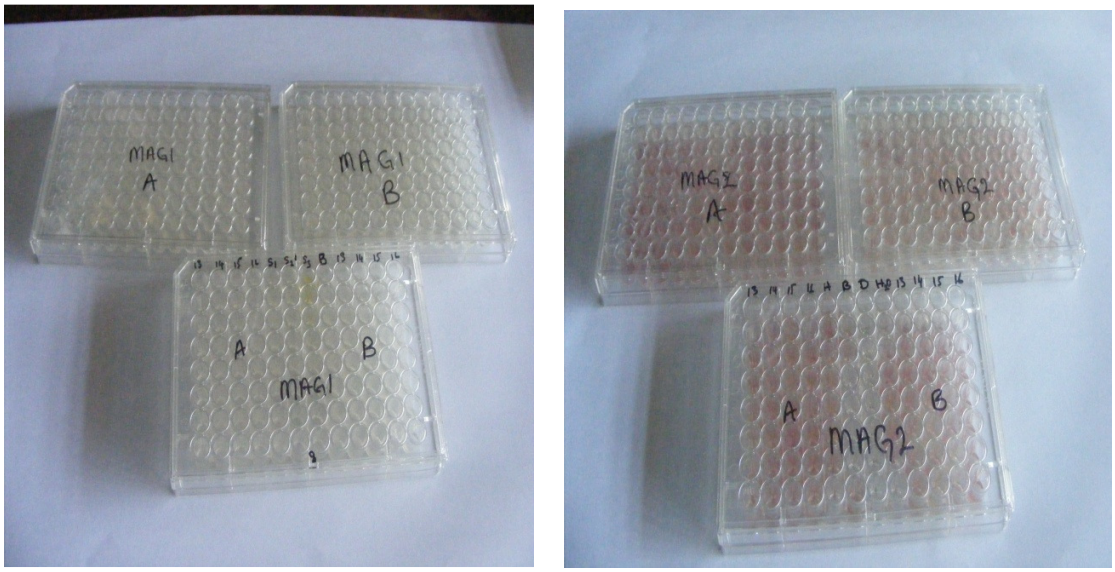


Figure 4.5: 96 Well plates (MIC) of the essential oils of *M. trichostachya* var. *compacta* (MAGI and MAG2)

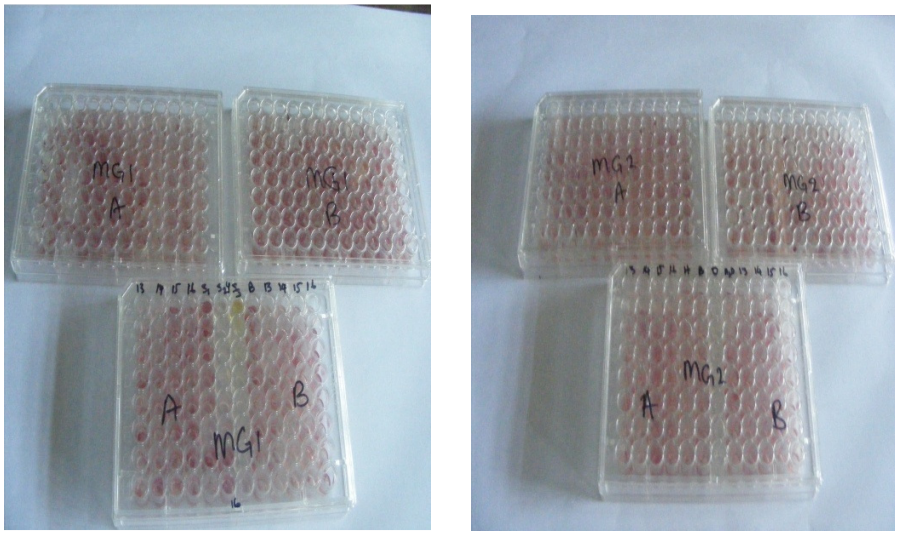


Figure 4.6: 96 Well plates (MIC) of the essential oil of *M. bracteata* var. *revolution green* (MGI and MG2)

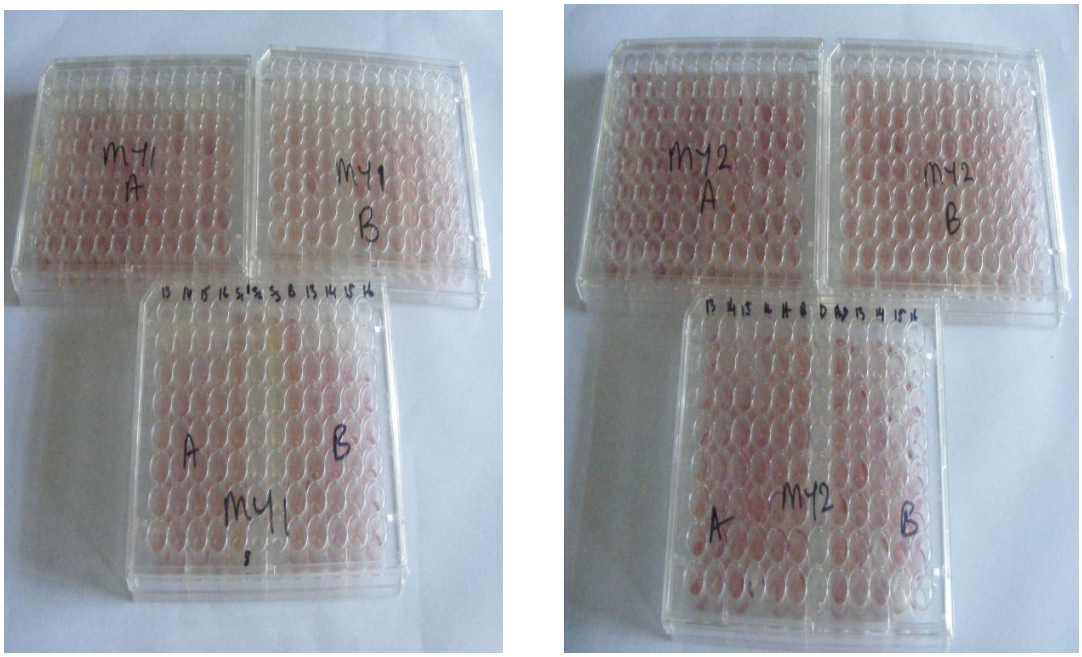


Figure 4.7: 96 Well plates (MIC) of the essential oil of *M. bracteata* var. *revolution gold* (MYGI and MYG2)

The zone of inhibition for all the six extracts ranged from 7.5-30.5 mm with MAG2 been the most active against all the bacteria (16.5-30.5 mm). Comparing the inhibition potential of MAG1 with MAG2, it was observed that MAG1 had a broader inhibitory effect on all the bacteria strains. The MIC result of MAG1 (1.25-0.030 mg/ml) followed similar pattern of success with very low inhibition concentration against most bacteria except for *E. coli* and *P. vulgaris.* Tetracycline is a known broad spectrum polyketide antibiotic, it is used for many bacteria infections; it is also known to be a protein synthesis inhibitor. It known to be used in treating cholera in developed countries of the world. Resistance to this antibiotic do arise from drug efflux, ribosomal protection proteins, rRNA mutation and drug inactivation.[50] Gentamycin is an amino glycoside antibiotic used for treating a wide range of bacterial infections with emphasis on Gram-negative bacteria.[51] The essential oils, MAG1 and MAG2, were more effective against these bacteria strain than tetracycline and gentamycin.

The inhibitory action of MG1 and MG2 were from 7.5-13.0 mm while the MIC ranges from 5.0 – 0.63 mg/ml. Similar pattern of activity was also notice for MYG1 and MYG2 but the activities of *M. bracteata var. revolution gold* was higher than *M. bracteata var. revolution green.* The IZ

activity of MYG1 and MYG2 ranged from 8.6-18.5 mm, while the MIC activity ranged from 2.50-0.63 mg/ml. The activity of MG2 was more pronounced than that of MG1. This pattern is similar to MAG1 and MAG2 discussed above. In general, the oils of *M. bracteata* var. *revolution gold and green* can be said to have moderate to low bioactivity.

The essential oil composition and antibacterial activities of *M. trichostachya* var. *compacta*, MAG1 and MAG2 are noteworthy as this is the first report on the chemical composition of the oils and their biological activity.

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CHAPTER FIVE

PHYTOCHEMICAL EXAMINATION OF *SYZYGIUM AROMATICUM* (L.) MERR. &PERRY

5.1 Introduction

Syzygium aromaticum (syn. *Eugenia caryophyllus, E. aromatica, Caryophyllus aromaticus*) belongs to the Myrtaceae family[1]. It is an evergreen tree that grows up to 10-20 m in height which is indigenous to India, Zanzibar, Indonesia, and Sir Lanka. The tree is best known for their bud which is often referred to as cloves[2, 3]. Figure 5.1 shows the whole tree, the leaves and the buds commonly known as cloves.

Figure 5.1: *Syzygium aromaticum* (L.) Merr. and Perry [4].: A – Whole tree. B – Leaves. C – Cloves.

5.2 Ethnobotanical Review of *Syzygium aromaticum*

Little is known about the ethnobotanical of the tree this is because the research has been more on the buds which is known as cloves. Cloves essential oil has eugenol as one of the major compounds and this compound is a powerful anti-inflammatory agent which is strongly anaesthetic. Eugenol inhibits prostaglandin E (PGE) synthesis which is responsible for creating

pain. Thus, clove oil gain popularity for pain relief from toothaches, mouth and gum sores or ulcers.[5]

Cloves are used traditionally to treat asthma [6], respiratory and digestive disorder. [1, 7] In Asia, cloves are used in treating dental illness, respiratory ailments, headaches, and sore throat complaints.[8] It is a common spice used in food preparation among certain tribes and believed to have anti-carcinogenic properties.[9, 10] The aqueous infusion of cloves has been reported to inhibit germination spores of *Bacillus subtilis* and other bacteria[11], thus often used as antiseptic[1]. It is also used as an antidote against oral bacteria often associated with dental caries and periodontal disease.[12, 13] It has been reported that *S. aromaticum* is among the major ingredient in toothpaste and mouth fresheners in India.[10] *S. aromaticum* has also been reported to show anti-inflammatory, cytotoxic and anaesthetic activity,[14] antifungal activity[15-16] antiviral and anti-hepatitis virus.[17-19] Eugenol and some other active components of the essential oil are used to prevent lipid peroxidation and have strong antioxidant properties.[1, 20-22]

Isolation of two flavonoid apigenin triglycosides from the seed of *S. aromaticum* has been reported by Nassar.[23] Eight compounds namely, gallic acid, ellagic acid, biflorin, kaempferol, rhamnocitrin, myricetin, D-glucopyranoside and oleanolic acid (OA) were isolated from methanol extract of cloves.[12]

5.3 Extractives of *Syzygium aromaticum*

5.3.1 Experimental

The buds of *S. aromaticum* were purchased from the spice market in Durban and were authenticated by Mr. Pravin Poorun a senior plant taxonomist, of the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Westville Campus. A voucher specimen OO4 was deposited at the University Herbarium.

5.3.2 Extraction and Isolation

The buds (1.5 kg) were milled using an industrial blender. The milled plant sample was extracted on a Labcom shaker with hexane (2 L) overnight and the solvent removed under reduced pressure to yield 137.76 g (9.2%) of plant extract. The plant residue (marc) was further extracted with ethyl

acetate (2 L) and removal of solvent was achieved under reduced pressure to give 68.20 g (4.6%) as the ethyl acetate extract.

4 g of the ethyl acetate extract was subjected to open column chromatography using Mapanga *et al* [24] method. Silica gel (70-230 mesh) in a column of 3 cm in diameter and hexane:ethyl acetate (80:20) was used as the eluting solvent. A total of 213 fractions were collected containing 20 ml of eluant, each. Each fraction was monitored using TLC and anisaldehyde/sulfuric acids as the spraying agent for spot development. Based on the results of the TLC, F_1-F_{22} (270 mg) was bulked together and further purified. Bulking and purification of F_{23}-F_{122} afforded a white powdery pure compound labelled **OO/55/A** (2.68 g).

Another 10 g of ethyl acetate extract was re-crystallized using methanol,[25] which gave 670 mg of pure oleanolic acid and 2.7 g a mixture of two compounds suspected to be oleanolic acid and maslinic acid. 0.69 g of the mixture was acetylated using pyridine and acetic anhydride with an aim to separate the compounds in the mixture, this was not successful. A portion (0.5 g) of the acetylated mixture was subjected to open column chromatography using silica gel (70-230 Mesh) as the stationary phase. Hexane:ethyl acetate (90:10 and 80:20) mixtures were used as the eluting solvents. Ten fractions of 20 ml each were collected for the 90:10 solvent, while 69 fractions of 10 mL each were collected for 80:20 eluting solvent. Each fraction was monitored by TLC and similar fractions were bulked together. F_{32}-F_{37} afforded a white powdery pure compound labelled **OO/55/B** (220 mg). This was subjected to NMR spectroscopic analysis for structural elucidation.

5.4 Results

Physical Data for Compound OO/55/A

Description: White powdery

Yield: 2.68 g

Melting point: 286-288 ^{O}C

Molecular weight (LC-MSD-Trap-VL):456.7

InfraRed Spectroscopy (Perkin Elmer ATR FTIR): $\nu_{C\text{-}OH}$ 3432, $\nu_{C=O}$1671, $\nu_{C=O\ (COOH)}$ 1721,$\nu_{C=C}$ 1641, $\nu_{C\text{-}O}$ 1008 cm^{-1}

Table 5.1 ^{1}H and ^{13}C NMR data of **OO/55/A**.

Position	δ_H	δ_H[26]	δ_C	δ_C[26]	δ_C[27]
1	0.87 (1H, *t*) 1.58 (1H, *t*)	NA	38.40 (*t*)	38.1	38.5
2	1.69 (1H, *m*) 1.84 (1H, *m*)	NA	27.20 (*t*)	27.2	27.4
3	3.25 (1H, *s*) (OH) 3.90 (1H, *m*)	3.23 (1H, *m*)	79.00 (*d*)	76.9	78.7
4	-	-	38.80 (*s*)	38.4	38.7
5	0.76 (1H, *t*)	NA	55.20 (*d*)	54.9	55.2
6	1.32 (1H, *m*) 1.57 (1H, *m*)	NA	18.30 (*t*)	18.0	18.3
7	1.73 (1H, *m*) 1.62 (1H, *m*)	NA	34.60 (*t*)	33.4	32.6
8	-	-	39.30 (*s*)	45.5	39.3
9	1.57 (1H, *m*)	NA	47.60 (*d*)	47.1	47.6
10	-	-	37.10 (*s*)	36.6	37.0
11	0.97 (1H, *m*) 1.68 (1H, *m*)	NA	23.60 (*t*)	22.9	23.1
12	5.25 (1H, *m*)	5.28 (1H, *m*)	122.70 (*d*)	121.5	122.1
13	-	-	143.60 (*s*)	143.7	143.4
14	-	-	41.60 (*s*)	41.3	41.6
15	1.07-1.11 (2H, *m*)	NA	27.70 (*t*)	26.9	27.7
16	1.59 (1H, *m*) 1.85(1H,*m*)	NA	23.40 (*t*)	22.6	23.4
17	-	-	46.50 (*s*)	45.5	46.6
18	2.80 (1H, *m*)	2.87(1H, *m*)	41.00 (*d*)	NA	41.3
19	1.23 (1H, *d*) 1.26 (1H, *d*)	NA	45.90 (*t*)	NA	45.8
20	-	-	30.70 (*s*)	30.4	30.6
21	1.23 (1H, *m*) 1.18 (1H, *m*)	NA	33.80 (t)	32.4	33.8
22	1.23 (1H, *m*) 1.80 (1H, *m*)	NA	32.40 (*t*)	NA	32.3
23	0.92 (3H, *s*)	0.89 (3H, *s*)	28.10 (*q*)	28.2	28.1
24	0.98 (3H, *s*)	0.87 (3H,*s*)	15.60 (*q*)	15.9	15.6
25	0.89 (3H,*s*)	0.91 (3H, *s*)	15.30 (*q*)	15.1	15.3
26	0.77 (3H, *s*)	0.75 (3H, *s*)	17.10 (*q*)	16.8	16.8
27	1.13 (3H, *s*)	1.12 (3H, *s*)	25.90 (*q*)	NA	26.0
28	-	-	182.30 (*s*)	178.6	181.0
29	0.90 (3H, *s*)	NA	33.10 (*q*)	32.9	33.1
30	0.91 (3H, *s*)	0.96 (3H, *s*)	23.00 (*q*)	23.4	23.6

Physical Data for Compound OO/55/B

Description: White powdery

Yield: 220 mg

Melting point: 226-228 ^{o}C

Molecular weight (LC-MSD-Trap-VL): 556.367

InfraRed Spectroscopy (Perkin Elmer ATR FT-IR): $\nu_{C\text{-}H}$ 2945, $\nu_{C=O}$1687, $\nu_{C=O\ (COOH)}$ 1746, $\nu_{C\text{-}O}$ 1107cm^{-1}

Table 5.2 ^{1}H and ^{13}C NMR data of **OO/55/B**

Position	δ_H	δ_H[28]	δ_C	δ_C[28]
1	1.32 (1H, *t*) 1.61(1H, *t*)	NA	43.80 (*t*)	44.8
2α	5.07 (1H,*s*)	5.10 (1H, ddd)	70.00 (*d*)	73.40
3β	4.73 (1H, *s*)	4.75 (1H)	80.60 (*d*)	81.5
4	-	-	38.20 (*s*)	40.1
5	0.95 (1H, *t*)	NA	54.90 (*d*)	55.50
6	1.36 (1H,*m*) 1.52 (1H,*m*)	NA	18.20 (*t*)	18.6
7	1.58 (1H,*m*) 1.98 (1H,*m*)	NA	33.80 (*t*)	32.8
8	-	-	39.30 (*s*)	39.7
9	1.60 (1H,*m*)	NA	47.50 (*d*)	48.0
10	-	-	39.40 (*s*)	38.7
11	1.59 (1H,*m*) 1.83 (1H,*m*)	NA	23.60 (*t*)	23.2
12	5.24 (1H, *m*)	5.25 (1H,br s)	122.20 (*d*)	123.0
13	-	-	143.70 (*s*)	144.0
14	-	-	41.60 (*s*)	42.0
15	1.06 (1H,*m*) 1.99 (1H,*m*)	NA	27.60 (*t*)	28.0
16	1.58 (1H,*m*) 1.93 (1H,*m*)	NA	22.80 (*t*)	23.3
17	-	-	46.50 (*s*)	46.90
18	2.78 (1H,*m*)	2.82 (1H,dd)	40.90 (*d*)	41.3
19		NA	45.80 (*t*)	46.20
20	-	-	32.40 (*s*)	31.0
21	1.44 (1H,*m*) 1.53 (1H,*m*)	NA	32.40 (t)	34.2
22	1.29 (1H,*m*) 1.74 (1H,*m*)	NA	30.70 (*t*)	30.0

Table 5.2 Continued.

23	0.90 (3H,*s*)	1.03 (3H,*s*)	28.40 (*q*)	28.9
24	0.75 (3H,*s*)	0.83 (3H,*s*)	17.10 (*q*)	17.0
25	1.05 (3H,*s*)	1.01 (3H,*s*)	16.40 (*q*)	16.7
26	0.84 (3H,*s*)	0.72 (3H,*s*)	17.60 (*q*)	17.4
27	1.09 (3H,*s*)	1.11 (3H,*s*)	25.90 (*q*)	26.3
28	-	-	180.70 (*s*)	185.0
29	0.88 (3H)	0.89 (3H,*s*)	33.10 (*t*)	33.5
30	0.94 (3H,*s*)	0.91 (3H,*s*)	23.60 (*q*)	24.0
1’	-		170.90 (*s*)	170.4
2’	2.03 (3H,*s*)	2.06 (3H,*d*)	20.90 (*q*)	21.8
3’	-		170.600 (*s*)	NA
4’	1.96 (3H,*s*)	NA	20.2 (*q*)	NA

5.5 Discussion

5.5.1 Compound OO/55/A

Proton NMR of compound **OO/55/A** gave signals of 48 protons with 30 carbons from the ^{13}C NMR (Figure 5.2.1 - 5.2.3 -Appendix 3). The presence of seven CH_3 groups, ten CH_2 groups and an olefinic proton prompted our suspicion of a triterpenoid. Two frequency absorption bands at 3463 and 1683 cm^{-1} in the IR spectrum (Figure 5.2.4 - Appendix 3) suggest the presence of a hydroxyl group OH and a carbonyl compound, respectively, indicating the presence of a carboxylic functionality as well. This was further strengthened by the OH proton signal at δ 3.25, a singlet implying that the OH is attached to a carbon and the presence of a carbonyl carbon at 180.7 ppm suggests the presence of an acid. A triterpenoid was therefore suspected to be **OO/55/A** with the help of the MS spectra (Figure 5.2.5-Appendix 3) which gave the molecular ion of 456.7 corresponding to $C_{30}H_{48}O_3$, the presence of an olefinic proton at C-12 (δ 5.24) and 2D NMR spectra (Figure 5.2.6 - 5.2.9 - Appendix 3) helped in assigning the protons and the carbons to an oleanane skeletal structure. Figure 5.2 is a representation of the suggested compound. These data presented in Table 5.1 correlated well with the literature report by Faycal *et al* [26] and Mahato, and Kundu,[27] thus Compound **OO/55/A** was identified as oleanolic acid.

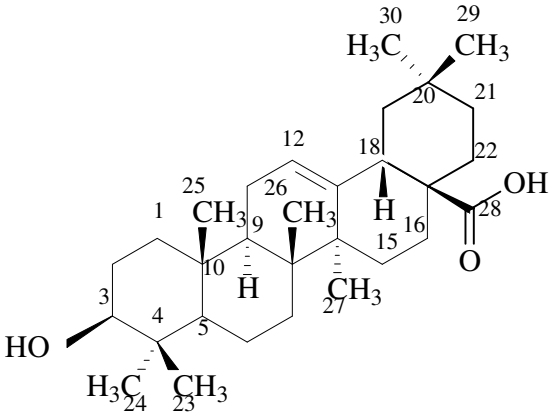

Figure 5.2 Structure of compound **OO/55/A** (Oleanolic acid)

5.5.2 Compound OO/55/B

The proton spectrum of OO/55/B (Figure 5.3.1-Appendix 3) was slightly different to that of **OO/55/A** with the nine CH_3 as compared to seven CH_3 while other protons were at similar position except for protons at C2 and C3. The ^{13}C NMR (Figure 5.3.2 and 5.3.3 - Appendix 3) helped in identifying three carbonyl carbons at 170.6, 170.9 and 180.7. The chemical shift of the two additional CH_3 at δ 1.96 and 2.03 suggest that they are methyl groups attached to carbonyl carbons. This was strengthened by the observed two carbonyl absorption frequencies at 1724 and 1695 cm^{-1} in the IR spectrum (Figure 5.3.4-Appendix 3) corresponding to an acid carbonyl and ester carbonyl functional group, respectively. Compound **OO/55/B** was suspected to be a diacetate of oleanolic acid derivative. The NMR data of synthesized diacetate of maslinic acid by Thomson *et al*[28] and Kojima and Ogura[29] correlate with the NMR data of the isolated compound **OO/55/B** (Figure 5.3.1 to 5.3.9 -Appendix 3) which assisted in unravelling the structure of **OO/55B** to be 2α,3β-diacetoxyloleanolic acid (Figure 5.3).

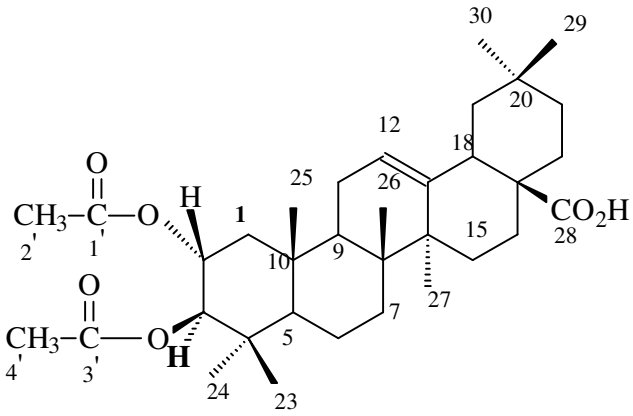

Figure 5.3: Structure of 2α,3β-diacetoxyloleanolic acid

5.6 Conclusion

Oleanolic acid and maslinic acid were successfully isolated from cloves in good to moderate yields. This study offers a method for large scale production of oleanolic and maslinic acids from cloves.

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CHAPTER SIX

PHYTOCHEMICAL EXAMINATION OF *TECTONA GRANDIS* L.F.

6.1 Introduction

Tectona grandis is a tropical hardwood tree in the Lamiaceae family (formerly Verbenaceae). It is native to South and southern Asia. The *Tectona* genus is made of three species namely, *T. grandis* (Common Teak), *T. hamiltoniana* (Dahat Teak) and *T. philippinensis* (Philippine Teak). The last two species are endangered.[1] *Tectona grandis* also known as Teak is a timber tree much sought-after because of the durability, strength, quality of its grain, quality of the wood, and resistance to various forms of mould and rot.[2] It is a large deciduous tree with maximum height of about 40 m and 2 m in diameter. The tree has large yellowish-green leaves with medium dense foliage and a straight trunk with tan –gray scaly bark (Figure 6.1). *T. grandis* is tolerant to wide range of climatic conditions.[3] Due to its enduring properties; Teak was introduced to some other parts of the world. 10% of the world teaks plantation comes from Africa and America.[2]

Figure 6.1: *Tectona grandis* [4-6]

T. grandis has excellent wide range of uses from flooring, decking, framing, cladding, to fascias and barge board. It is also used for lining, panelling, carving, turnery, furniture, rails and boat building.[7-8]

6.2 Ethnobotanical Review of *T. grandis*

The bark of *T. grandis* is traditionally used in the treatment of diabetes. [9] Warrier[10] reported the use of *T. grandis* as a traditional medicinal plant in treating diabetic, lipid disorder, inflammation, ulcer, and bronchitis illness. Further studies on *T. grandis* have revealed that the plant has antiucler[11], antimicrobial[12], wound healing,[13] and anticancer[14] activities. The leaves of *T. grandis* with or without *Jatropha curcas* and *Flacourtia flavenscens* are traditionally used in Togo to treat malaria and other anaemia diseases.[15] It has been reported that the leaves of *T. grandis* are widely used in the India folklore in the treatment of various kinds of wounds especially burn wound. [16]

Isolation of 2α,3β-dihydroxylup-12-en-28-oic acid, 3β-hydroxylup-12-en-28-oic acid, friedlin, 3β-friedelinol, betulinic acid, oleanolic acid and β-sitosterol from *T. grandis* has been reported.[16] 5-Hydroxylapachol, dehydro-α-lapachone, methylquinizarin and squalene were isolated by Khan and Mlungwana from the root of *T. grandis* and, 5-hydrxylapachol and dehydro-α-lapachone were found to be cytotoxic to *Artemia salina* (brime shrimp) and may find use in the treatment of cancer.[14] Bioactive apocarotenoids – tectoinols A and B have been isolated from the dried leaves of *T. grandis* and found to process strong allelopathic potential.[17] Quinones isolated from the sawdust extract of *T. grandis* has some good inhibitory activity against fungal cells - *Aspergillus niger*.[12] The ethanolic extract of *T. grandis* bark was found to be effective in treating Type-II induced diabetic rats.[9]

6.3 Extractives of *T. grandis*

6.3.1 Experimental

Stem bark of *T. grandis* (436.64 g) was collected from Obafemi Awolowo University Farm, Nigeria, in October 2007 and a voucher specimen (FOS/UKZN) was deposited at the University Herbarium.

6.3.2 Extraction and Isolation

T. grandis (stem bark, 436.64 g) was macerated in ethyl acetate (2 L) at room temperature on on a Labcon shaker for 5 days. The solvent was recovered at reduced pressure using a rota vapor. An ethyl acetate extract (2.47 g) was obtained. Another 2 L ethyl acetate solvent was further used to extract the marc (plant residue from the first extraction) on a Labcon shaker for 5 days. This procedure was repeated for the 3rd and 4th extraction. Combination of all the four extractions gave a light yellowish ethyl acetate extract which was charcoaled and recrystallised using methanol. The recrystallised product was filtered and dried to give a white solid tagged OO/21/EA with a weight of 710 mg (0.2%).

6.4 Results

Physical Data for Compound OO/21/EA

Description: White powdery solid

Yield: 710 mg

Melting point: 280-282 ^{o}C

Molecular weight (LC-MSD-Trap-VL): 456.7

InfraRed Spectroscopy (Perkin Elmer ATR FT-IR);$\nu_{C\text{-}OH}$ 3463, $\nu_{C\text{-}H}$ 2938, $\nu_{C=O}$1683, $\nu_{C=O\ (COOH)}$ 1644cm^{-1}

Table 6.1: ^{1}H and ^{13}C-NMR data of Compound **OO/21/EA**

Position	δ_H	δ_H [18]	δ_C	δ_C [18]	δ_C [19]
1	0.88 (1H,*t*) 1.03 (1H,*t*)	0.98 (*m*) 1.05 (1H,*m*)	38.70 (*t*)	37.1 (*t*)	38.7
2	1.93 - 1.95 (2H,*m*)	1.83 (*m*) 1.96 (*m*)	27.40 (*t*)	25.4(*t*)	27.4
3	3.50(1H, *s*) 3.15-3.18 (1H,*t*)	3.40(*br,s*)	79.02(*d*)	76.3(*d*)	78.9
4			38.96(*s*)	37.3(*s*)	38.8
5	0.64(1H,*t*)	0.68(*t*)	55.34(*d*)	50.3(*d*)	55.3
6	1.39(1H,*m*) 1.58 (1H,*m*)	1.40(*m*) 1.6(*m*)	18.28(*t*)	18.3(*t*)	18.3
7	1.35(1H,*m*) 1.41(1H,*m*)	1.35(*m*) 1.41(*m*)	34.32(*t*)	34.2(*t*)	34.3
8	-	-	40.69(*s*)	40.9(*s*)	40.7
9	1.66(1H,*m*)	1.68(*m*)	50.52(*d*)	49.3(*d*)	50.5

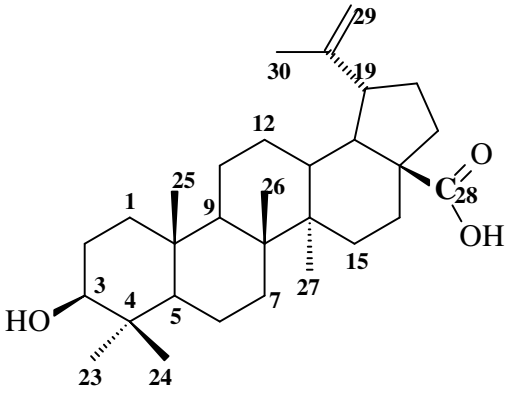
Table 6.1 Continued.

10	-	-	37.21(*s*)	37.5(*s*)	37.2
11	1.24(1H,*m*) 1.44(1H,*m*)	1.26(*m*) 1.42(*m*)	20.85(*t*)	20.7(*t*)	20.8
12	1.40(1H,*m*) 1.58(1H,*m*)	1.41(*m*) 1.52(*m*)	25.49(*t*)	25.5(*t*)	25.5
13	0.67(1H,*m*)	0.69(*m*)	38.39(*d*)	38.4(*d*)	38.4
14	-	-	42.44(*s*)	42.5(*s*)	42.4
15	1.35(2H,*m*)	1.41(*m*) 1.52(*m*)	30.55(*t*)	29.7(*t*)	30.5
16	1.35(1H,*m*) 1.40(1H,*m*)	1.33(*m*) 1.42(*m*)	32.16(*t*)	30.6(*t*)	32.1
17	-	-	56.28(*s*)	56.4(*s*)	56.3
18	1.67(1H,*m*)	1.77(*dd*)	46.89(*d*)	49.1(*d*)	46.8
19	3.14(1H,*ddd*)	3.01(*dt*)	49.27(*d*)	46.9(*d*)	49.2
20	-	-	150.42(*s*)	150.4(*s*)	150.3
21	1.42(1H,*m*) 1.51(1H,*m*)	1.41(*m*) 1.51(*m*)	29.70(t)	30.6(t)	29.7
22	1.35(1H,*m*) 1.42(1H,*m*)	1.35(*m*) 1.41(*m*)	37.03(*t*)	33.2(*t*)	37.0
23	0.80(3H,*s*)	0.81(*s*)	27.99(*q*)	16.0(*q*)	27.9
24	0.73(3H,*s*)	0.83(*s*)	15.35(*q*)	28.2(*q*)	15.3
25	0.91(3H,*s*)	0.93(*s*)	16.03(*q*)	15.9(*q*)	16.0
26	0.94(3H,*s*)	0.99(*s*)	16.13(*q*)	16.0(*q*)	16.1
27	0.95(3H,*s*)	1.02(*s*)	14.69(*q*)	14.8(*q*)	14.7
28	-	-	180.40(*s*)	181.5(*s*)	180.5
29	4.77(1H,*d*) 4.58(1H,*d*)	4.74(*br,s*) 4.65(*br,s*)	109.69(*t*)	109.7(*t*)	109.6
30	1.68(3H,*s*)	1.69(*s*)	19.37(*q*)	19.4(*q*)	19.4

6.5 Discussion

The ^{1}H NMR (Figure 6.1.1 - Appendix 4) of Compound OO/21/EA showed signals corresponding to 48 protons with OH proton at δ 3.50, two olefinic protons at δ 4.77 (1H,*d*) and 4.58 (1H,*d*). ^{13}C NMR and DEPT (Figure 6.1.2 and 6.1.3 - Appendix 4) gave 11 CH_2 (C*t*), 6 CH_3 (C*q*), 6 CH (C*d*), 6 quaternary C (C*s*) and a carbonyl C (C*s*) at 180 ppm which gave a total of 30 carbons (Table 6. 1). The molecular weight of the compound (456) (Figure 6.1.5 – Appendix 4) also suggests a pentacyclic compound with three oxygen atoms ($C_{30}H_{48}O_3$). Significant IR peaks (Figure 6.1.4 - Appendix 4) at 3463 cm^{-1} corresponding OH peak , 1683 cm^{-1} (C = O), 1449 cm^{-1}, 1375 cm^{-1},1237 cm^{-1},748 cm^{-1} strengthens our suspicion. A literature survey of spectroscopic data confirmed that

compound **OO/21/EA** was betulinic acid[18-19]. The melting point of compound **OO/21/EA** was in agreement with that found in report of Kweyu *et al*.[19] Assignment of protons to their carbons was achieved with the help of COSY (Figure 6..1.6 - Appendix 4), HSQC (Figure 6.1.7 - Appendix 4), HMBC (Figure 6.1.8 - Appendix 4) and NOSEY (Figure Figure 6.1.9 - Appendix 4)



Betulinic acid

6.6 Conclusion

The isolated compound designated as **OO/21/EA** was betulinic acid. This was confirmed by its spectroscopic data.

6.7 References

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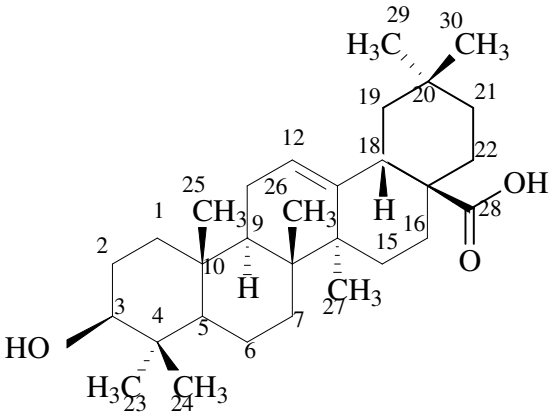
CHAPTER SEVEN

SOME DERIVATIVES OF OLEANOLIC ACID

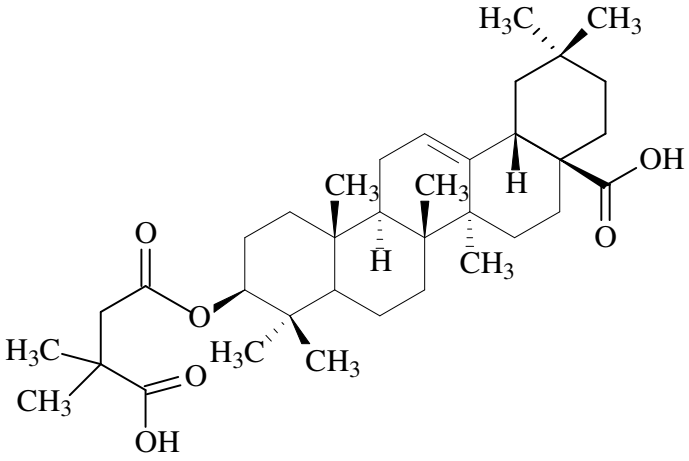
7.1 Introduction

Pentacyclic triterpenoids of which oleanolic acid is a member are compounds found commonly in most plants. There are about 4000 known triterpenes with various wide biological properties.[1] Oleanolic acid (**7.1**) has been reported isolated from various plant sources and identified as anti-HIV agents with EC_{50} and TI values of 3.7 and 4.4,.respectively.[2-3]



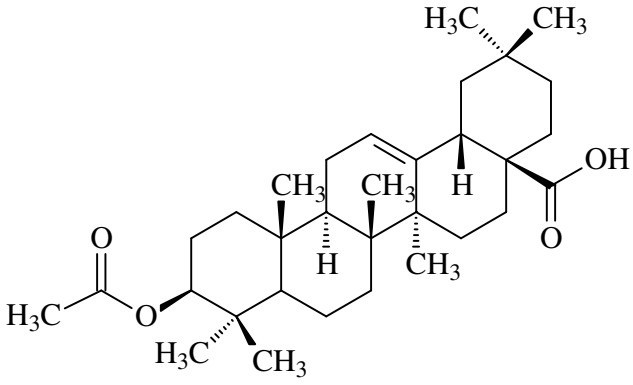
7.1

Most of the syntheses of oleanolic acid derivatives reported were compounds in which positions 3, 28 and 12 of oleanolic acid have been modified in one way or another. Kashiwada Y *et al*.[4] have synthesized the esters of oleanolic acid (**7.2**) (3-*O*-(3′,3′-dimethyl)-succinyloleanolic acid) in reasonable yield. The semi-synthetic derivative was found to be more active as an anti-HIV agent than its parent compound.[4]



7.2

Hichri, F. *et al*., [2] also reported some synthesized derivatives of oleanolic acid and their biological activities. In his report, acetylation of oleanolic acid (**7.1**) gave acetyloleanolic acid (**7.3**) while oxidation of the acetyloleanolic acid yielded 12α-hydroxyl-δ-lactone (**7.4**) and 12-oxo-δ-lactone (**7.5**) after 4 hours. However, **7.4**, **7.5** and 3-acetyl-11-oxo-oleanolic acid (**7.6**) was obtained after 12 hours.



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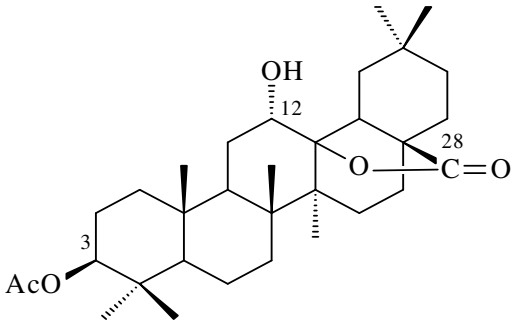

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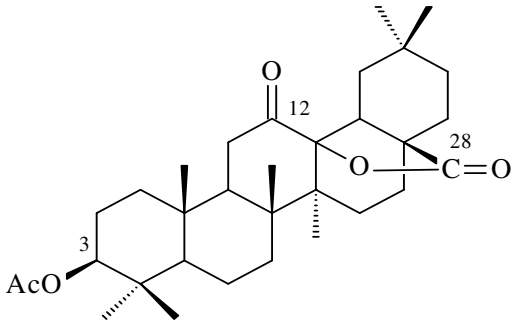

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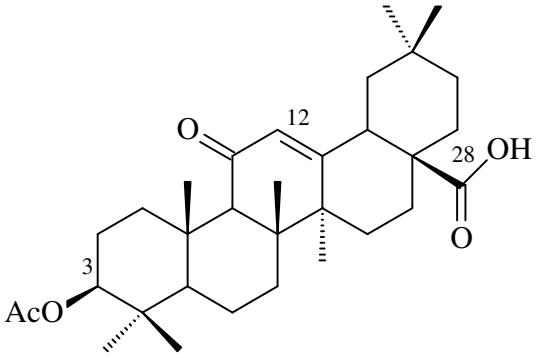
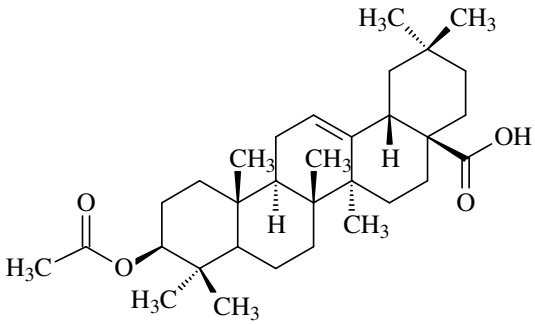
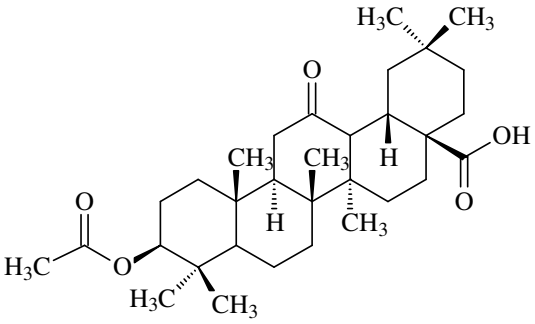


7.6

Li, J.F, *et al*. [5] have reported the synthesis of two acetyl derivatives of oleanolic acid (**7.3** and **7.8**) and an oxidative derivative of oleanolic acid at position 3 to give a 3-keto oleanolic acid (**7.9**). These three derivatives are known to be anti-osteocast formation agents.



7.3



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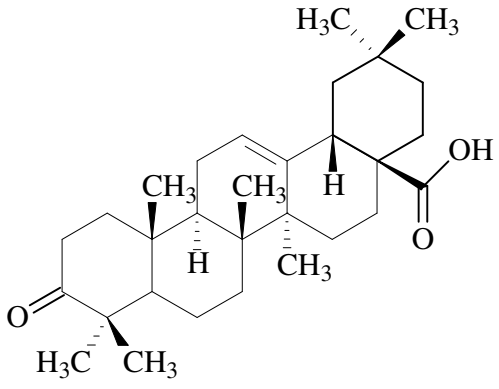
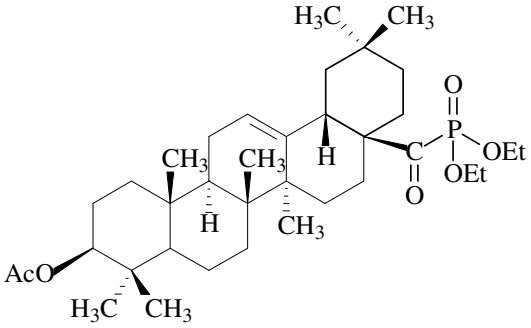
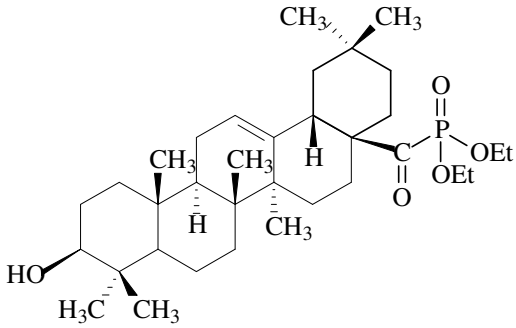


7.9

Phosphate derivatives of acetyloleanoic acid; diethyl-3-acetylolean-12-en-28-oxyphosphate (**7.10**) and diethyl-3-hydroxyolean-28-oxyphosphonate (**7.11**) were also prepared by Hirch *et al*.[2]

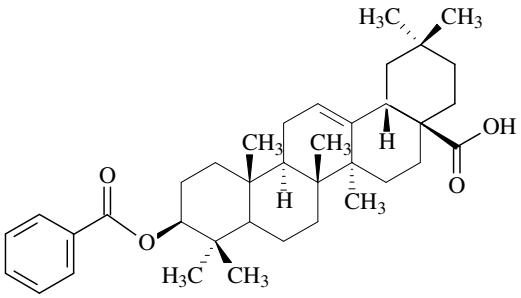


7.10

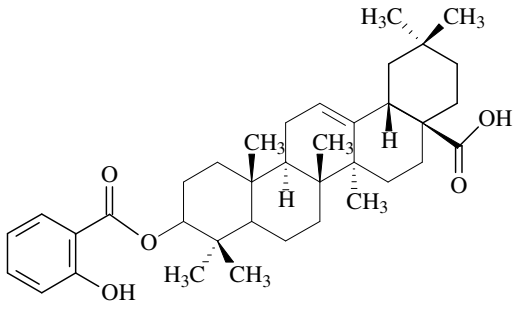


7.11

Also three ester derivatives of oleanolic acid (**7.12-7.14**) have been reportedly synthesized by Hirch *et al*.[2]



7.12



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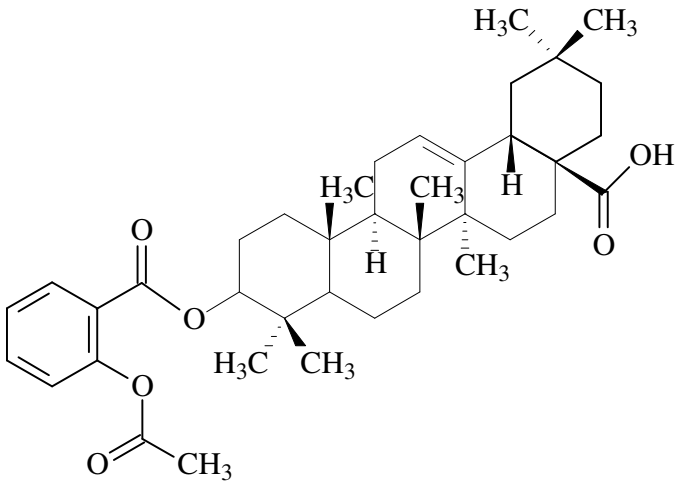


7.14

Compounds **7.4**-**7.6**, **7.9**-**7.11** are known to be good anti-inflammatory agents.[2, 5] Several other derivatives of oleanolic acid have been synthesized by various researchers.[7] Some of them are promising bioactive lead compounds.[7] During a literature review of known bioactive oleanolic acid derivatives, it was observed that there are still many derivatives of OA which have not yet been

synthesized and which could have useful pharmacological properties. As a second phase of our chemical prospecting of medicinal plants for drug discovery purposes and the great promises shown by oleanolic acid as anti-diabetic [6] and anti-viral drug,[4] it was decided to synthesize some known and unknown derivatives of OA for biological evaluation.

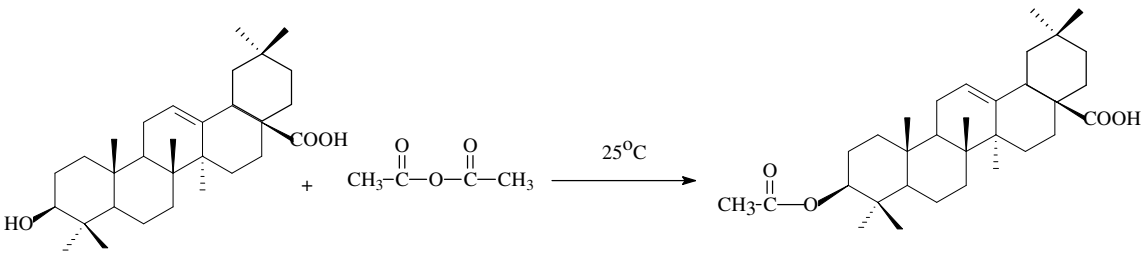
7.2 Semi-synthesis of Oleanolic Acid Derivatives

7.2.1 3-Acetoxyoleanolic Acid

Experimental Procedure

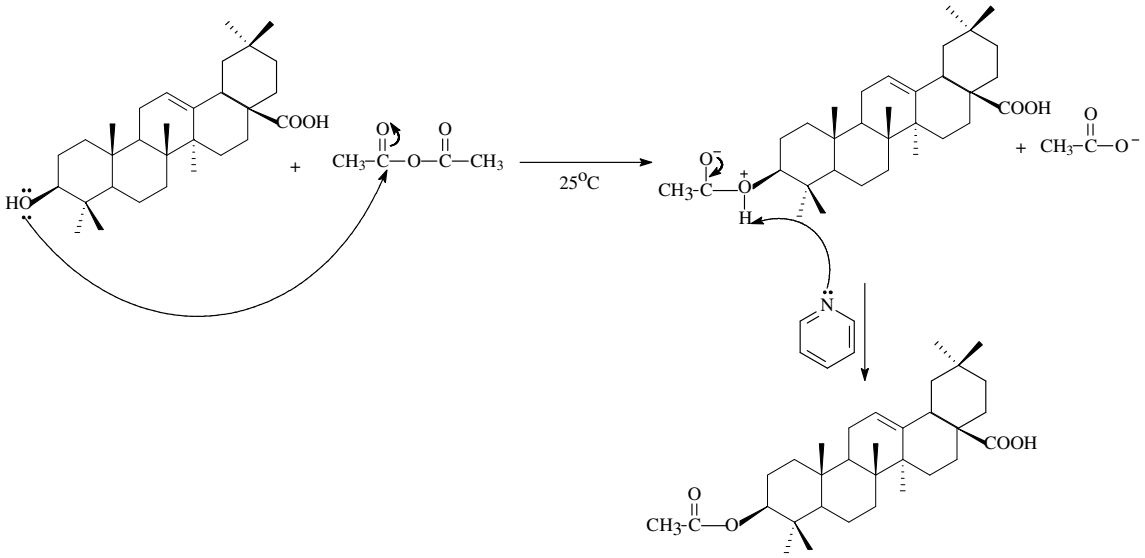
The synthesis of 3-acetoxyoleanolic acid was carried out using the procedure in Hichri, F. *et al.*[2] Oleanolic acid (OA) (2 g, 0.004 mol.) was dissolved in pyridine (5 mL) and excess acetic anhydride (10 mL) was added in a 100mL round bottom flask. The mixture was stirred for about 12 hrs at room temperature. The product was poured into 100 mL of water to hydrolyse excess acetic anhydride. The final product was separated by suction filtration and purified by column chromatography to give the compound OO/52.

Equation of the reaction



Oleanolic acid 3-Acetyloleanolic acid

Mechanism of Reaction



During the hydrolysis excess anhydride was converted to acetic acid which reacts with pyridine to form a salt. The salt was soluble in the aqueous layer while the acetate was filtered off.

Results and Discussion

Physical Data for Compound OO/52 (3-Acetoxyoleanolic acid)

Description: White powdery solid

Yield: 1.67 g (83.5%)

Melting point: 260-262 ^{o}C

Molecular weight (LC-MSD-Trap-VL): 498.7

InfraRed Spectroscopy (Perkin Elmer ATR FT-IR); $\nu_{C\text{-}OH}$ 3200 $\nu_{C\text{-}H}$ 2941, $\nu_{C=O}$1679, $\nu_{C=O\ (COOH)}$ 1721, $\nu_{C\text{-}O}$ 1077cm^{-1}

Table 7.1 ^{1}H and ^{13}C-NMR data of 3-Acetoxyoleanolic acid

Position	δ_H(OA)	δ_H (Acetate of OA)	δ_C(OA)	δ_C (Acetate of OA)	$\delta_C{}^2$ (Acetate of OA)
1	0.87(1H,*t*) 1.58(1H,*t*)	1.05(1H,*m*) 1.58(1H,*m*)	38.41(*t*)	38.07(*t*)	38.0
2	1.69(1H,*m*) 1.84 (1H,*m*)	1.06(1H.m) 1.70(1H,m)	27.18(*t*)	27.66(*t*)	27.7

Table 7.1 Continued.

3	3.25(1H, *s*)(OH) 3.90 (1H,*m*)	4.24(1H,*m*)	79.03(*d*)	80.93(*d*)	80.9
4	-	-	38.76(*s*)	39.28(*s*)	39.0
5	0.76(1H,*t*)	0.79(1H,m)	55.21(*d*)	55.29(*d*)	55.3
6	1.32(1H,*m*) 1.57 (1H,*m*)	1.30(1H,m) 1.51(1H,m)	18.30(*t*)	18.18(*t*)	18.1
7	1.73(1H,*m*) 1.62(1H,*m*)	1.45(1H,m) 1.51(1H,m)	34.63(*t*)	32.55(*t*)	32.8
8	-	-	39.27(*s*)	36.97(*s*)	NA
9	1.57(1H,*m*)	1.58(1H,m)	47.63(*d*)	47.55(*s*)	48.0
10	-	-	37.07(*s*)	37.69(*s*)	37.7
11	0.97(1H,*m*) 1.68(1H,*m*)	1.94-1.95 (2H,m)	23.57(*t*)	23.52(*t*)	24.0
12	5.25 (1H,*m*)	5.15(1H,m)	122.65(*d*)	122.57(*d*)	122.5
13	-	-	143.58(*s*)	143.59(*s*)	143.6
14	-	-	41.63(*s*)	41.59(*s*)	41.5
15	1.07-1.11(2H,*m*)	1.09(1H,m) 1.63(1H,m)	27.68(*t*)	28.04(*t*)	28.0
16	1.59(1H,*m*) 1.85(1H,*m*)	1.56(1H,m) 1.86(1H,m)	23.40(*t*)	23.39(*t*)	23.4
17	-	-	46.49(*s*)	46.51(*s*)	46.6
18	2.80(1H,*m*)	2.79(1H,*m*)	41.04(*d*)	41.00(*d*)	NA
19	1.23(1H,*d*) 1.26(1H,*d*)	1.55(1H,m) 1.69(1H,m)	45.88(*t*)	45.84(*t*)	NA
20	-	-	30.67(*s*)	30.67(*s*)	30.7
21	1.23(1H,*m*) 1.18(1H,*m*)	1.30(1H,m) 1.38(1H,m)	33.80(*t*)	33.79(*t*)	33.8
22	1.23(1H,*m*) 1.80(1H,*m*)	1.54(1H,m) 1.76(1H,m)	32.43(*t*)	32.43(*t*)	NA
23	0.92(3H,*s*)	0.89(3H,m).	28.10(*q*)	22.98(*q*)	29.7
24	0.98(3H,*s*)	0.72(3H,*s*)	15.55(*q*)	16.66(*q*)	16.7
25	0.89(3H,*s*)	0.99(3H,*s*)	15.32(*q*)	15.38(*q*)	15.5
26	0.77(3H,*s*)	0.84(3H,*s*)	17.11(*q*)	17.13(*q*)	17.2
27	1.13(3H,*s*)	1.16(3H,*s*)	25.92(*q*)	25.88(*q*)	NA
28	-	-	182.27(*s*)	182.75(*s*)	178.6
29	0.90(3H,*s*)	0.87(3H,s)	33.06(*q*)	33.05(*q*)	33.1
30	0.91(3H,*s*)	0.90(3H,s)	22.96(*q*)	23.57(*q*)	23.6
1′		-	-	171.04(*s*)	171.0
2′		2.06(3H,*s*)	21.31(*q*)	22.93(*q*)	22.80

NA = Not available in literature

Significant changes were noticed when the ^{1}H NMR and ^{13}C NMR of oleanolic acid (Figure 5.2.1 – 5.2.3 Appendix 3) were compared with the spectra data (Figure 7.1.1 – 7.1.3 Appendix 5) obtained from the synthesized acetate of OA. Only one proton was identified in the ^{1}H NMR spectra at position 3 while the hydroxyl proton was lost. Furthermore, a carbonyl carbon was observed at 171.04 ppm in addition to carbonyl carbon of the carboxylic acid and a methyl group at δ 2.06 (*s*), these suggesting the introduction of an acetyl group at position 3. This was further substantiated with the increase in number of proton and carbon atoms (MS spectrum – Figure 7.1.5 Appendix 5) and the IR spectra (Figure 7.1.4 Appendix 5). Using the 2D-NMR spectra (Figure 7.1.6-7.1.9 Appendix 5) it was ascertained that the synthesised compound OO/52 was 3-Acetoxyoleanolic acid (Figure 7.1) and the NOESY correlation is presented in Figure 7.2. Table 7.1 displays the significant differences of the proton and carbon NMR data of oleanolic acid and 3-acetoxyoleanolic acid.

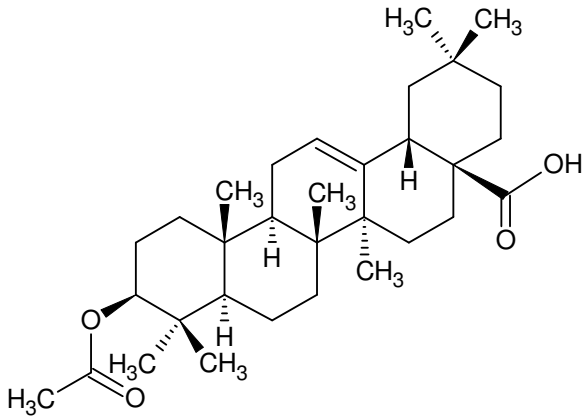


Figure 7.1: Chemical Structure of **OO/52** (3β-Acetoxyoleanolic acid)

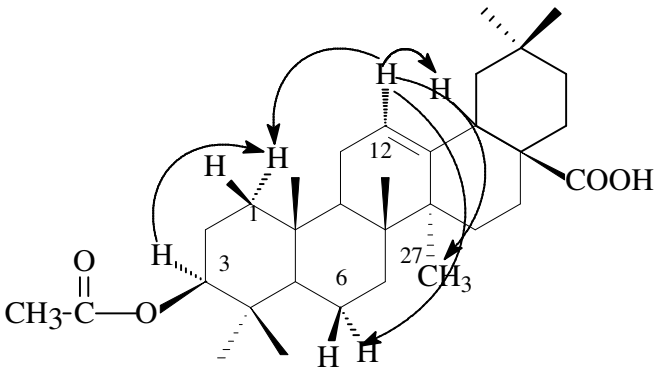


Figure 7.2: Significant NOESY correlation of 3-Acetoxyoleanolic acid

7.2.2 3-Acetyloleanolic Hydrazide

Experimental Procedure

3-Acetyloleanolic acid (0.4 g, 0.008 mol) and triethylamine (0.1 g, 0.001 mol) were dissolved in dichloromethane (20 mL). A solution of thionyl chloride (0.1 g, 0.001 mol) in dichloromethane (10 ml) was added to the mixture dropwise while cooling under ice and continuous stirring. About 0.25 g of hydrazine hydrate was added to the mixture. A solution of triethylamine (0.1 g, 001 mol) in 10ml of dichloromethane was prepared and added to the mixture drop wise under ice and continuous stirring for 3 h. On completion of the reaction, the dichloromethane soluble fraction was separated and the solvent was distilled off to give a crude reaction product. The product was purified using column chromatography with silica gel as the stationary phase. 46 fractions were collected and analysed with thin layer chromatography (TLC), fractions 1-33 were combined as unreacted acetoxyoleanolic acid while fractions 34-46 contained the expected product (95 mg) which was labelled OO/48(TT/027). Compound **OO/48** was subjected to a full 1D and 2D NMR analysis.

Results and Discussion

Physical Data for Compound OO/48 (3-Acetyloleanolic hydrazide**)**

Description: White powdery solid

Yield: 95 mg (23.75%)

Melting point: 210-212 ^{o}C

Molecular weight (LC-MSD-Trap-VL): 512

InfraRed Spectroscopy (Perkin Elmer ATR FT-IR); $\nu_{C=O}$1729, ν_{N-H} 3308, ν_{C-N} 1251, ν_{C-H} 2939, $\nu_{C=O}$1618, ν_{C-O} 1075, $\nu_{C=C}$1655 cm^{-1}

The 1D and 2D NMR spectra along with the IR spectra and Mass spectra (Figure 7.3.1-7.3.9 Appendix 5) confirmed the transformation of the C 28 from a carboxylic acid to a hydrazide as presented in Figure 7.3. Compound **OO/48** was therefore confirmed to be a synthesized hydrazide of 3-acetyloleanolic acid. Table 7.2 presents the ^{1}H and ^{13}C NMR data of the synthesized 3-acetyloleanolic hydrazide.

Table 7.2 ^{1}H and ^{13}C-NMR data of Compound **OO/48** (3-Acetyloleanolic hydrazide)

Position	δ_H (OA)	δ_H (Acetate of OA)	δ_H (Hydrazide o OA)	δ_C (OA)	δ_C (Acetate of OA)	δ_C (Hydrazide of OA)
1	0.87(1H,*t*) 1.58(1H,*t*)	1.05(1H,*m*) 1.58(1H,*m*)	1.05(1H,*m*) 1.60(1H,*m*)	38.41(*t*)	38.07(*t*)	38.11(*t*)
2	1.69(1H,*m*) 1.84 (1H,*m*)	1.06(1H.*m*) 1.70(1H,*m*)	1.09(1H,*m*) 1.48(1H,*m*)	27.18(*t*)	27.66(*t*)	27.20(*t*)
3	3.25(1H,*s*)(OH) 3.90 (1H,*m*)	4.24(1H,*m*)	4.47(1H,*m*)	79.03(*d*)	80.93(*d*)	80.81(*d*)
4	-	-	-	38.76(*s*)	39.28(*s*)	39.35(*s*)
5	0.76(1H,*t*)	0.79(1H,*m*)	0.85(1H,*m*)	55.21(*d*)	55.29(*d*)	55.18(*d*)
6	1.32(1H,*m*) 1.57(1H,*m*)	1.30(1H,*m*) 1.51(1H,*m*)	1.46(1H,*m*) 1.73(1H,*m*)	18.30(*t*)	18.18(*t*)	18.13(*t*)
7	1.73(1H,*m*) 1.62(1H,*m*)	1.45(1H,*m*) 1.51(1H,*m*)	1.49(1H,*m*) 1.54(1H,*m*)	34.63(*t*)	32.55(*t*)	32.95(*t*)
8	-	-	-	39.27(*s*)	36.97(*s*)	36.83(*s*)
9	1.57(1H,*m*)	1.58(1H,*m*)	1.58(1H,*m*)	47.63(*d*)	47.55(*s*)	47.45(*d*)
10	-	-	-	37.07(*s*)	37.69(*s*)	37.68(*s*)
11	0.97(1H,*m*) 1.68(1H,*m*)	1.94-1.95 (2H,*m*)	1.63(1H,*m*) 1.91(1H,*m*)	23.57(*t*)	23.52(*t*)	23.48(*t*)
12	5.25(1H,*m*)	5.15(1H,*m*)	5.35(1H,*m*)	122.65(*d*)	122.57(*d*)	123.32(*d*)
13	-	-	-	143.58(*s*)	143.59(*s*)	144.73(*s*)
14	-	-	-	41.63(*s*)	41.59(*s*)	41.89(*s*)
15	1.07-1.11 (2H,*m*)	1.09(1H,*m*) 1.63(1H,*m*)		27.68(*t*)	28.04(*t*)	27.99(*t*)
16	1.59(1H,*m*) 1.85(1H,*m*)	1.56(1H,*m*) 1.86(1H,*m*)	1.60(1H,*m*) 1.90(1H,*m*)	23.40(*t*)	23.39(*t*)	23.50(*t*)
17	-	-	-	46.49(*s*)	46.51(*s*)	46.39(*s*)
18	2.80(1H,*m*)	2.79(1H,*m*)	2.47-2.43 (1H,*m*)	41.04(*d*)	41.00(*d*)	41.41(*d*)
19	1.23(1H,*d*) 1.26(1H,*d*)	1.55(1H,*m*) 1.69(1H,*m*)	1.18(1H,*m*) 1.72(1H,*m*)	45.88(*t*)	45.84(*t*)	45.86(*t*)
20	-	-	-	30.67(*s*)	30.67(*s*)	30.71(*s*)
21	1.23(1H,*m*) 1.18(1H,*m*)	1.30(1H,*m*) 1.38(1H,*m*)	1.20(1H,*m*) 1.38(1H,*m*)	33.80(t)	33.79(*t*)	33.93(*t*)
22	1.23(1H,*m*) 1.80(1H,*m*)	1.54(1H,*m*) 1.76(1H,*m*)	1.24(1H,*m*) 1.75(1H,*m*)	32.43(*t*)	32.43(*t*)	32.12(*t*)
23	0.92(3H,*s*)	0.89(3H,*m*)	0.87(3H,*s*)	28.10(*q*)	22.98(*q*)	21.05(*q*)
24	0.98(3H,*s*)	0.72(3H,*s*)	0.72(3H,*s*)	15.55(*q*)	16.66(*q*)	16.65(*q*)
25	0.89(3H,*s*)	0.99(3H,*s*)	0.98(3H,*s*)	15.32(*q*)	15.38(*q*)	15.39(*q*)

Table 7.2 Continued

26	0.77(3H,*s*)	0.84(3H,*s*)	0.82(3H,*s*)	17.11(*q*)	17.13(*q*)	16.64(*q*)
27	1.13(3H,*s*)	1.16(3H,*s*)	1.15(3H,*s*)	25.92(*q*)	25.88(*q*)	25.78(*q*)
28	-	-	-	182.27(*s*)	182.75(*s*)	179.17(*s*)
29	0.90(3H,*s*)	0.87(3H,*s*)		33.06(*q*)	33.05(*q*)	32.95(*q*)
30	0.91(3H,*s*)	0.90(3H,*s*)	0.89(3H,*s*)	22.96(*q*)	23.57(*q*)	23.74(*q*)
1′	-	-	-	-	171.04(*s*)	171.04(*s*)
2′	-	2.06(3H,*s*)	2.08(3H,s)	-	22.93(*q*)	21.30(*q*)
O=^{28}C-NH	-	-	7.02(1H,*m*)	-	-	-
O=^{28}C-NH-NH$_2$	-	-	2.6-4.0 (2H,*b* *m*)	-	-	-

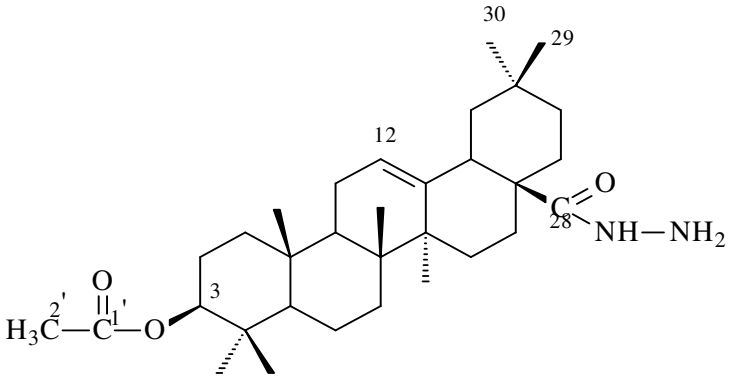


Figure 7.3: Chemical Structure of compound OO/48 (3-Acetoxyoleanolic hydrazide)

7.2.3 3-Acetoxyoleanolic Hydrazone

Experimental Procedure

A mixture of 3-acetoxyoleanolic hydrazide (200 mg), benzaldehyde (0.05 g), glacial acetic acid (2 drops), and methanol (20 mL) in a round bottom flask was refluxed for 2 hours during which the desired product crystallized out. The crystals were filtered by suction from the solution, washed, and dried. The yield was 165 mg (82.5%). The product was coded as **OO/56**.

Results and Discussion

Physical Data for Compound OO/56 (3-Acetoxyoleanolic hydrazone**)**

Description: White powdery solid

Yield: 165 mg

Melting point: 168-170 ^{o}C

Molecular weight (LC-MSD-Trap-VL): 587

InfraRed Spectroscopy (Perkin Elmer ATR FTIR); $\nu_{C\text{-}H}$ 2945, $\nu_{C\text{-}N}$ 2163, $\nu_{C=O}$1661, $\nu_{C=O\ (COOH)}$ 1728, $\nu_{C\text{-}O}$ 1071cm^{-1}

Comparison of the ^{1}H and ^{13}C NMR (Figure 7.4.1- 7.4.9- Appendix 5) of the synthesized 3-acetoxyoleanolic hydrazone with those of oleanolic acid (Table 7.2) confirmed the product as 3-acetoxyoleanolic hydrazone (Figure 7.4).

Table 7.3 ^{1}H and ^{13}C NMR data of compound **OO/56** (3-Acetyloleanolic acid hydrazone)

Position	δ_H (OA)	δ_H (Hydrazone of OA)	δ_C (Hydrazide of OA)	δ_C (Hydrazone of OA)
1	0.87(1H,*t*) 1.58(1H,*t*)	1.05(1H,*m*) 1.58(1H,*m*)	38.11(*t*)	38.17(*t*)
2	1.69(1H,*m*) 1.84 (1H,*m*)		27.20(*t*)	27.21(*t*)
3	3.25(1H,*s*)(OH) 3.90 (1H,*m*)	4.46(1H,*m*)	80.81(*d*)	80.81(*d*)
4	-	-	39.35(*s*)	39.50(*s*)
5	0.76(1H,*t*)		55.18(*d*)	55.13(*d*)
6	1.32(1H,*m*) 1.57 (1H,*m*)		18.13(*t*)	18.11(*t*)
7	1.73(1H,*m*) 1.62(1H,*m*)		32.95(*t*)	
8	-	-	36.83(*s*)	36.82(*s*)
9	1.57(1H,*m*)		47.45(*d*)	47.46(*d*)
10	-	-	37.68(*s*)	37.67(*s*)
11	0.97(1H,*m*) 1.68(1H,*m*)		23.48(*t*)	23.49(*t*)
12	5.25 (1H,*m*)	5.53(1H,m)	123.32(*d*)	123.47(*d*)
13	-	-	144.73(*s*)	145.49(*s*)
14	-	-	41.89(*s*)	42.05(*s*)
15	1.07- 1.11(2H,*m*)		27.99(*t*)	28.01(*t*)

Table 7.3 Continued

16	1.59(1H,*m*) 1.85(1H,*m*)		23.50(*t*)	23.69(t)
17	-	-	46.39(*s*)	46.69 (s)
18	2.80(1H,*m*)	2.79(1H,*m*)	41.41(*d*)	42.17(d)
19	1.23(1H,*d*) 1.26(1H,*d*)		45.86(*t*)	46.47(t)
20	-	-	30.71(*s*)	30.73(s)
21	1.23(1H,*m*) 1.18(1H,*m*)		33.93(*t*)	34.01(t)
22	1.23(1H,*m*) 1.80(1H,*m*)		32.12(*t*)	32.07(t)
23	0.92(3H,*s*)		21.05(*q*)	21.31(*q*)
24	0.98(3H,*s*)	0.98(3H,*s*)	16.65(*q*)	16.96(q)
25	0.89(3H,*s*)	0.89(3H,*s*)	15.39(*q*)	15.44(q)
26	0.77(3H,*s*)	0.82(3H,*s*)	16.64(*q*)	16.63(q)
27	1.13(3H,*s*)	1.16(3H,*s*)	25.78(*q*)	25.79(q)
28	-	-	179.17(*s*)	174.82(s)
29	0.90(3H,*s*)		32.95(*q*)	32.93(q)
30	0.91(3H,*s*)		23.74(*q*)	24.16(q)
1′		-	171.04(*s*)	171.06(q)
2′		2.06(3H,*s*)	21.30(*q*)	
O=C-NH			-	-
3′		8.98(1H,s)	-	147.69(d)
4′		-	-	134.19(s)
5′		7.71(1H,d)	-	128.62(d)
6′		7.37(1H,d)	-	127.73(d)
7′		7.36(1H,m)	-	127.71(d)
8′		7.37(1H,m)	-	127.73(d)
9′		7.71(1H,d)	-	128.62(d)

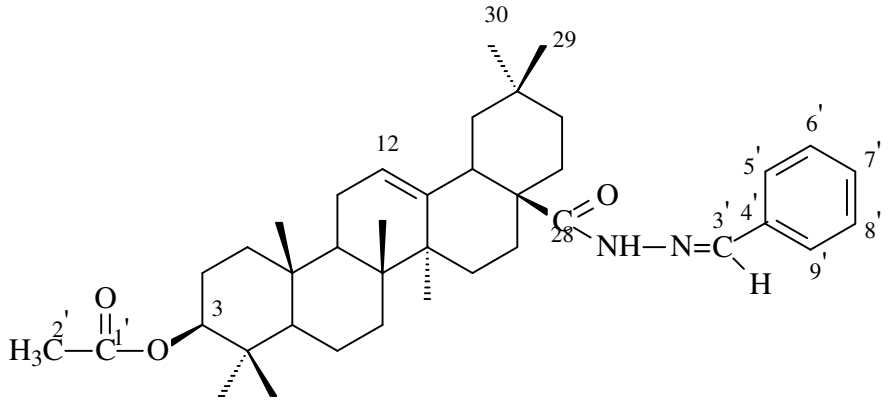


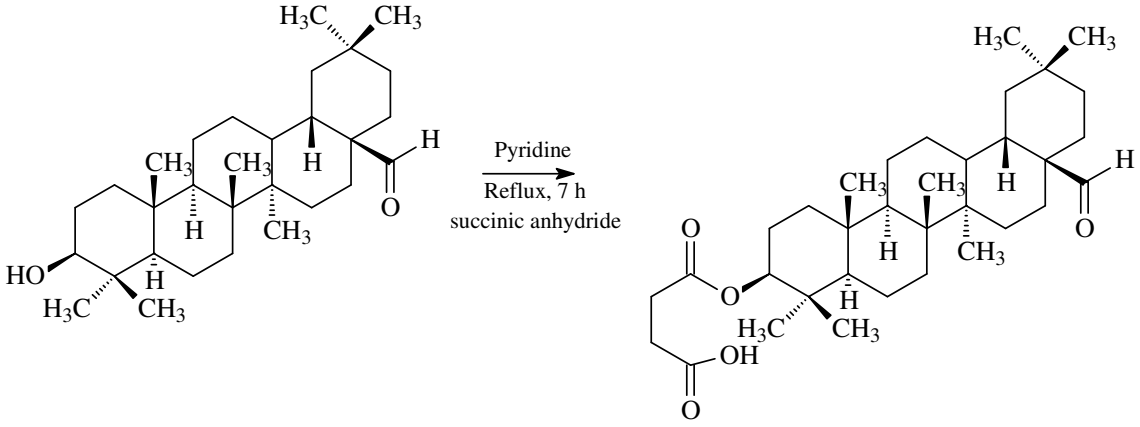
Figure 7.4: Chemical Structure of compound **OO/56** (3-Acetyloleanolic hydrazone).

7.2.4 3-Succinyloleanolic acid

Experimental Procedure

A modified method of Wrzeciono *et al*.,[2] was used for the synthesis of 3-succinyloleanolic acid. A mixture of oleanolic acid (1 g, 0.002 mol), pyridine (20mL), and succinic anhydride (2.2 g, 0.022 mol) was refluxed for 7 h in a round bottom flask. The mixture was then concentrated to about one fifth the original volume. The concentrate was then decanted into 50 ml of water in a 100mL beaker. This was allowed to stand for another 72 hr at room temperature. The product was filtered off and purified by column chromatogram to give compound **OO/59**.

Equation of the reaction



Result and Discussion

Physical Data for Compound OO/59 (3-Succinyloleanolic acid)

Description: White powdery solid

Yield: 0.26 g (22%)

Melting point: 252-254 ^{o}C

Molecular weight (LC-MSD-Trap-VL): 556

InfraRed Spectroscopy (Perkin Elmer ATR FT-IR); $\nu_{C\text{-}H}$ 2945, $\nu_{C=O}$ 1704 $\nu_{C=O\ (COOH)}$ 1737, $\nu_{C\text{-}O}$ 1077cm^{-1}

It was observed in the proton NMR spectra (Figure 7.5.1 Appendix 5) that the OH signal at δ 3.25 was completely absent when compared with the ^{1}H NMR spectrum of oleanolic acid. The ^{13}C NMR (Figure 7.5.2 and 7.5.3 Appendix 5) revealed the presence of three carbonyl groups, the carboxyl group was noticed at 184 ppm while the two other carbonyl groups were found at 171 ppm and 178 ppm suggesting two different types of carbonyl functional group. The IR absorption frequencies (Figure 7.5.4 Appendix 5) at 1704 cm^{-1} and 1077 cm^{-1} suggested an ester and carboxyl functional groups. This was further strengthened by the increase in the molecular ion of the mass spectra of the synthesized compound (Figure 7.5.5 Appendix 5). 2D NMR (Figure 7.5.6 – 7.5.9 Appendix 5) helped in confirming the presence of a succinyl group in the product. The ^{1}H and ^{13}C NMR data are presented in Table 7.4 and compound **OO/59** was confirmed to be 3-succinyloleanolic acid (Figure 7.5).

Table 7.4: ^{1}H and ^{13}C NMR data of compound **OO/59** (3-Succinyloleanolic acid)

Position	**δ_H (OA)**	**δ_H (Succinate of OA)**	**δ_C (OA)**	**δ_C (Succinate of OA)**
1	0.87(1H,*t*) 1.58(1H,*t*)		38.41(*t*)	37.04(*t*)
2	1.69(1H,*m*) 1.84 (1H,*m*)		27.18(*t*)	
3	3.25(1H, *s*)(OH) 3.90 (1H,*m*)	4.49(1H,m)	79.03(*d*)	81.43(*d*)
4	-		38.76(*s*)	
5	0.76(1H,*t*)		55.21(*d*)	55.12(*d*)
6	1.32(1H,*m*) 1.57 (1H,*m*)		18.30(*t*)	
7	1.73(1H,*m*) 1.62(1H,*m*)		34.63(*t*)	
8	-		39.27(*s*)	
9	1.57(1H,*m*)		47.63(*d*)	
10	-		37.07(*s*)	
11	0.97(1H,*m*) 1.68(1H,*m*)		23.57(*t*)	
12	5.25 (1H,*m*)	5.24(1H,m)	122.65(*d*)	122.62(*d*)
13	-		143.58(*s*)	143.66(*s*)
14	-		41.63(*s*)	
15	1.07-1.11(2H,*m*)		27.68(*t*)	
16	1.59(1H,*m*) 1.85(1H,*m*)		23.40(*t*)	
17	-		46.49(*s*)	

Table 7.4 Continued.

18	2.80(1H,*m*)		41.04(*d*)	
19	1.23(1H,*d*) 1.26(1H,*d*)		45.88(*t*)	
20	-		30.67(*s*)	
21	1.23(1H,*m*) 1.18(1H,*m*)		33.80(t)	
22	1.23(1H,*m*) 1.80(1H,*m*)		32.43(*t*)	
23	0.92(3H,*s*)		28.10(*q*)	
24	0.98(3H,*s*)		15.55(*q*)	
25	0.89(3H,*s*)		15.32(*q*)	
26	0.77(3H,*s*)		17.11(*q*)	
27	1.13(3H,*s*)		25.92(*q*)	
28	-		182.27(*s*)	184.57(s)
29	0.90(3H,*s*)		33.06(*q*)	
30	0.91(3H,*s*)		22.96(*q*)	
1’				171.37(s)
2’				
3’				
4’				178.19(s)

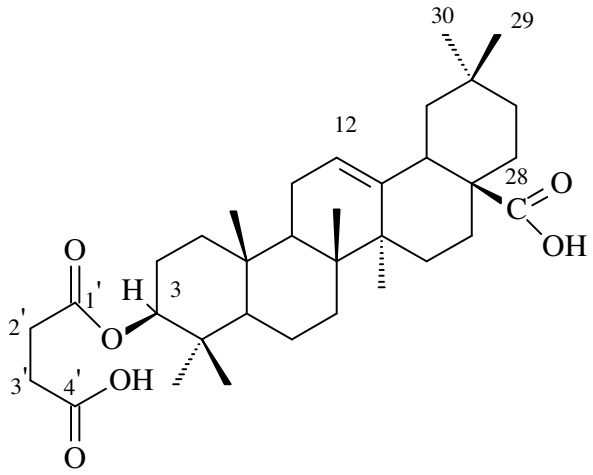


Figure 7.5: Chemical Structure of compound **OO/59** (3-Succinyl oleanolic acid)

7.3 Conclusion

3-acetyloleanolic acid, 3-acetyloleanolic hydrazide, 3-acetyloleanolic hydrazone, and 3-succinyloleanolic acid were synthesized and structurally elucidated. To the best of my knowledge,

two of these compounds, 3-acetyloleanolic hydrazide, 3-acetyloleanolic hydrazone, are now being reported for the first time as new derivatives of oleanolic acid.

7.4 Future Work

The biological activity evaluation of the synthesized compounds will be an objective in the future.

7.5 References

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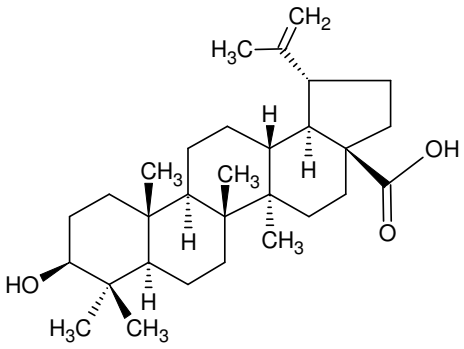
CHAPTER EIGHT

DERIVATIVES OF BETULINIC ACID

8.1 Introduction

Betulinic acid (**8.1**) is a promising pentacyclic compound as it can act by inducing apoptosis in cancer cells. Due to this significant biological property on melanoma cells, betulinic acid seems to be a more promising anticancer agent than drugs like Taxol.[1] Thus, a lot of research activity is focussed on betulinic acid and its derivatives in search for a perfect anticancer agent. Betulinic acid and its derivatives constitute a class of compounds that has the potential to protect the cells of human immunological system in vitro from the attack of HIV virus.[2]

Kashiwada *et al*, [3] and Hashimoto, F. *et al*., [4], first synthesised 3-*O*-(3′, 3′-dimethyl)-succinylbetulinic acid (**8.2**), 3′,3′-dimethylglutaryl (**8.3**) and diglycoryl (**8.4**) derivatives which had significant anti-HIV activities. It was found that the biological potentials of these derivatives inhibited the life cycle of HIV virus in its early phase thereby defending the surrounding cells from HIV proliferation. The study of Kashiwada Y, *et al*.,[5] on the activities of some triterpenic acids revealed that ring E in betulinic acid played an important role in the anti-HIV study.



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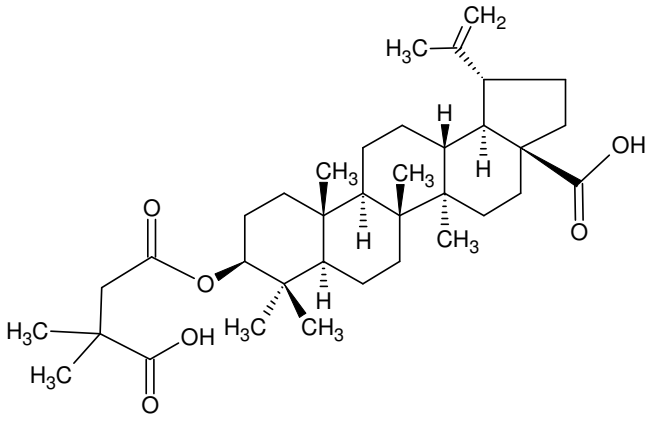

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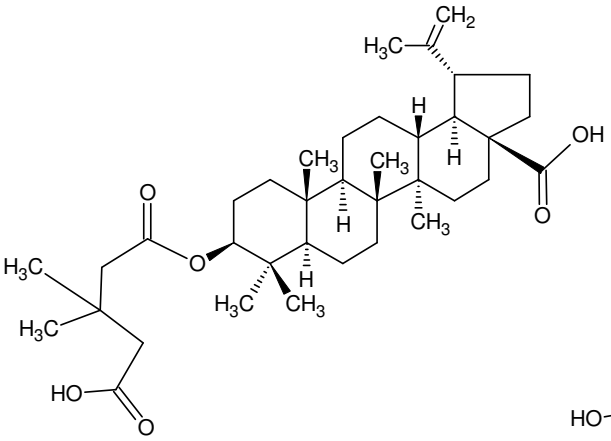

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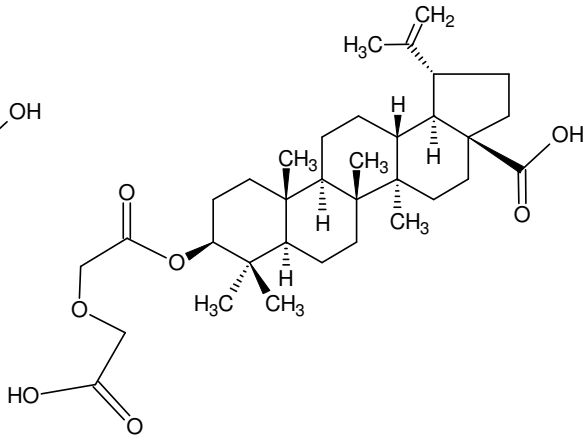
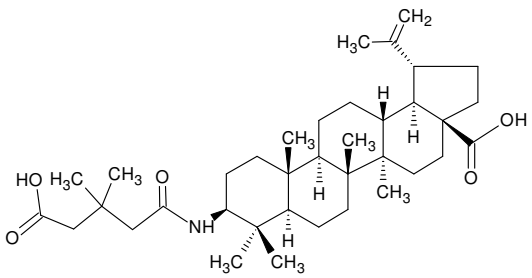


8.4

Further study by Kashiwada Y, *et al*.,[5] on the derivatives of betulinic acid ester lead to the synthesis of β-and α- derivatives of 3-alkylamido-3-deoxy-betulinic acid (**8.5** and **8.6**), 3β- and 3α-(3′,3′-dimethylsuccinyl)amido-3-deoxy-betulinic acid (**8.7** and **8.8**), and, 3β- and 3α-diglycorylamido-3-deoxy-betulinic acid (**8.9** and **8.10**) in which the ester group was replaced by an amido group. The biological potency of these amido derivatives against HIV virus was less or inactive at all indicating that the ester group at C-3 is essential for potent anti-HIV activity.



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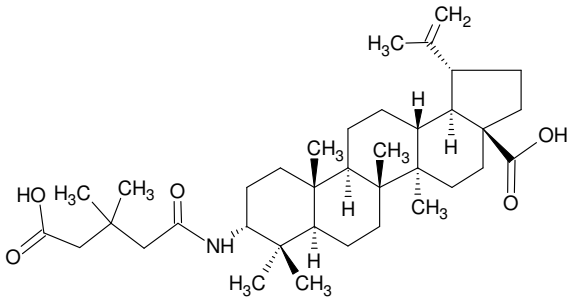

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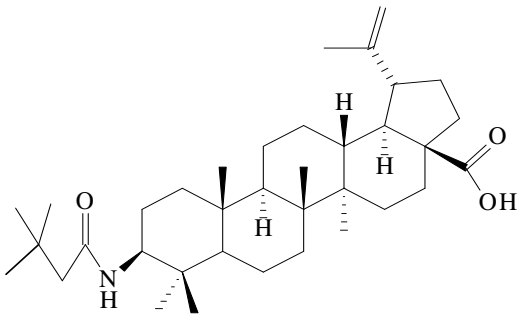

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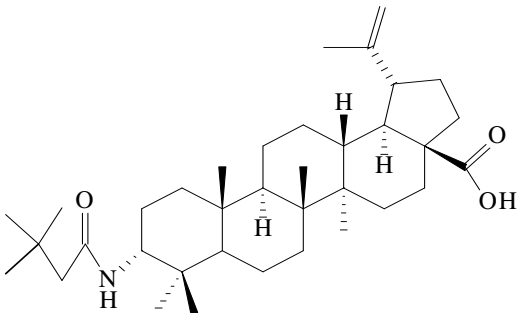
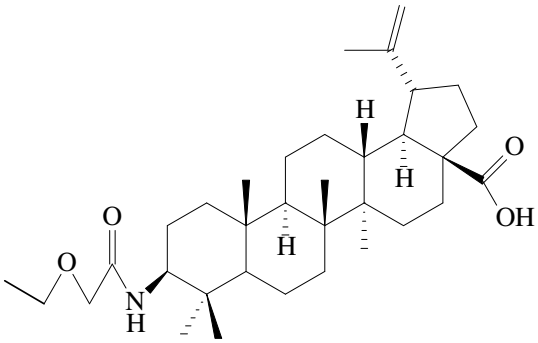


8.8



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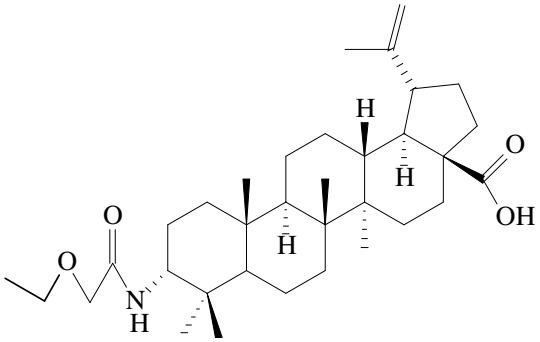
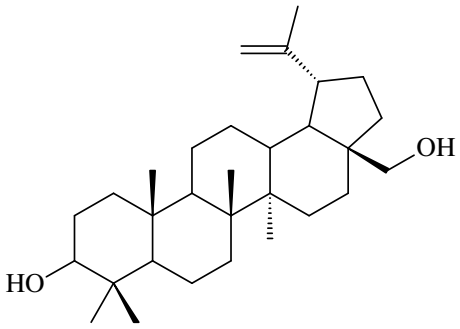


8.10

Derivatives via transformations of the carboxylic acid at position 28 to an hydroxyl group (**8.11**) and an amide group (**8.12**) along with the introduction of a methyoxy group (**8.13**) and bromide (**8.14**) at position 30 and; the conversion of the methylene group at position 29 to an aldehyde (**8.12** and **8.15**) have been recently reported by Santos *et al*.[6] Also synthesis of diacetylated betulinic acid (**8.16**) has been reported.[6]



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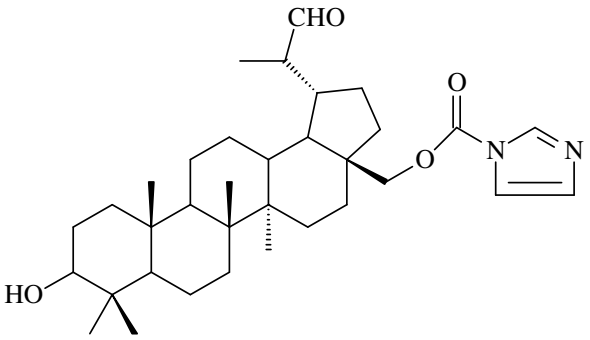

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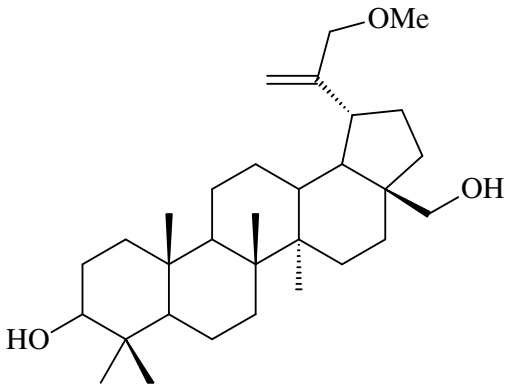

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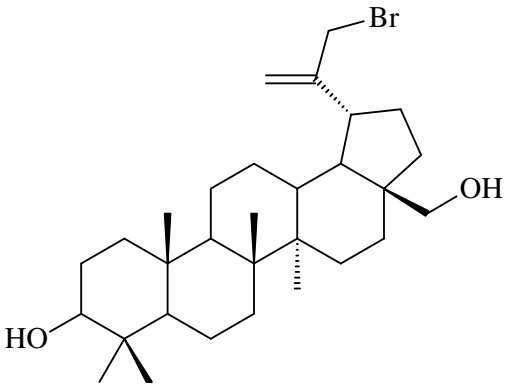

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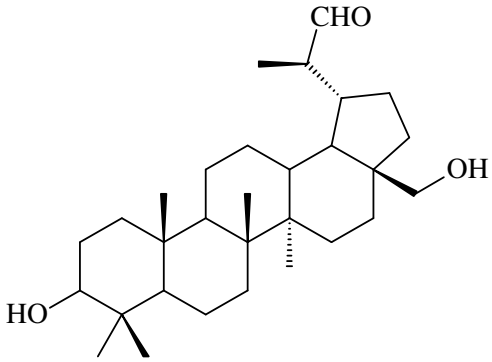

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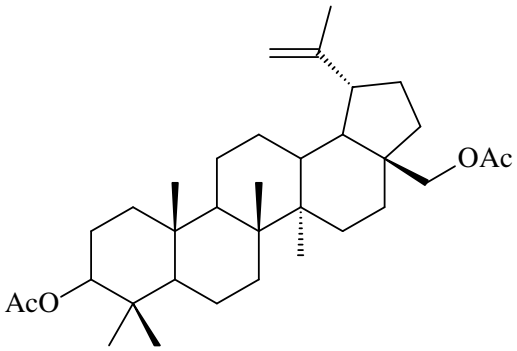


9.16

Several reviews have emerged in the chemical and pharmacological literature on the natural occurrence, isolation, structural elucidation, synthesis, pharmacology, toxicology, and pharmaceutical applications of BA and many of its derivatives.[8-28] In fact, one author referred to BA as the "White Gold".

In recognition of the great potential of BA and its derivatives as lead compounds for drug discovery and the availability of BA, it was decided to bio-source it from common and readily available medicinal plants and use it as starting material for the semi-synthesis of some known and unknown derivatives of BA for bio-evaluation purposes.

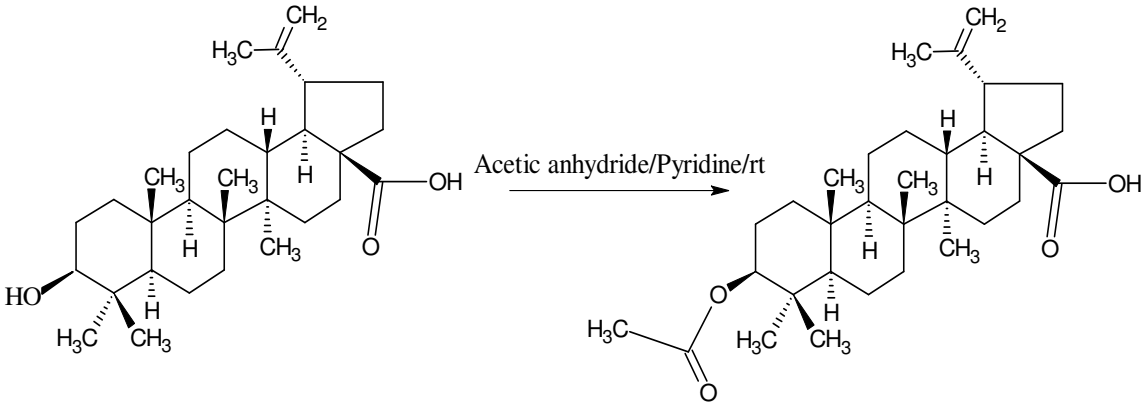
8.2. Semi-synthesis of Betulinic Acid Derivatives

8.2.1 3-Acetylbetulinic Acid

Experimental Procedure:

A mixture of betulinic acid (1.07 g, 0.002 mol), pyridine (5 mL) and excess acetic anhydride (10 mL) in a 100 mL round bottom flask was stirred for about 10-12 h at room temperature.The reaction mixture was poured into a mixture of ice and water and stirred for 30 min. The solid that was separated was separated by suction filtration and purified by column chromatography using silica gel as the stationary phase. A pure compound of 0.95 g (88.9%) was obtained after bulking and was tagged **OO/67**.

Equation of Reaction



Betulinic acid Compound **OO/67**

Results and Discussion

Physical Data for Compound OO/67(3-acetoxybetulinic acid)

Description: White powdery solid

Yield: 0.95 g (88.8%)

Melting point: 270-272 ^{o}C

Molecular weight (LC-MSD-Trap-VL): 498.7

Infra Red Spectroscopy (Perkin Elmer ATR FT-IR); $\nu_{C\text{-}OH}$ 3072, $\nu_{C\text{-}H}$ 2939, $\nu_{C=O}$1691, $\nu_{C=O\ (COOH)}$ 1733cm^{-1}

Analysis of the spectroscopic data of synthesized product OO/67 (Figure 8.1.1 – 8.1.9 Appendix 6) and comparison with aforementioned betulinic acid in chapter 6 confirmed that compound **OO/67** was, indeed, a 3-acetoxybetulinic acid (Figure 8.1) and the data are presented in Table 8.1. Comparison of our spectral data with that of Salaman *et al*[7] reported in literature were in close agreement.

Table 8.1: ^{1}H and ^{13}C-NMR data of compound **OO/67** (3-Acetoxybetulinic acid)

Position	δ_H (BA)	δ_H (Acetate of BA)	δ_H (BA)	δ_C (Acetate of BA)	δ_C [7] (Acetate of BA)
1				38.37	38.37
2				18.16	18.09
3		4.46(1H,d)		80.96	80.93
4		-		37.79	37.74
5				55.42	55.34
6				20.85	20.79
7				34.24	34.16
8		-		40.69	40.62
9				50.40	50.32
10				37.03	37.02
11				23.69	23.64
12				25.44	25.37
13				38.39	38.37
14				42.42	42.34
15				30.55	30.52
16				32.14	32.10
17				56.35	56.65
18				49.26	49.20
19				46.92	46.89
20				150.37	150.35
21				29.68	29.64
22				37.11	37.02
23		1.03(3H,*s*)		27.94	27.89
24		1.00(3H,*s*)		16.45	15.42
25		0.99(3H,*s*)		16.17	16.12
26		0.97(3H,*s*)		16.03	15.99
27		0.75(3H,*s*)		14.64	14.60
28		-		181.21	182.55
29		4.71(1H,*d*) 4.70(1H,*d*)		109.73	109.69
30		1.64(3H,*s*)		19.33	19.30
1′		-		171.05	171.12
2′		2.14(3h,*s*)		21.31	21.27

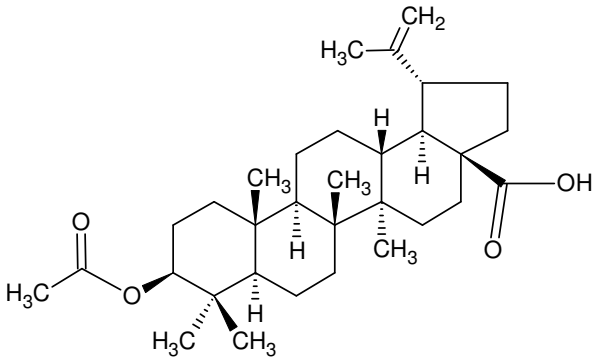


Figure 8.1: Chemical Structure of Compound **OO/67** (3-Acetylbetulinic acid)

8.2.2 3-Succinylbetulinic acid

Experimental Procedure

A mixture of betulinic acid (1.0g, 0.002 mol), succinic anhydride (2.0 g, 0.02 mol), and pyridine (20 mL) was refluxed for 7 h in a round bottom flask. The reaction mixture was concentrated to about 1/5th its original volume and then poured in 50 mL of water. The whole content was allowed to stand for 72 h at room temperature. The solid product obtained was filtered under vacuum and purified by column chromatography. A pure solid (0.24 g, 20% yield) was obtained and was labelled as **OO/61**.

Result and Discussion

Physical Data for Compound OO/61 (3-Succinylbetulinic acid)

Description: White powdery solid

Yield: 0.24 g (20%)

Melting point: 260-262 ^{o}C

Molecular weight (LC-MSD-Trap-VL): 556

InfraRed Spectroscopy (Perkin Elmer ATR FT-IR); ν_{OH} 3071, ν_{C-H} 2941, $\nu_{C=O}$1686, 1642, $\nu_{(COOH)}$ 1708cm^{-1}

Analysis of the spectroscopic data of compound **OO/61** synthesized was done by the comparison of its spectral data (Figure 8.2.1 – 8.2.9 Appendix 6) with aforementioned betulinic acid in chapter

6 and reported data of synthesized derivative of BA by Salaman *et al*[7]. Compound **OO/61** was confirmed to be a 3-succinylbetulinic acid (Figure 8.2) and the data are presented in Table 8.2.

Table 8.2: ^{1}H and ^{13}C NMR data of compound **OO/61** (3-Succinylbetulinic acid)

Position	δ_H (BA)	δ_H (Acetate of BA)	δ_C (BA)	δ_C (Succinate of BA)	δ_C[7] (Acetate of BA)
1			38.7	38.23	38.37
2			27.4	28.00	18.09
3		4.46(1H,d)	79.0	81.53	80.93
4		-	38.9	38.39	37.74
5			55.3	55.33	55.34
6			18.3	18.19	20.79
7			34.3	34.14	34.16
8		-	40.7	40.65	40.62
9			50.5	50.22	50.32
10			37.2	37.11	37.02
11			20.9	20.88	23.64
12			25.5	25.36	25.37
13			38.4	37.88	38.37
14			42.4	42.39	42.34
15			30.6	30.55	30.52
16			32.2	32.13	32.10
17			56.3	56.45	56.65
18			46.9	46.94	49.20
19			49.3	49.23	46.89
20			150.4	150.35	150.35
21			29.7	29.70	29.64
22			37.0	37.07	37.02
23		1.03(3H,*s*)	28.0	29.17	27.89
24		1.00(3H,*s*)	15.3	14.64	15.42
25		0.99(3H,*s*)	16.0	16.26	16.12
26		0.97(3H,*s*)	16.1	16.57	15.99
27		0.75(3H,*s*)	14.7	14.19	14.60
28		-	180.4	182.61	182.55
29		4.71(1H,*d*) 4.70(1H,*d*)	109.7	109.75	109.69
30		1.64(3H,*s*)	19.4	19.34	19.30
1′		-		171.73	171.12
2′					21.27
3′					
4′				178.09	

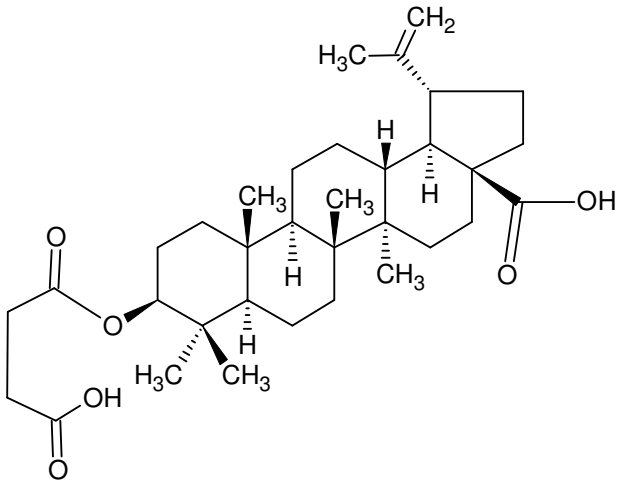


Figure 8.2: Chemical Structure of compound **OO/61** (3-Succinylbetulinic acid)

8.3 Conclusion

In this study, 3-acetoxybetulinic acid and 3-succinylbetulininc acid were synthesized and their structures elucidated by spectroscopic methods.

8.4 Future Work

Since 3-succinyloleanolic acid has been reported to be an antiulcer agent, the biological study of the newly synthesized succinylbetulinic acid will be a future work in the antiulcer and wound healing therapy.

8.5 References

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APPENDIX 1

GC–MS Chromatogram of *Callistemon* Species

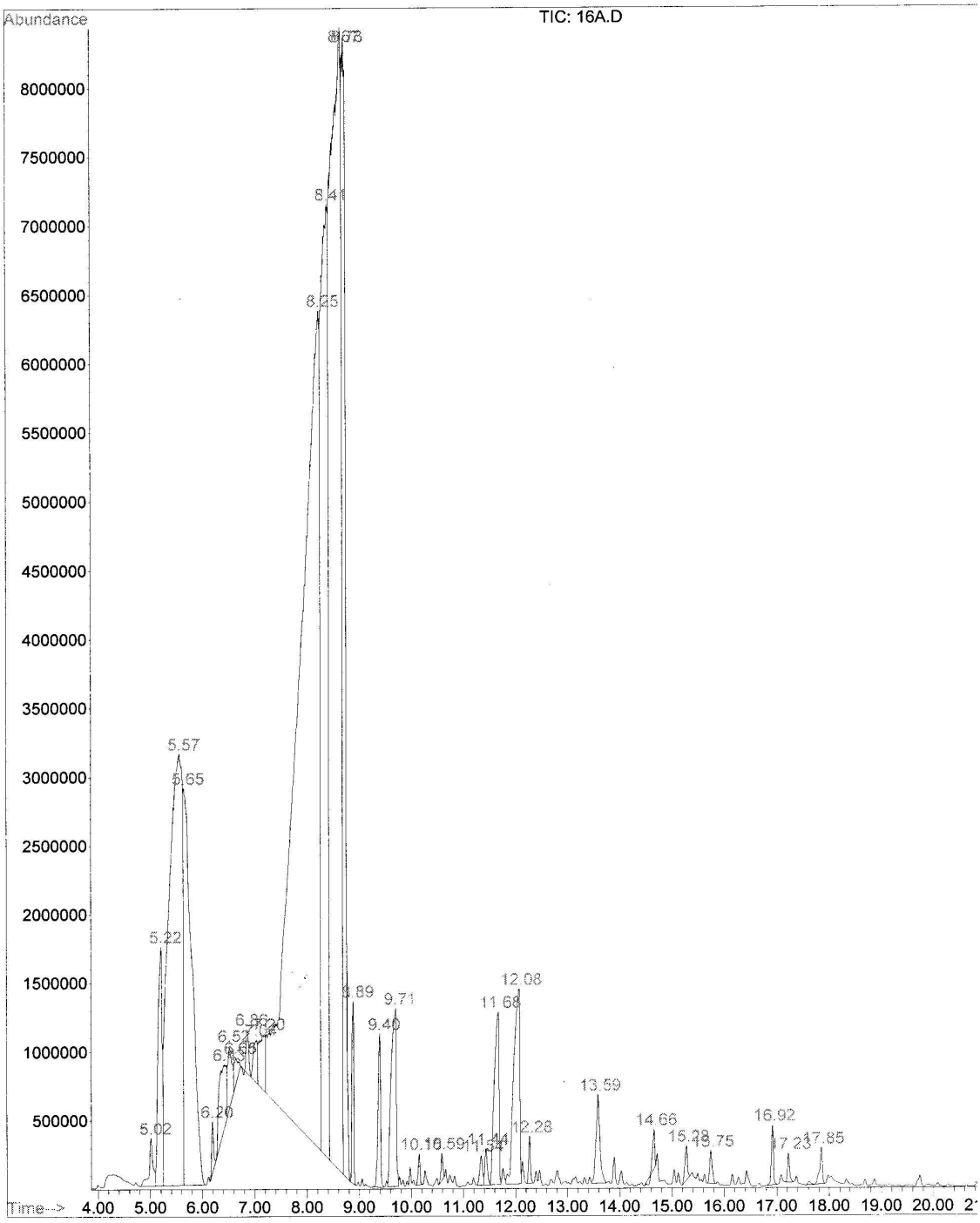


Figure 3.1.1: GC-MS Chromatogram of the essential oil of *C. salignus* from KwaDlangezwa campus (Sample A)

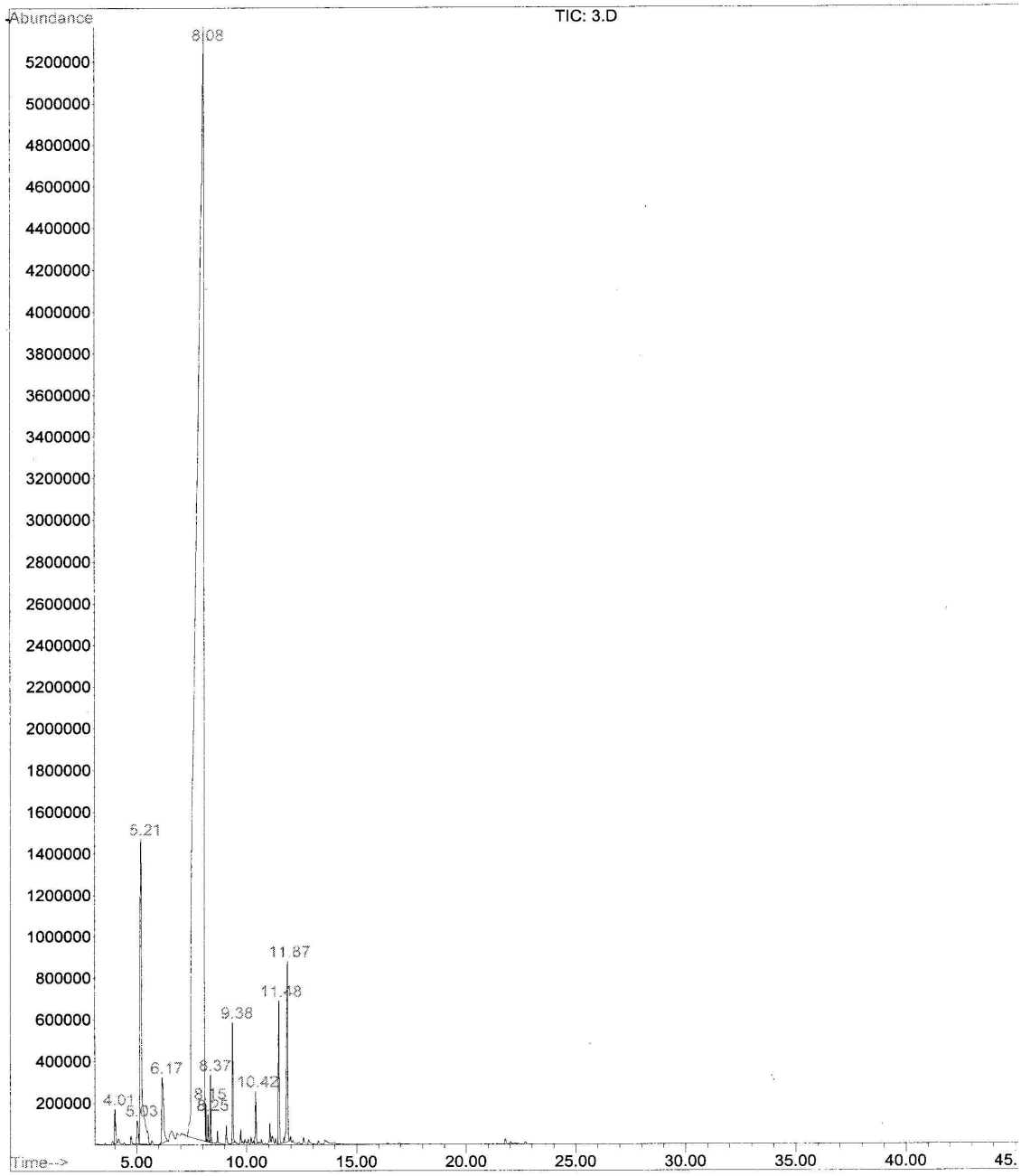


Figure 3.1.2: GC-MS Chromatogram of the essential oil of *C. salignus* from Empangeni (Sample B)

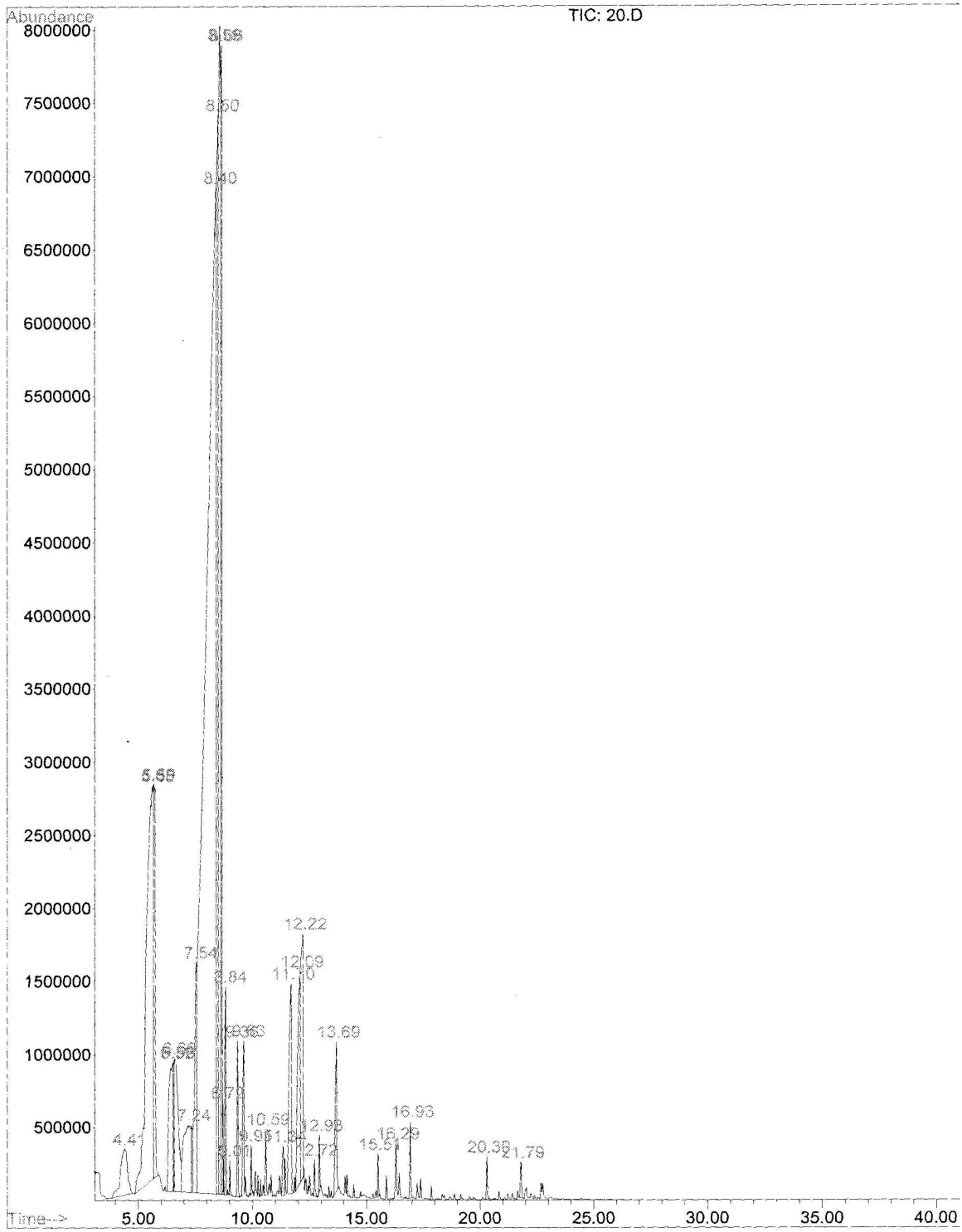


Figure 3.1.3: GC-MS Chromatogram of the essential oil of *C. salignus* from Johannesburg (Sample C)

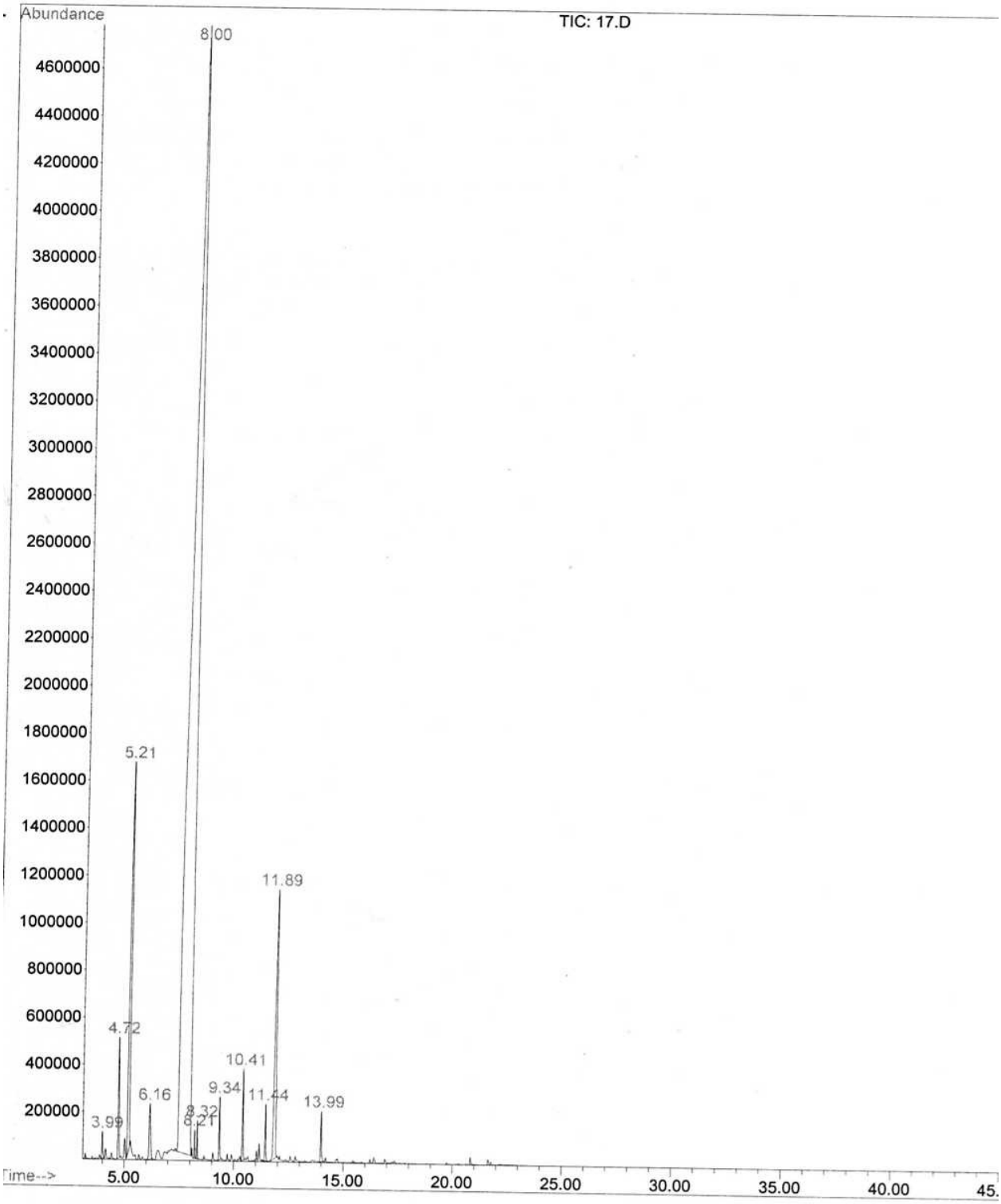


Figure 3.2.1: GC-MS Chromatogram of the essential oil of *C. viminalis* from Durban

APPENDIX 2

GC–MS Chromatogram of *Melaleuca* Species

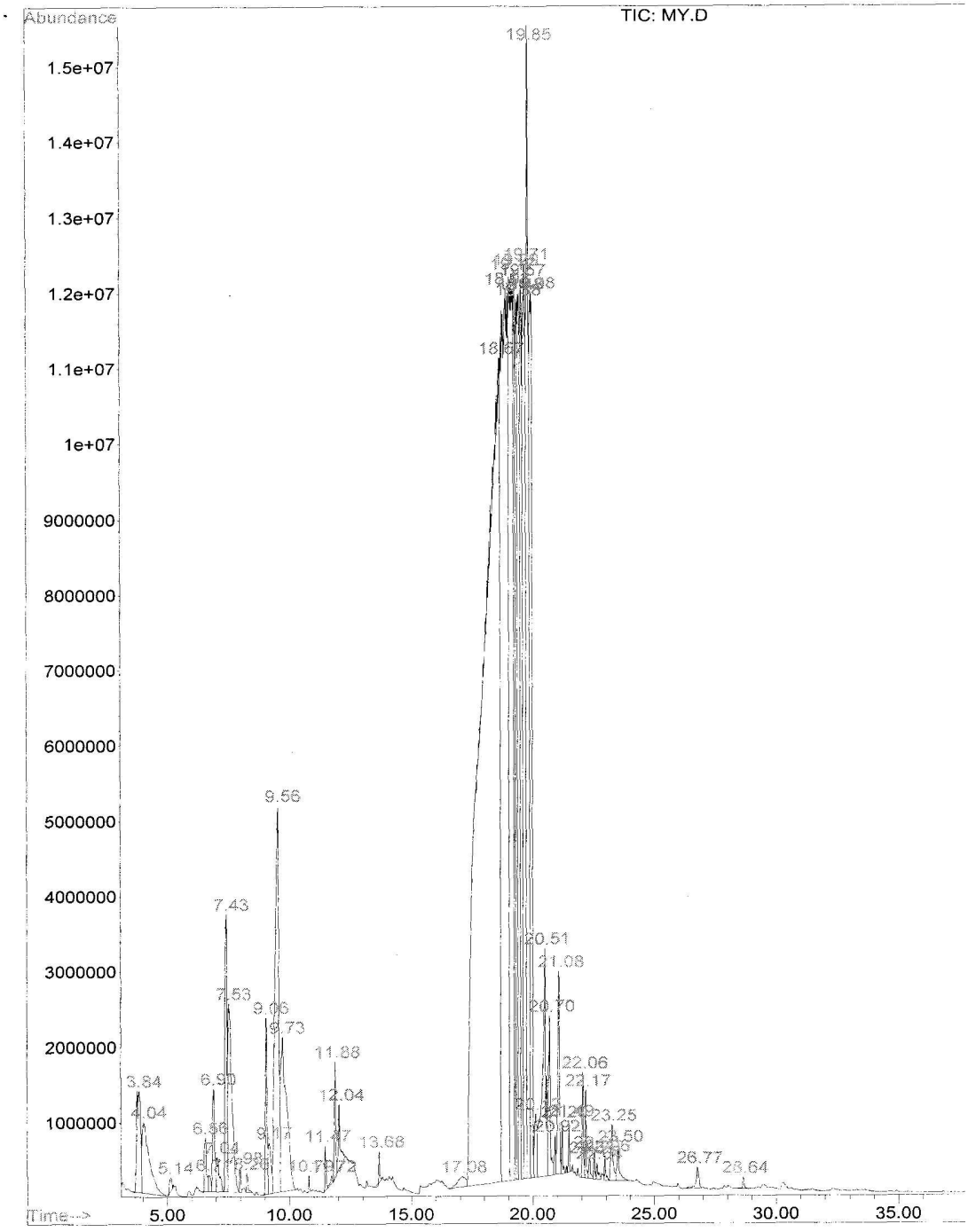


Figure 4.1.1: G-CMS Chromatogram of the essential oil of *M. bracteata* var. *revolution gold* (MYG1)

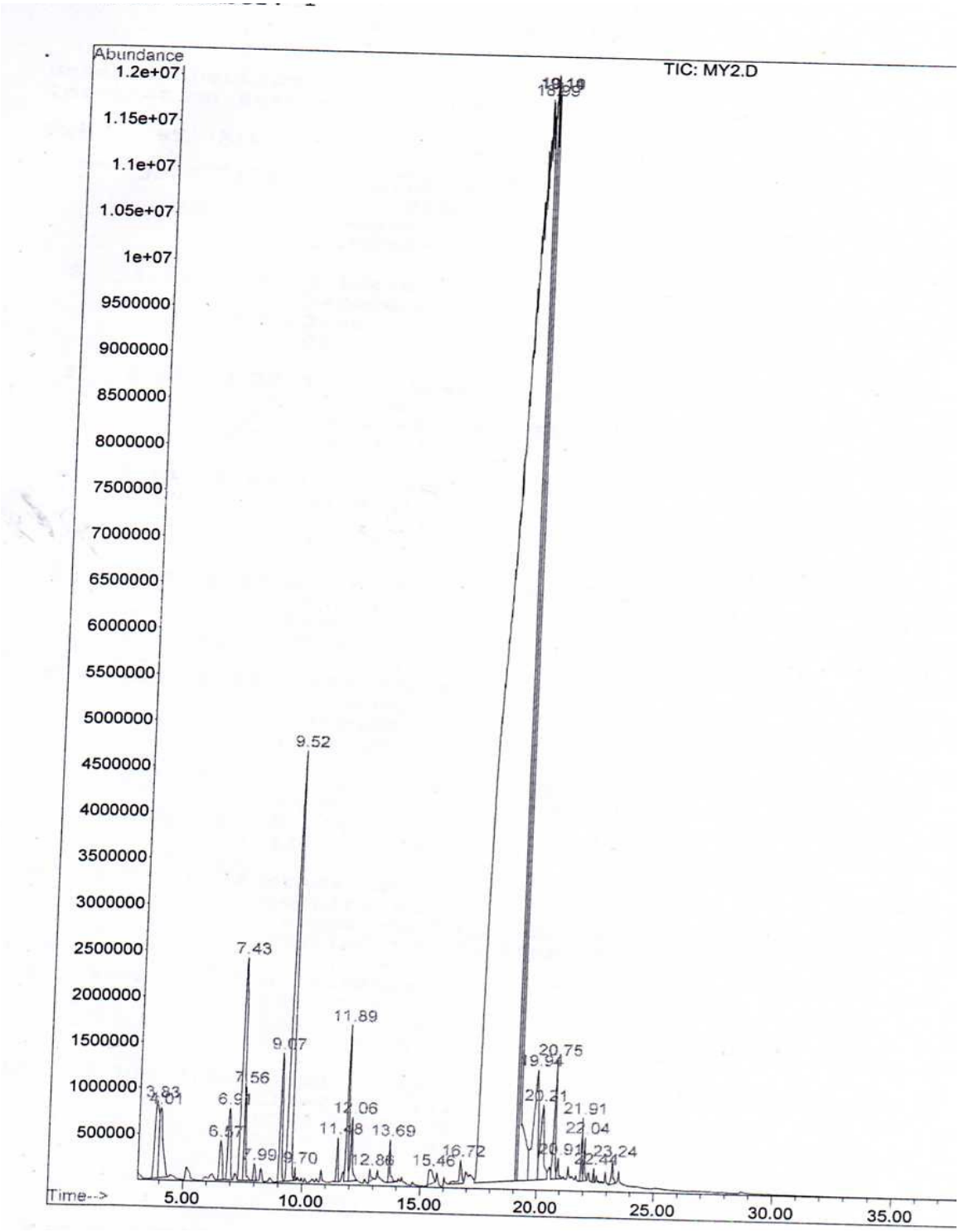


Figure 4.1.2: GC-MS Chromatogram of the essential oil of *M. bracteata* var. *revolution gold* (MYG2)

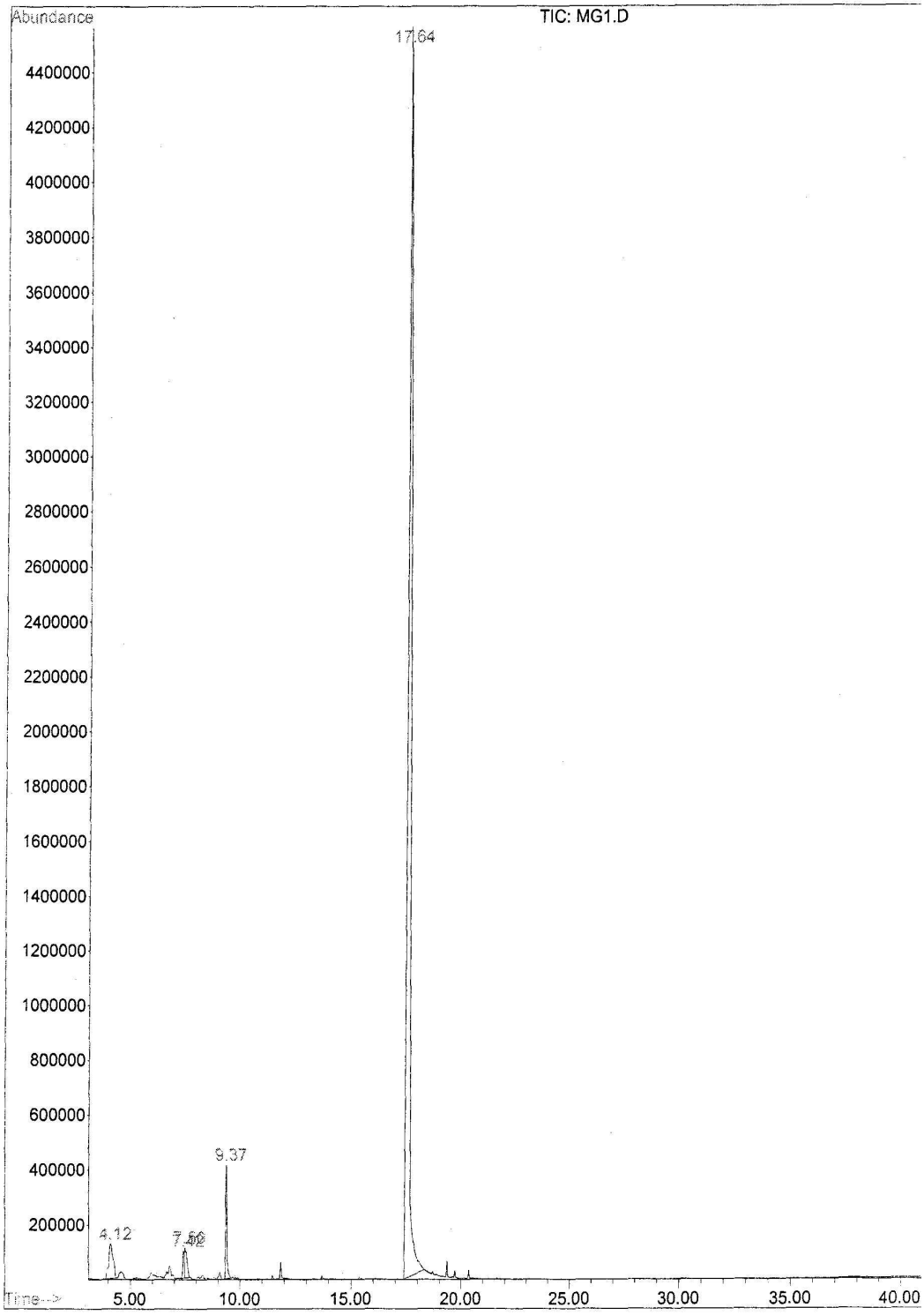


Figure 4.2.1: GC-MS Chromatogram of the essential oil of *M. bracteata* var. *revolution green* (MG1)

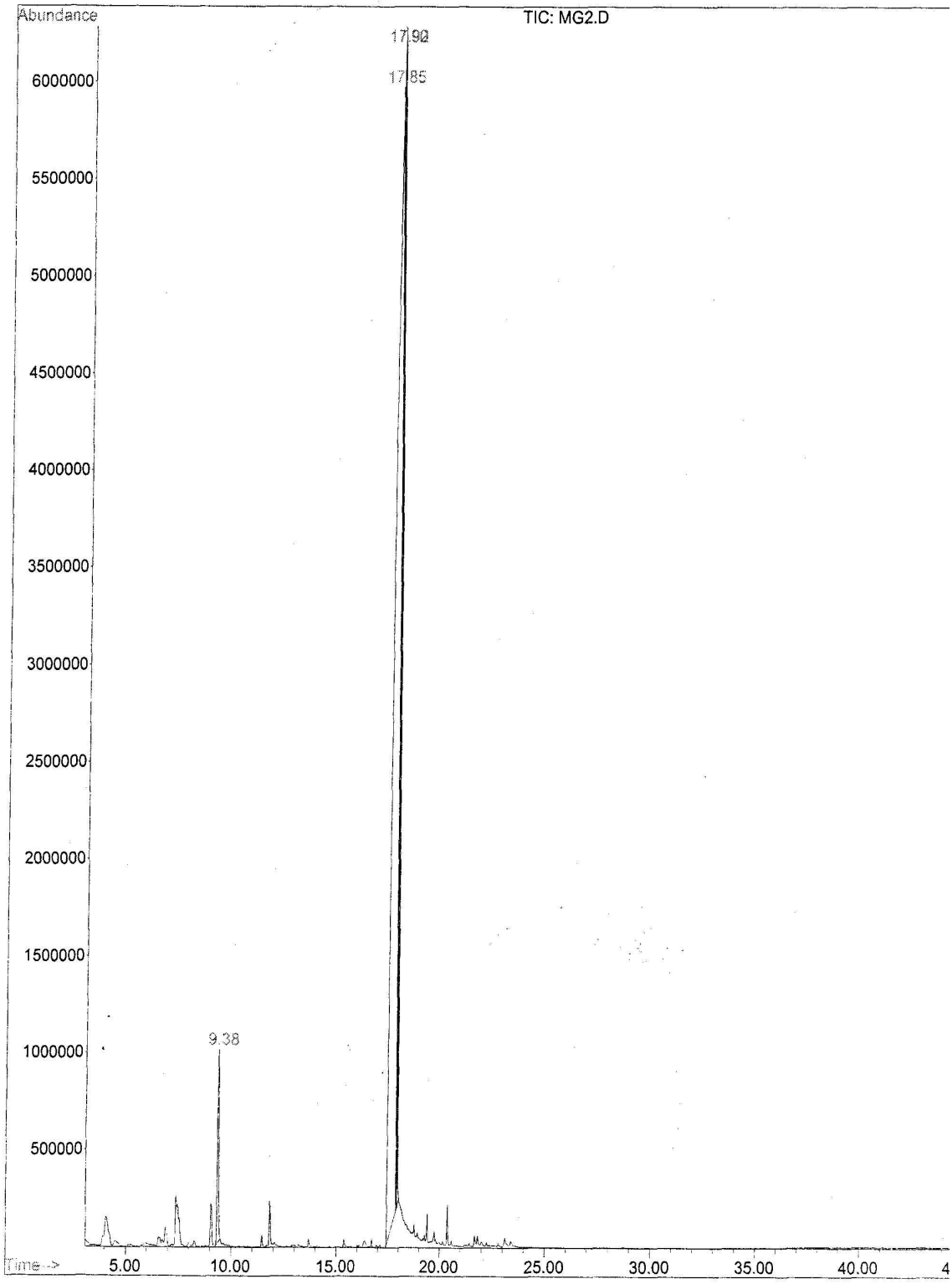


Figure 4.2.2: GC-MS Chromatogram of the essential oil of *M. bracteata* var. *revolution green* (MG2)

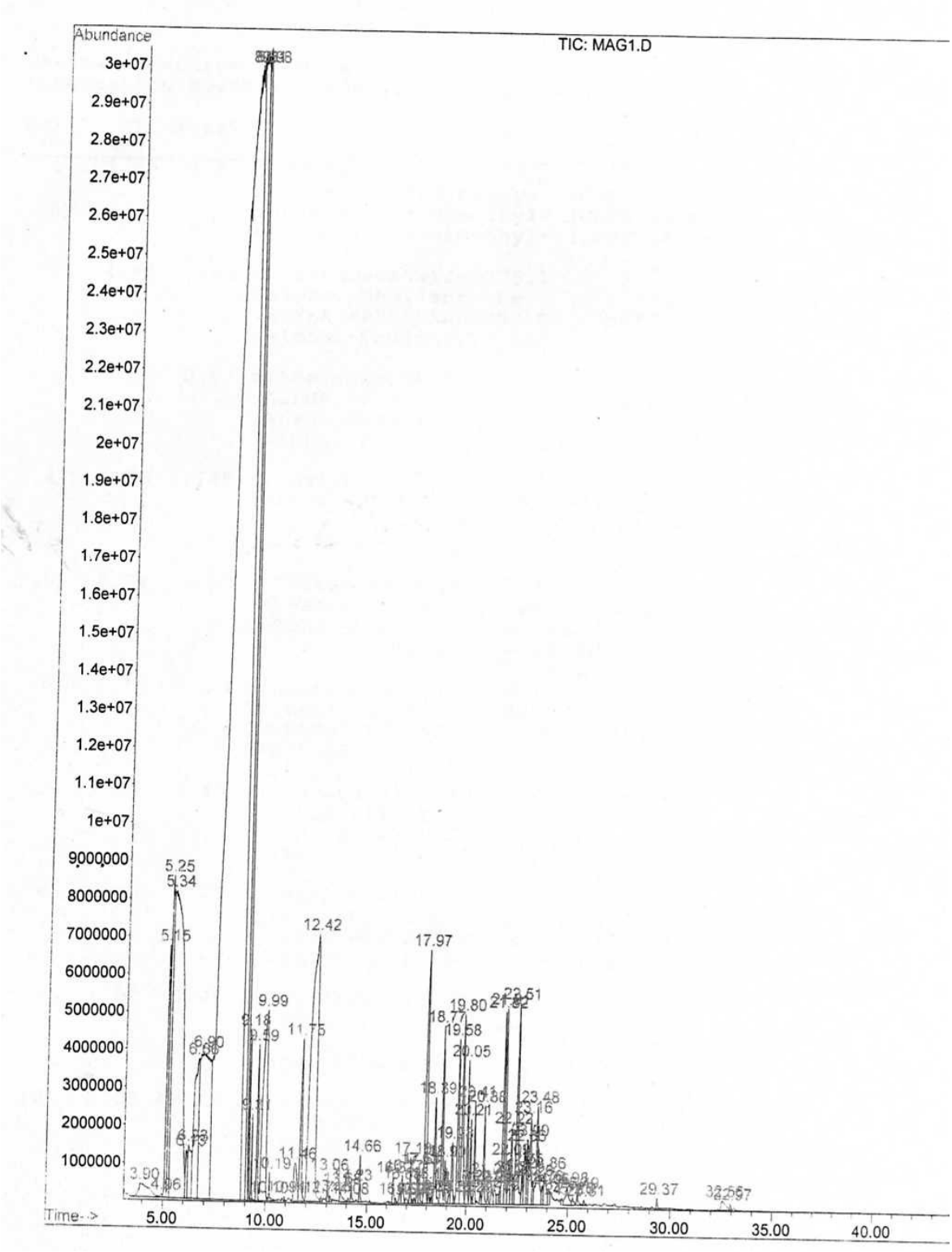


Figure 4.3.1: GC-MS Chromatogram of the essential oil of *M. trichostachya* var. *compacta* (MAG1)

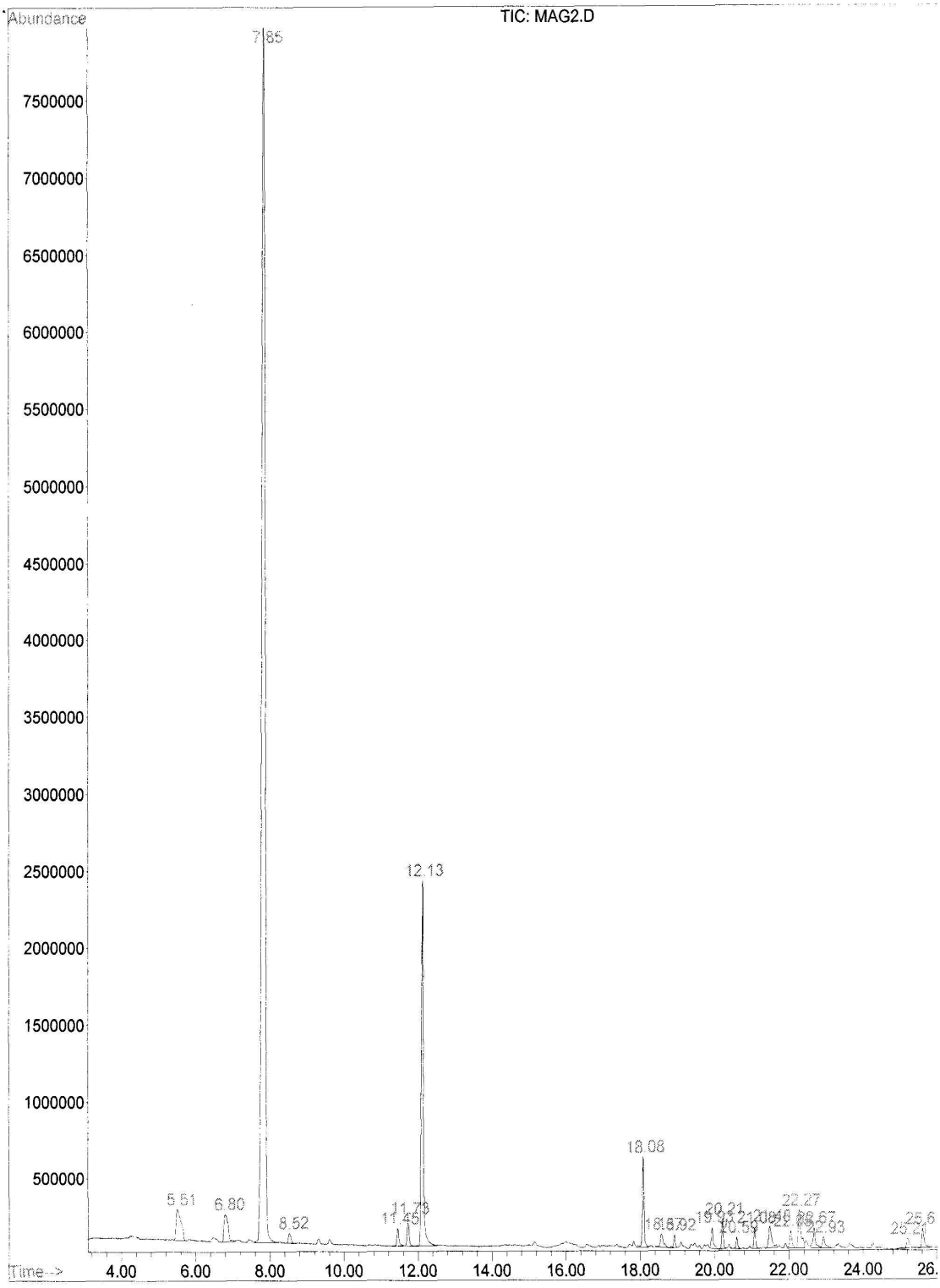


Figure 4.3.2: GC-MS Chromatogram of the essential oil of *M. trichostachya* var. *compacta* (MAG2

APPENDIX 3

Spectra of Oleanolic Acid And 2,3-Diacetoxyoleanolic Acid

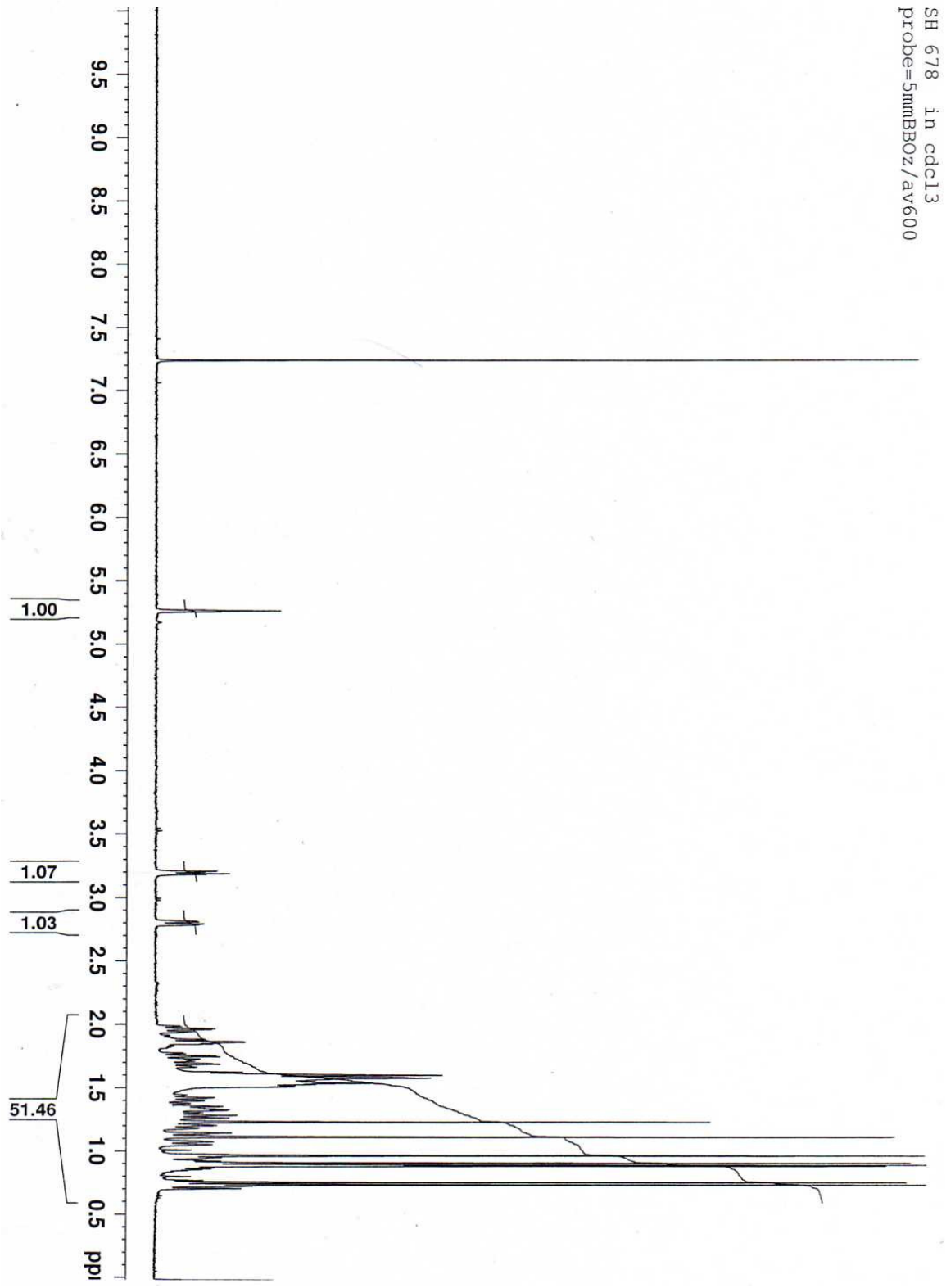


Figure 5.2.1: ^{1}H NMR spectrum of **OO/55/A**

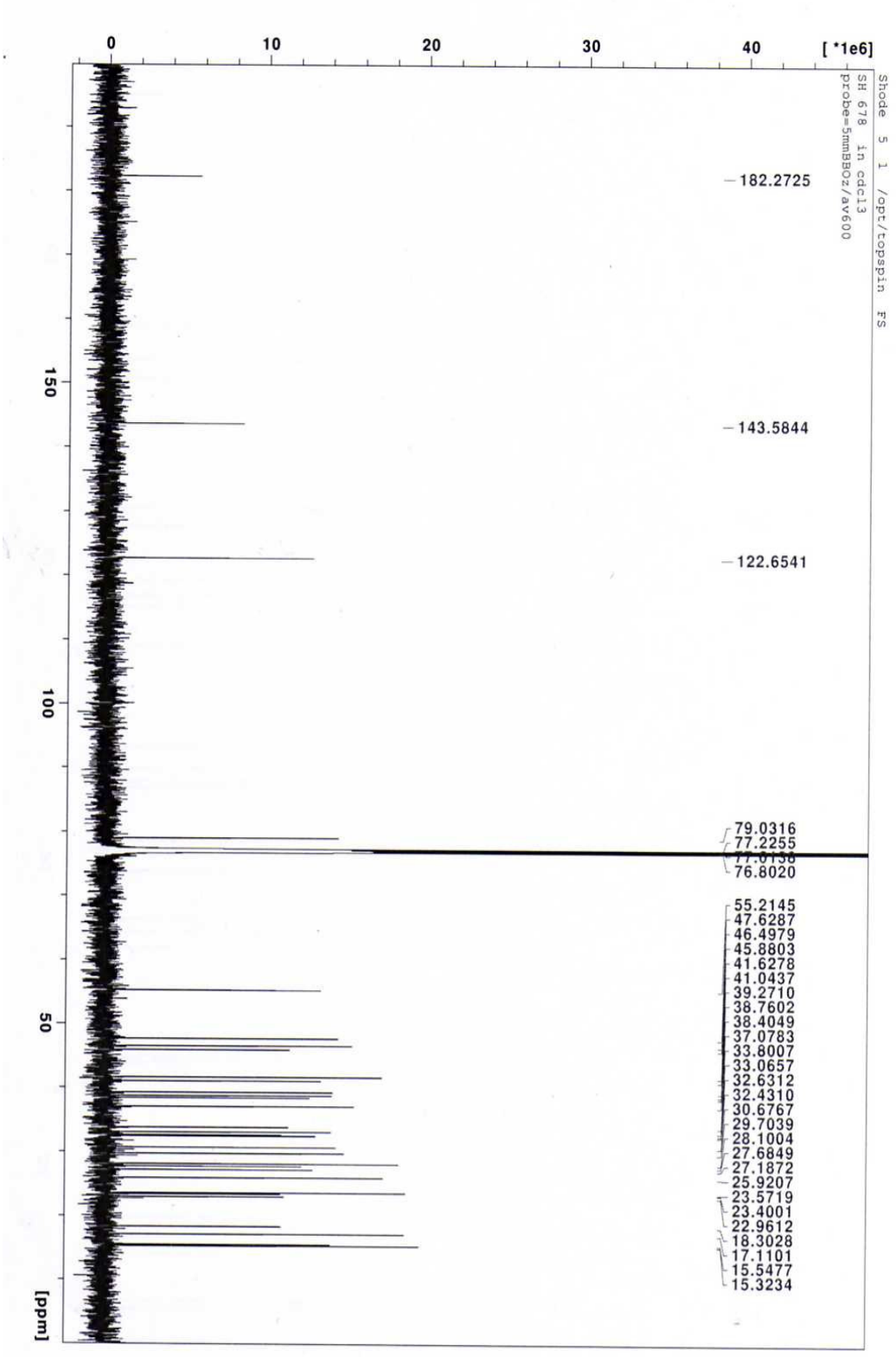


Figure 5.2.2: ^{13}C NMR spectrum of **OO/55/A**

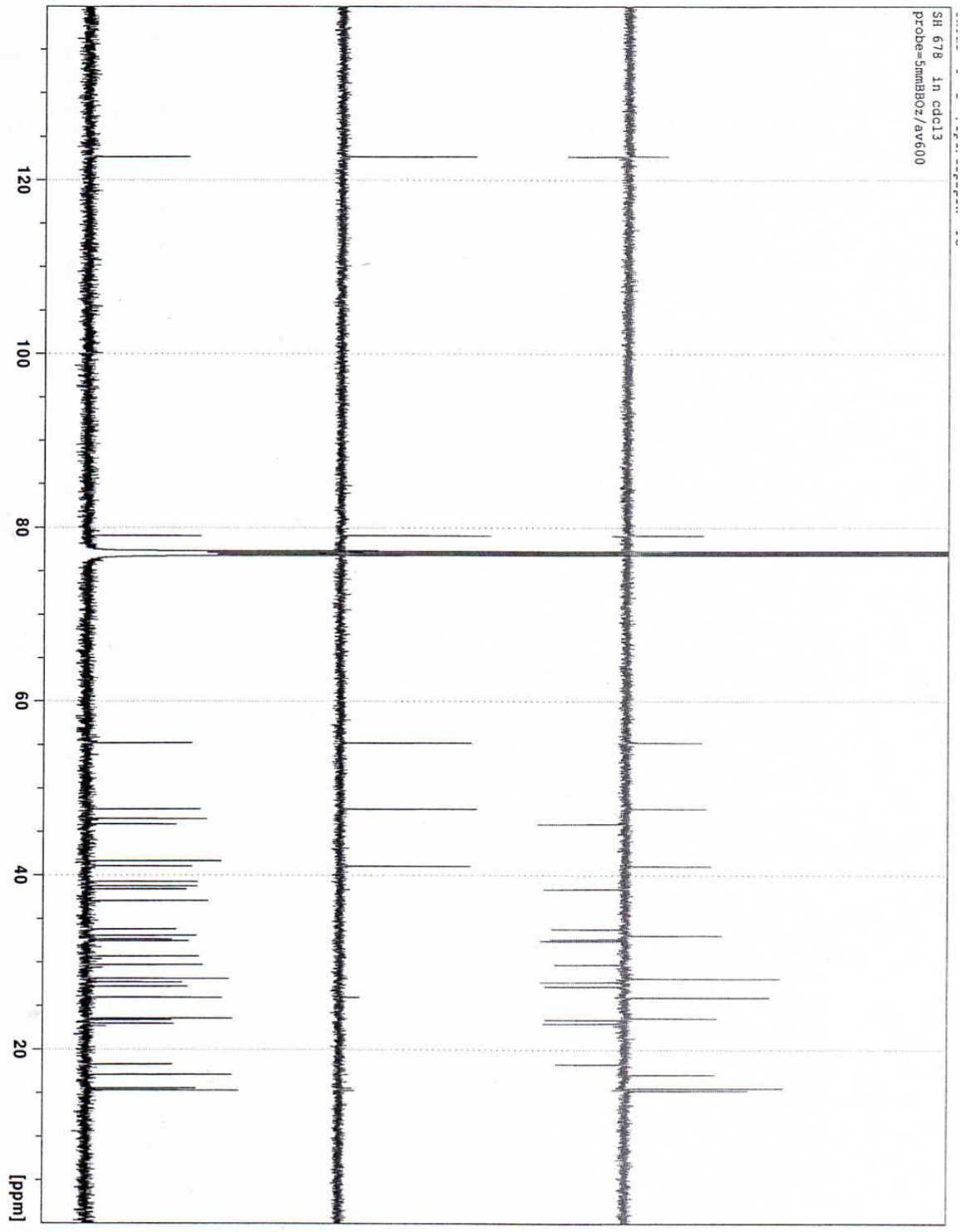


Figure 5.2.3: ^{13}C DEPT NMR spectrum of **OO/55/A**

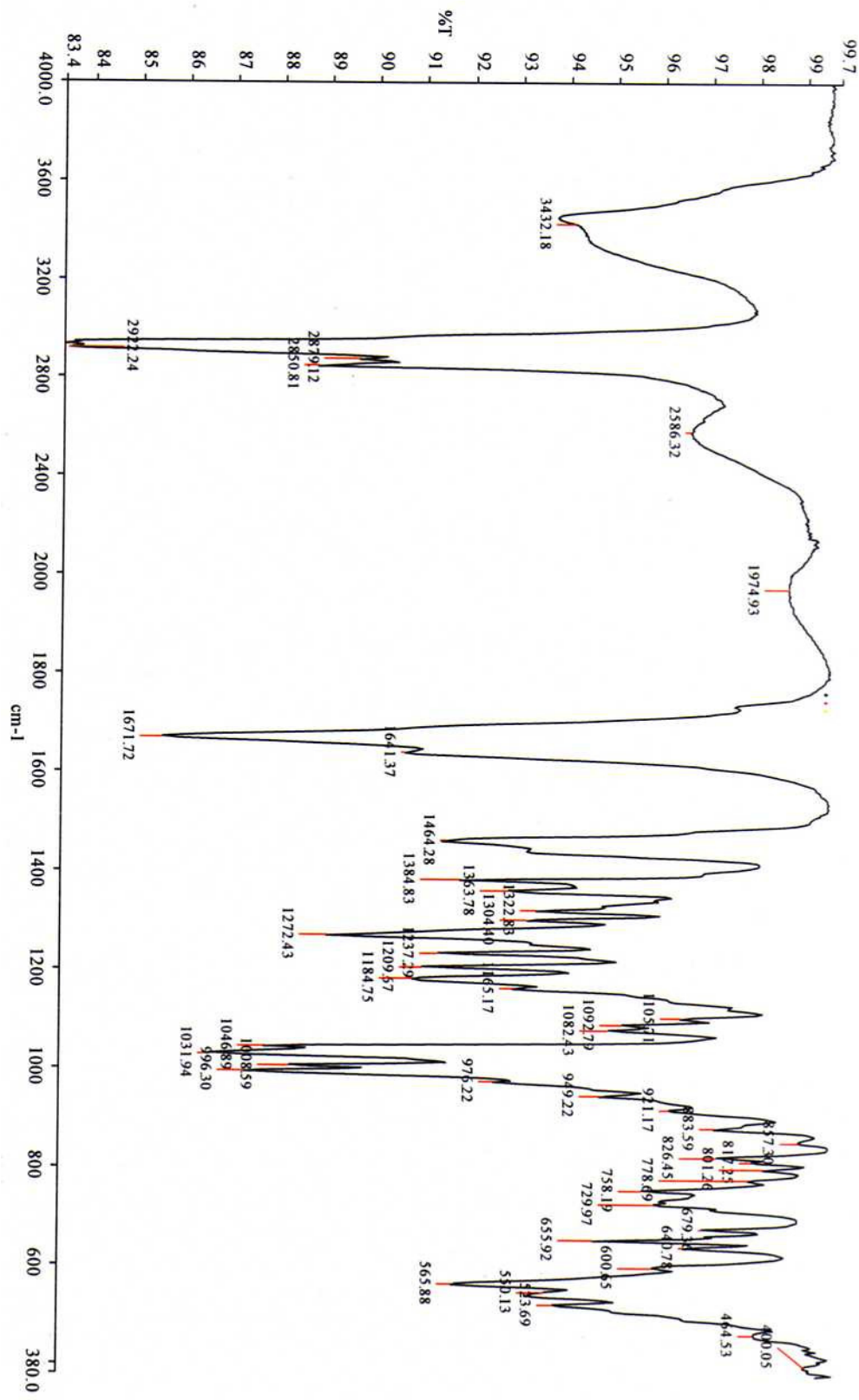


Figure 5.2.4: FTIR Spectrum of **OO/55/A**

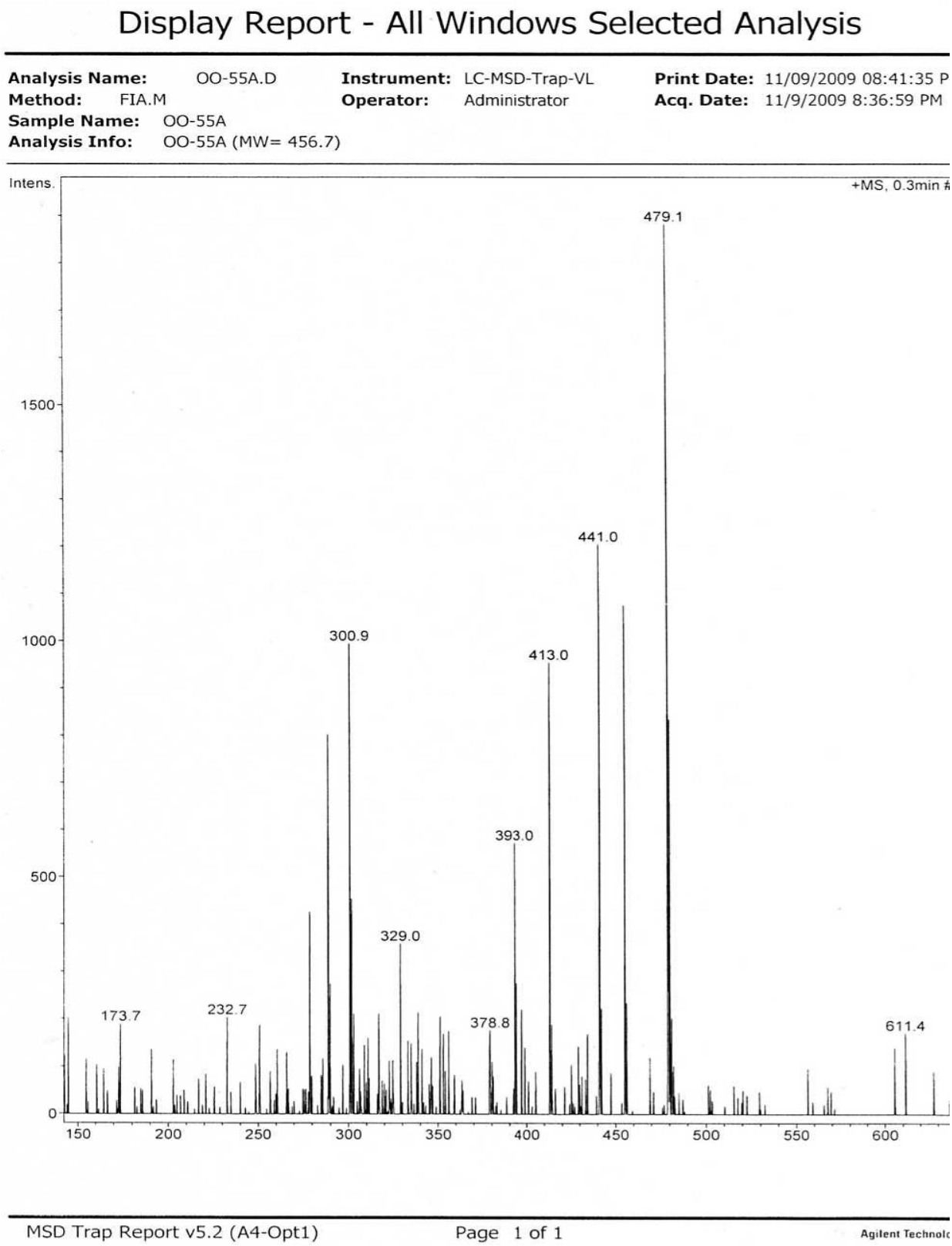


Figure 5.2.5: Mass Spectrum of **OO/55/A**

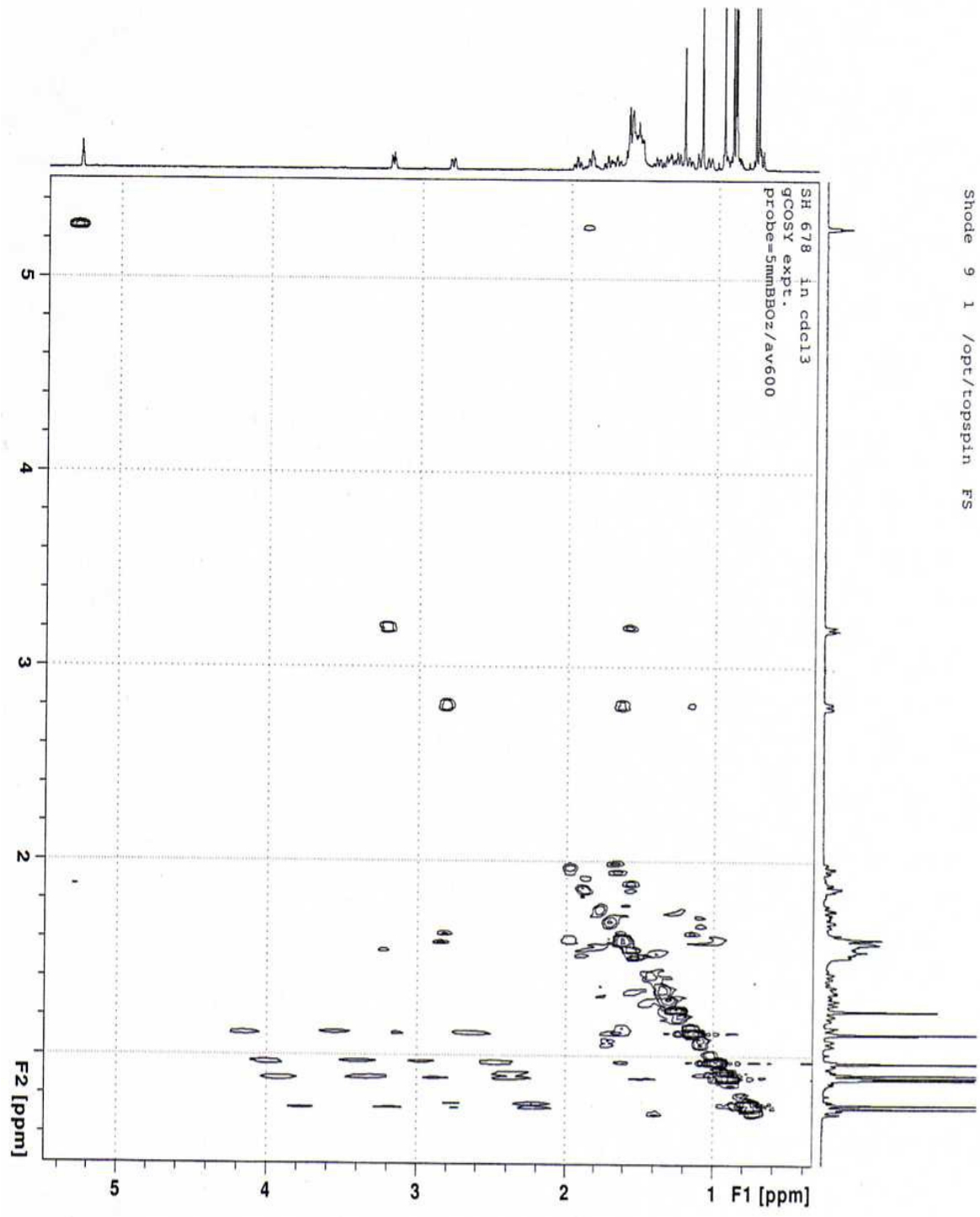


Figure 5.2.6: ^{1}H-^{1}H COSY NMR Spectrum of **OO/55/A**

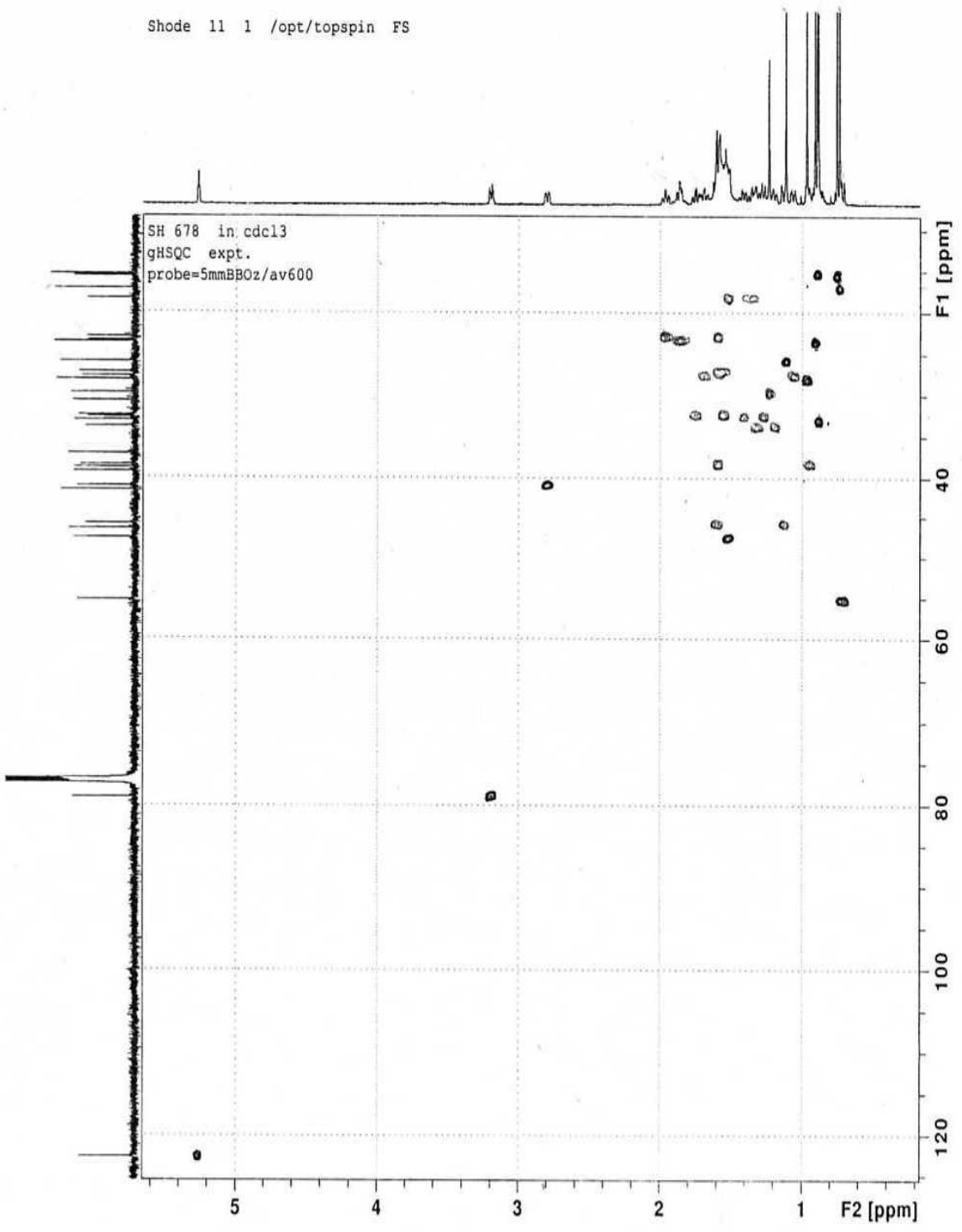


Figure 5.2.7: ^{1}H ^{13}C HSQC NMR Spectrum of **OO/55/A**

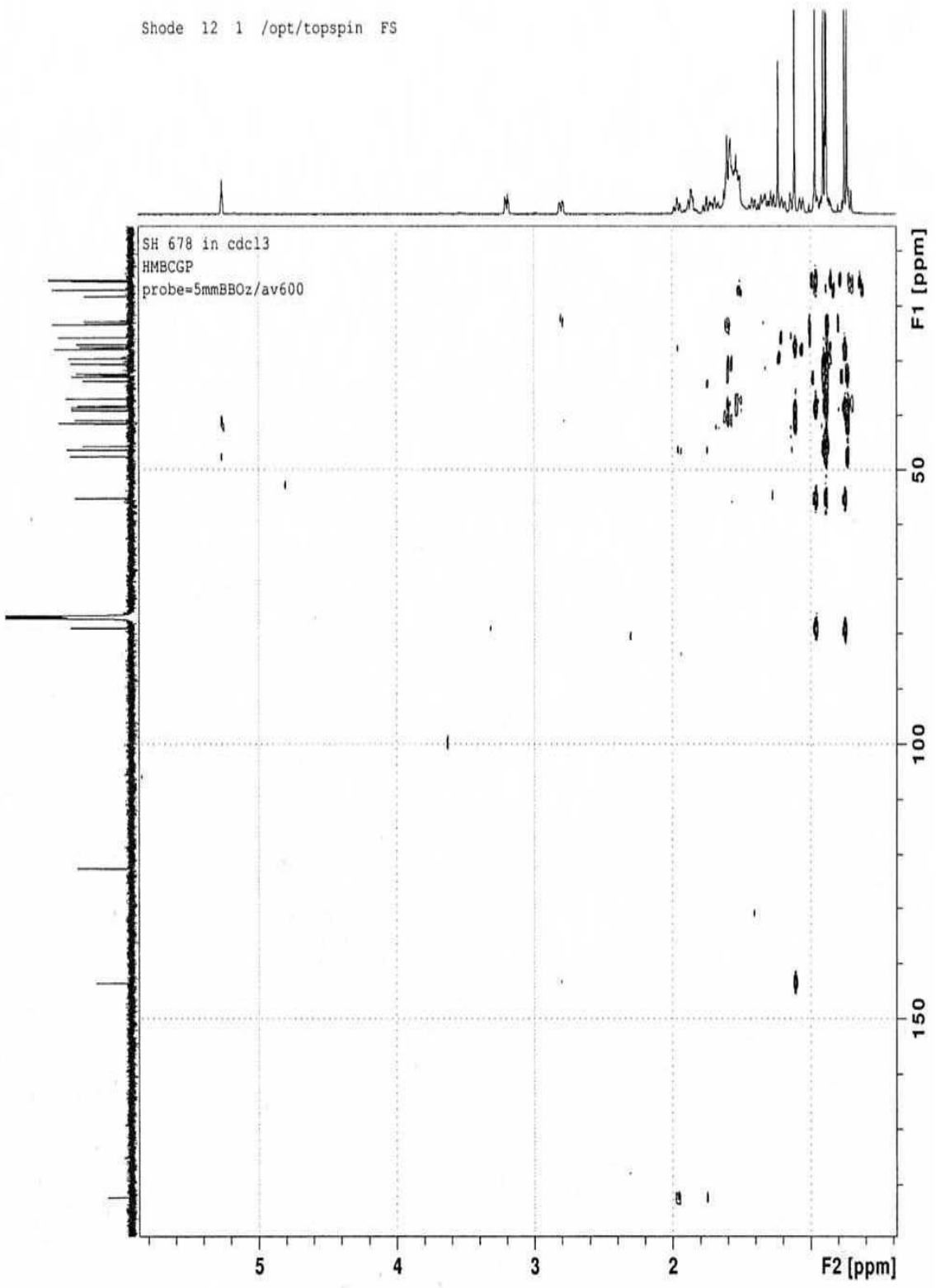


Figure 5.2.8: ^{1}H ^{13}C HMBC NMR Spectrum of **OO/55/A**

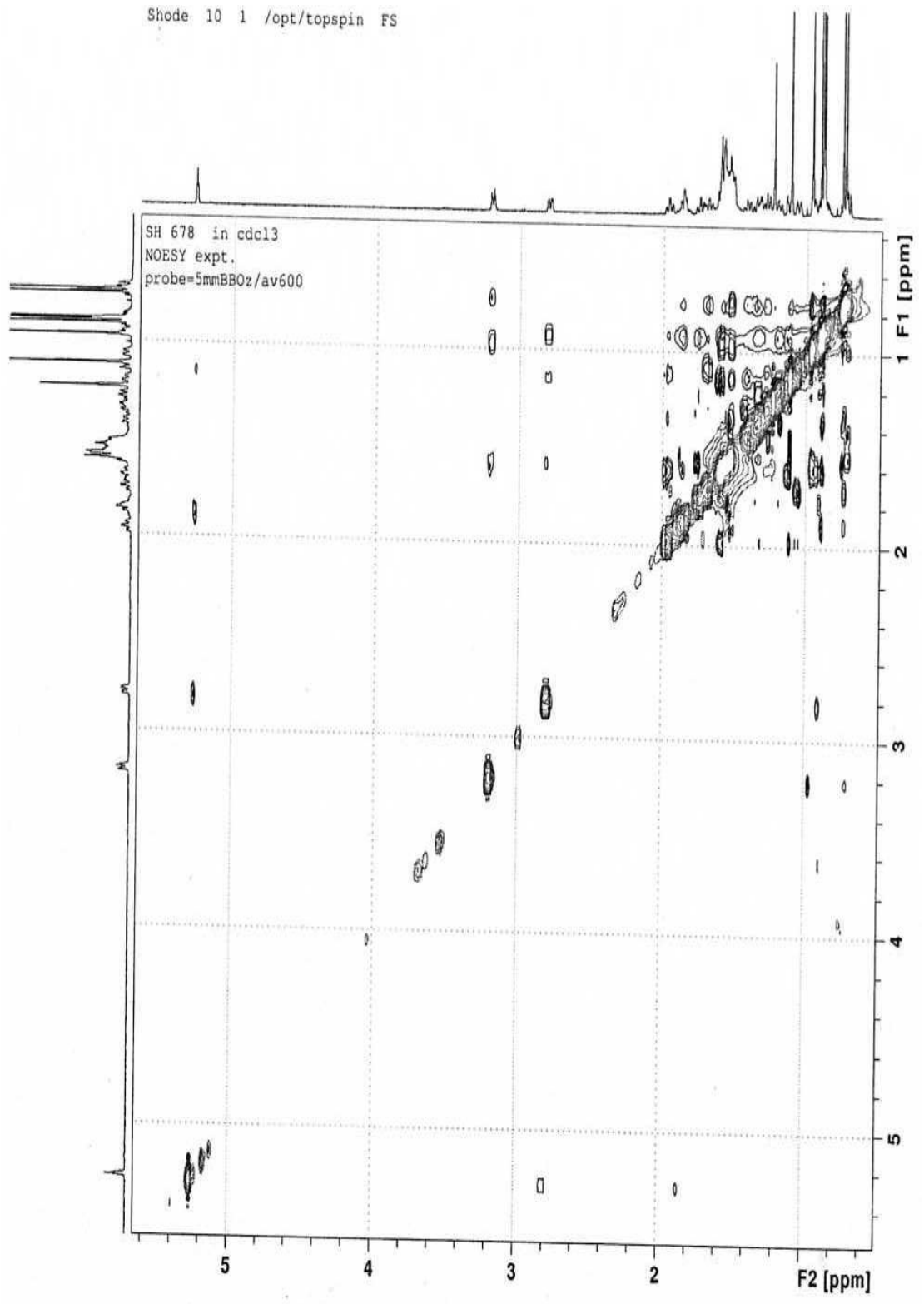


Figure 5.2.9: ^{1}H ^{1}H NOESY NMR of **OO/55/A**

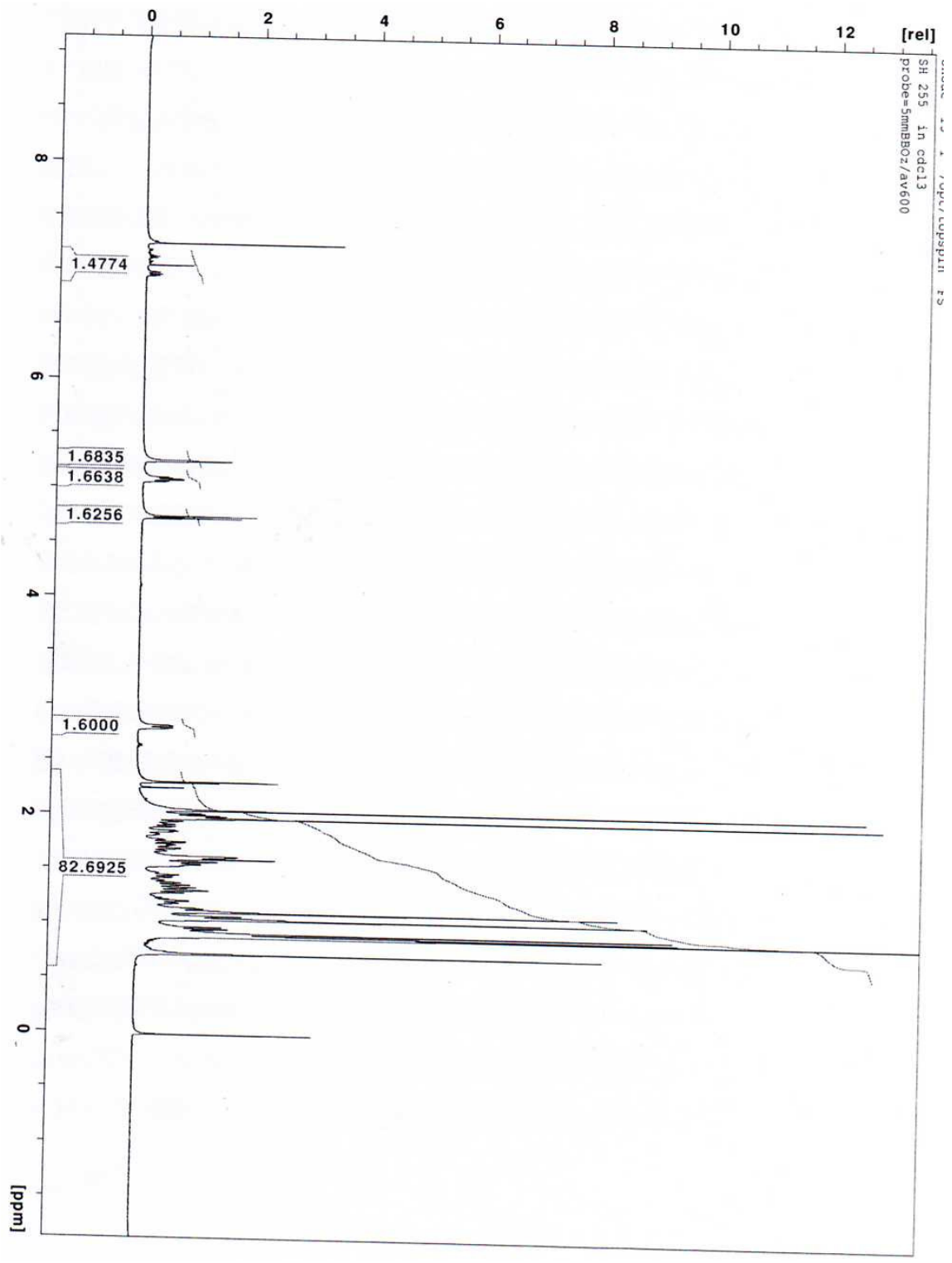


Figure 5.3.1: ^{1}H NMR spectrum of **OO/55/B**

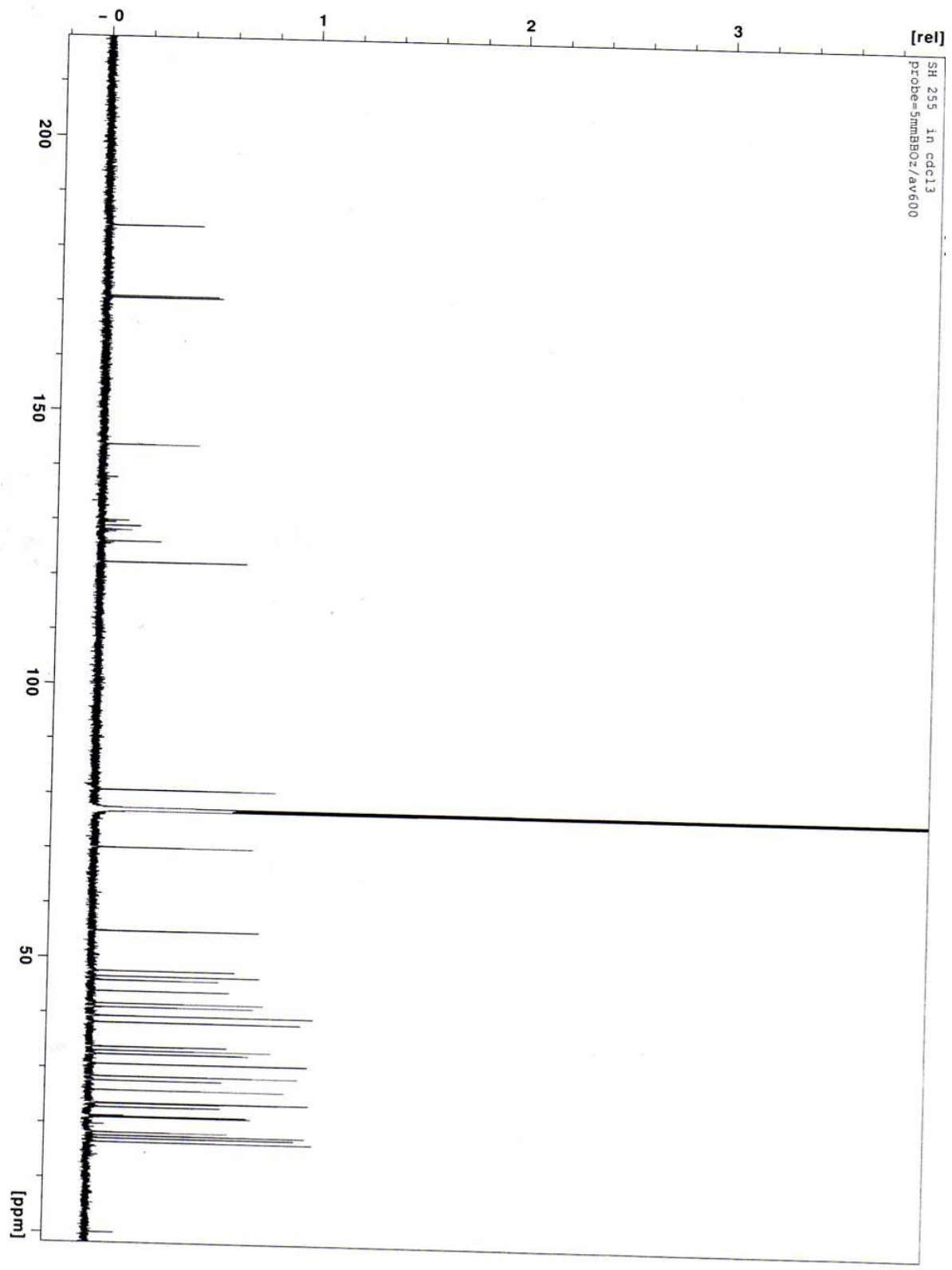


Figure 5.3.2: ^{13}C NMR spectrum of **OO/55/B**

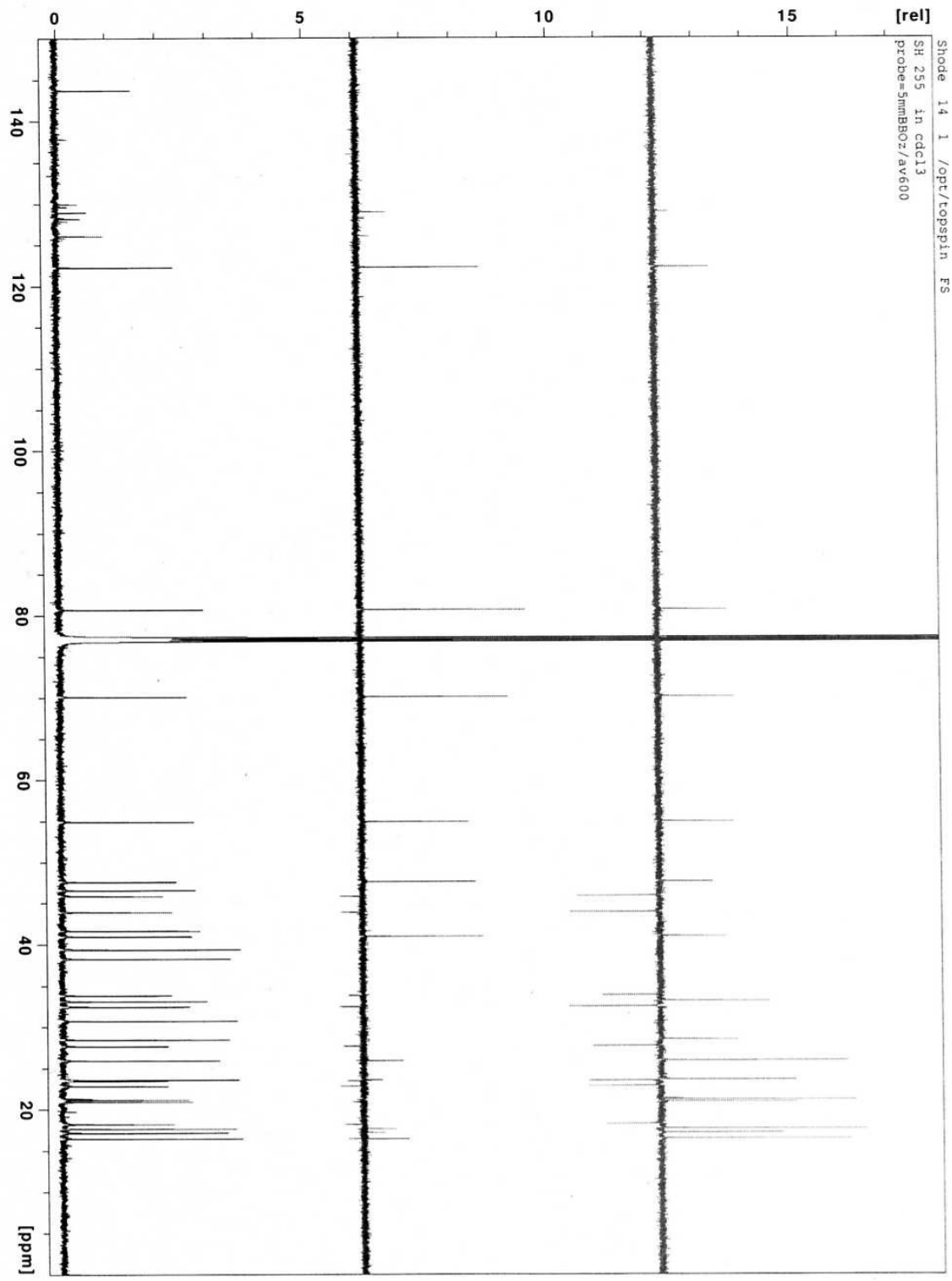


Figure 5.3.3: ^{13}C DEPT NMR spectrum of **OO/55/B**

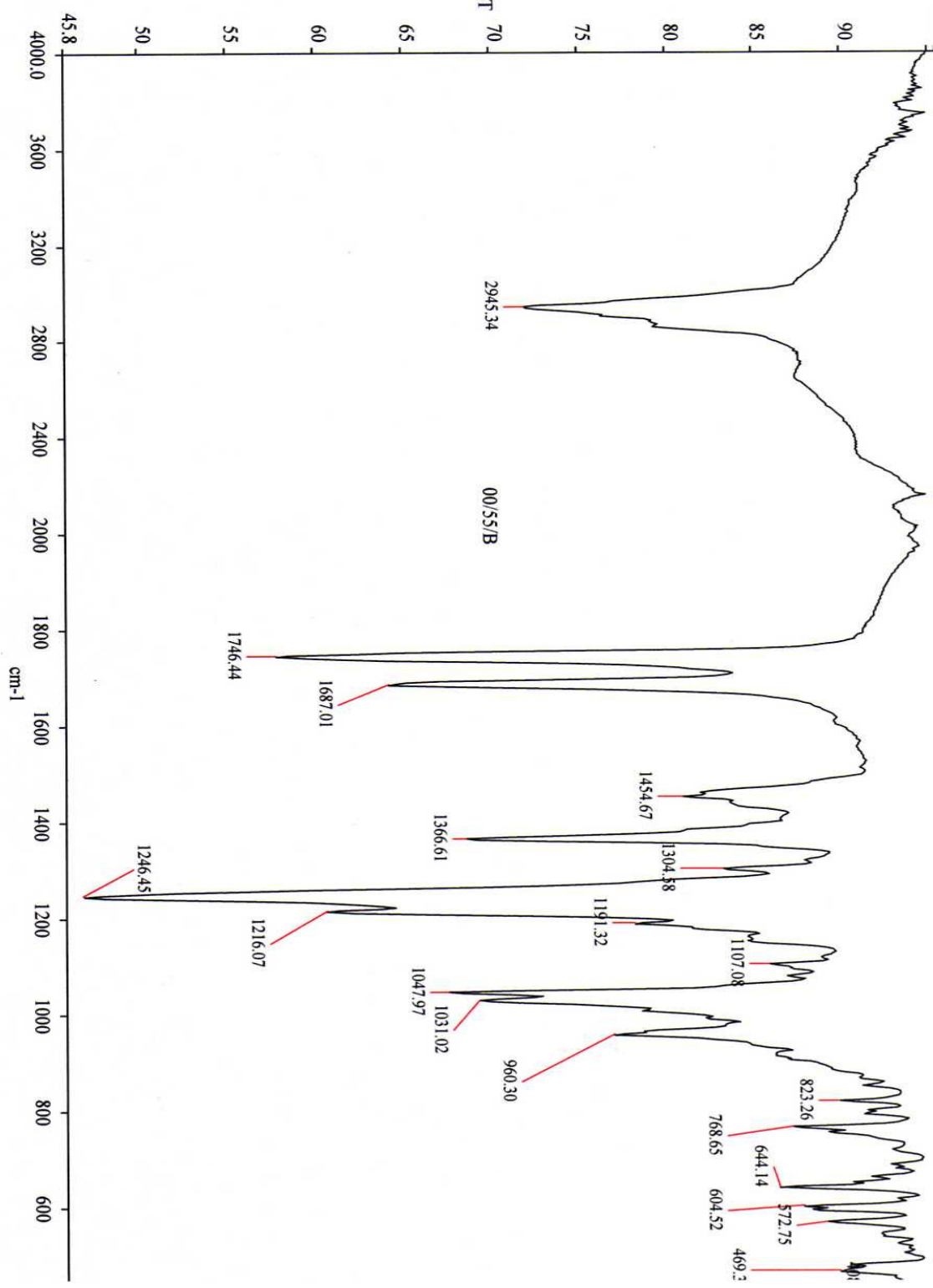


Figure 5.3.4: FTIR Spectrum of **OO/55/B**

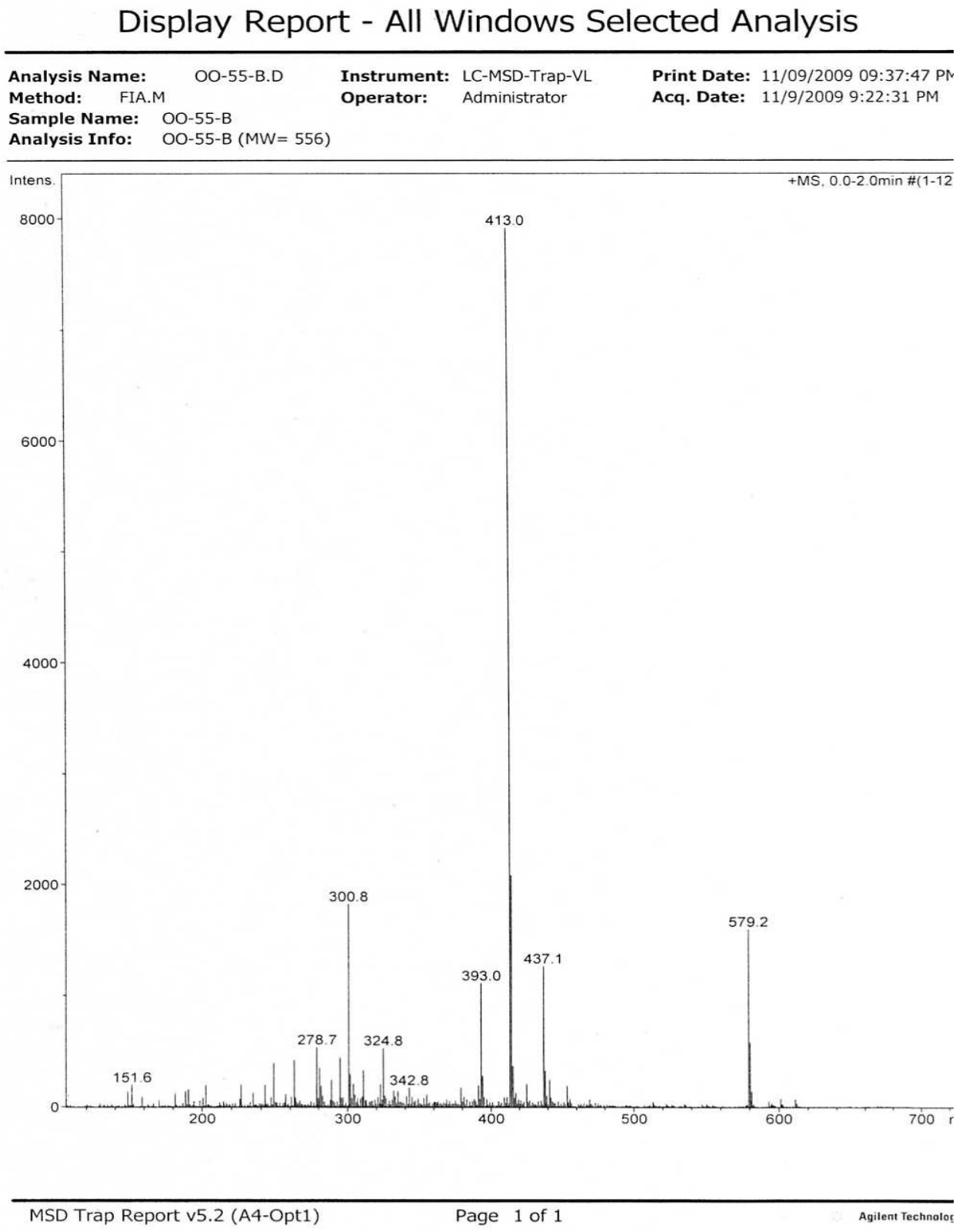


Figure 5.3.5: Mass Spectrum of **OO/55/B**

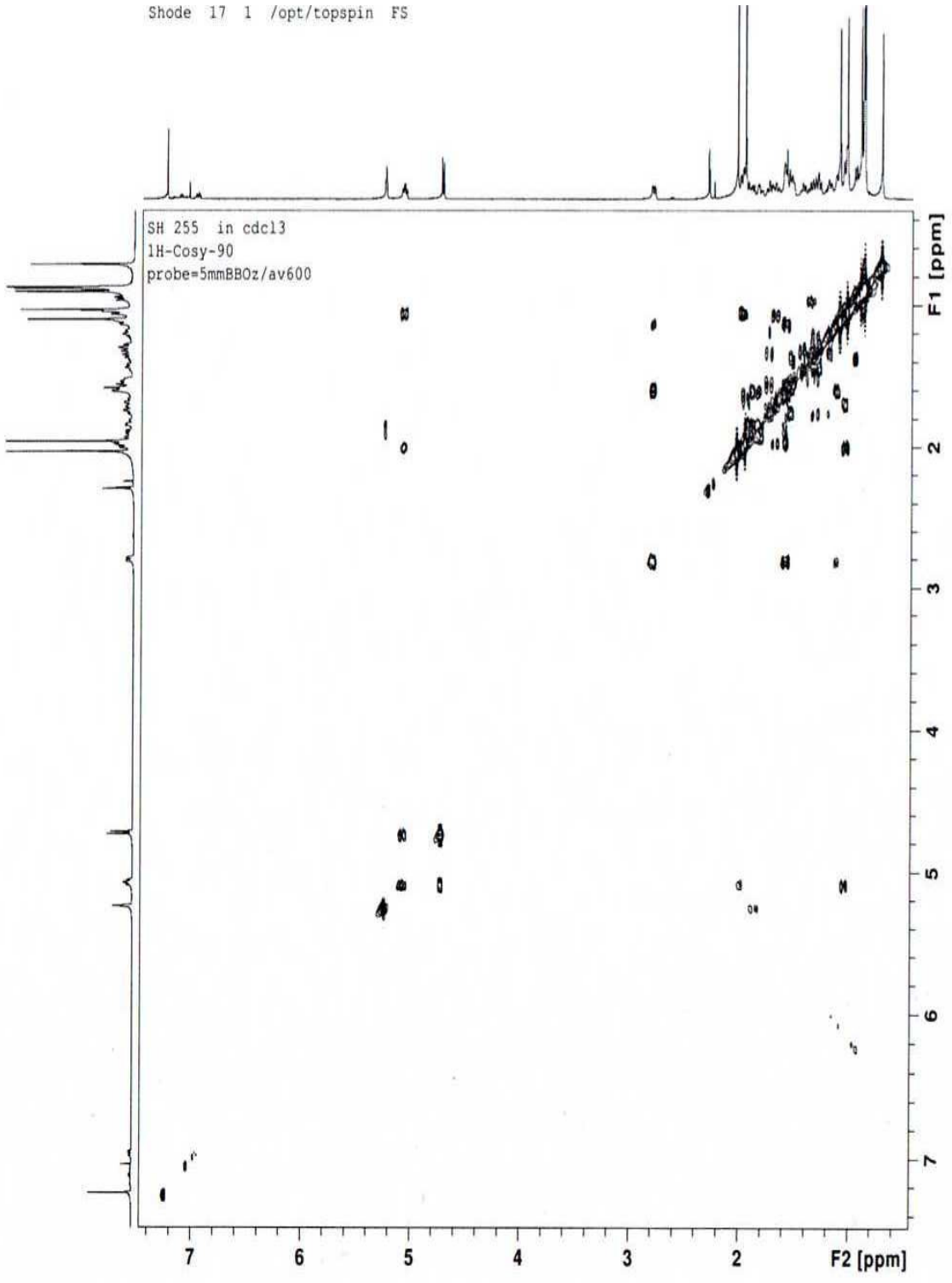


Figure 5.3.6: ^{1}H ^{1}H COSY NMR spectrum of **OO/55/B**

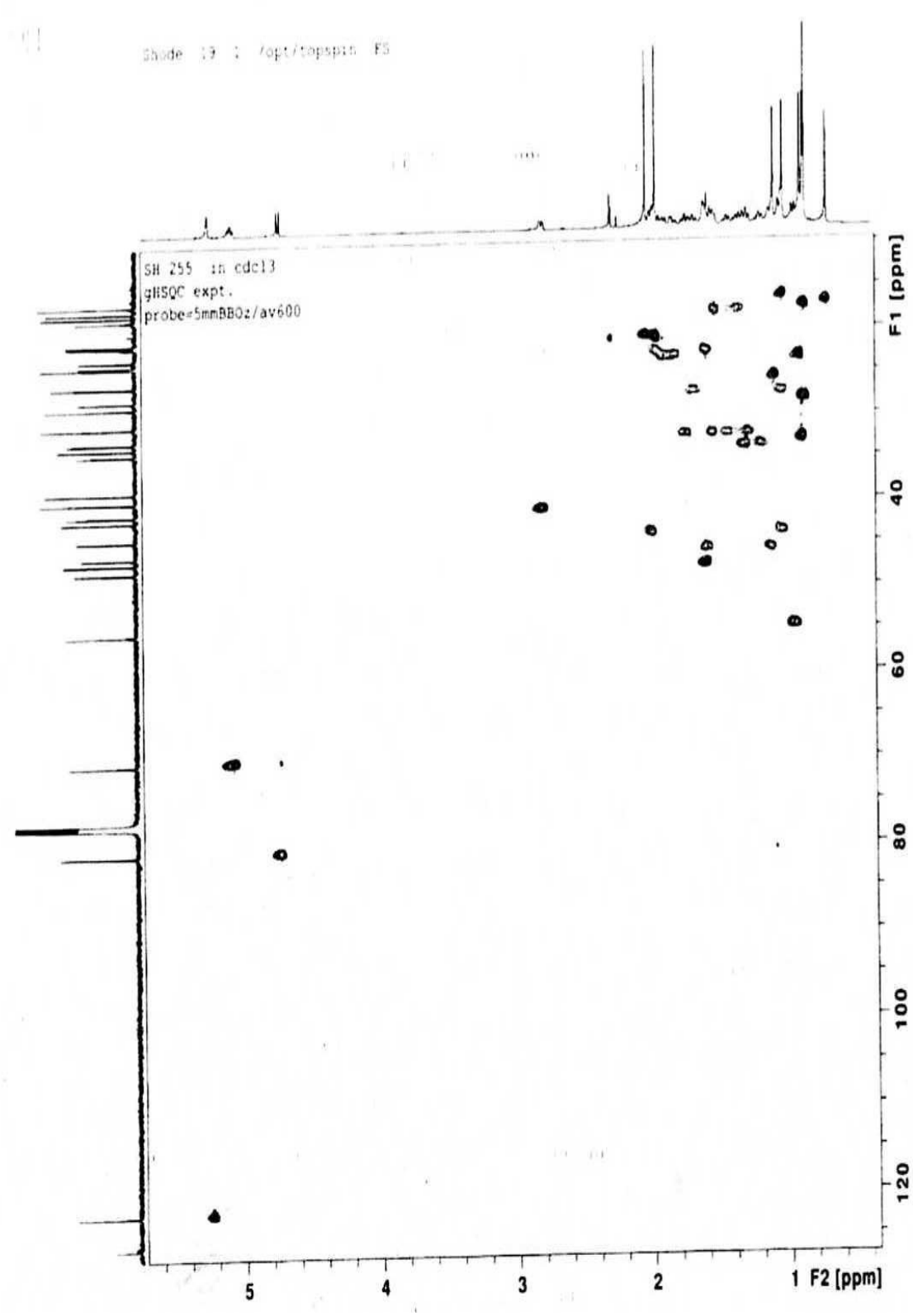


Figure 5.3.7: ^{1}H ^{13}C HSQC NMR spectrum of **OO/55/B**

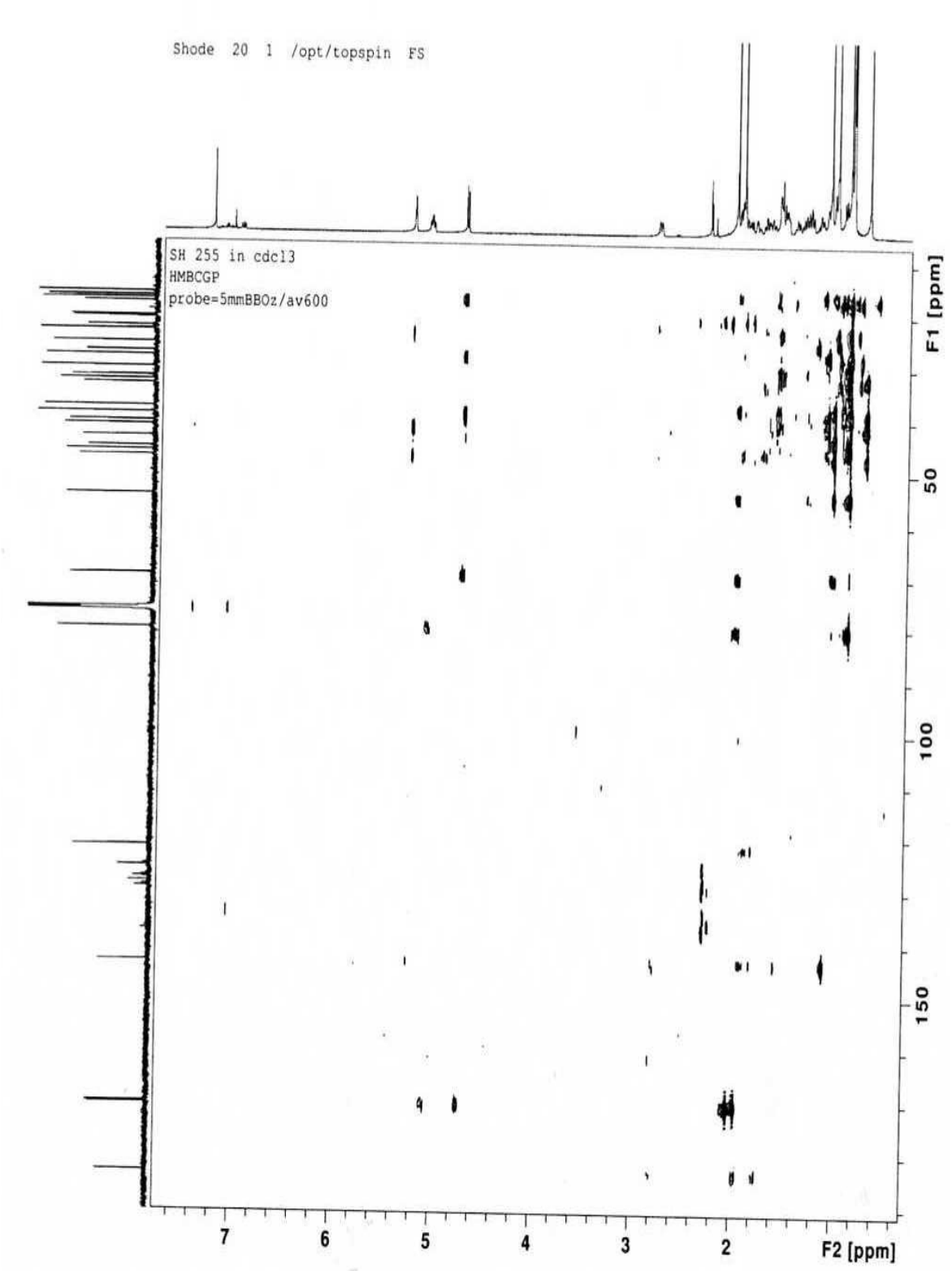


Figure 5.3.8: ^{1}H ^{13}C HMBC NMR spectrum of **OO/55/B**

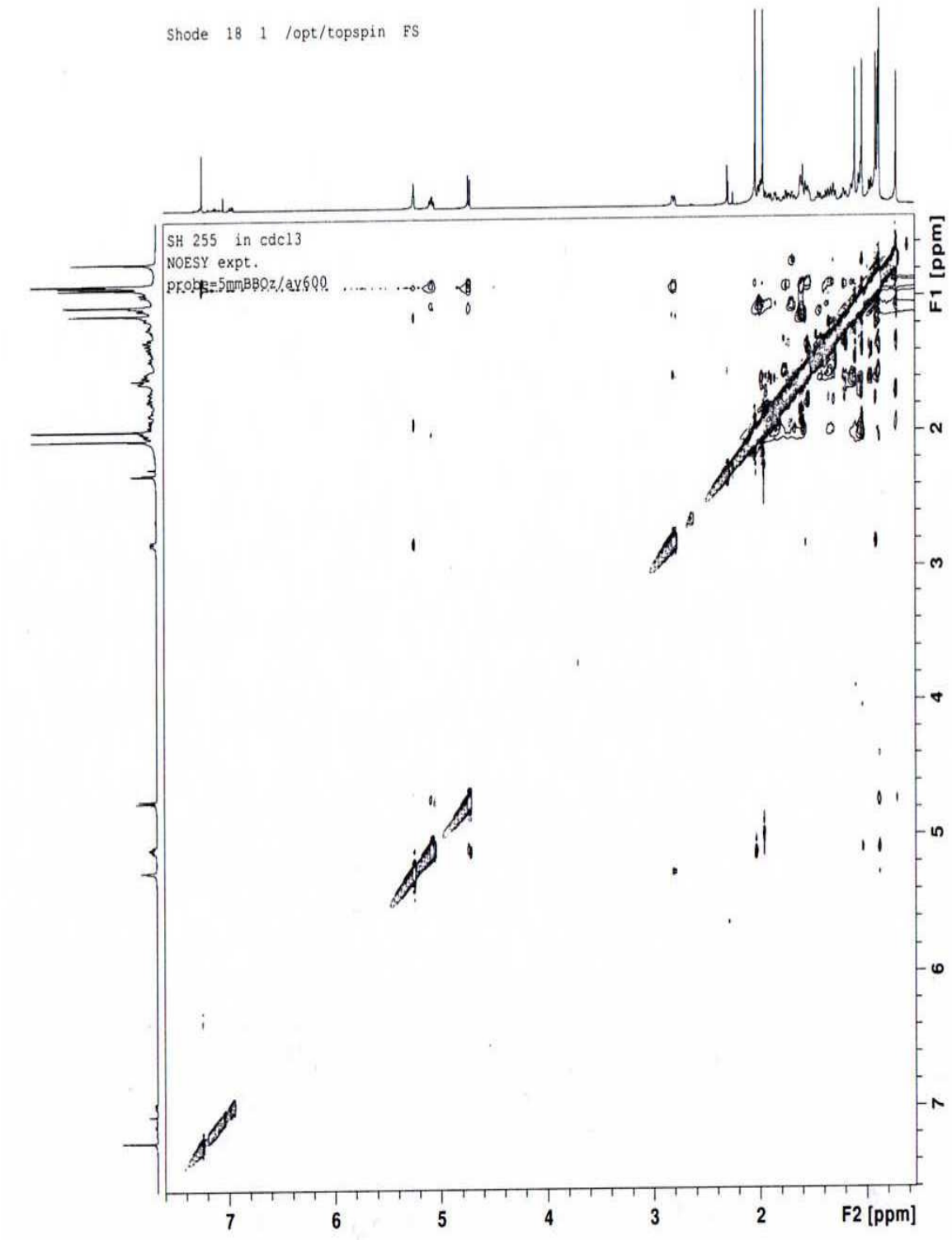


Figure 5.3.9: ^{1}H ^{1}H NOESY NMR spectrum of **OO/55/B**

APPENDIX 4

Spectra of Betulinic Acid

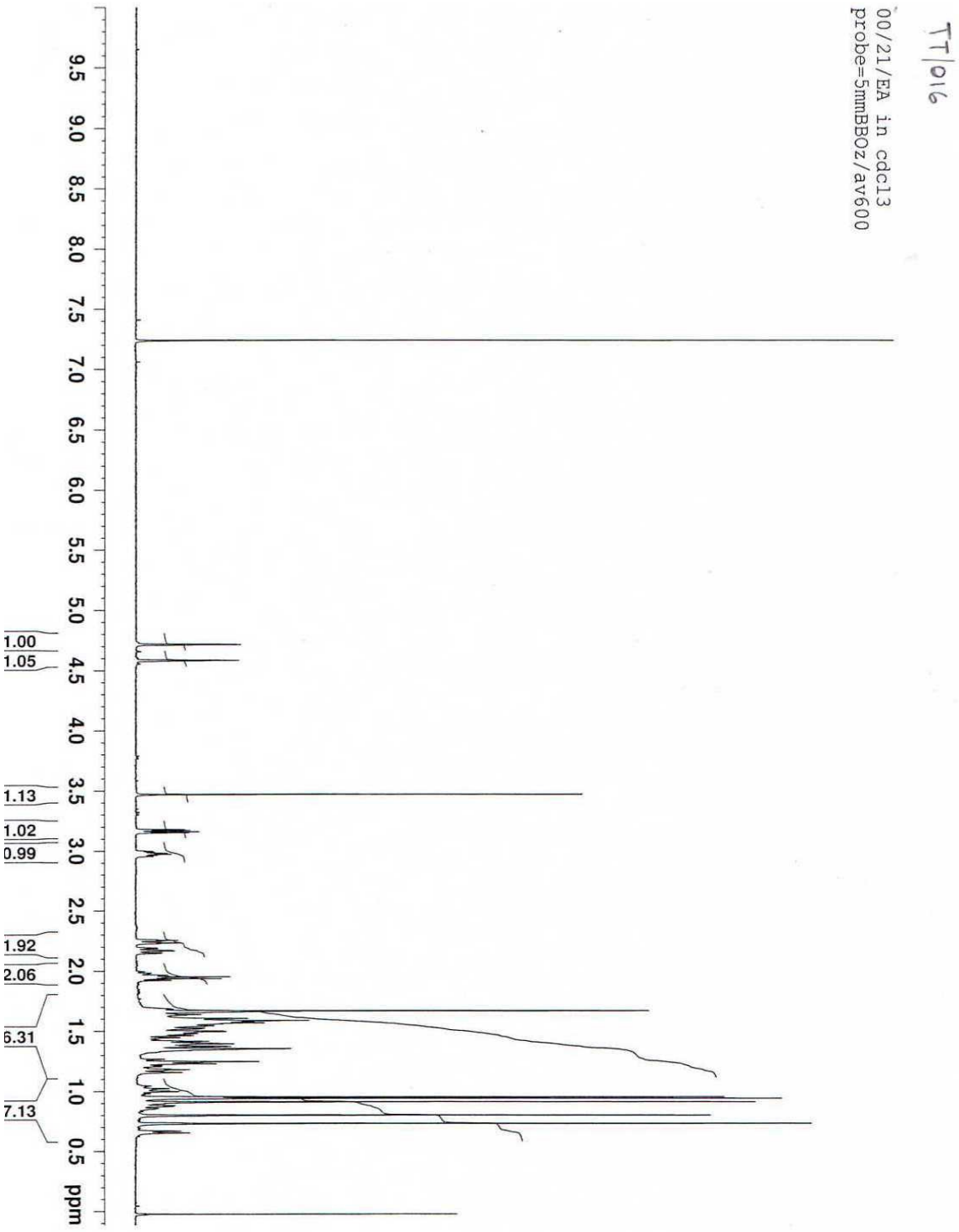


Figure 6.1.1: ^{1}H NMR Spectrum of **OO/21/EA**

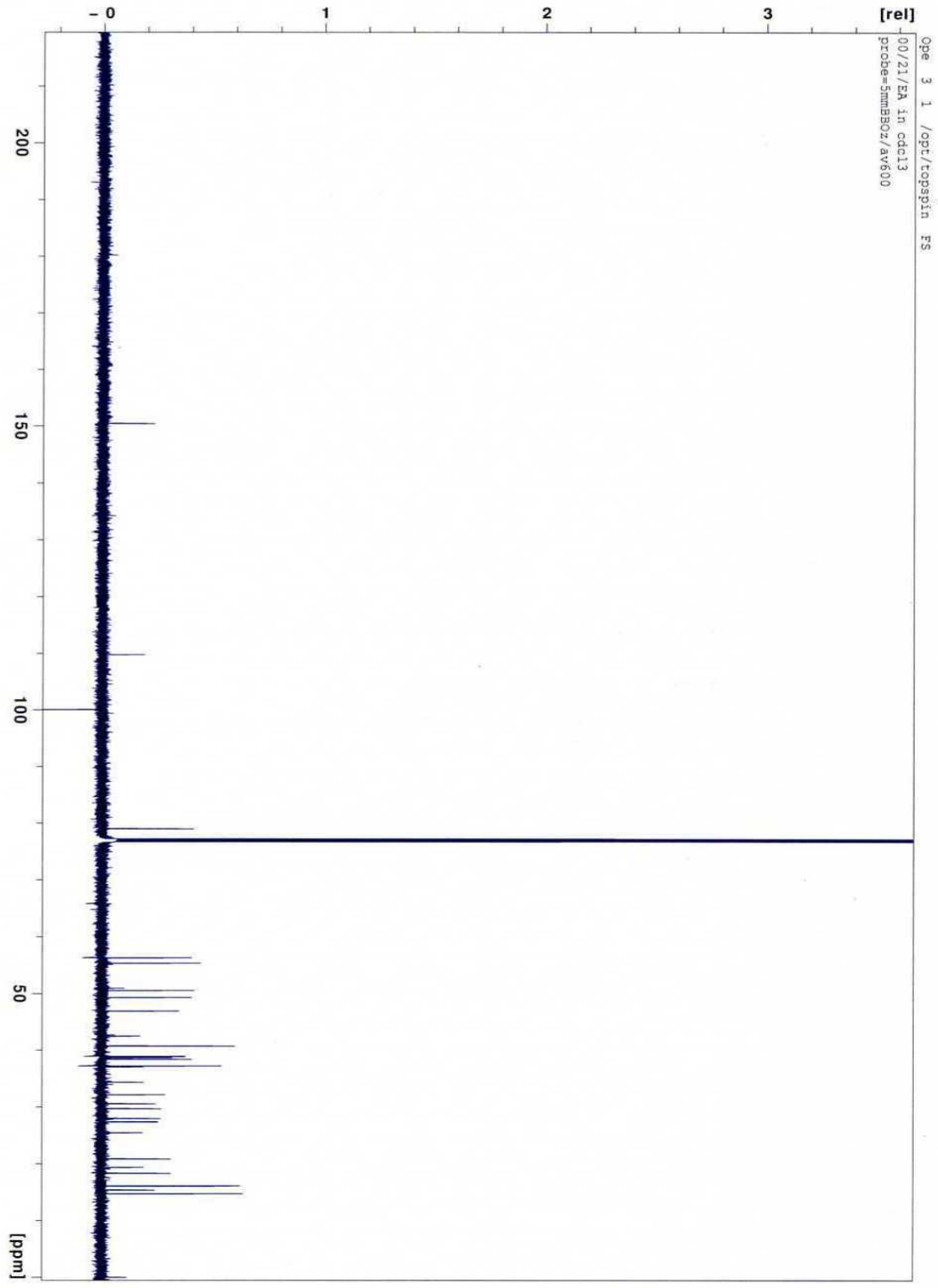


Figure 6.1.2: ^{13}C NMR Spectrum of **OO/21/EA**

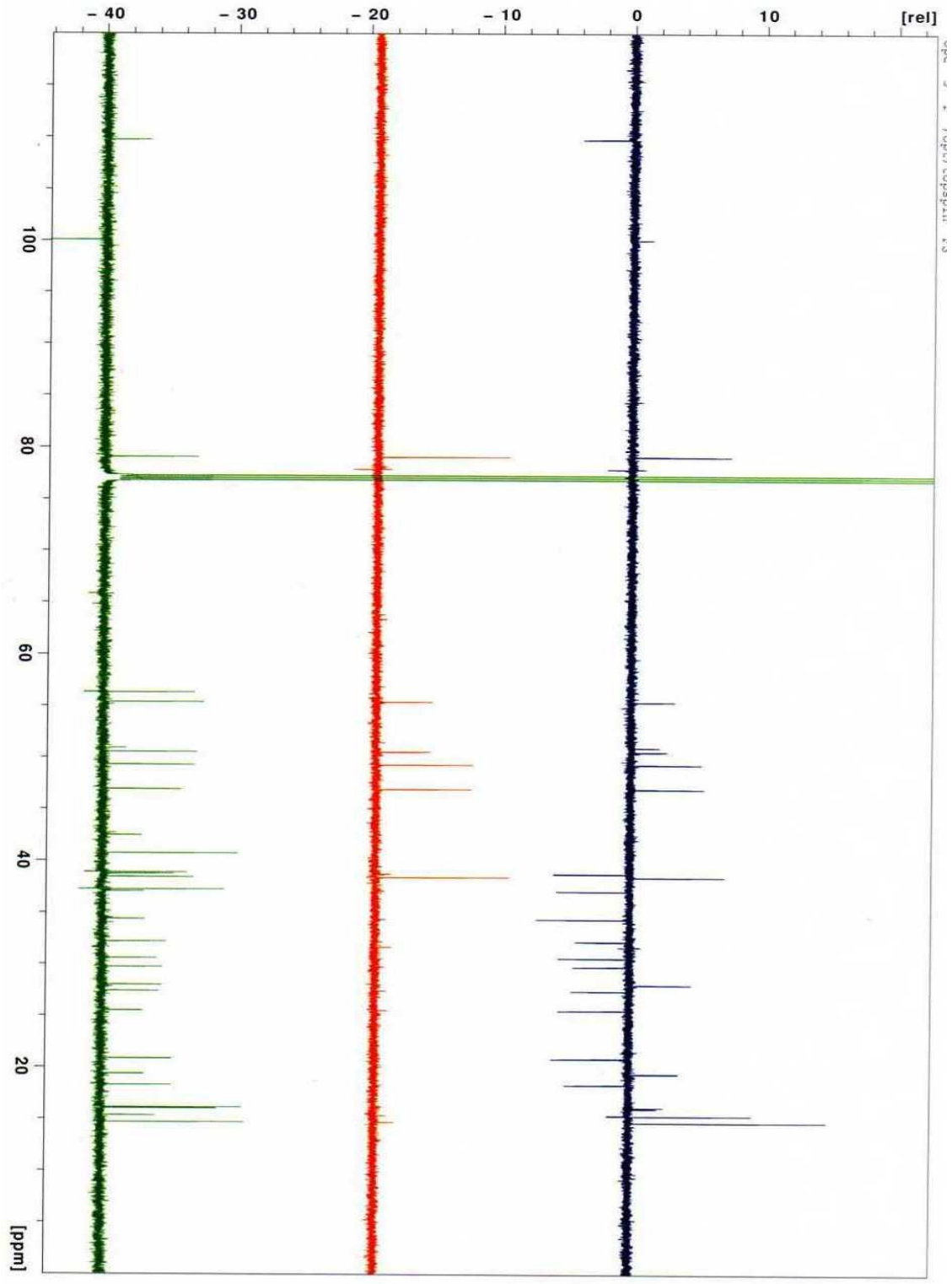


Figure 6.1.3: ^{13}C DEPT NMR Spectrum of **OO/21/EA**

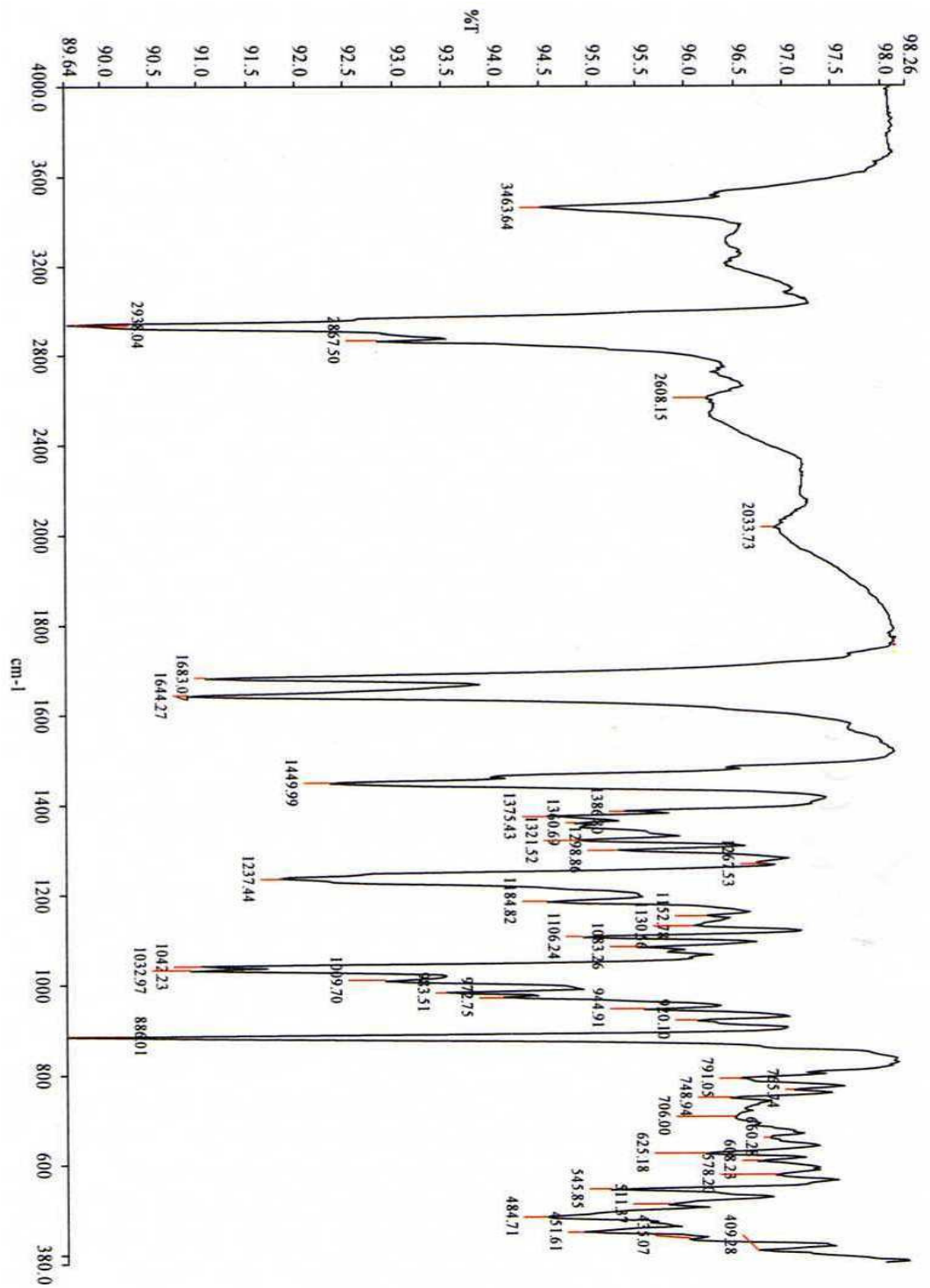

%T
cm-1
3463.64
2938.04
2867.50
2608.15
2033.73
1683.07
1644.27
1449.99
1375.43
1340.69
1321.52
1237.44
1184.82
1106.24
1042.23
1032.97
1009.70
944.91
884.01
791.05
748.94
706.00
625.18
545.85
484.71
451.61
409.28

Figure 6.1.4: FTIR Spectrum of **OO/21/EA**

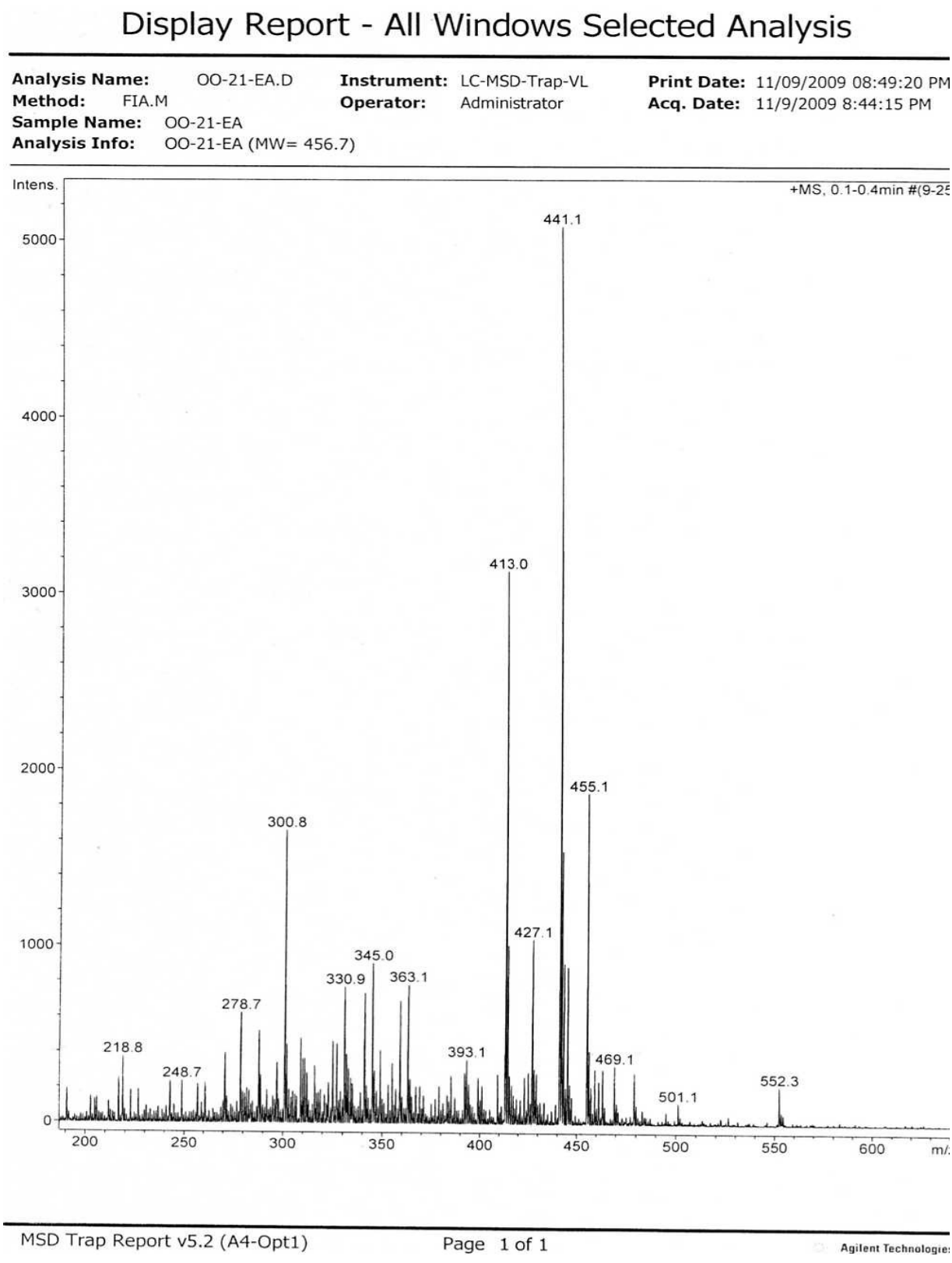


Figure 6.1.5: Mass Spectrum of **OO/21/EA**

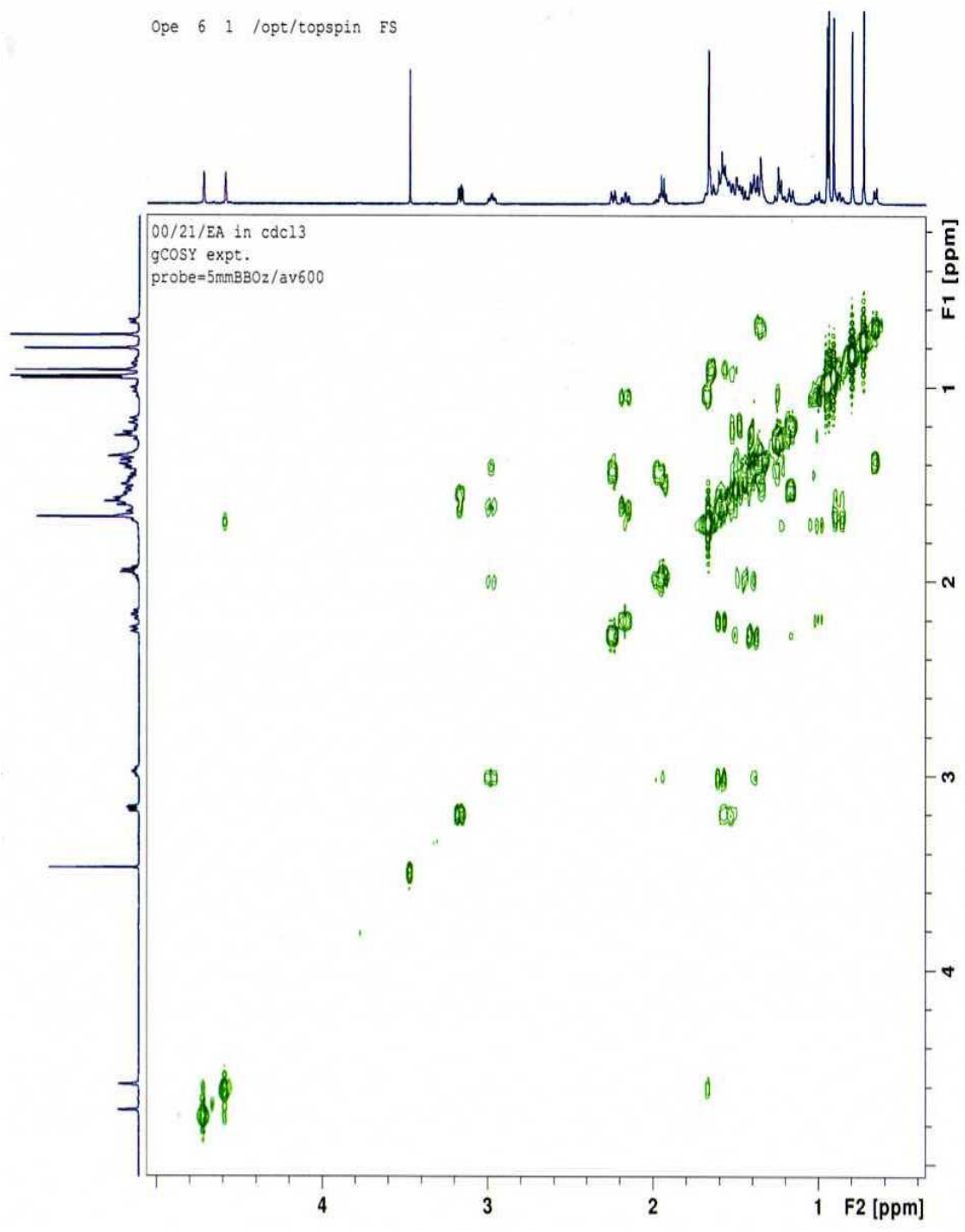


Figure 6.1.6: ^{1}H-^{1}H COSY NMR Spectrum of **OO/21/EA**

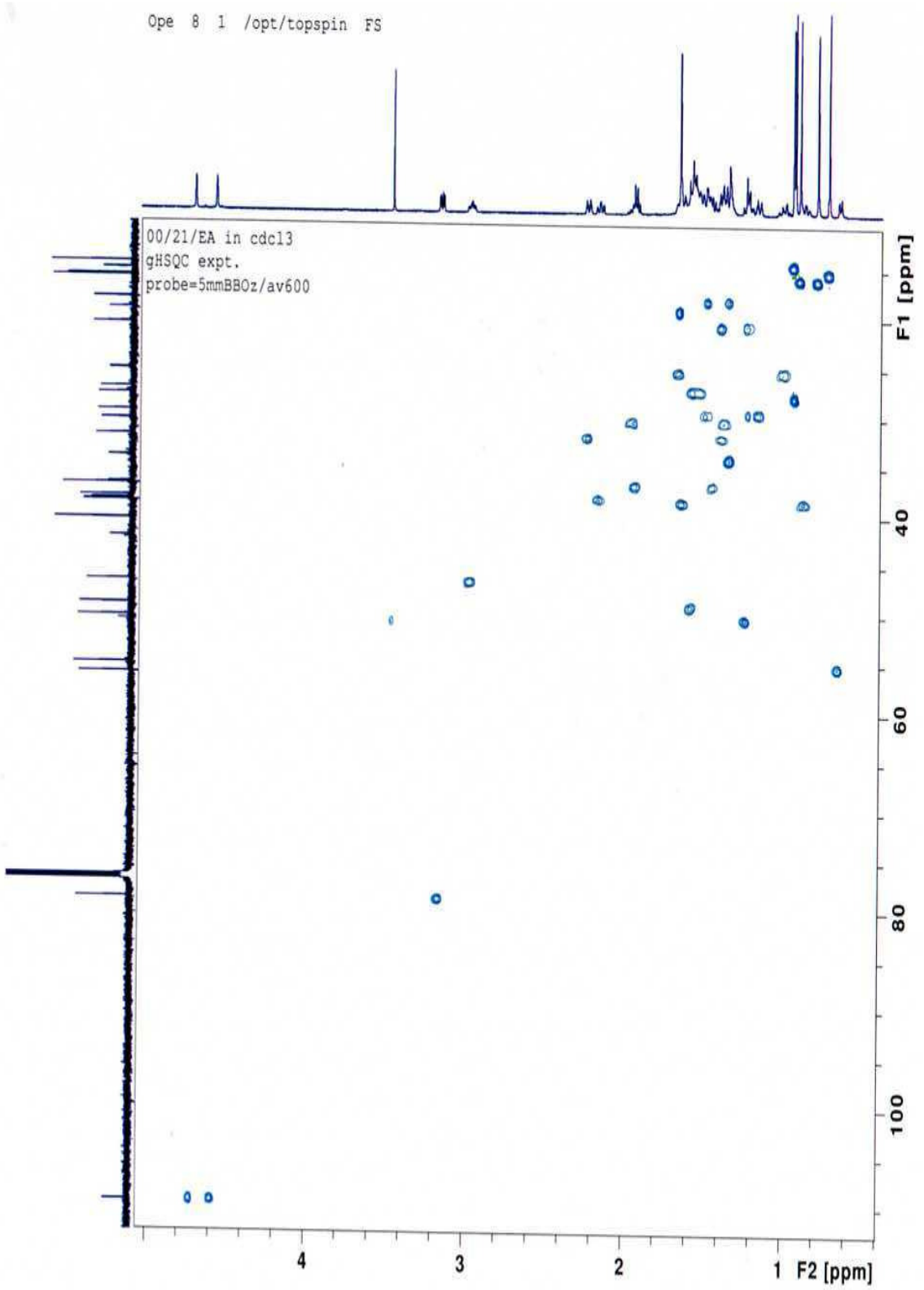


Figure 6.1.7: ^{1}H ^{13}C HSQC NMR Spectrum of **OO/21/EA**

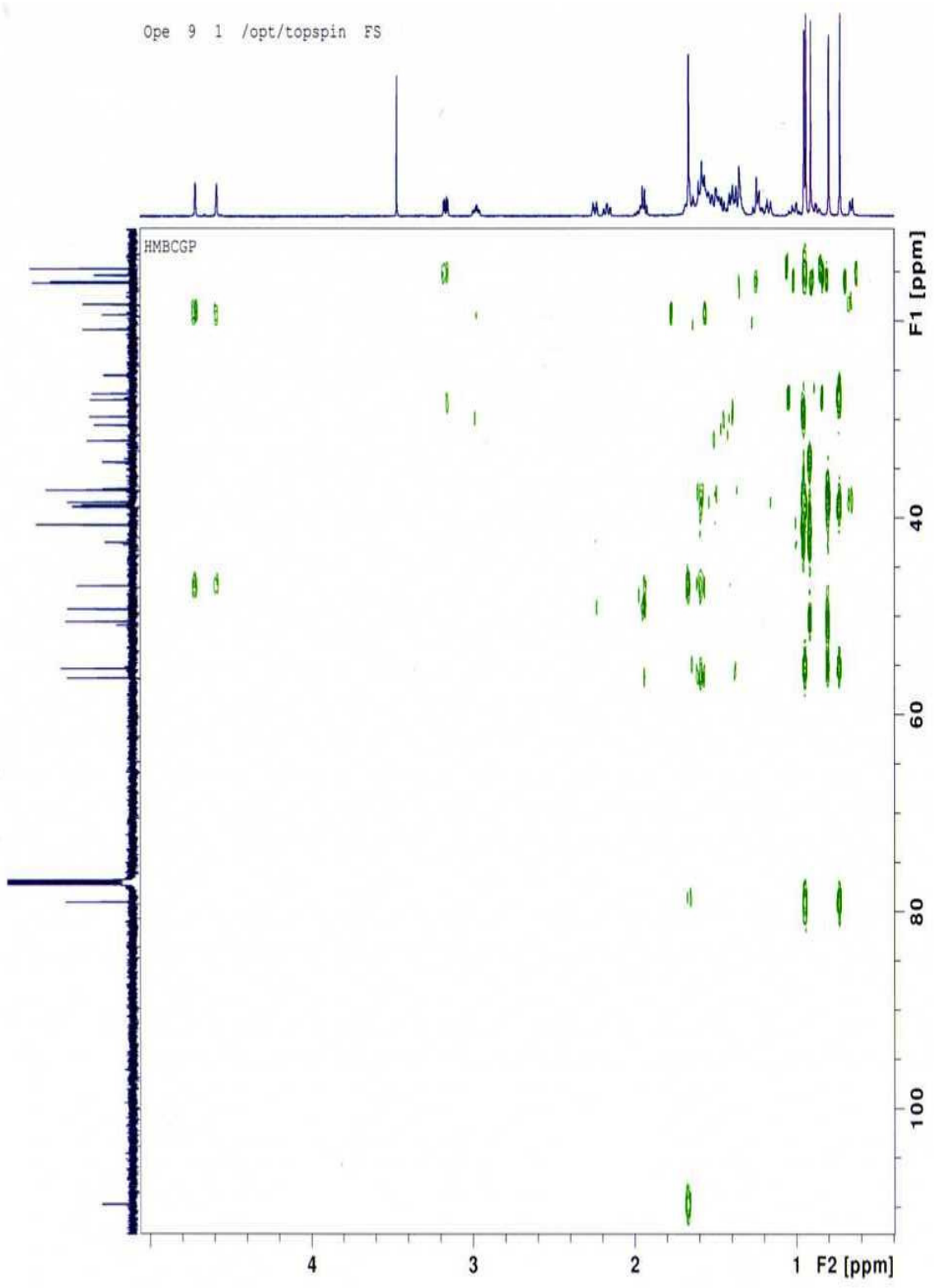


Figure 6.1.8: ^{1}H ^{13}C HMBC NMR Spectrum of **OO/21/EA**

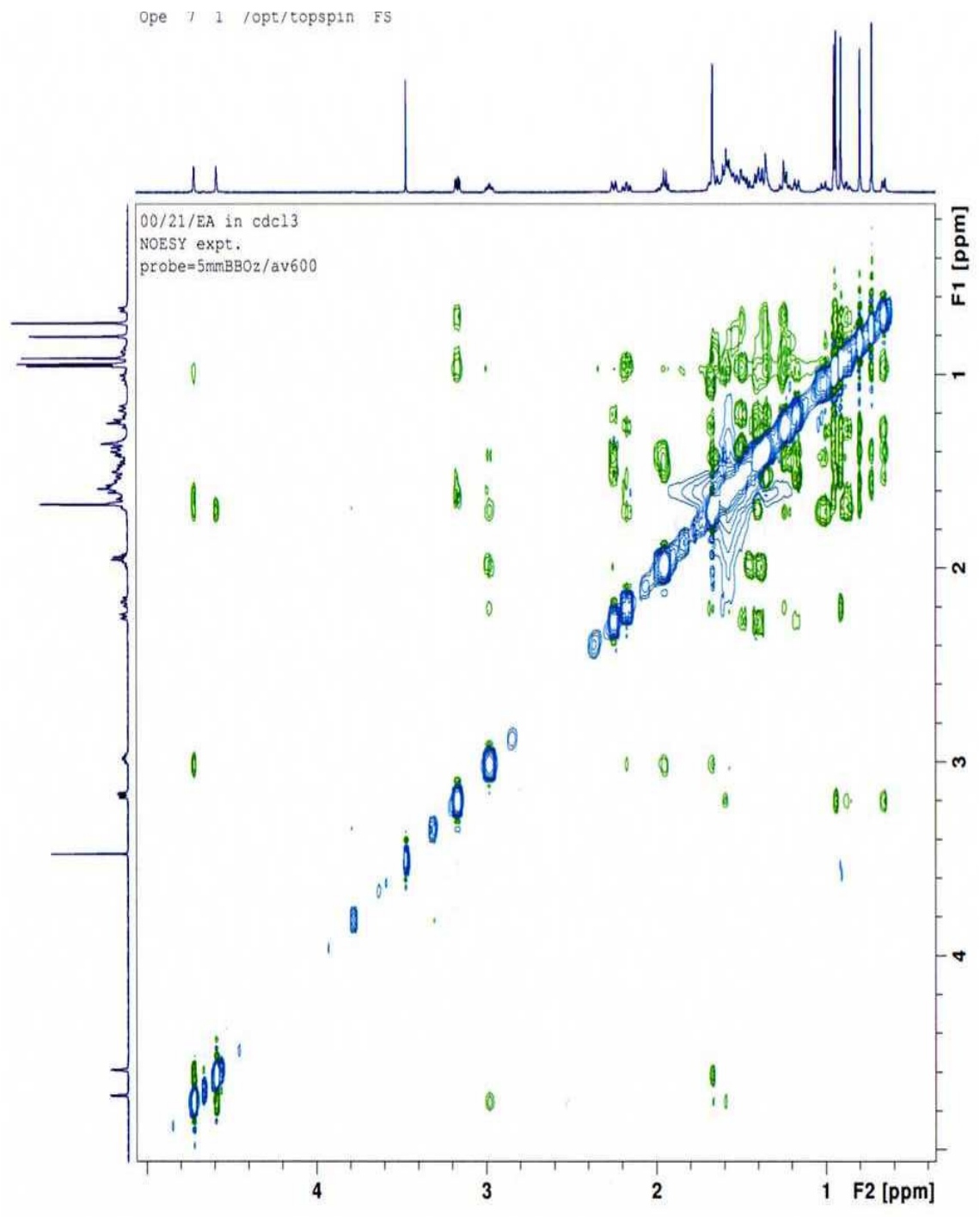


Figure 6.1.9: ^{1}H ^{1}H NOESY NMR Spectrum of **OO/21/EA**

APPENDIX 5

Spectra of the Derivatives of Oleanolic Acid

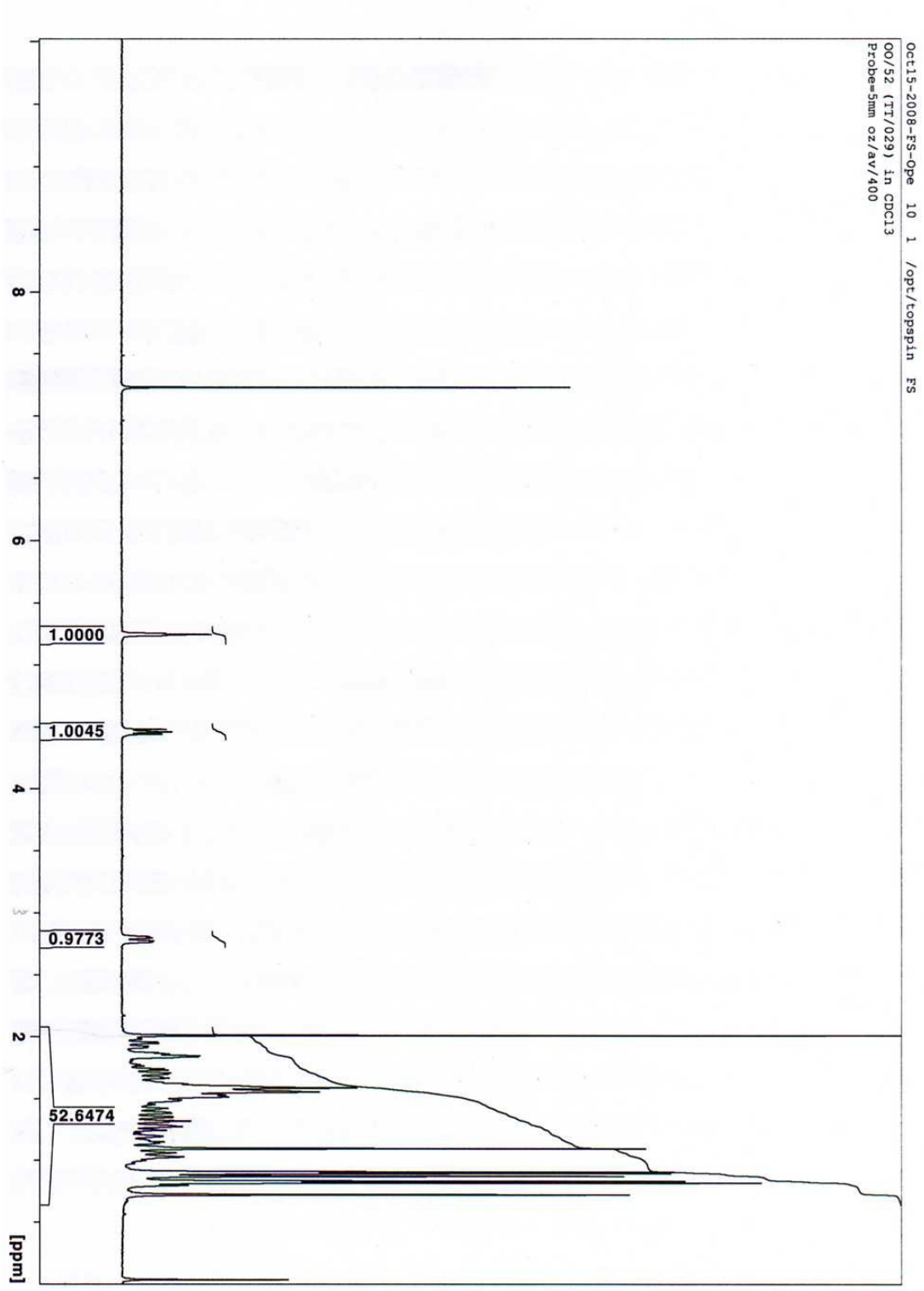


Figure 7.1.1: ^{1}H NMR Spectrum of **OO/52**

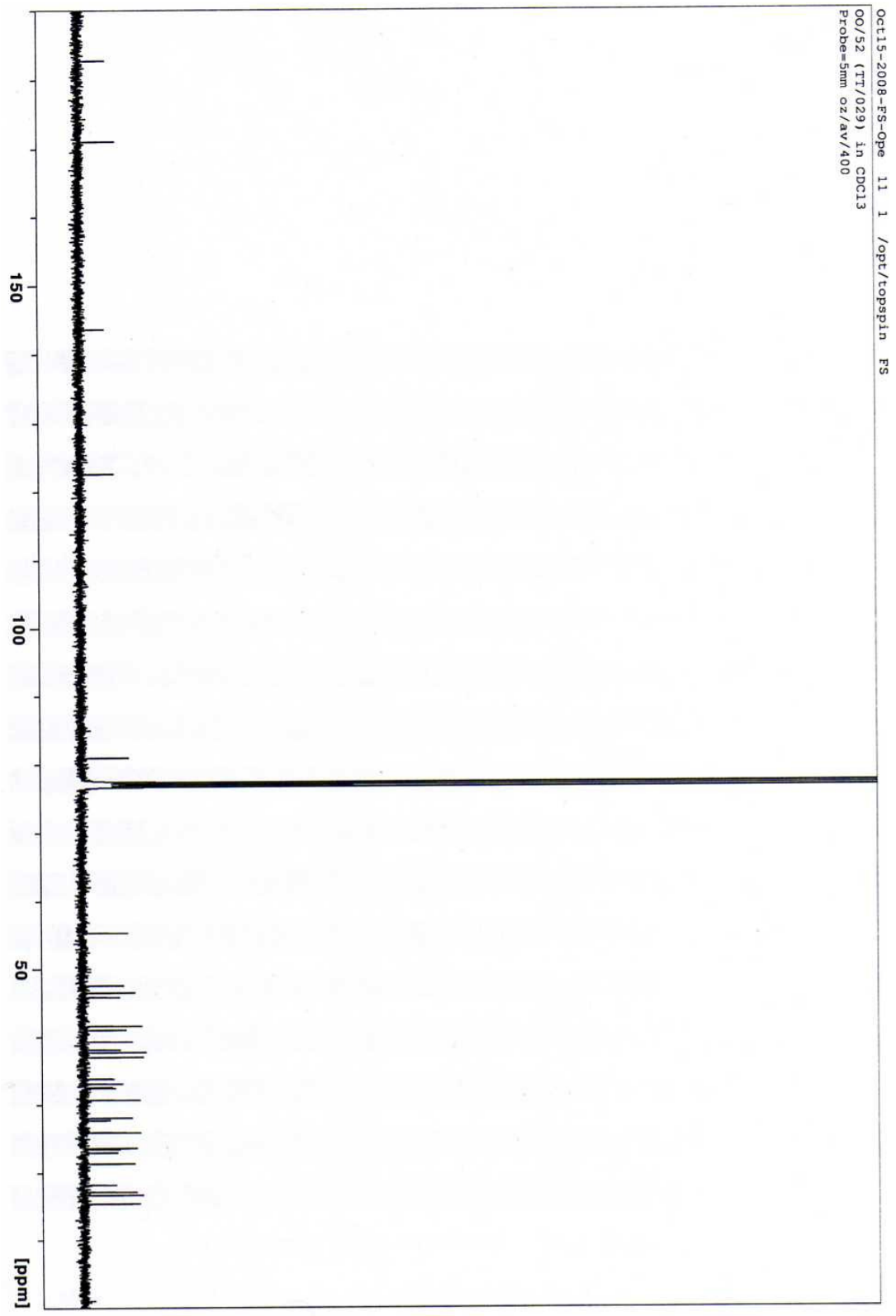


Figure 7.1.2: ^{13}C NMR Spectrum of **OO/52**

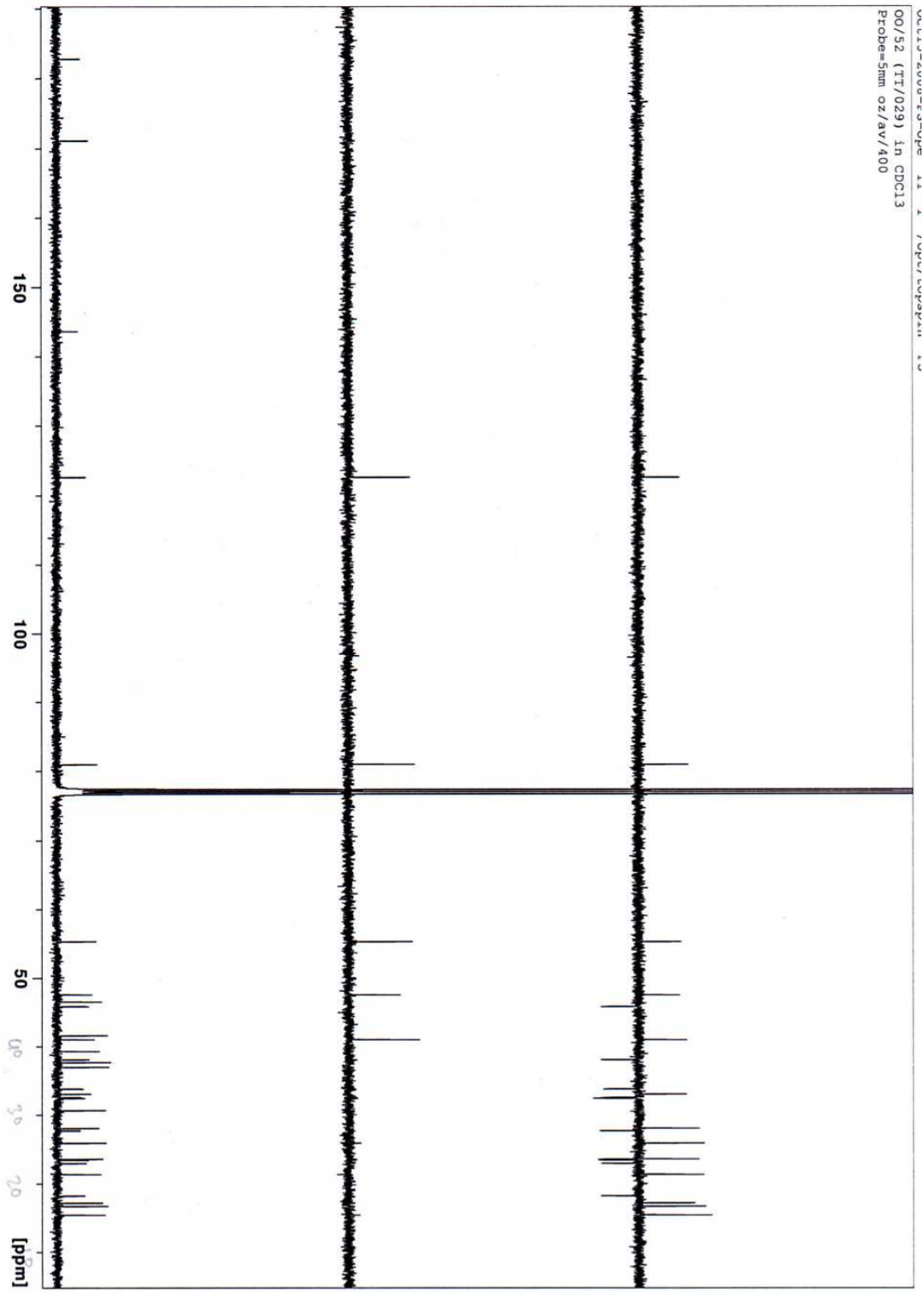


Figure 7.1.3: ^{13}C DEPT NMR Spectrum of **OO/52**

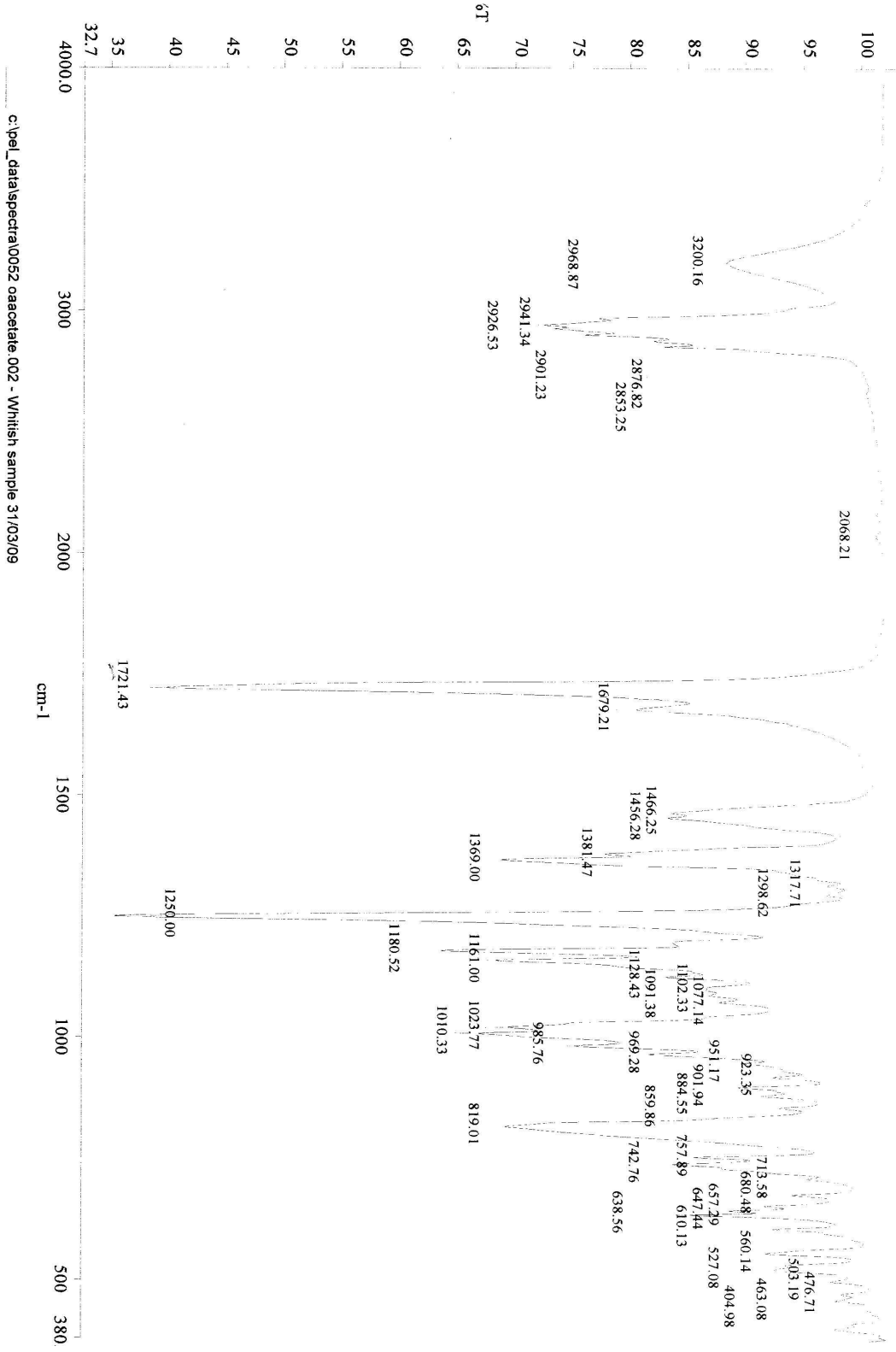


Figure 7.1.4: FTIR Spectrum of **OO/52**

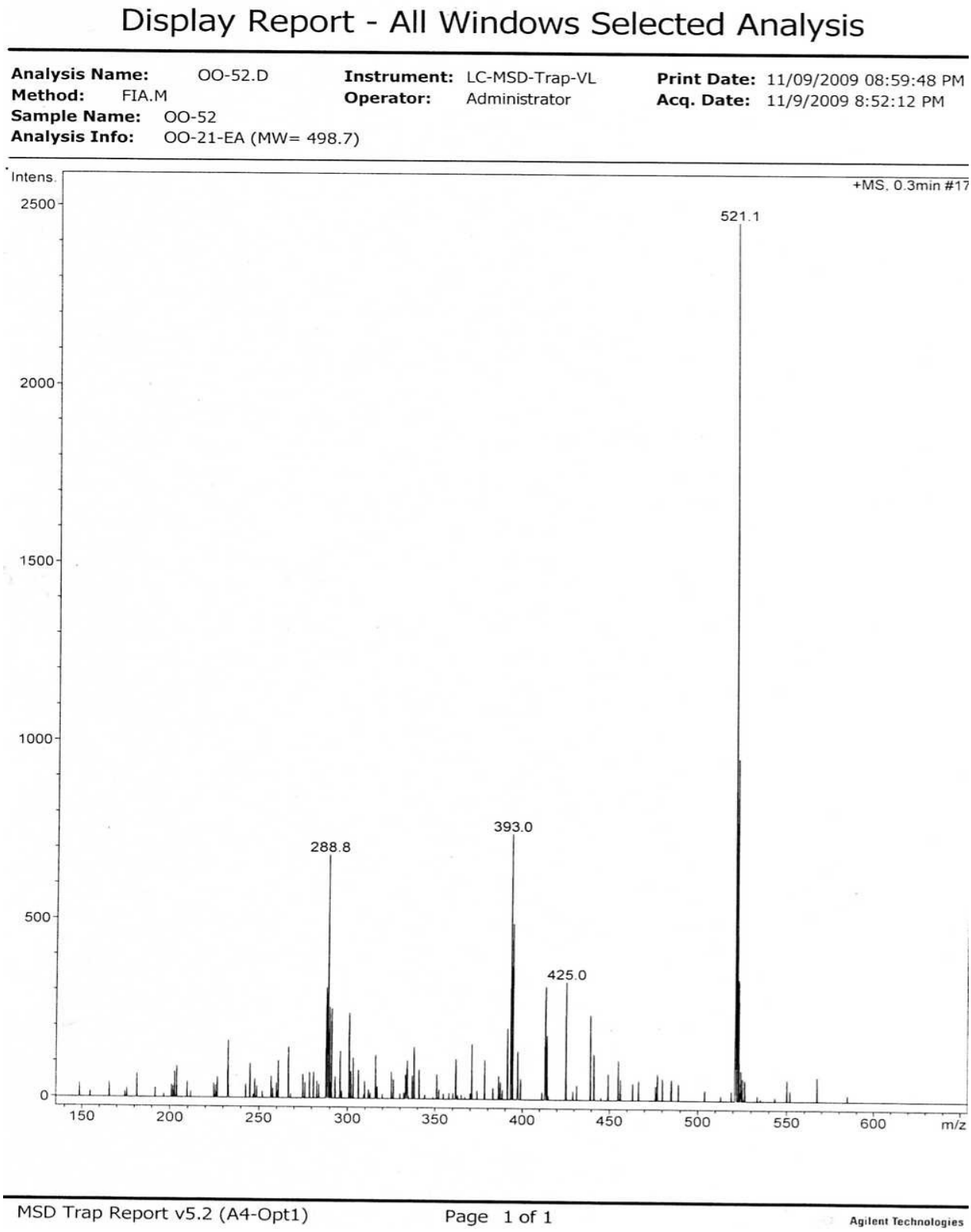


Figure 7.1.5: Mass Spectrum of **OO/52**

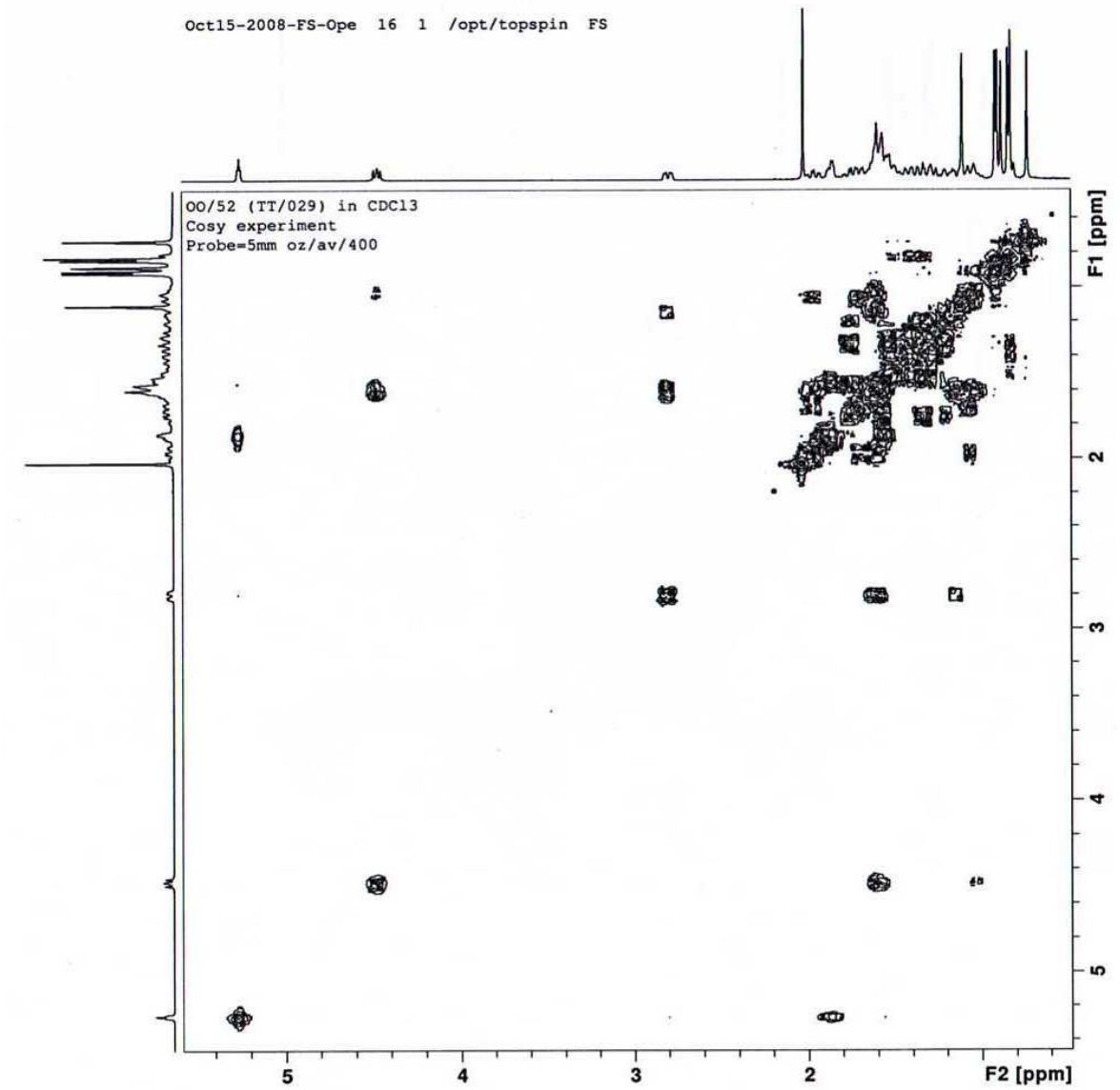


Figure 7.1.6: ^{1}H - ^{1}H COSY NMR Spectrum of **OO/52**

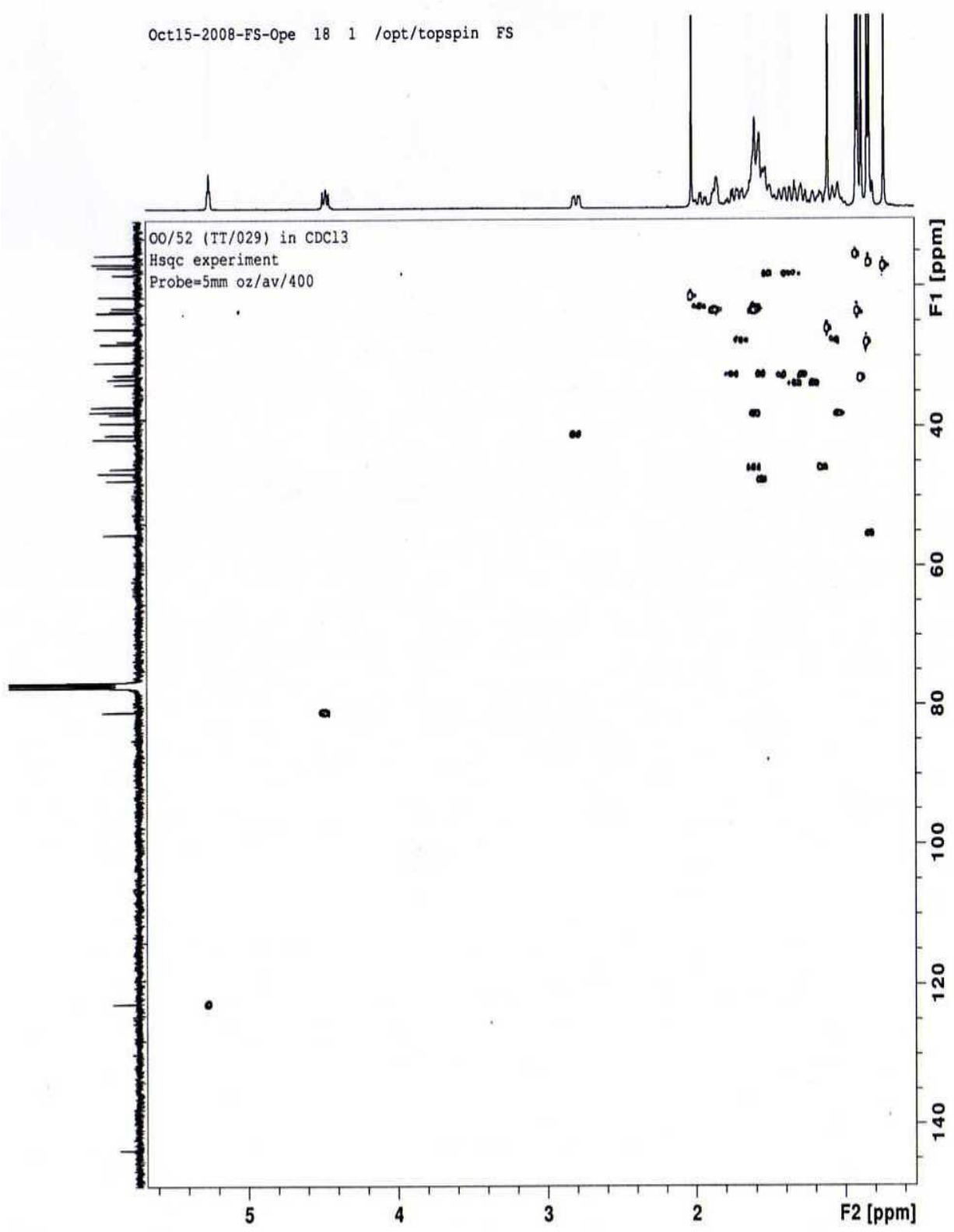


Figure 7.1.7: ^{1}H ^{13}C HSQC NMR Spectrum of **OO/52**

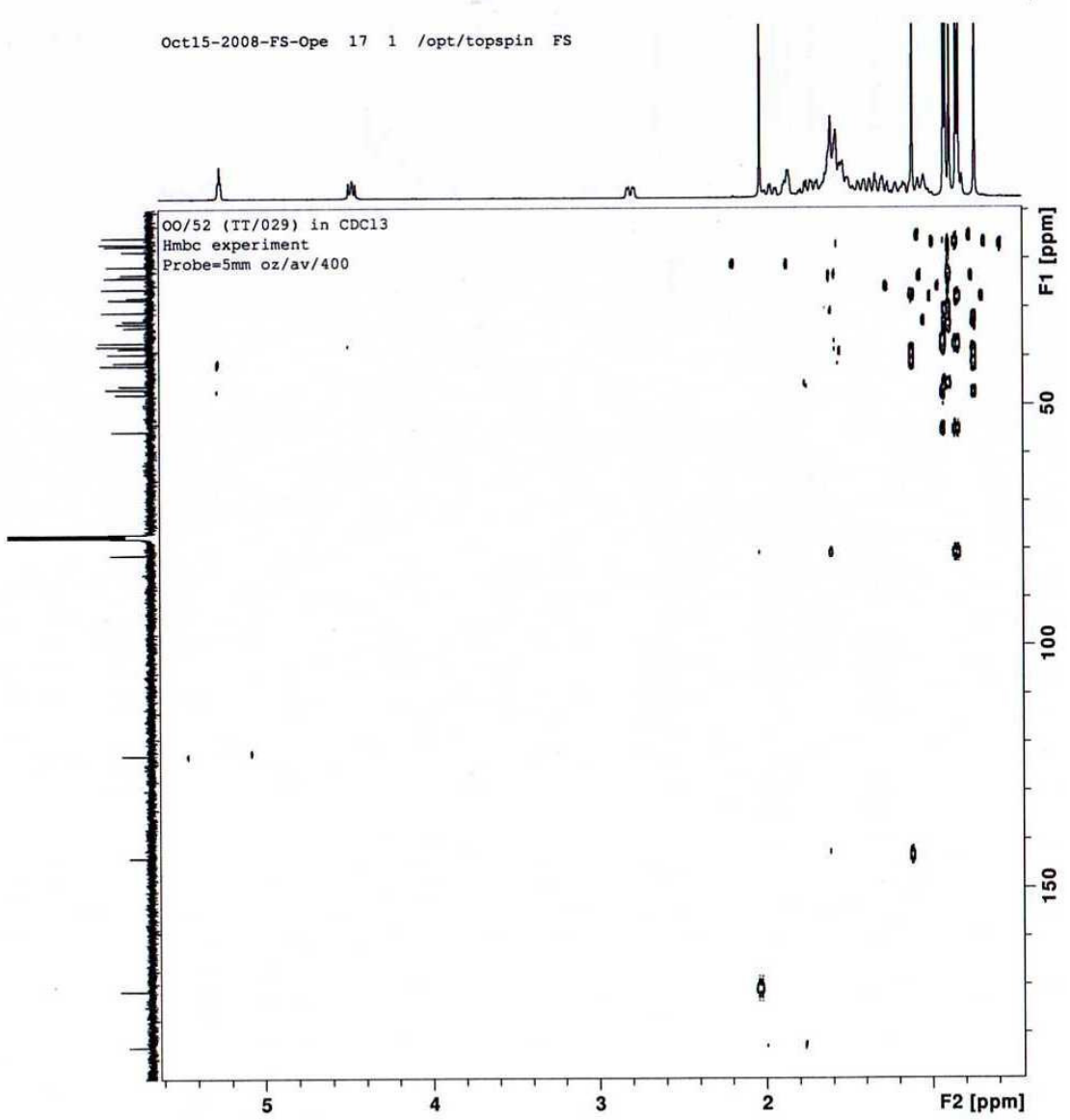


Figure 7.1.8: ^{1}H ^{13}C HMBC NMR Spectrum of **OO/52**

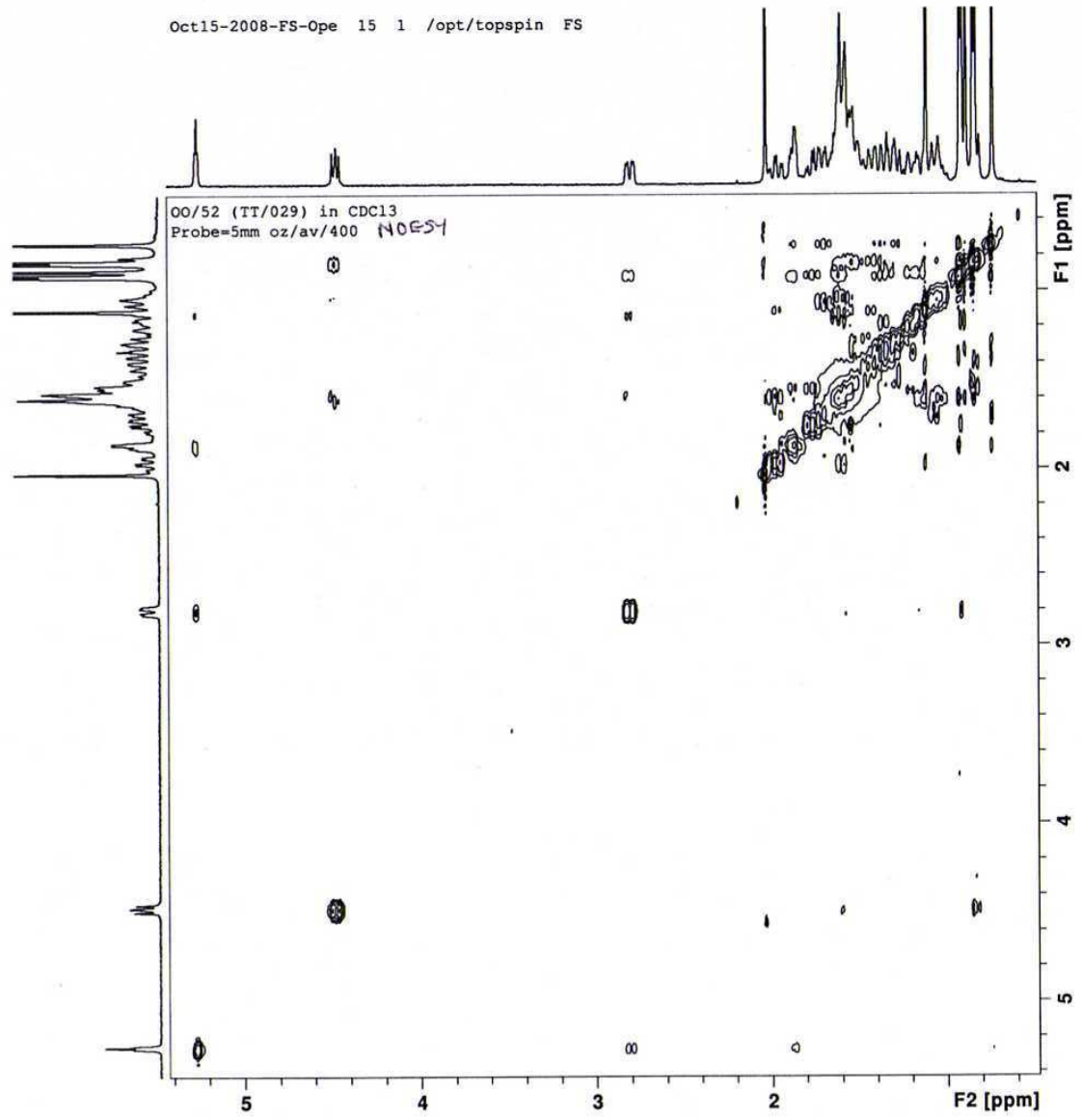


Figure 7.1.9: ^{1}H ^{1}H NOESY NMR Spectrum of **OO/52**

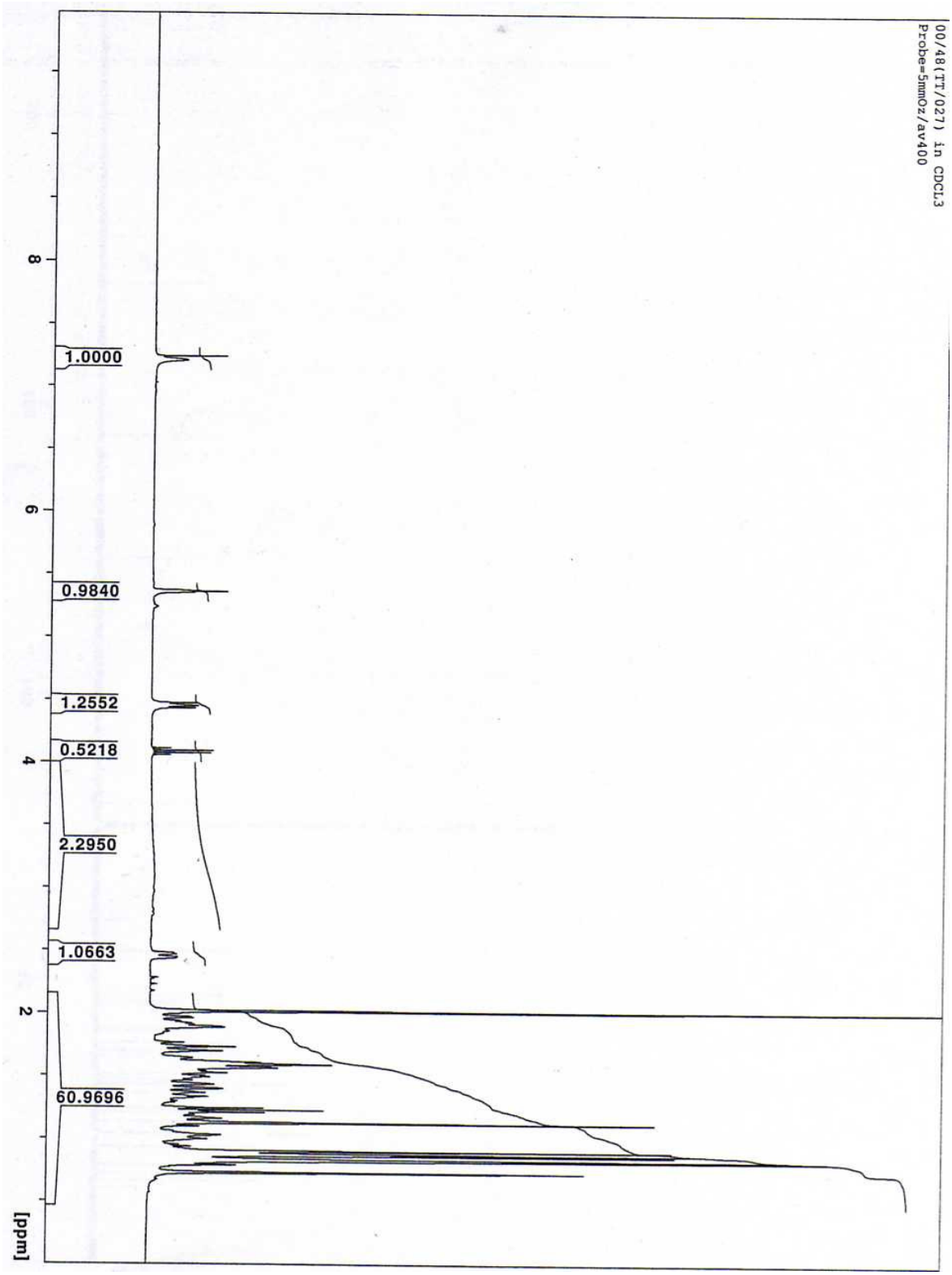


Figure 7.3.1: ^{1}H NMR Spectrum of **OO/48**

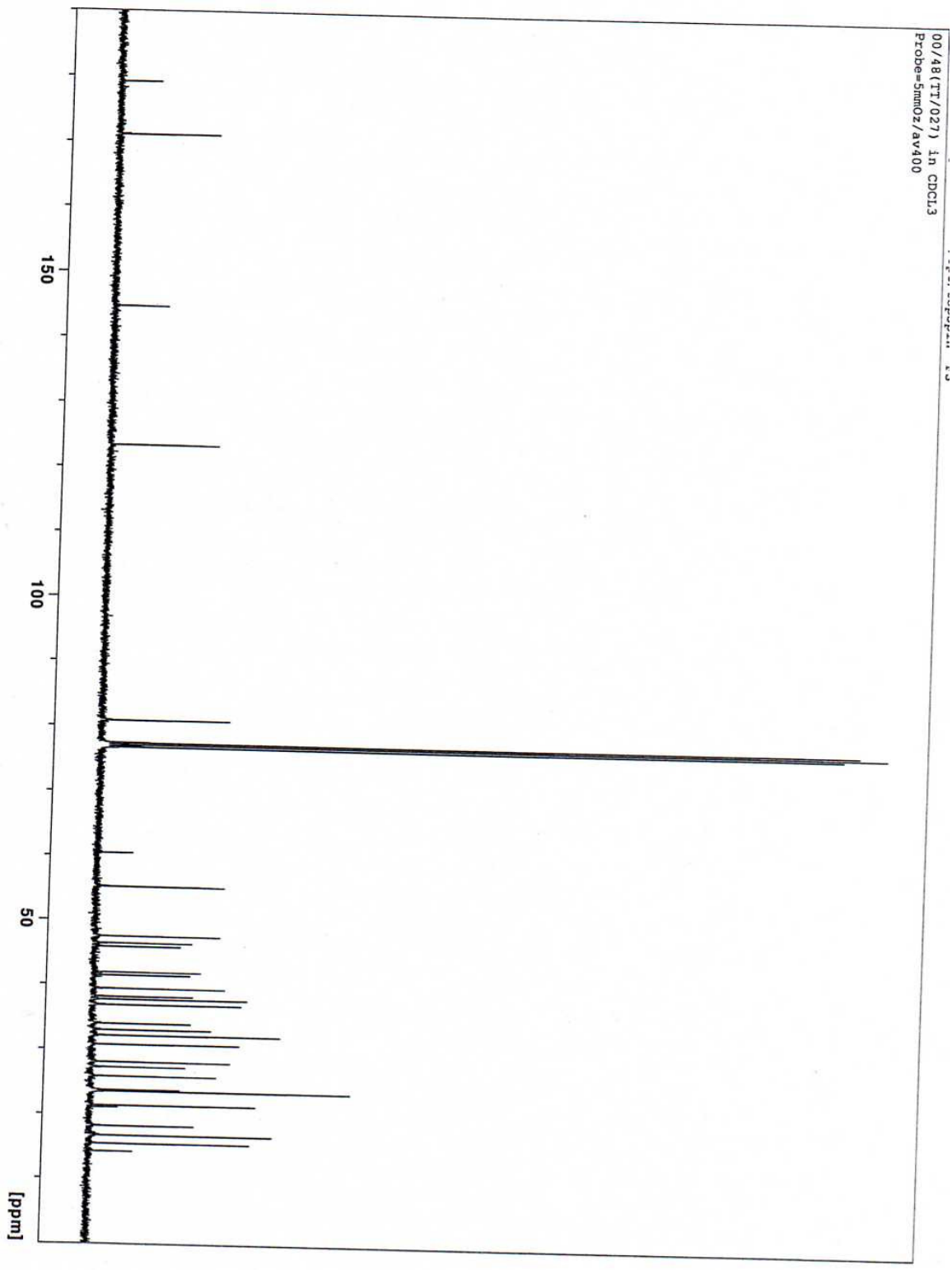


Figure 7.3.2: ^{13}C NMR Spectrum of **OO/48**

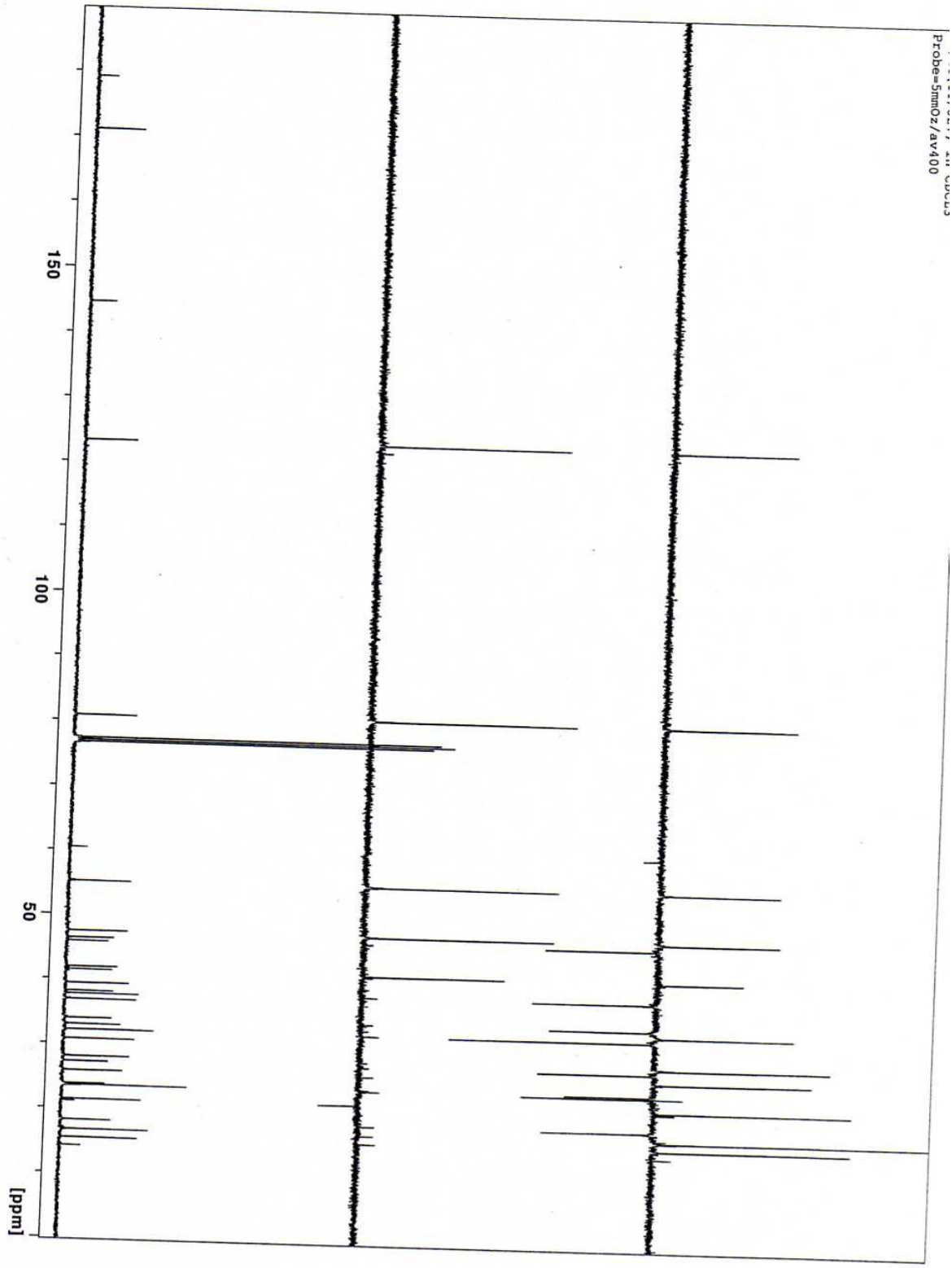


Figure 7.3.3: ^{13}C DEPT NMR Spectrum of **OO/48**

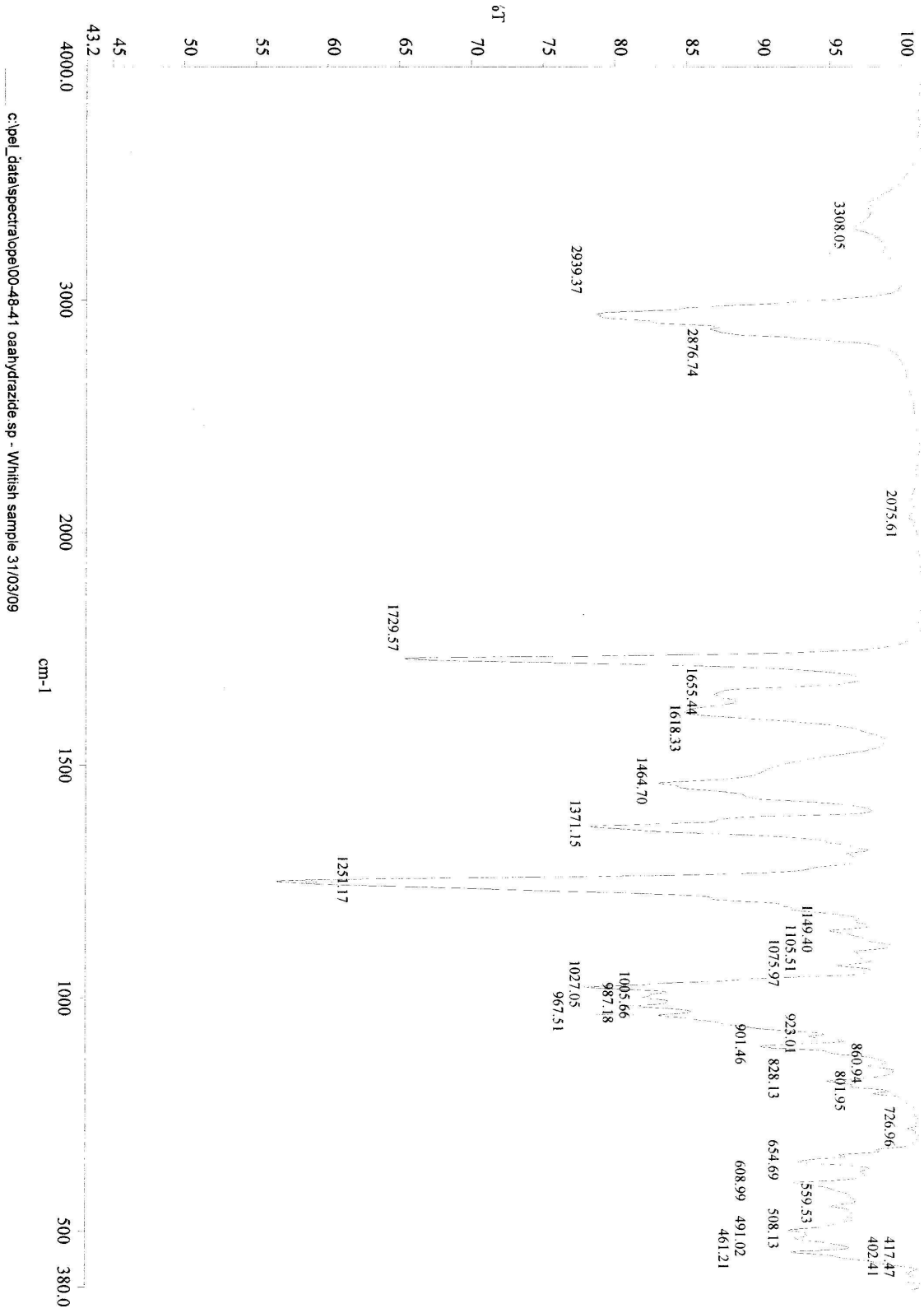


Figure 7.3.4: FTIR Spectrum of **OO/48**

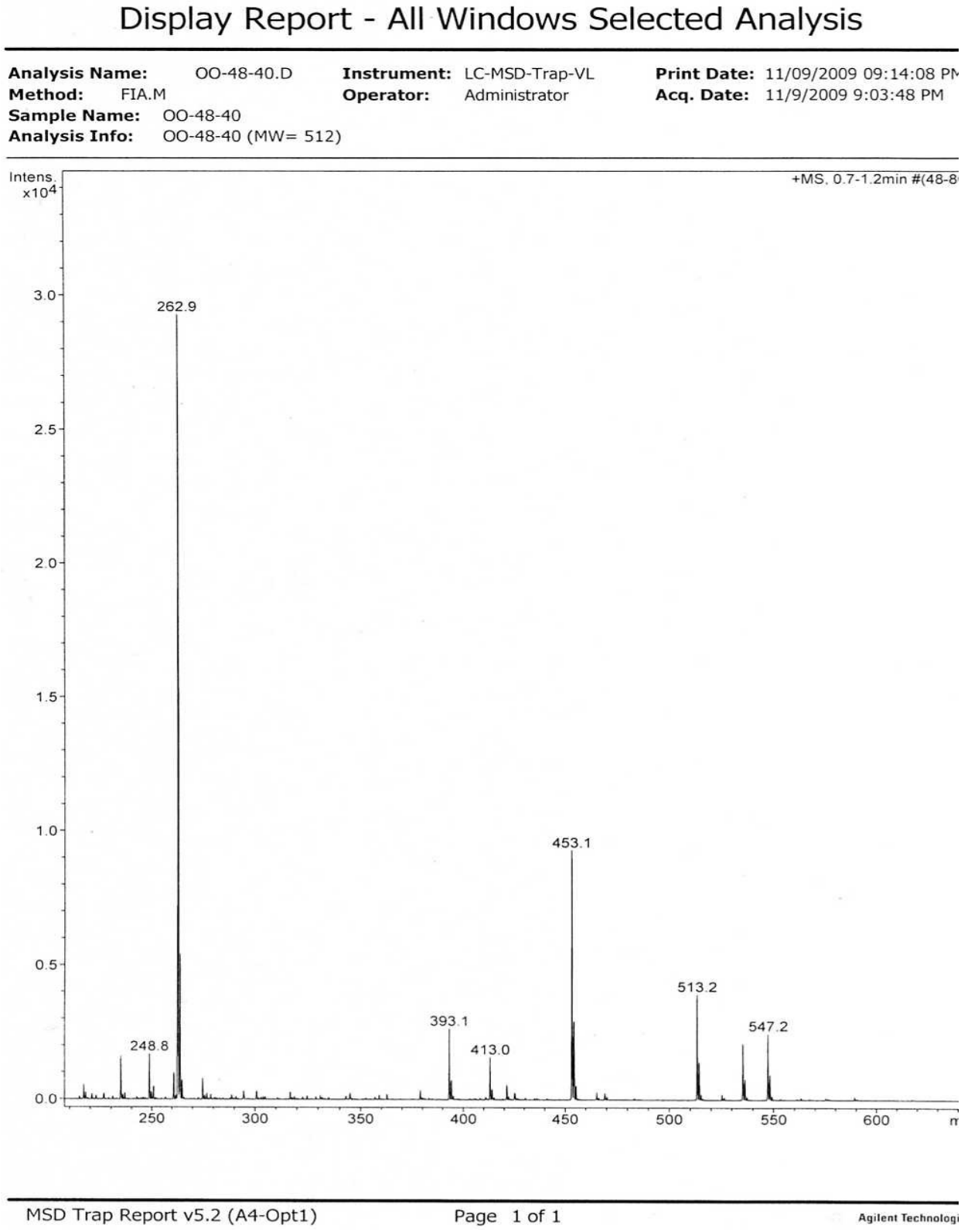


Figure 7.3.5: Mass Spectrum of **OO/48**

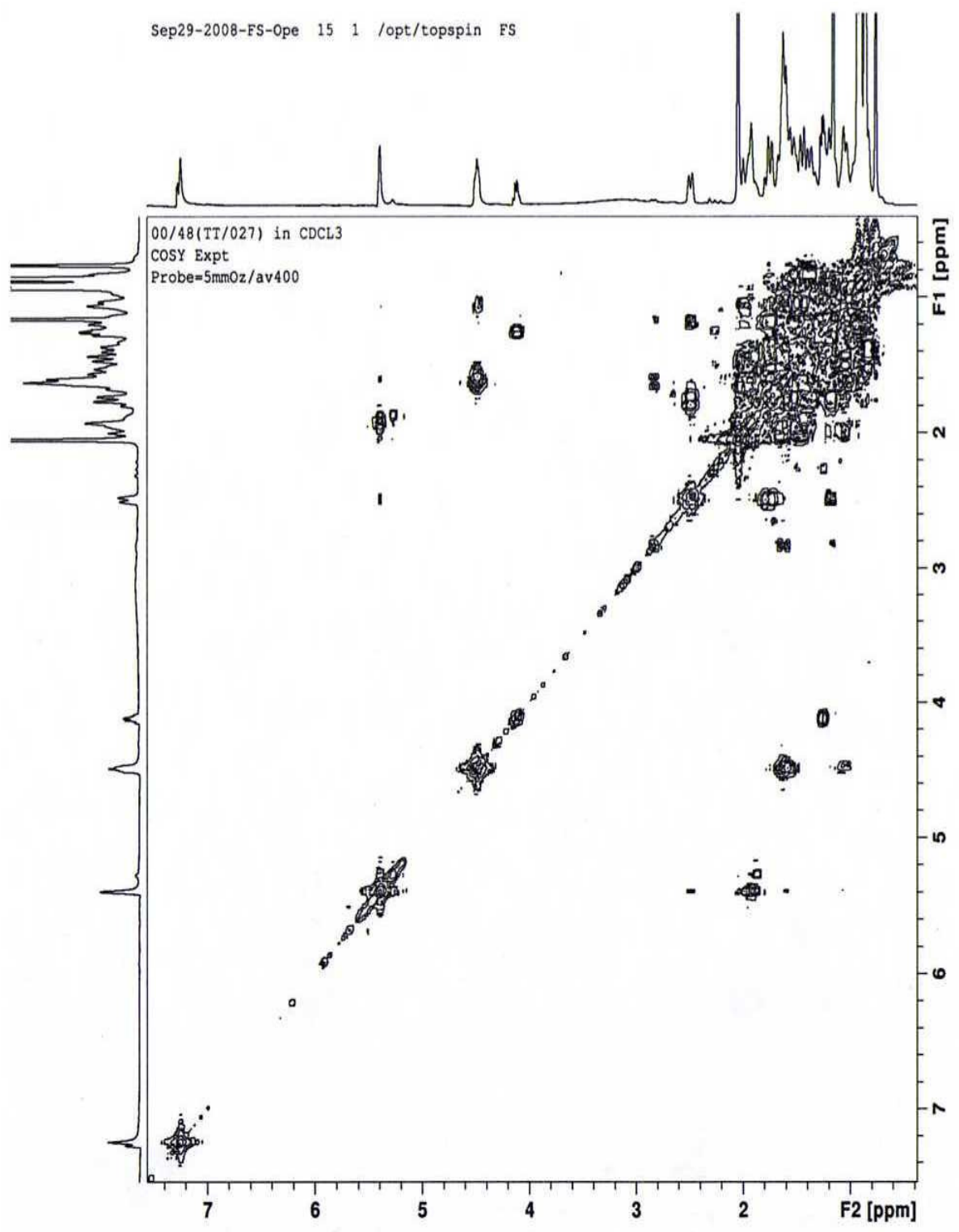


Figure 7.3.6: ^{1}H ^{1}H COSY NMR Spectrum of **OO/48**

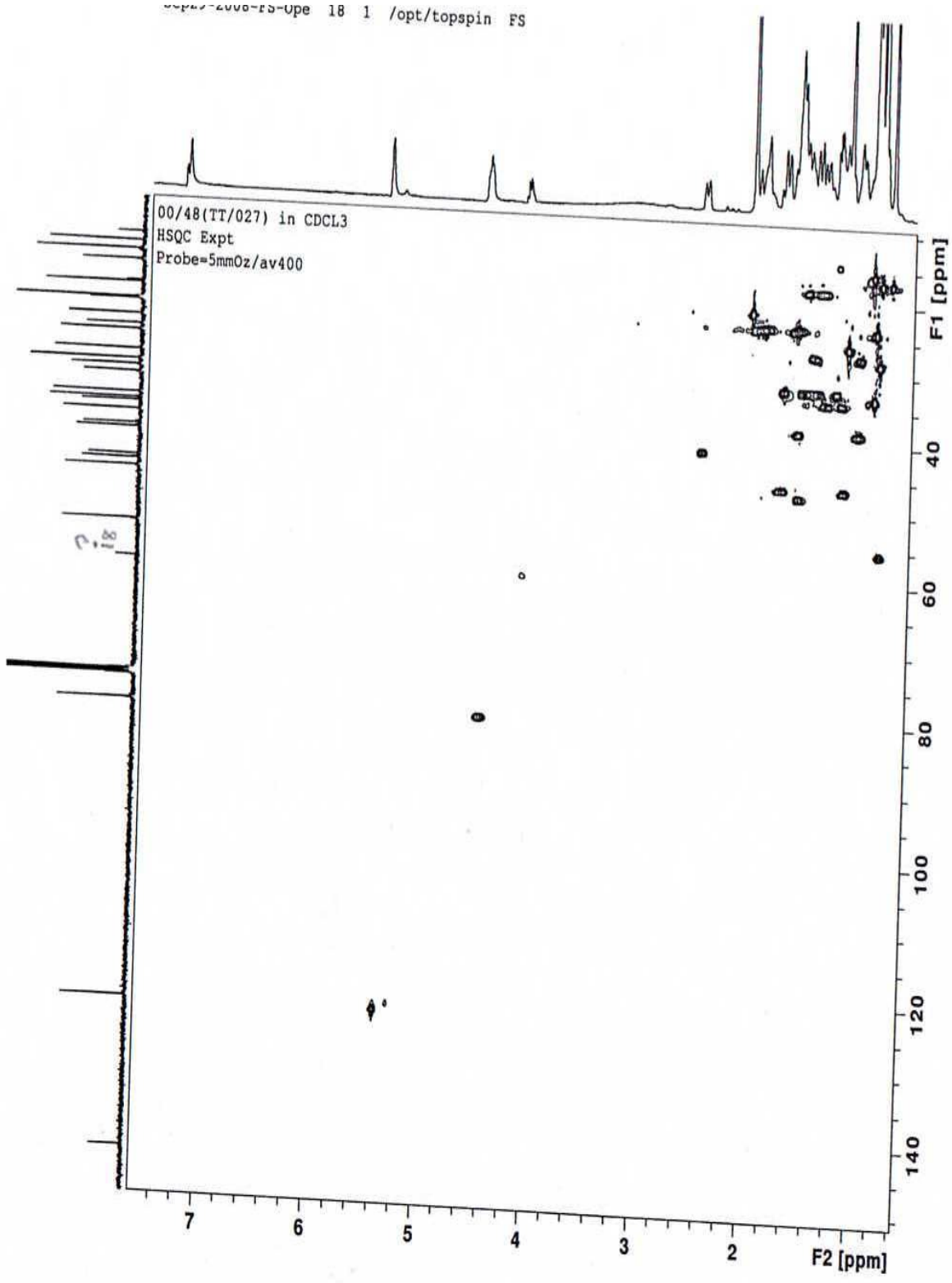


Figure 7.3.7: ^{1}H ^{13}C HSQC NMR Spectrum of **OO/48**

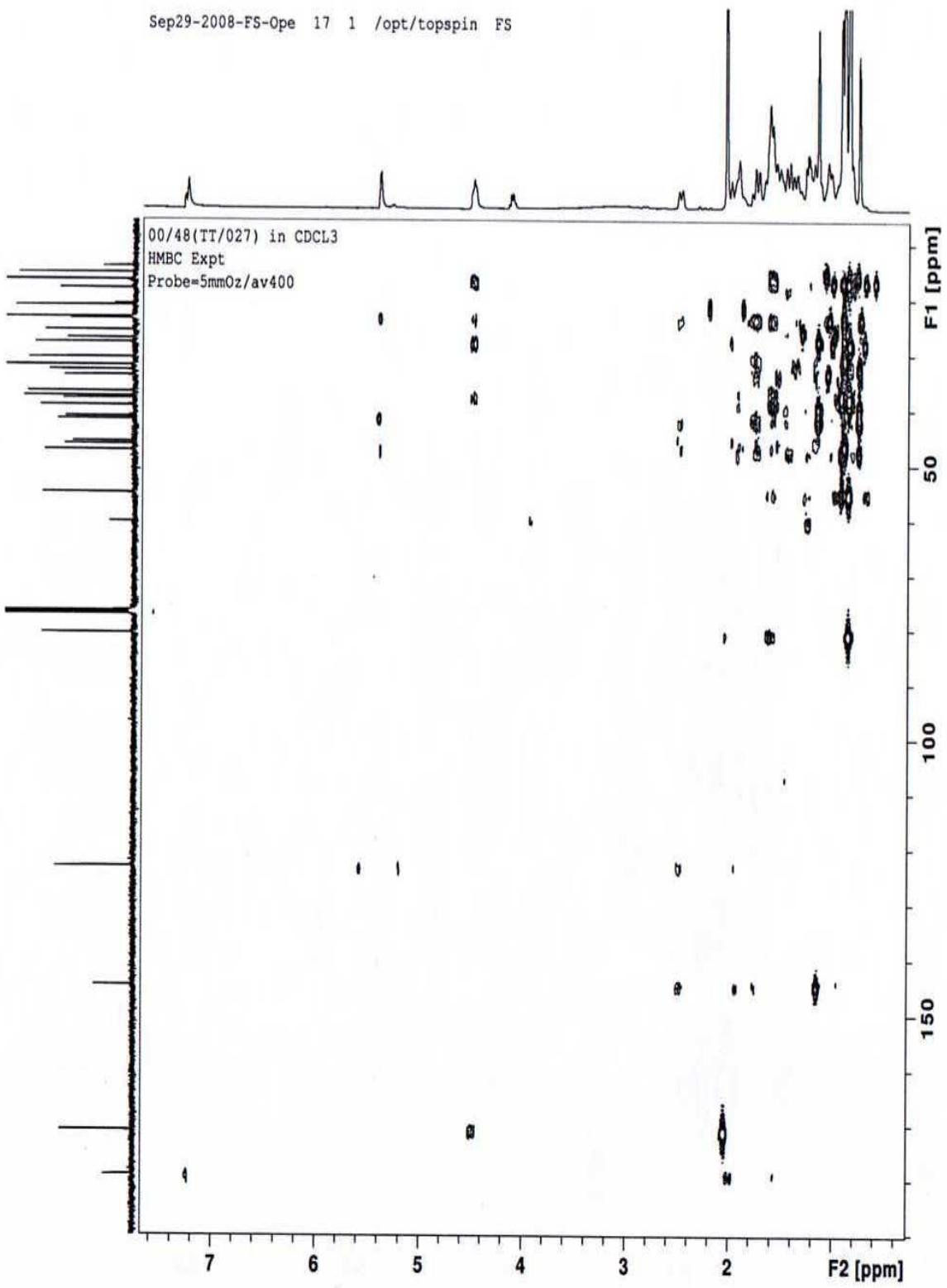


Figure 7.3.8: ^{1}H ^{13}C HMBC NMR Spectrum of **OO/48**

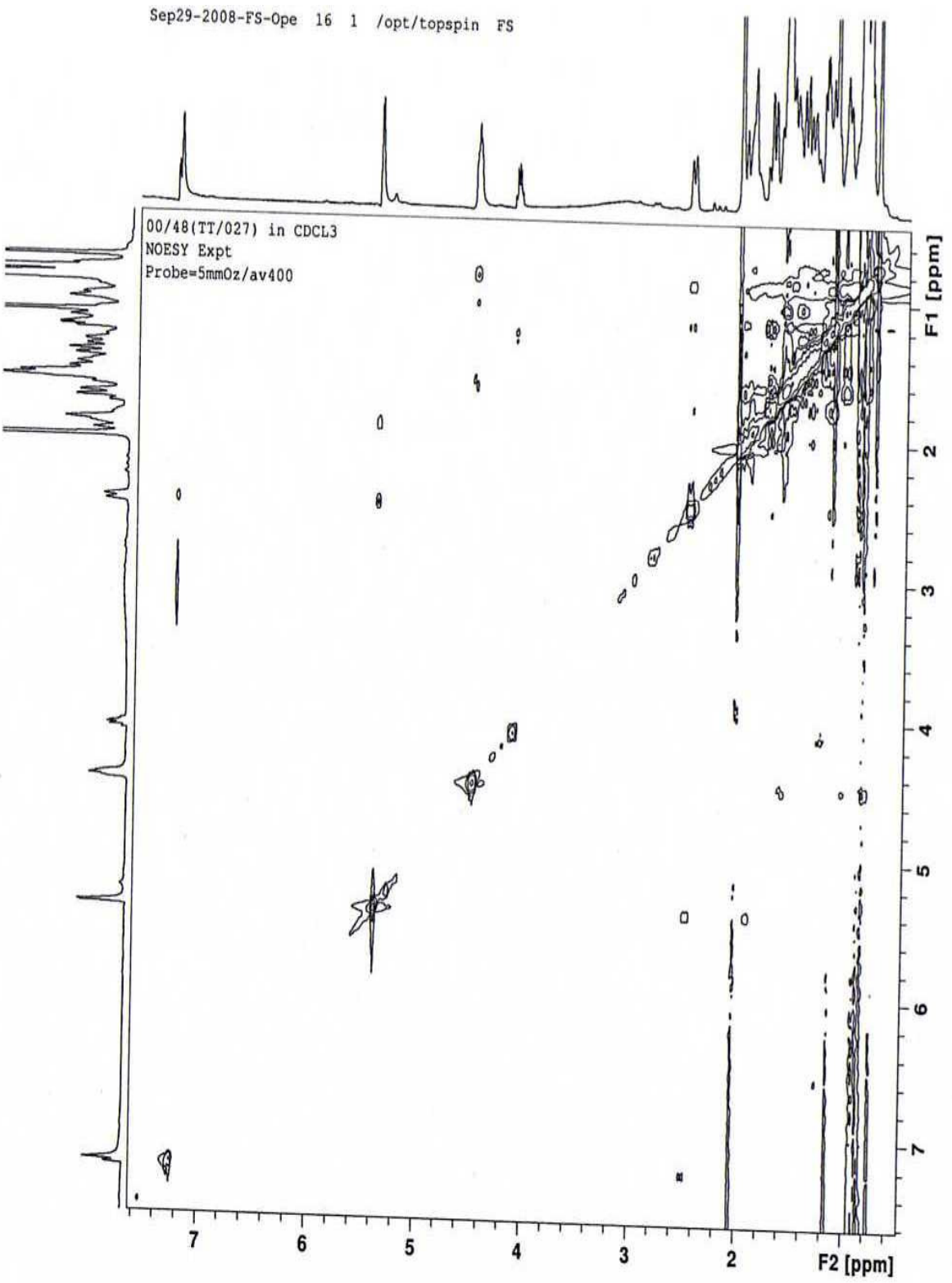


Figure 7.3.9: ^{1}H ^{1}H NOSEY NMR Spectrum of **OO/48**

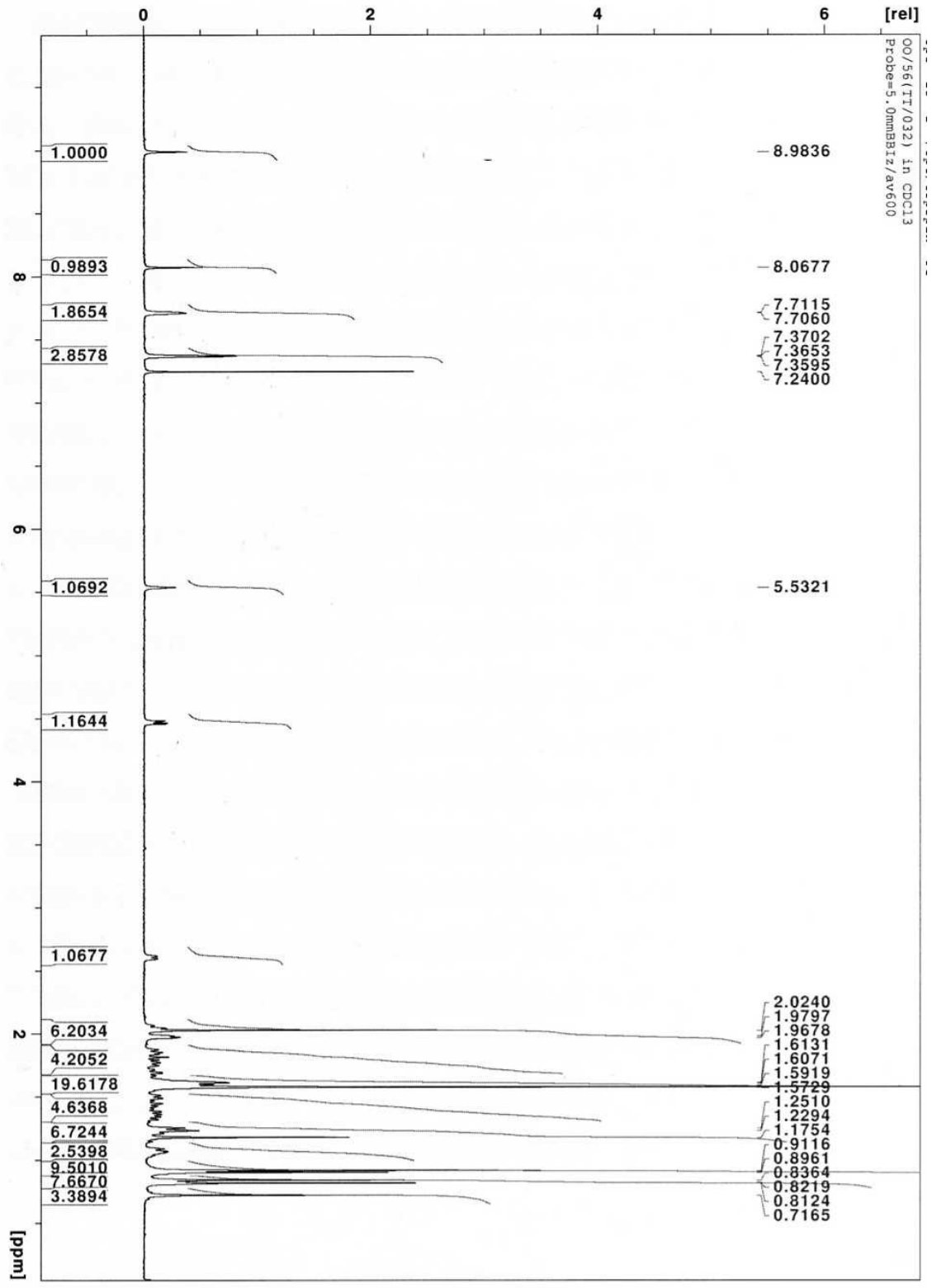


Figure 7.4.1: ^{1}H NMR Spectrum of **OO/56**

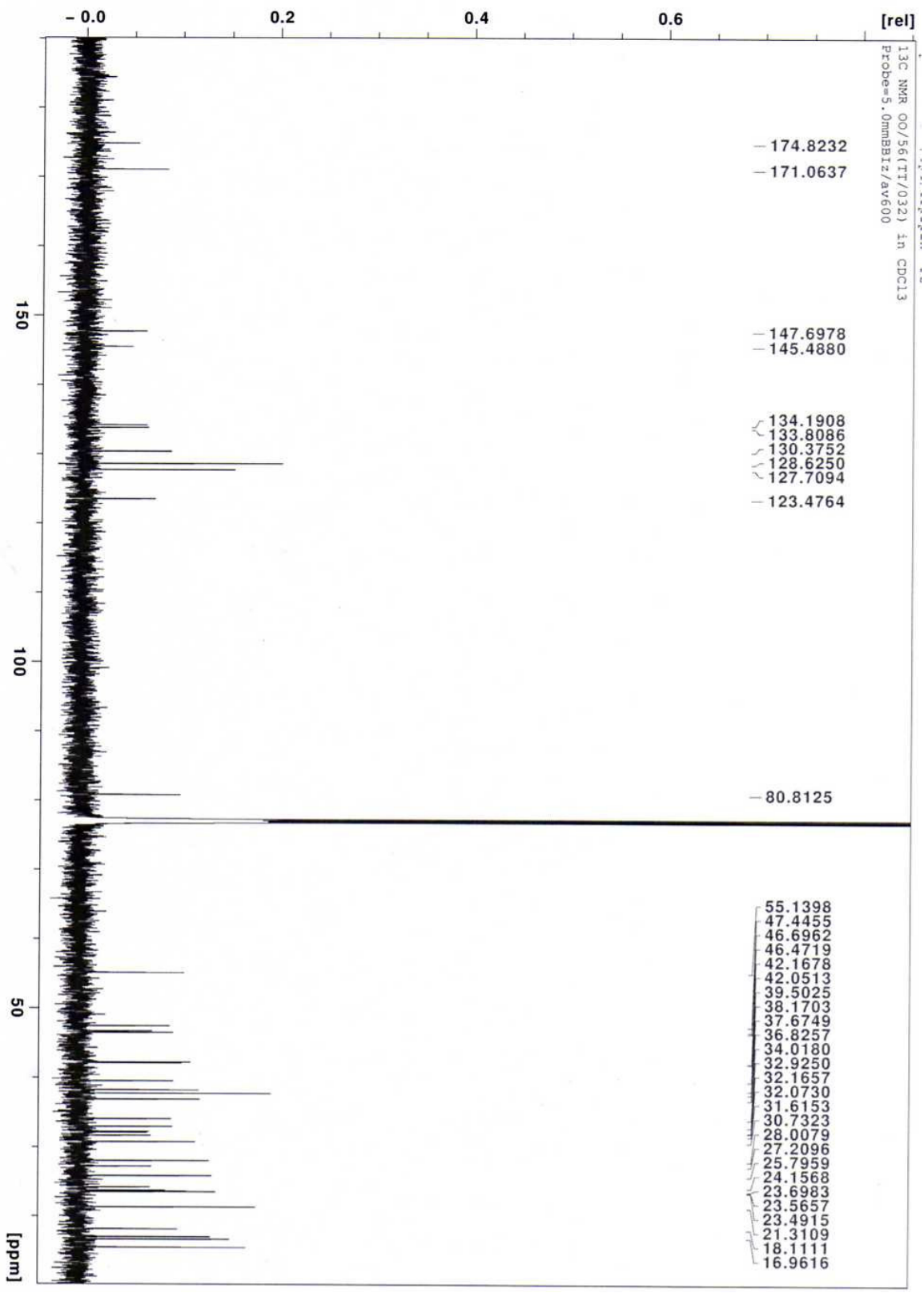


Figure 7.4.2: ^{13}C - NMR of OO/56

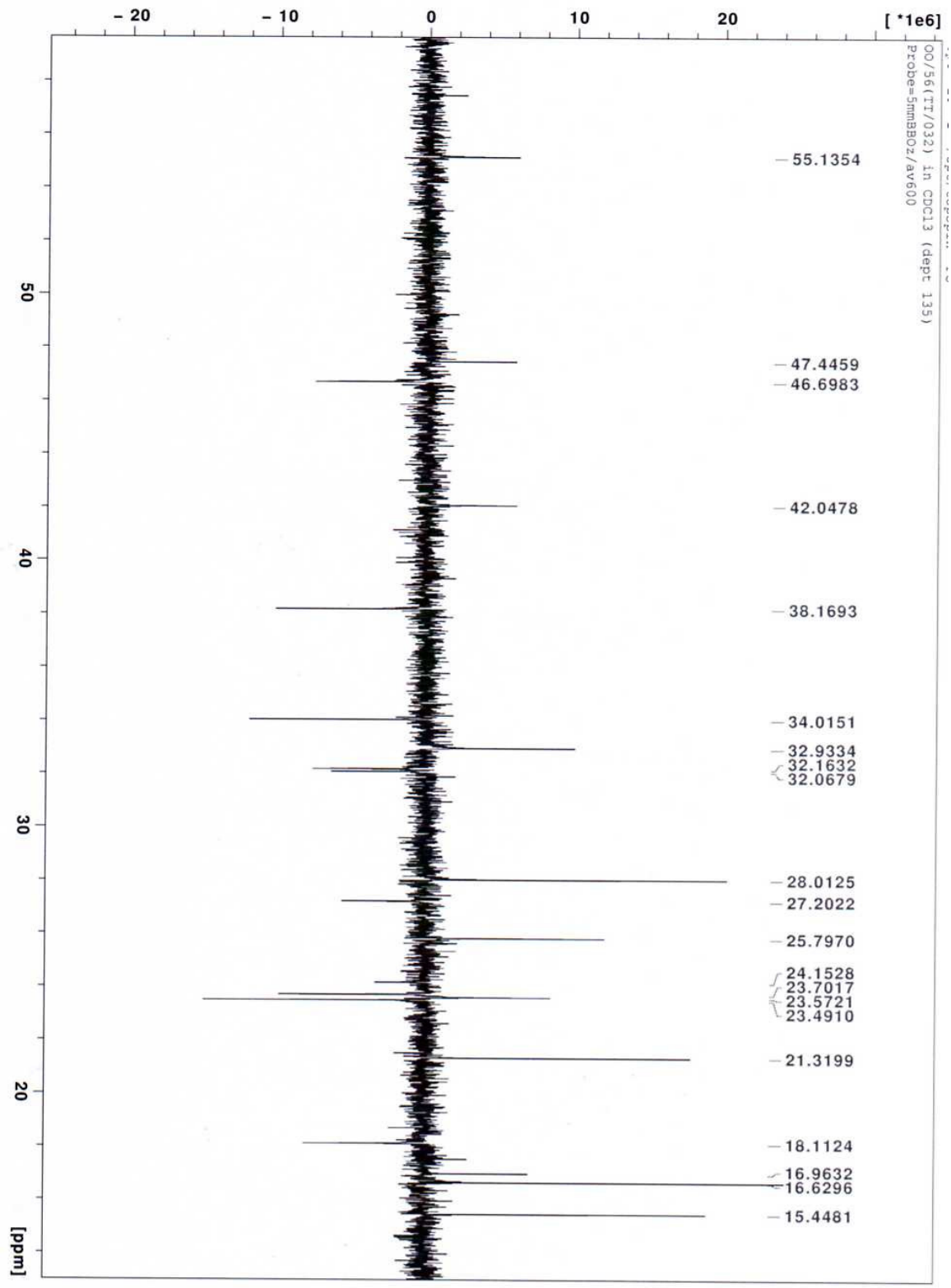


Figure 7.4.3: ^{13}C DEPT NMR Spectrum of **OO/56**

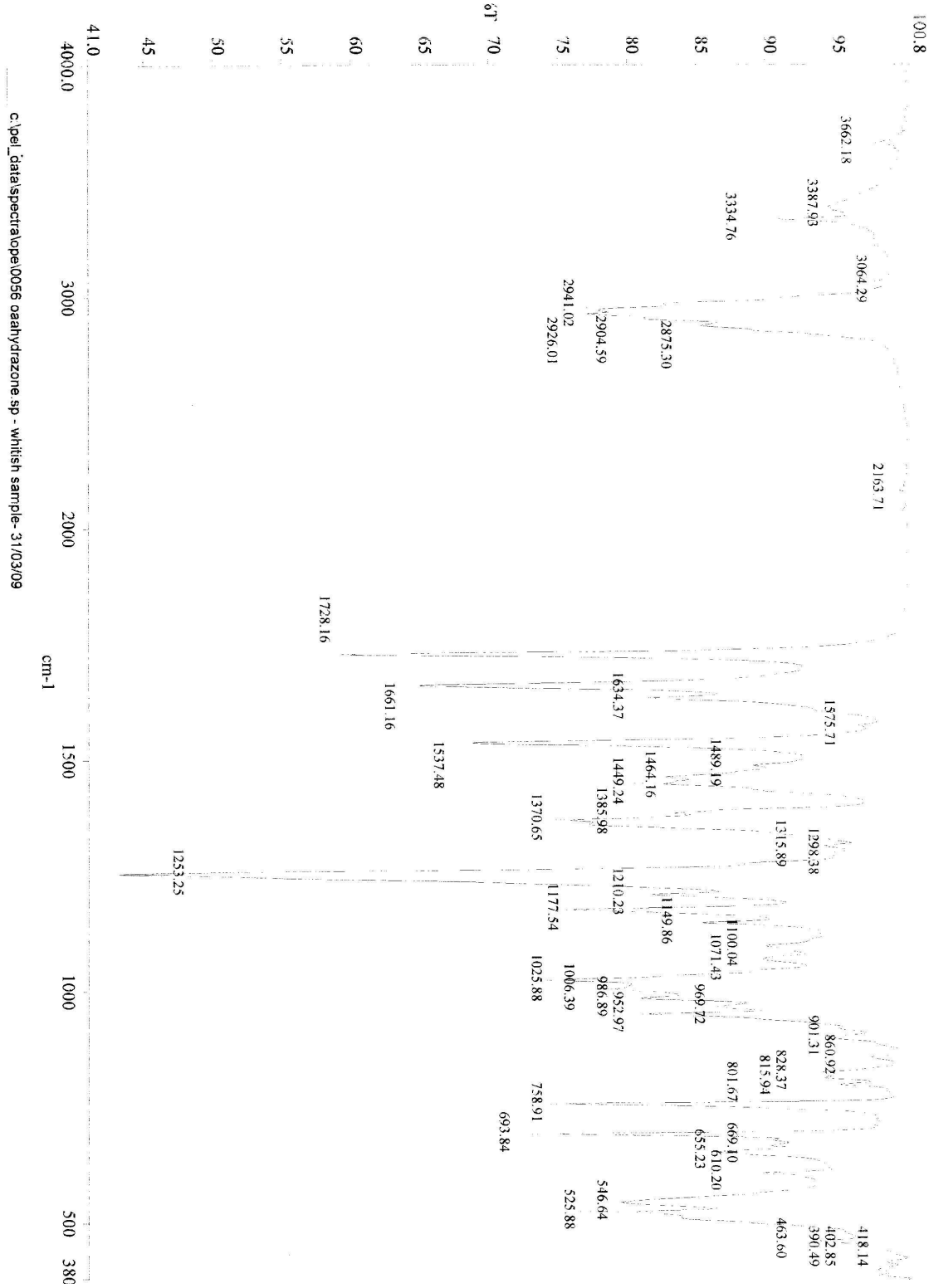


Figure 7.4.4: FTIR Spectrum of **OO/56**

Display Report - All Windows Selected Analysis

Analysis Name: 00-56-.D
Method: FIA.M
Sample Name: OO-56-
Analysis Info: OO-56 (MW = 587)

Instrument: LC-MSD-Trap-VL
Operator: Administrator

Print Date: 11/14/2009 07:09:33 PM
Acq. Date: 11/14/2009 6:58:40 PM

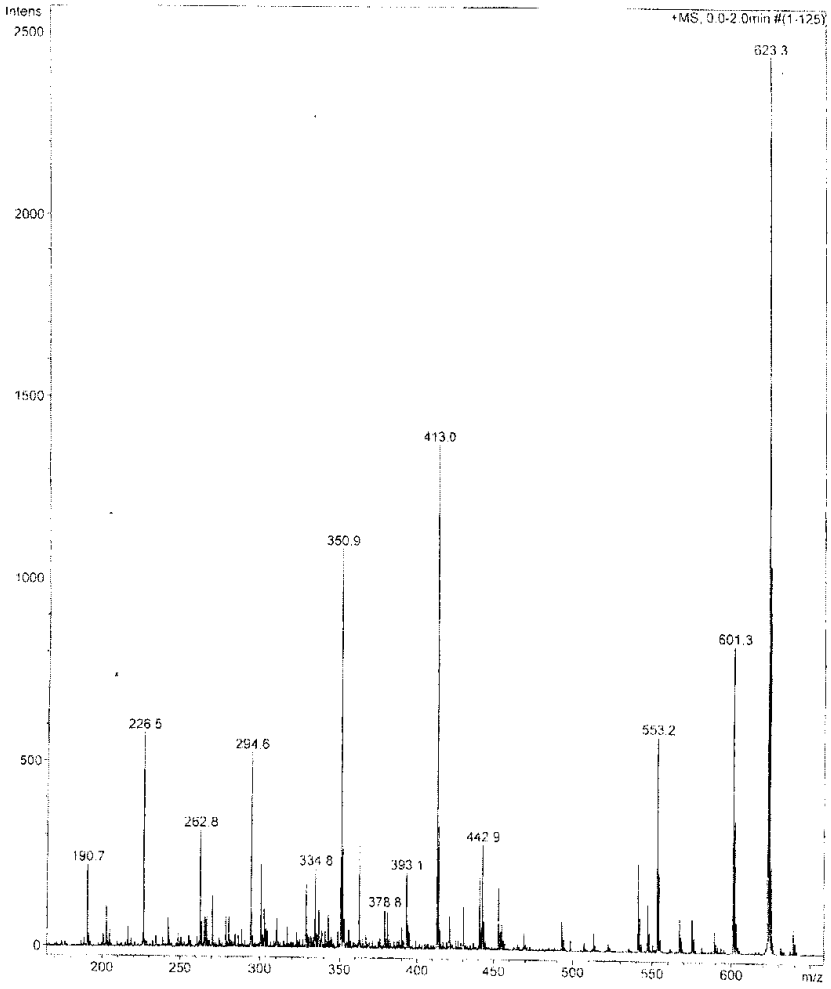


Figure 7.4.5: Mass Spectrum of **OO/56**

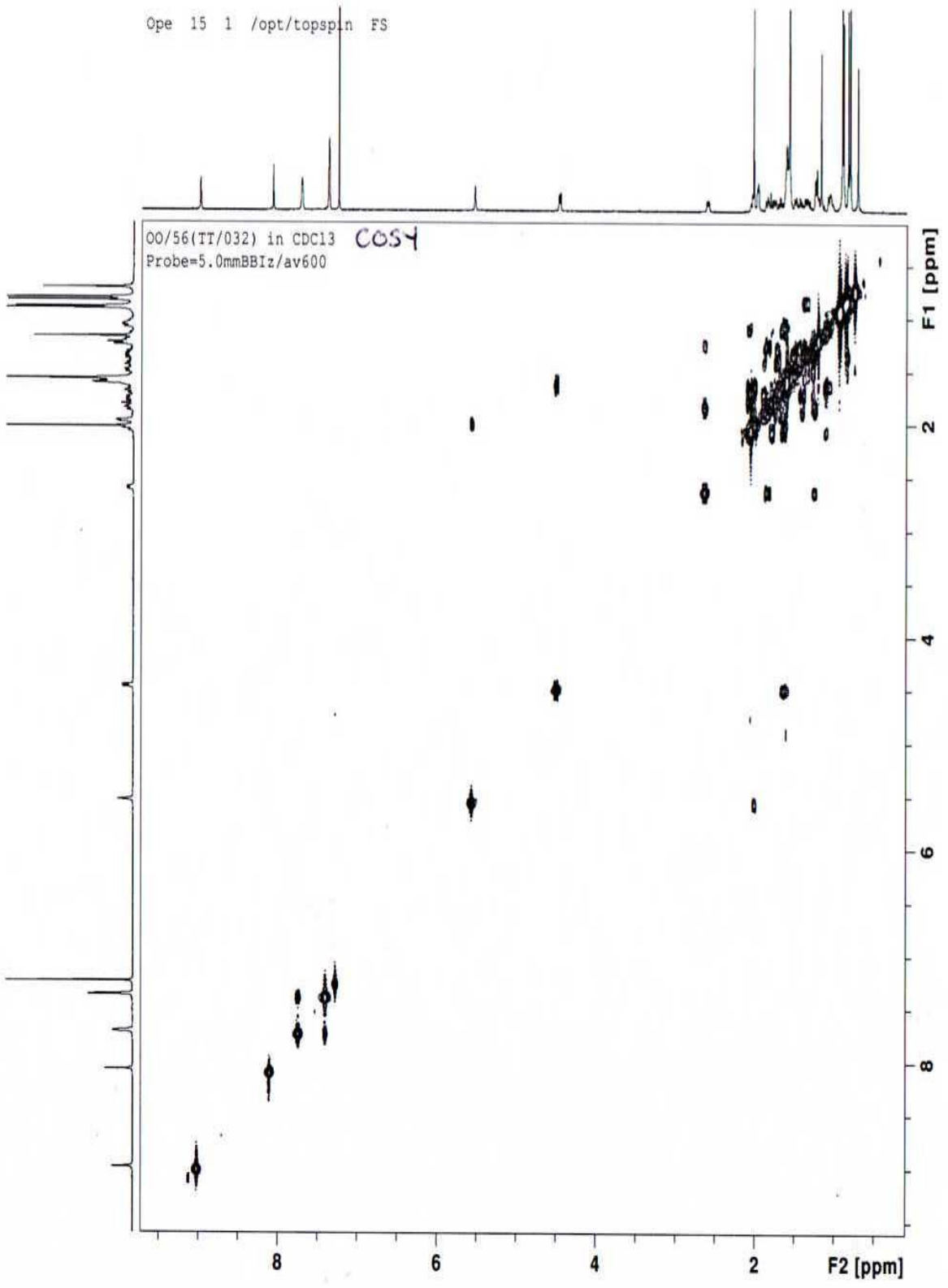


Figure 7.4.6: ^{1}H ^{1}H COSY NMR Spectrum of **OO/56**

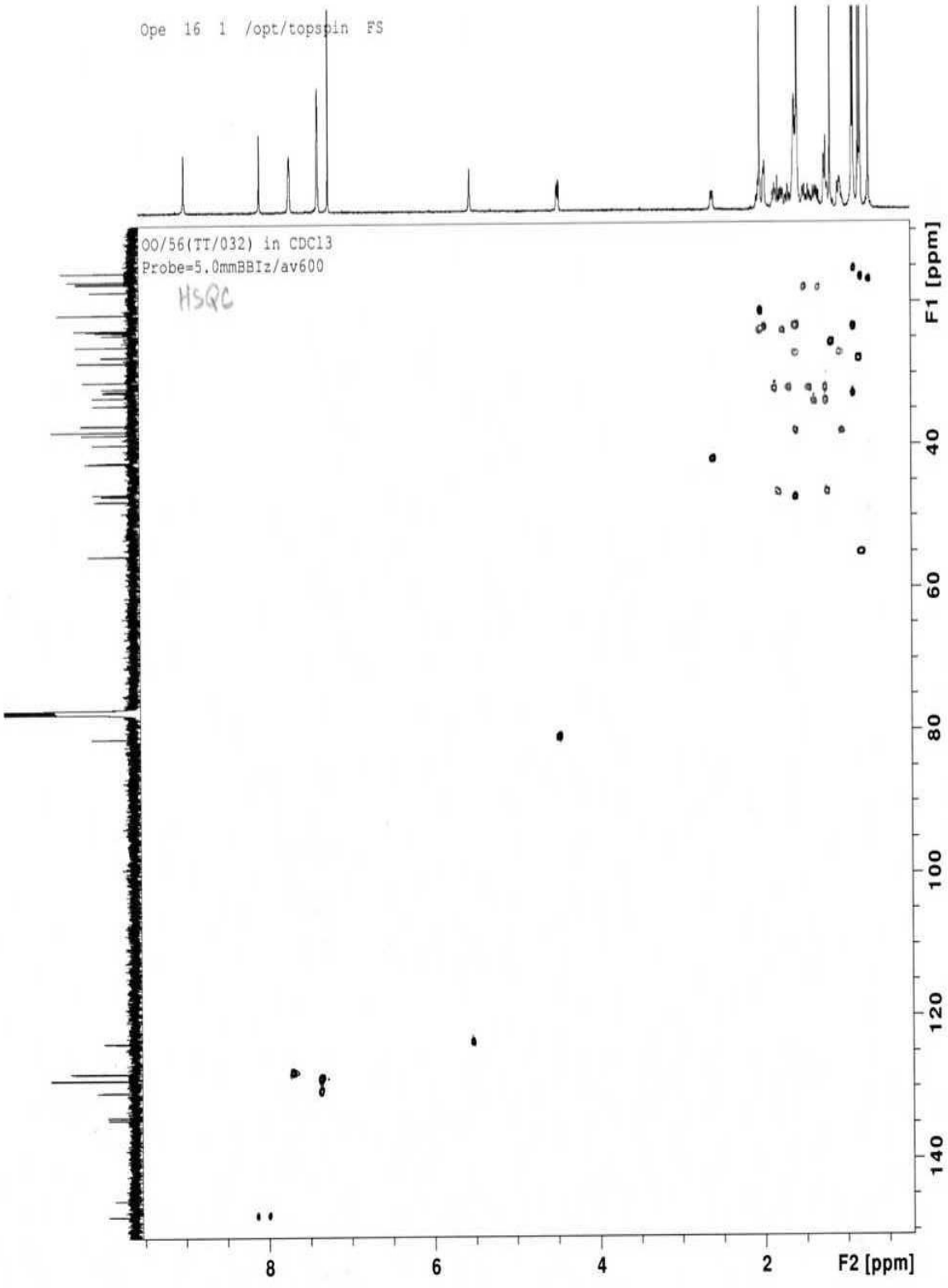


Figure 7.4.7: ^{1}H ^{13}C HSQC NMR Spectrum of **OO/56**

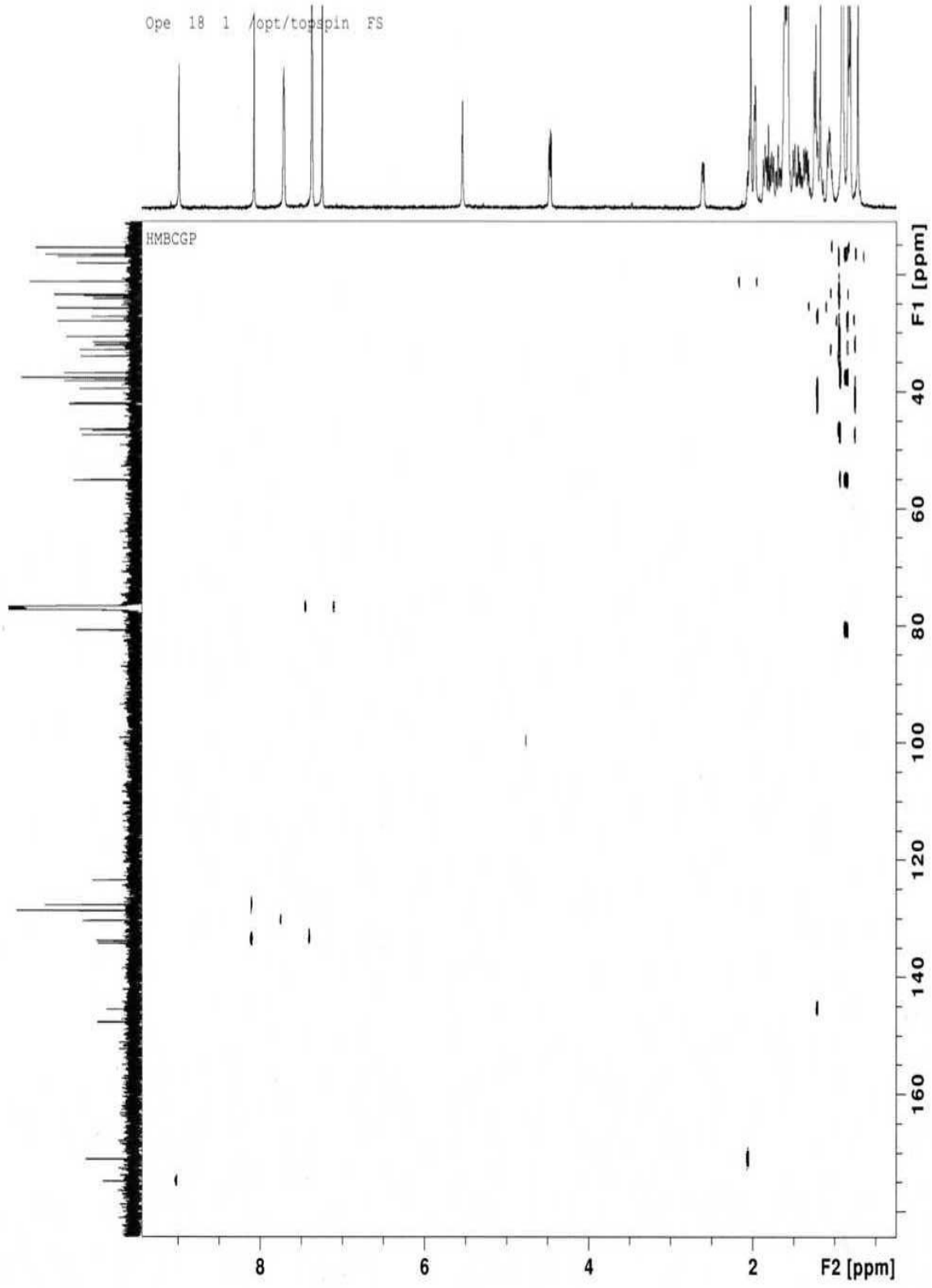


Figure 7.4.8: ^{1}H ^{13}C HMBC NMR Spectrum of **OO/56**

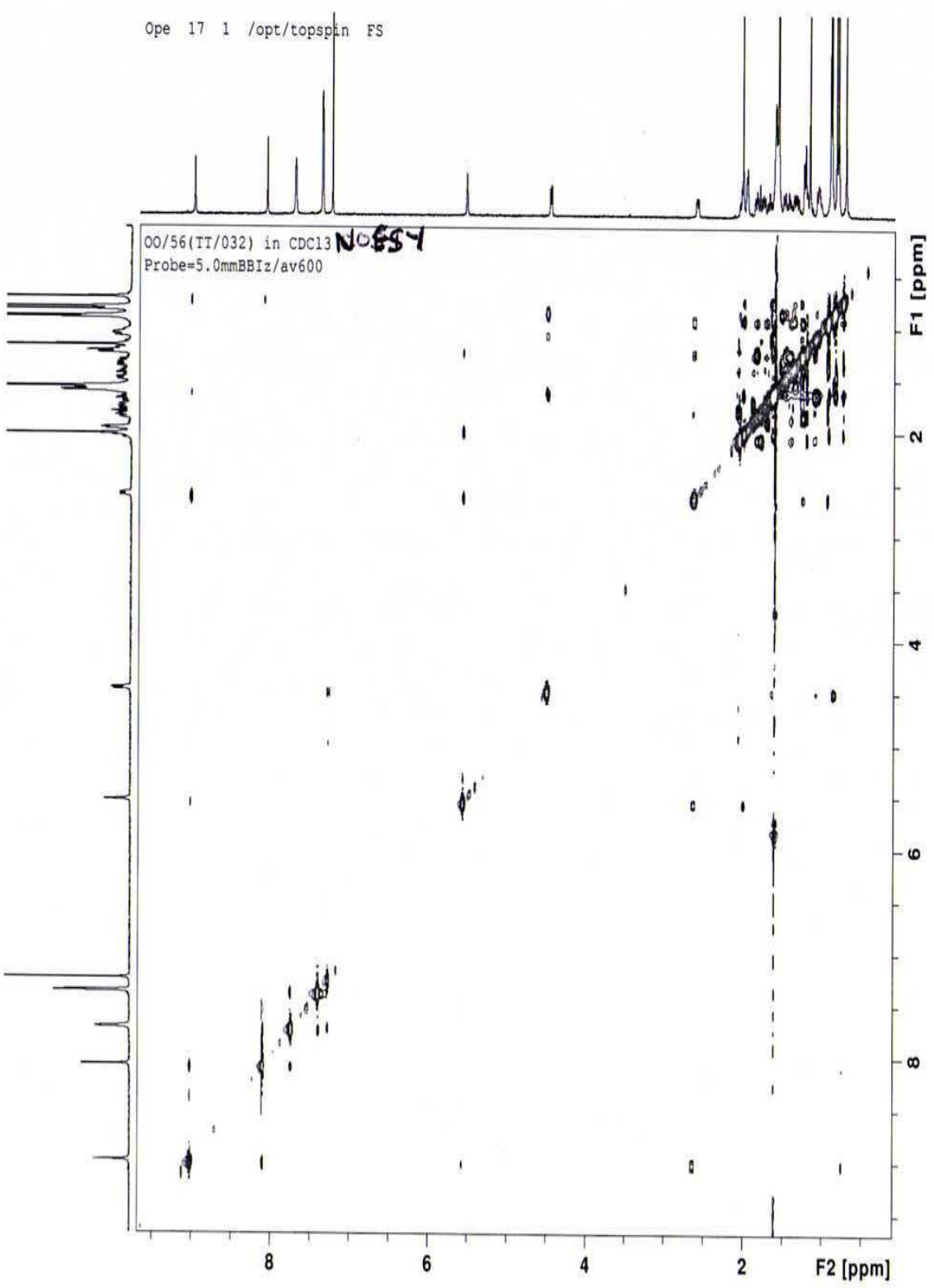


Figure 7.4.9: ^{1}H ^{1}H NOSEY NMR Spectrum of **OO/56**

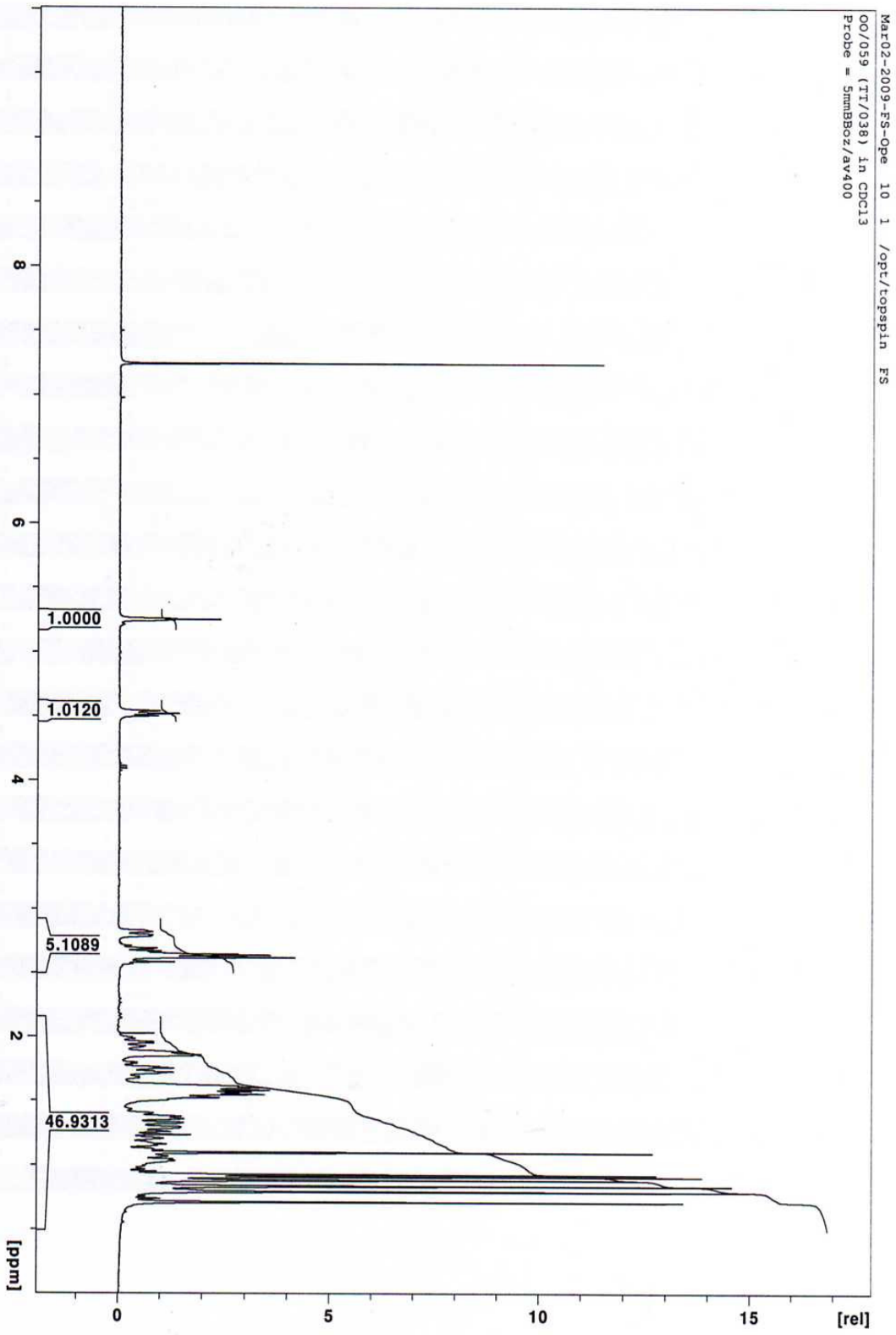


Figure 7.5.1: ^{1}H NMR Spectrum of **OO/59**

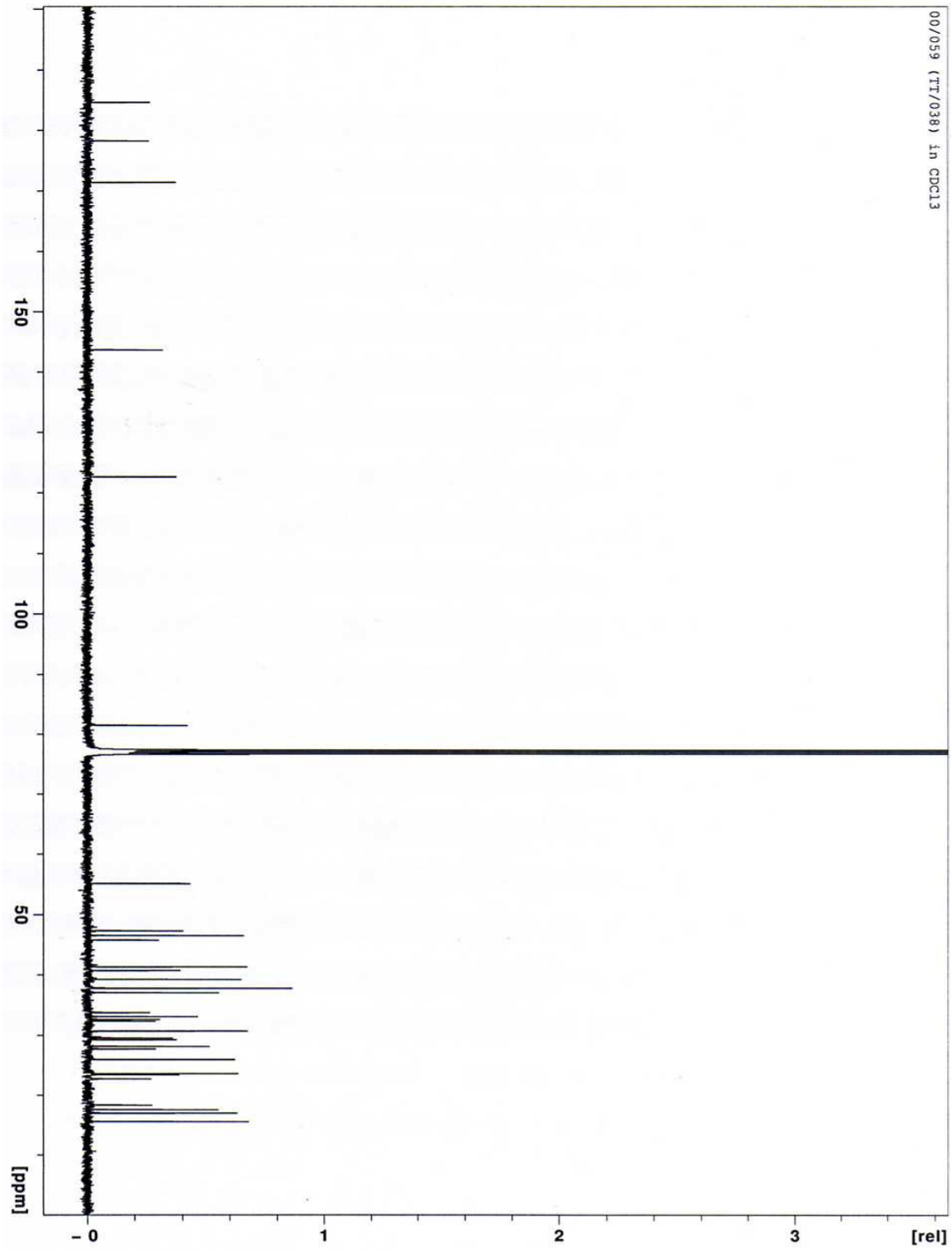


Figure 7.5.2: ^{13}C NMR Spectrum of **OO/59**

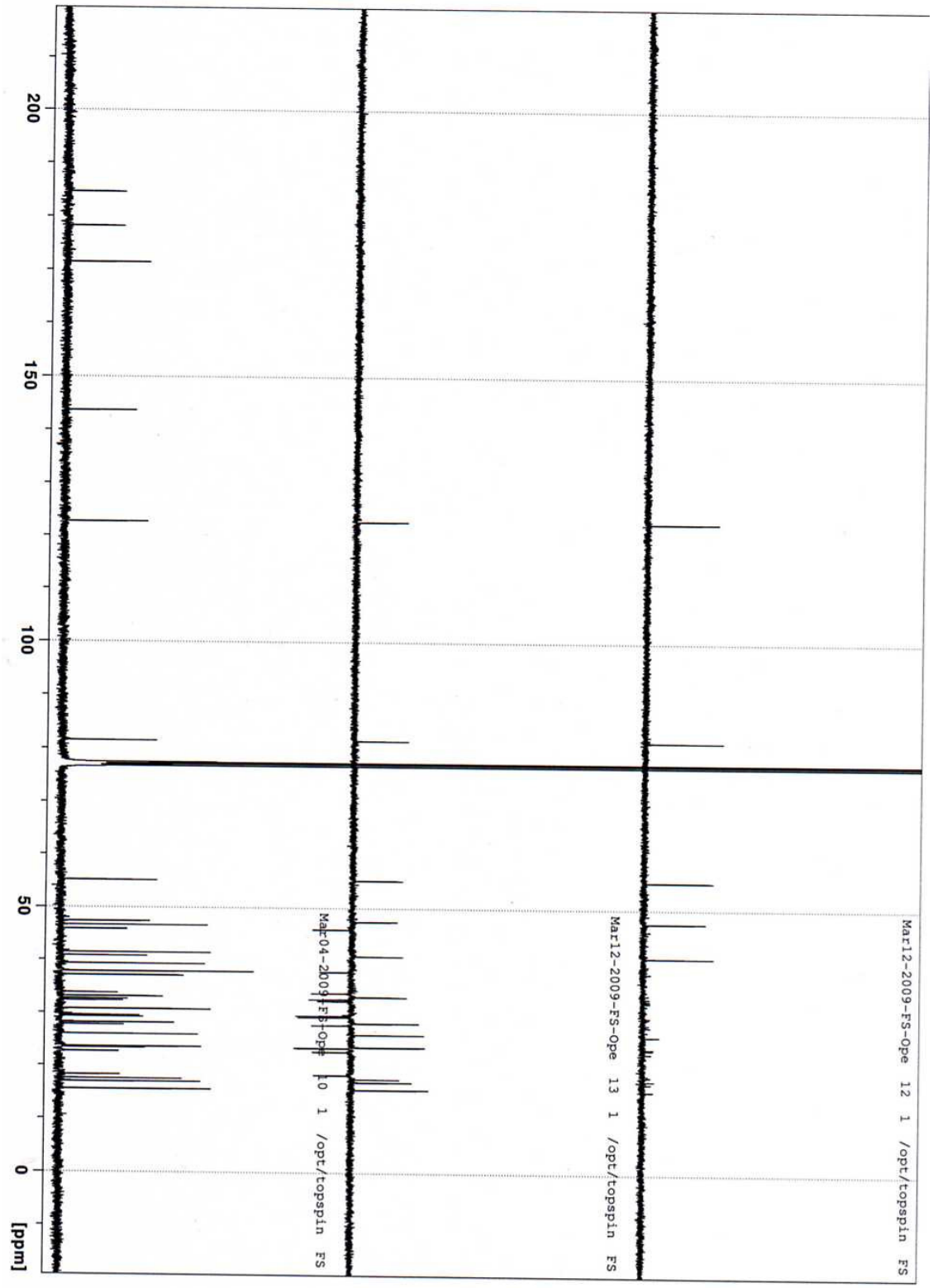


Figure 7.5.3: ^{13}C DEPT NMR Spectrum of **OO/59**

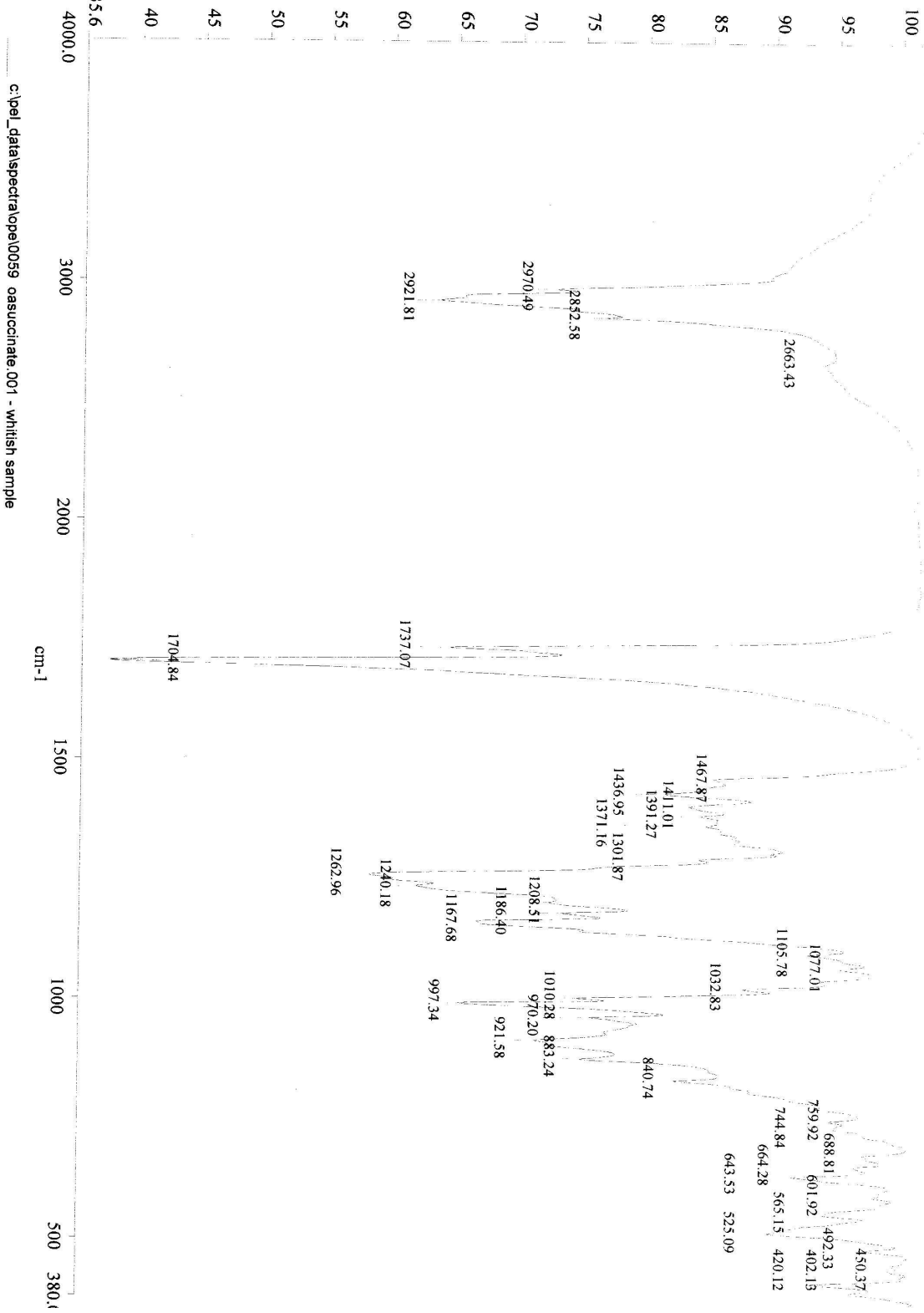


Figure 7.5.4: FTIR Spectrum of **OO/59**

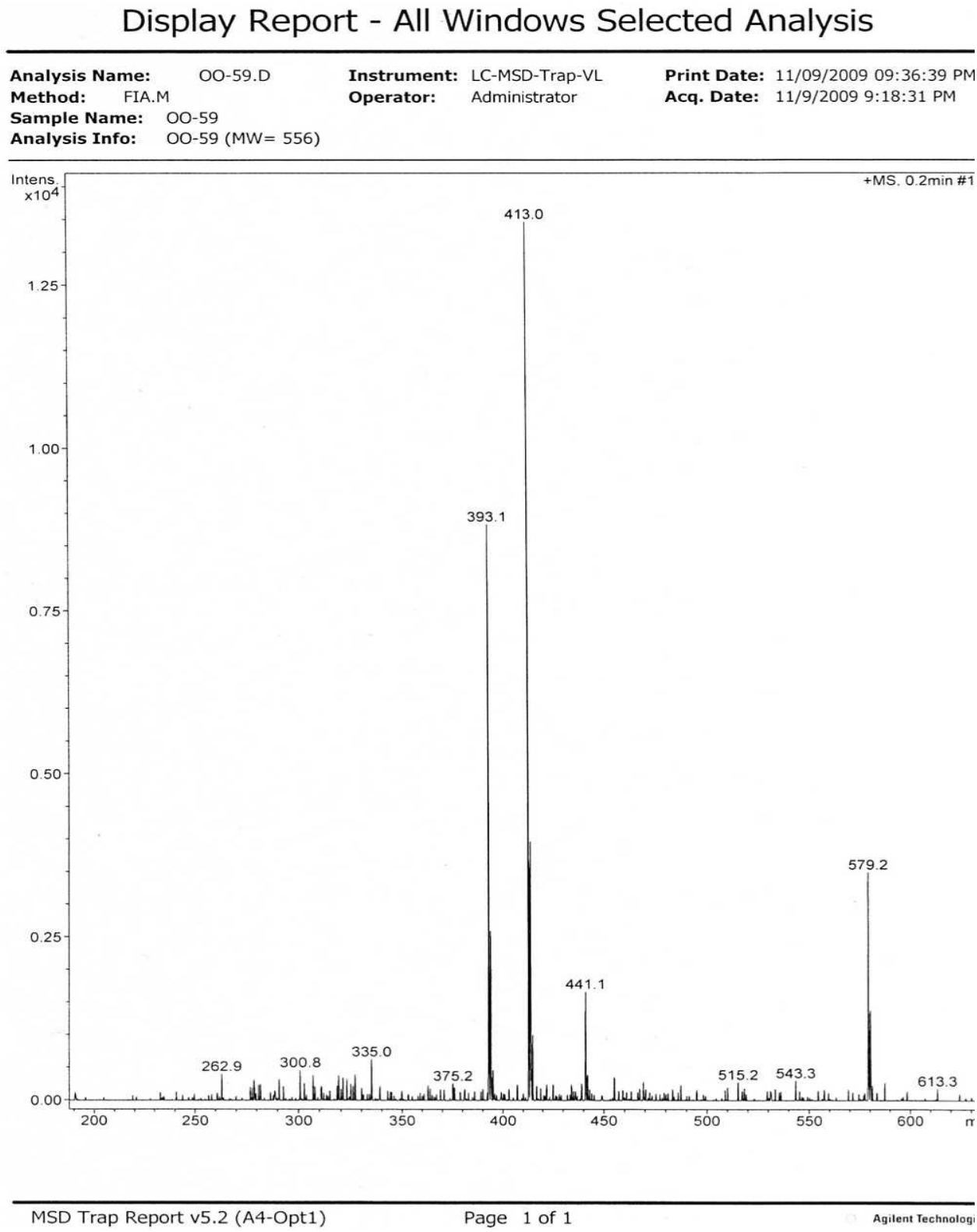


Figure 7.5.5: Mass Spectrum of **OO/59**

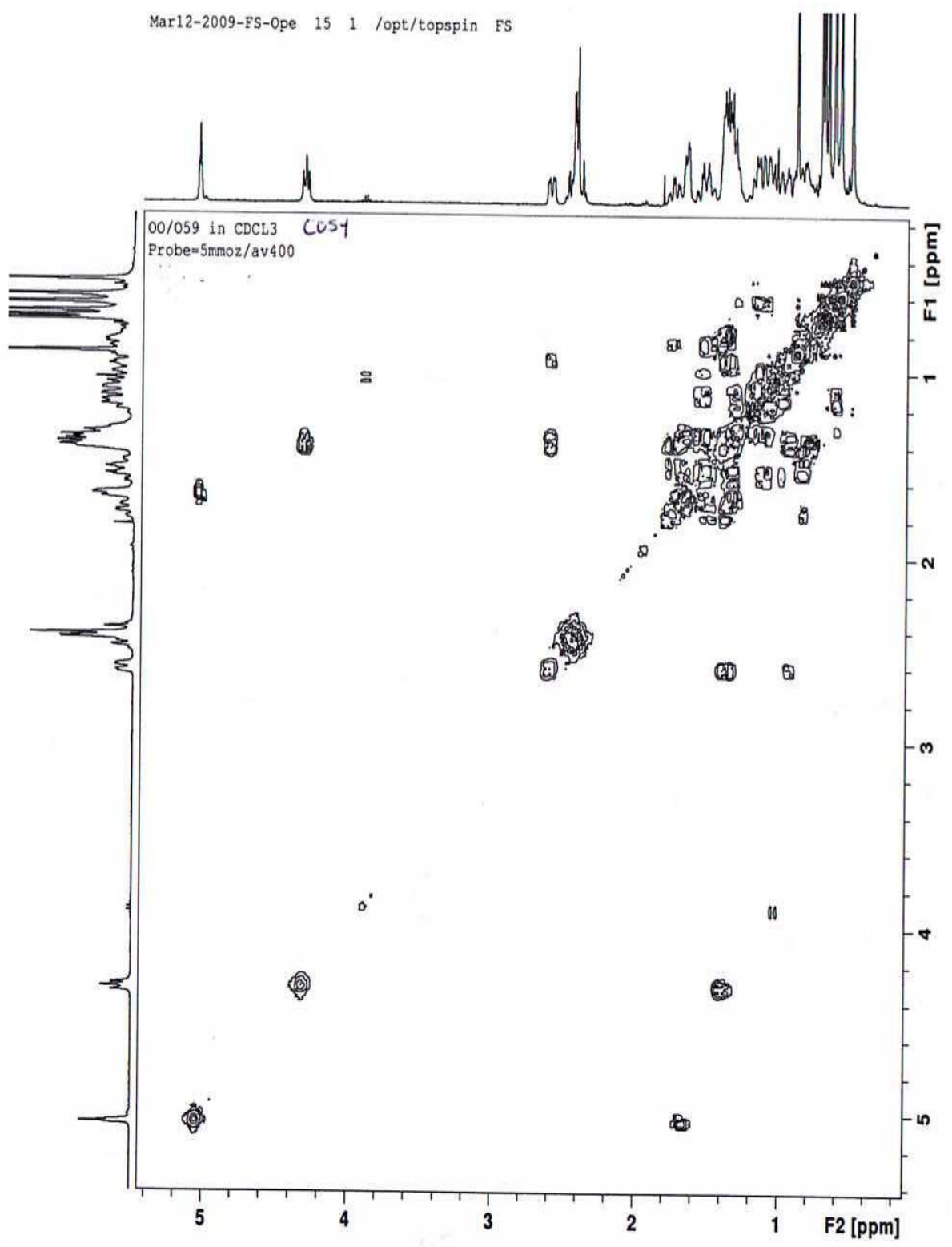


Figure 7.5.6: ^{1}H ^{1}H COSY NMR Spectrum of **OO/59** (Expanded).

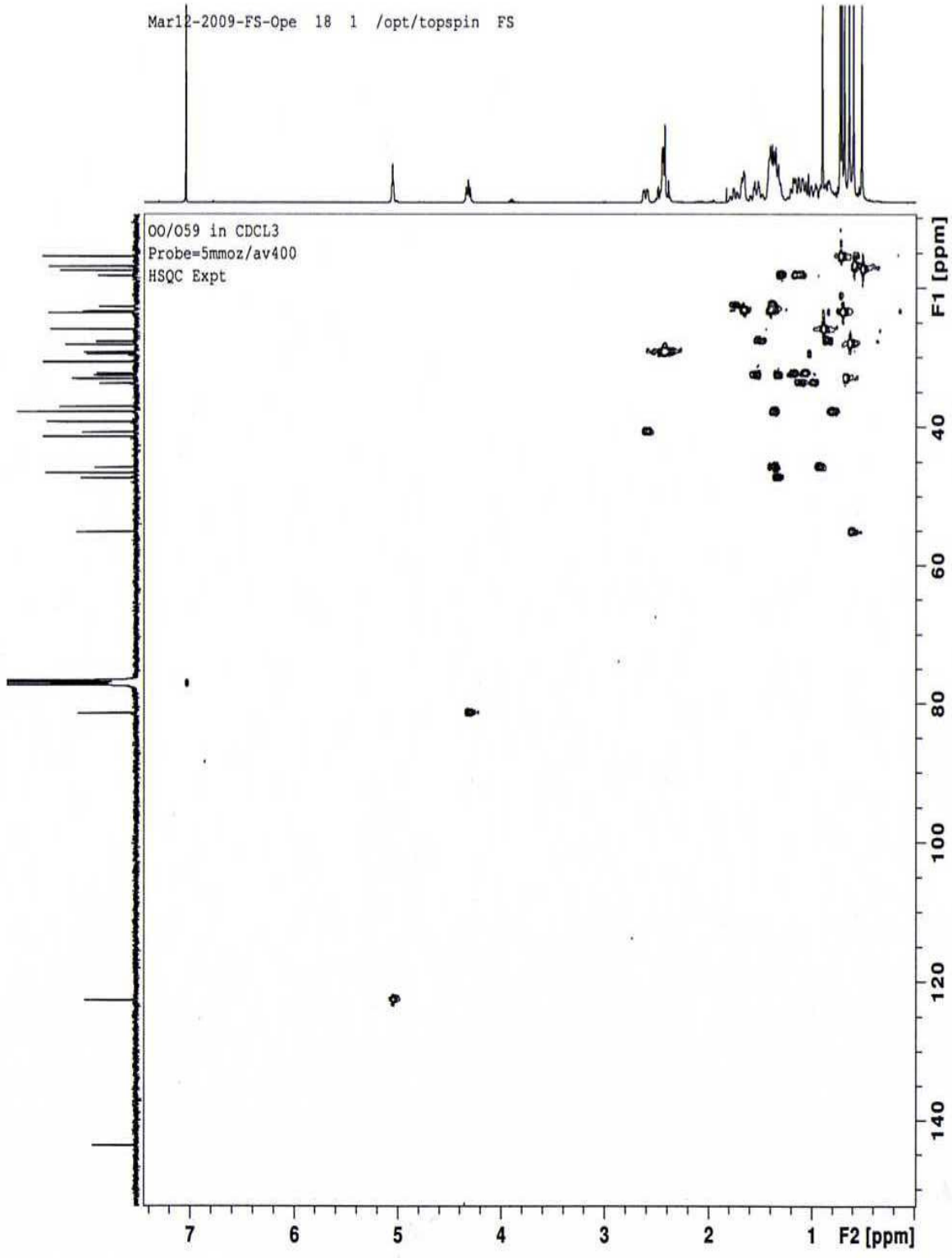


Figure 7.5.7: ^{1}H ^{13}C HSQC NMR Spectrum of **OO/59**

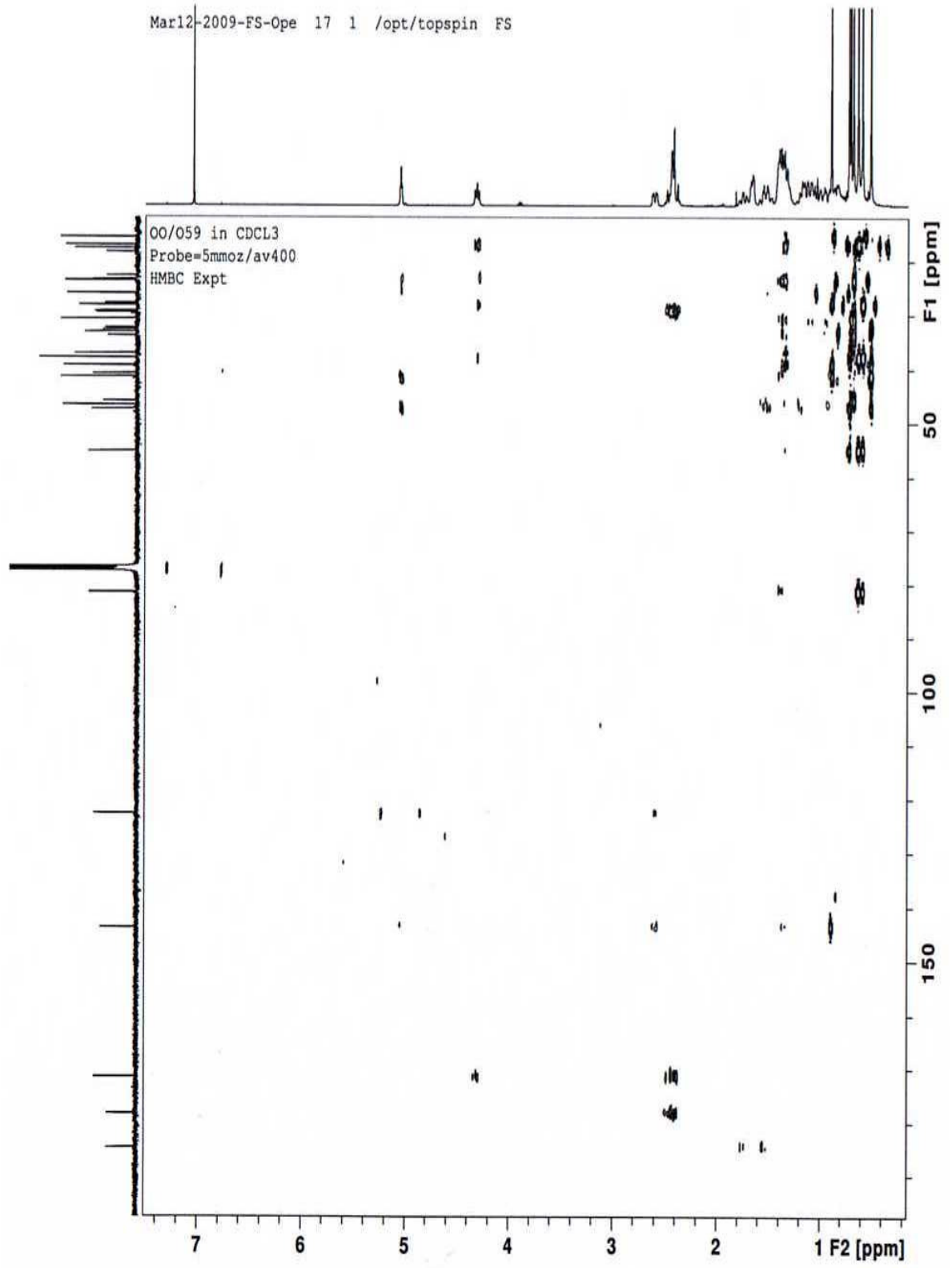


Figure 7.5.8: ^{1}H ^{13}C HMBC NMR Spectrum of **OO/59**

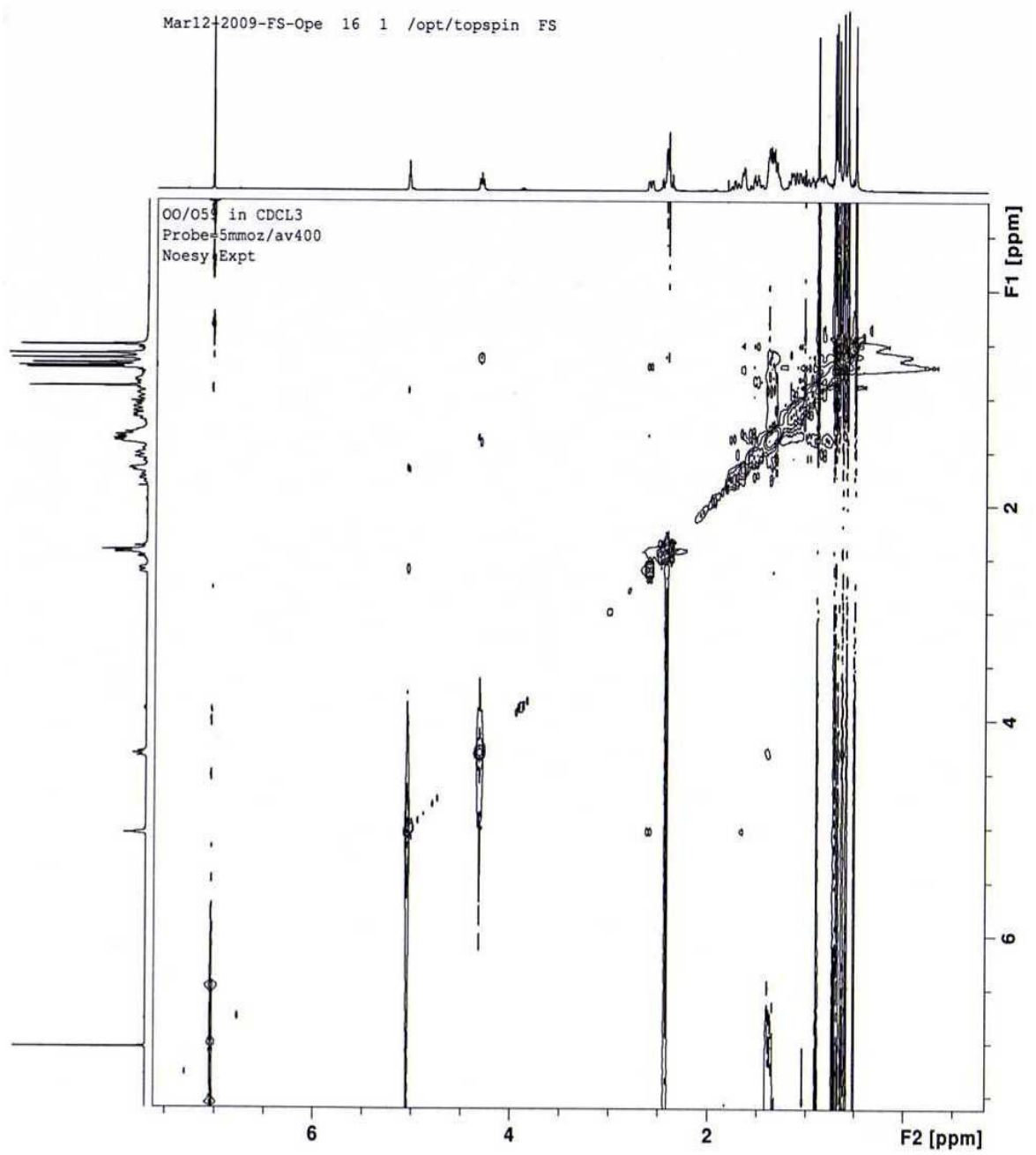


Figure 7.5.9: ^{1}H ^{1}H NOESY NMR Spectrum of **OO/59**

APPENDIX 6

Spectra of the Derivatives of Betulinic Acid

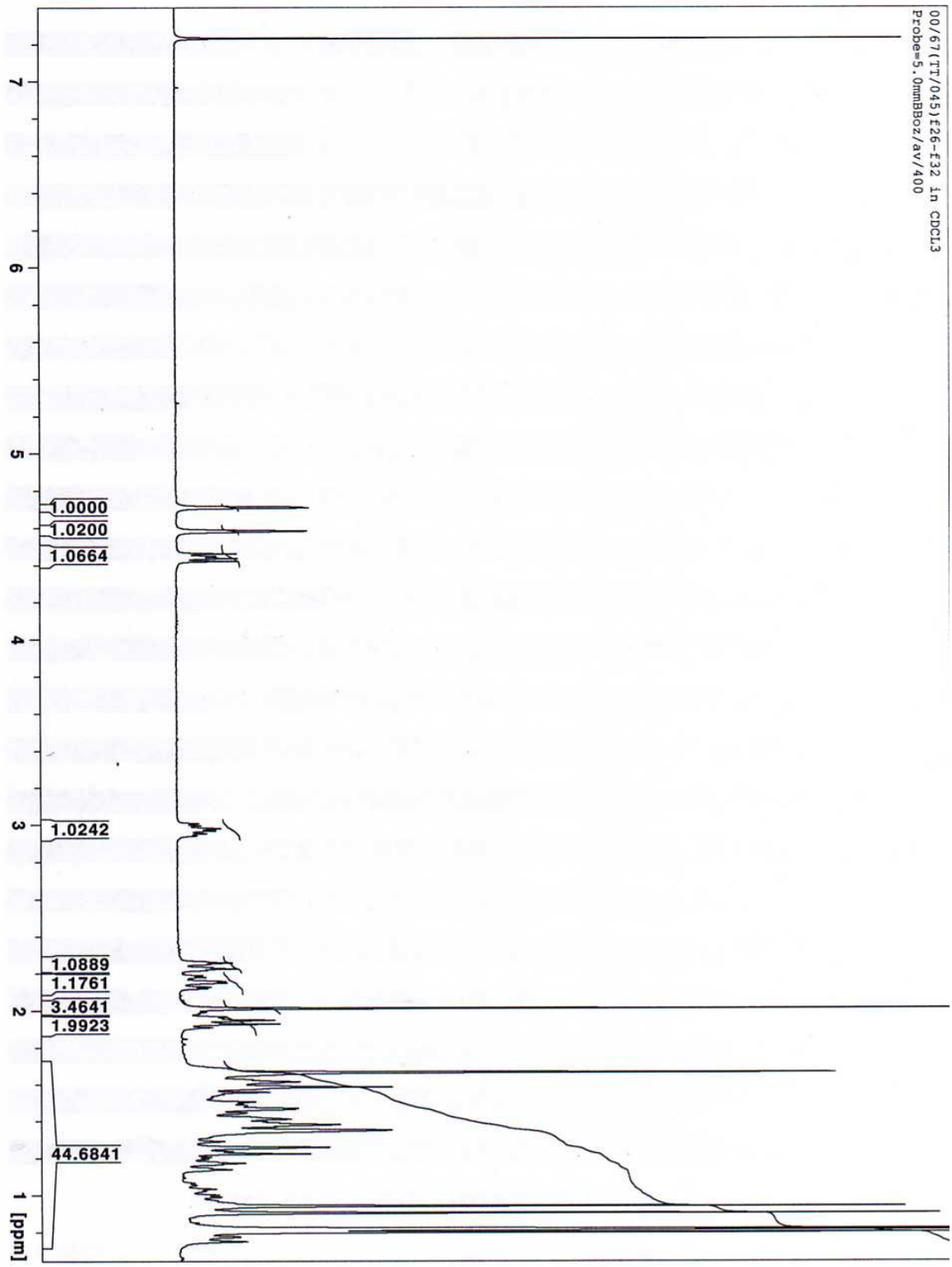


Figure 8.1.1: ^{1}H - NMR Spectrum of OO/67

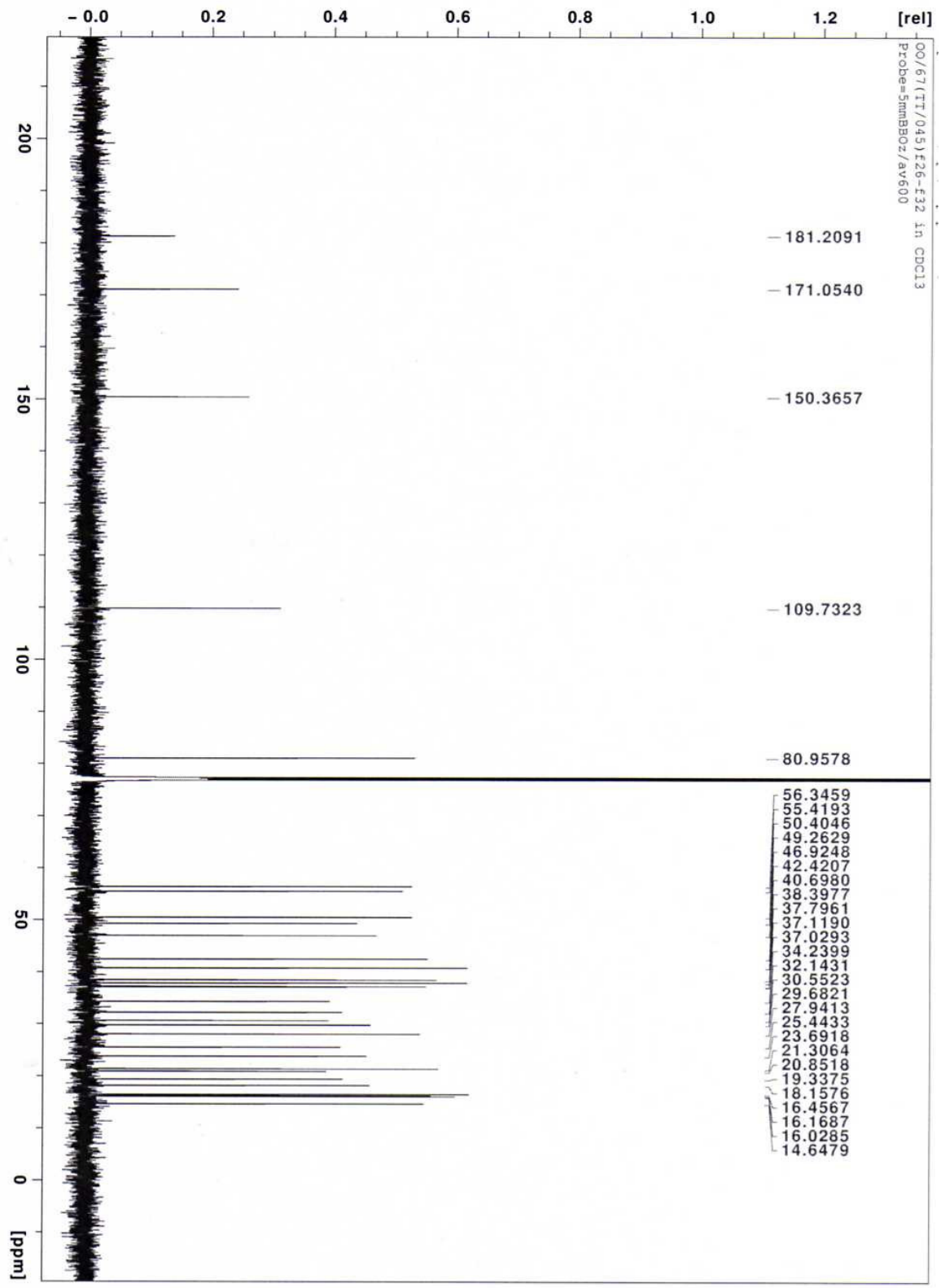


Figure 8.1.2: ^{13}C – NMR Spectrum of OO/67

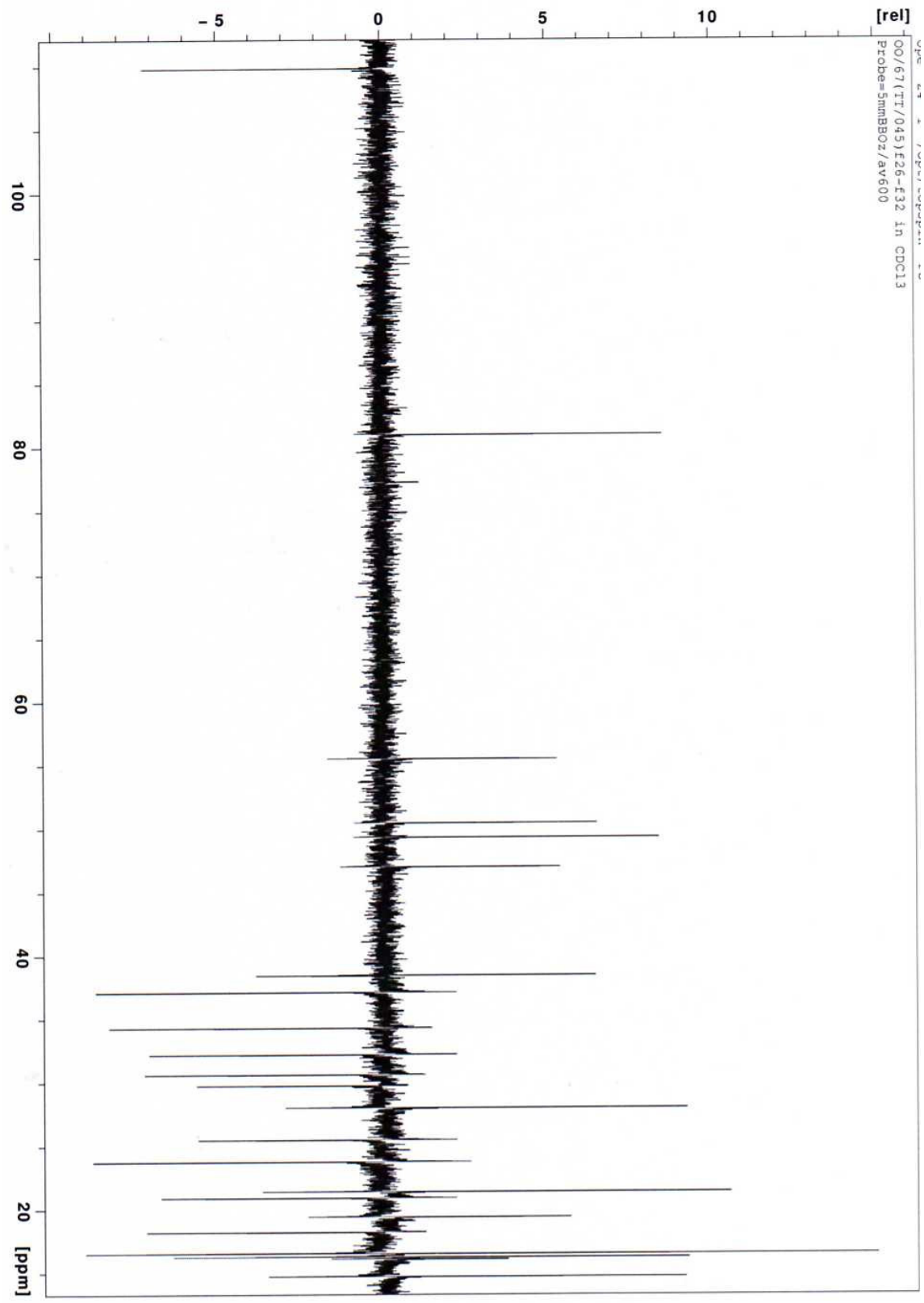


Figure 8.1.3: ^{13}C DEPT - NMR Spectrum of OO/67

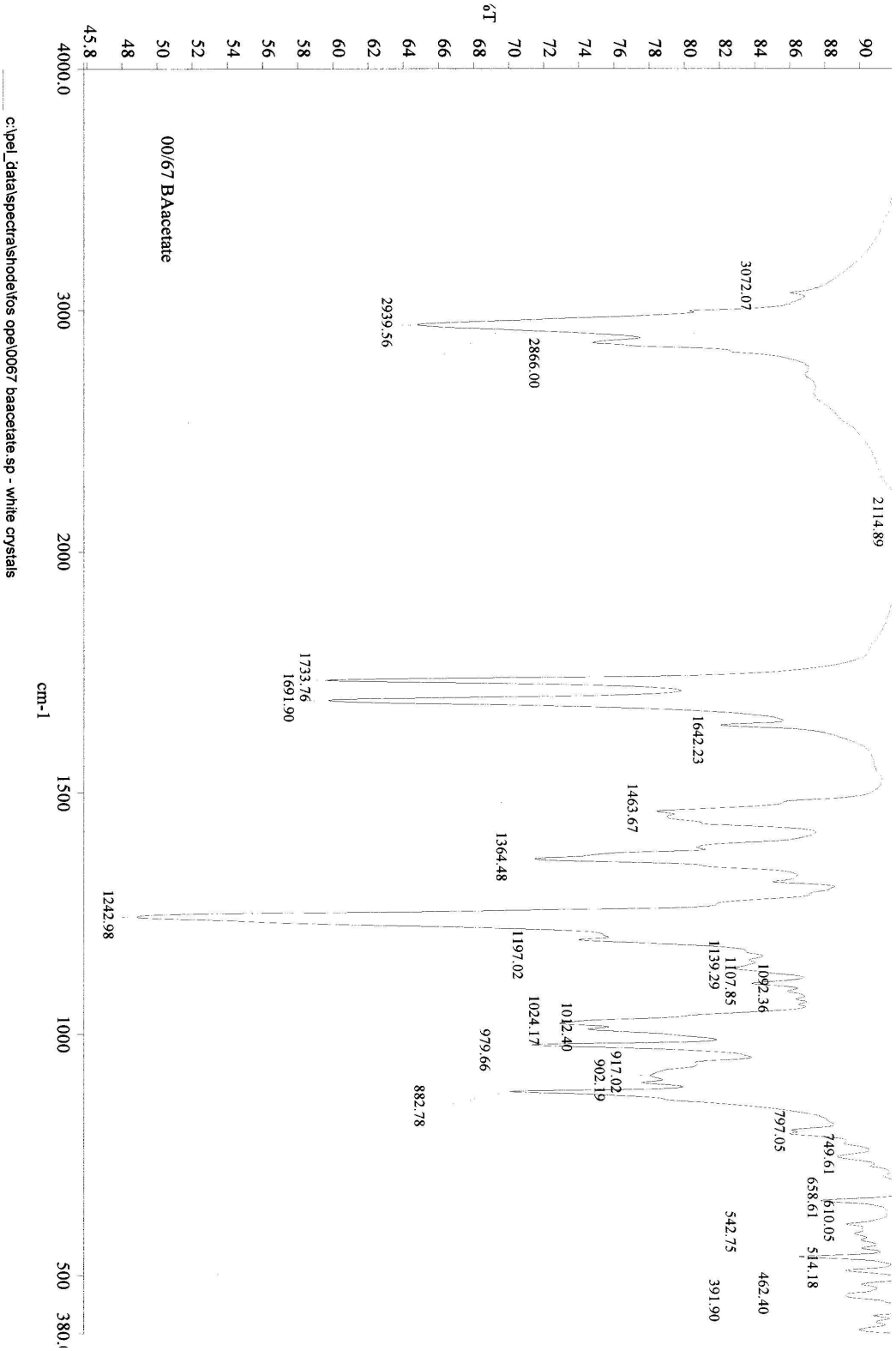
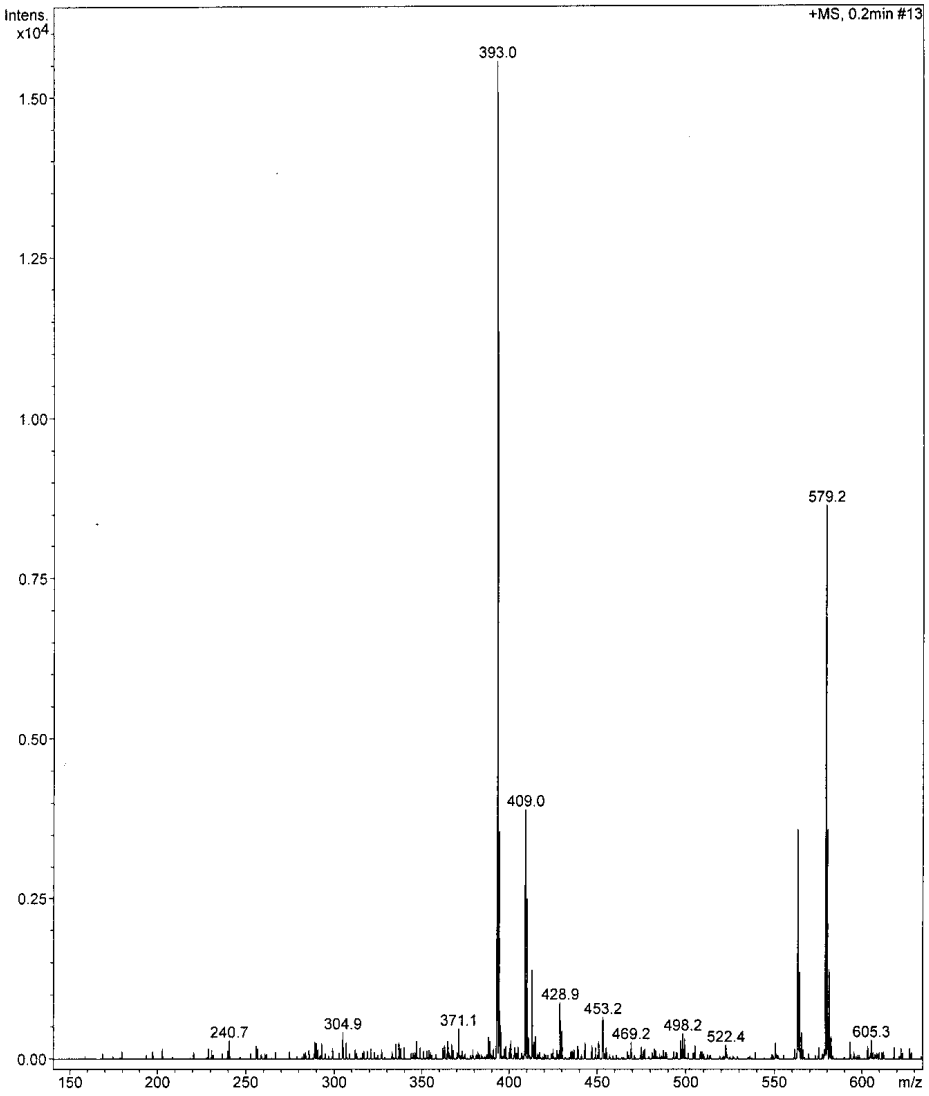


Figure 8.1.4: FTIR Spectrum of OO/67

Display Report - All Windows Selected Analysis

Analysis Name: OO-67.D **Instrument:** LC-MSD-Trap-VL **Print Date:** 11/10/2009 04:33:36 PM
Method: FIA.M **Operator:** Administrator **Acq. Date:** 11/10/2009 4:26:23 PM
Sample Name: OO-67
Analysis Info: OO-67 (MW = 498.7)



MSD Trap Report v5.2 (A4-Opt1) Page 1 of 1 Agilent Technologies

Figure 8.1.5: Mass Spectrum of OO/67

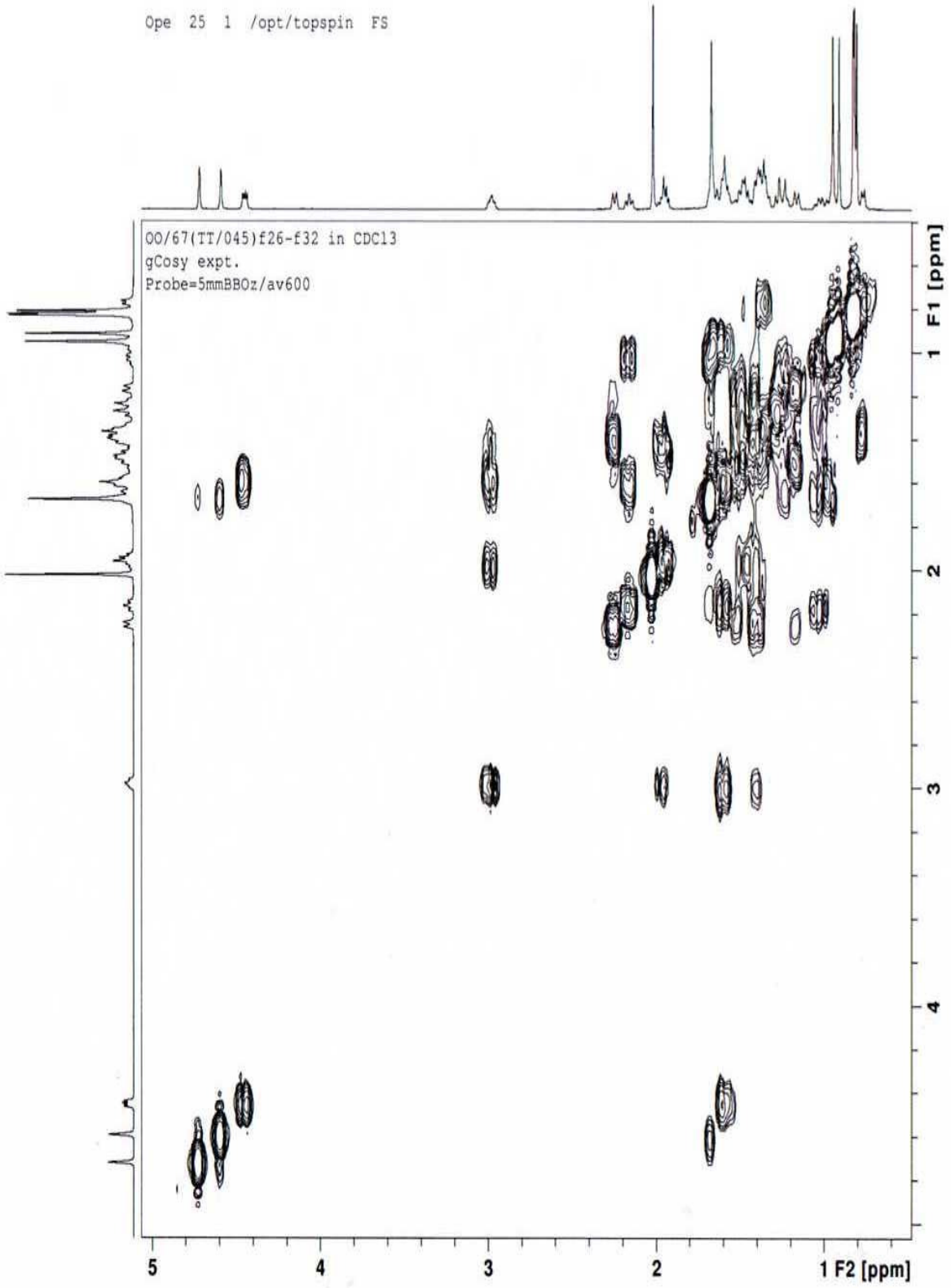


Figure 8.1.6: ^{1}H-^{1}H COSY NMR Spectrum of OO/67

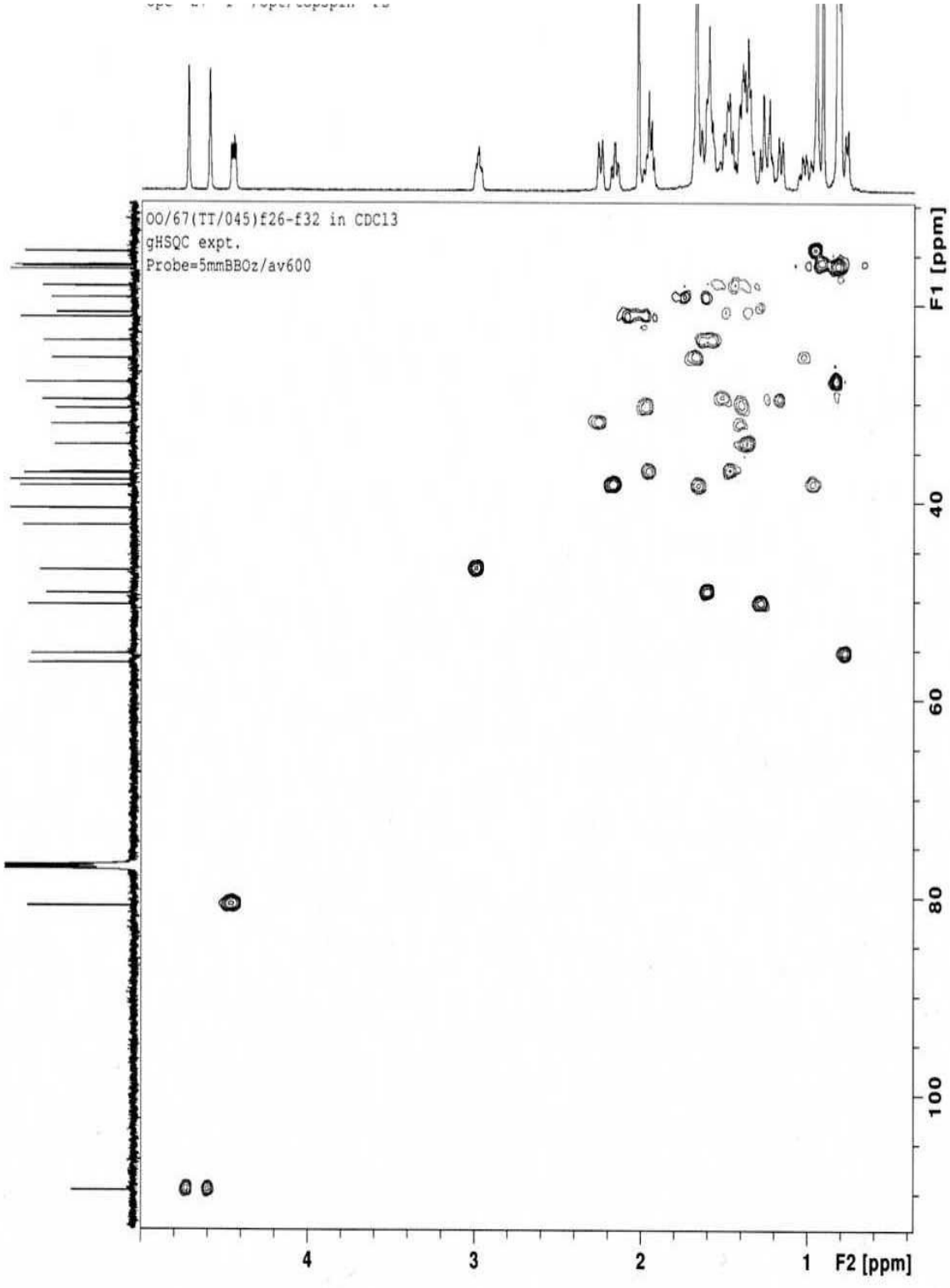


Figure 8.1.7: ^{1}H-^{13}C HSQC NMR Spectrum of OO/67

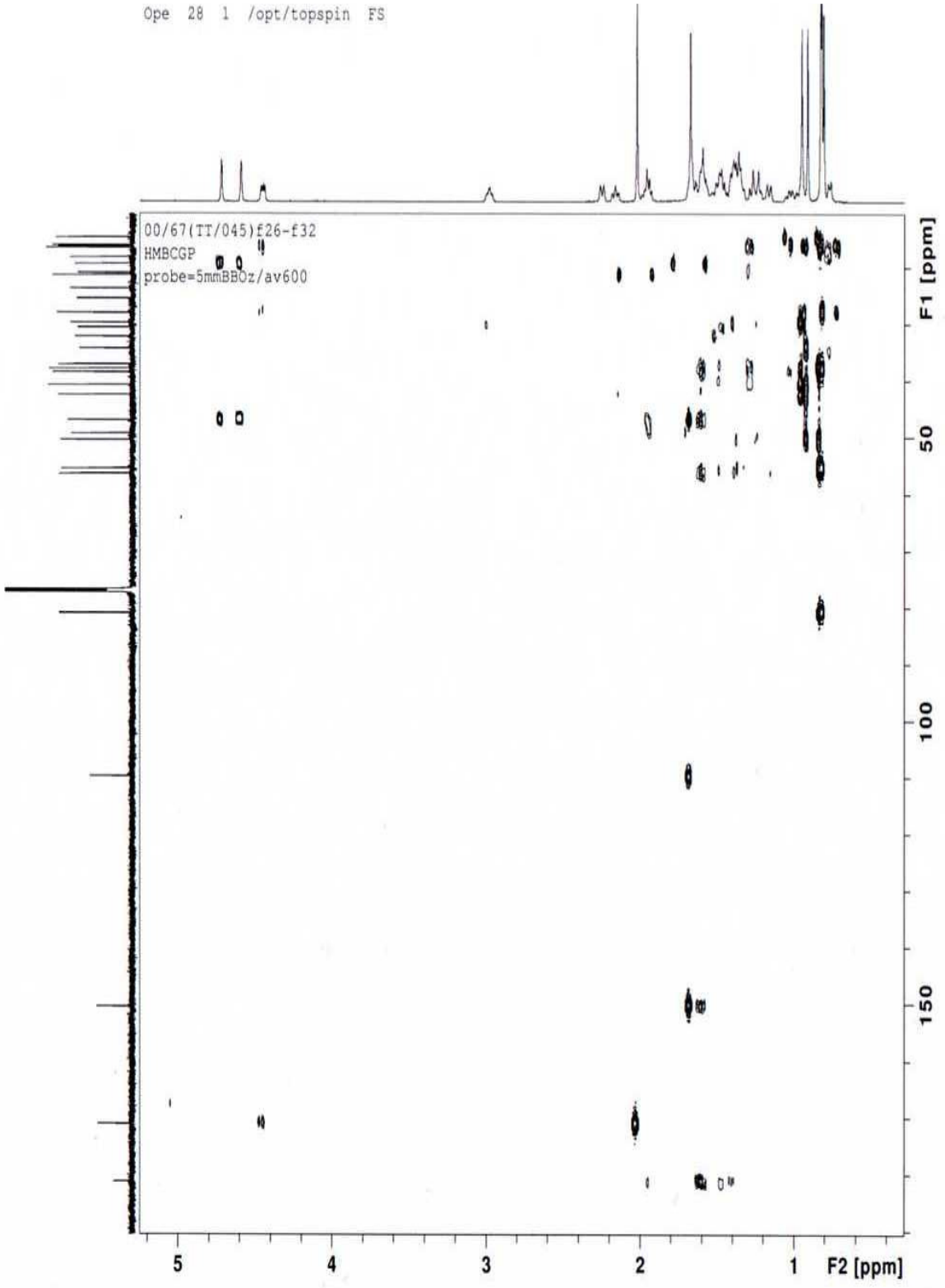


Figure 8.1.8: ^{1}H-^{13}C HMBC NMR Spectrum of OO/67

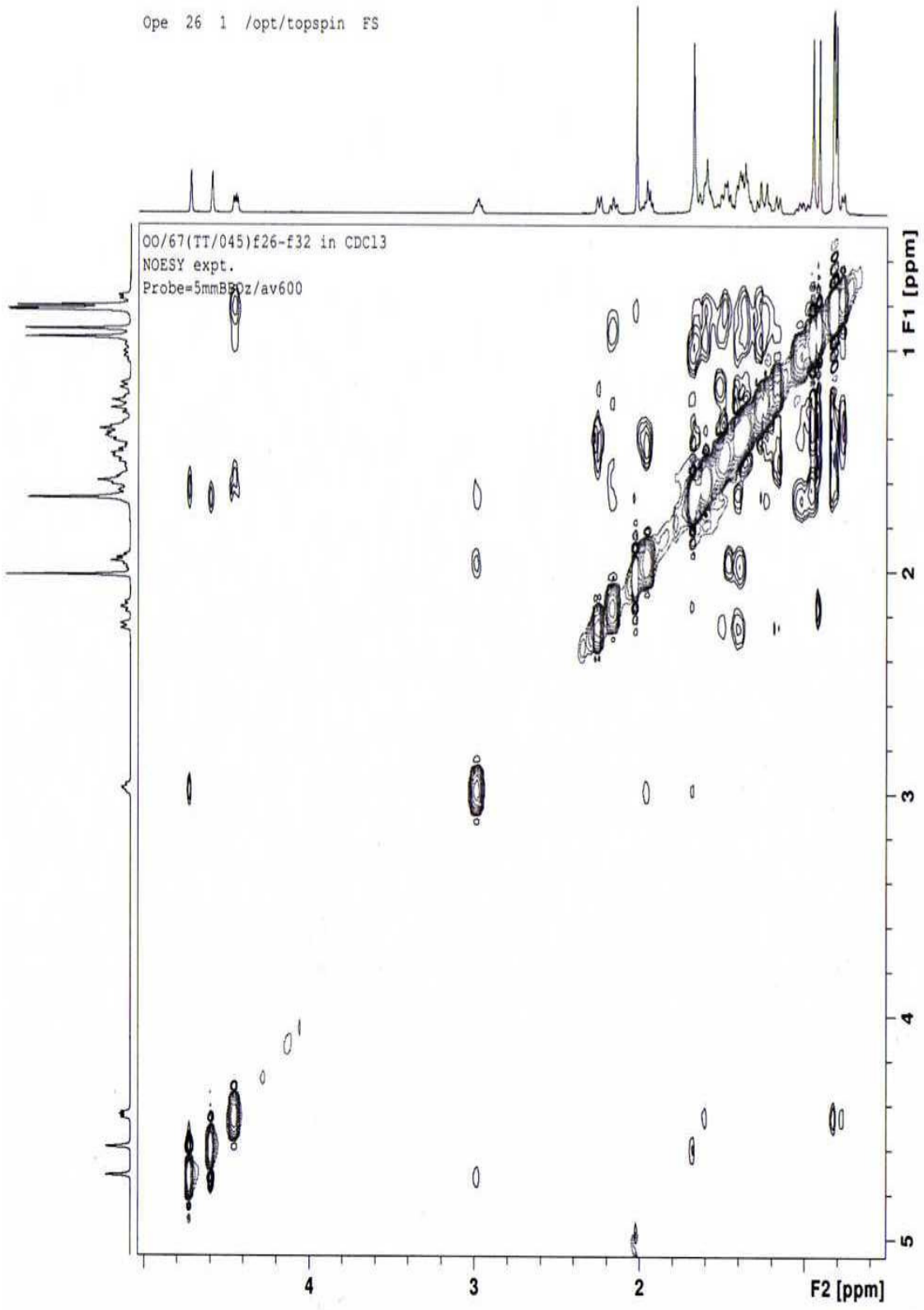


Figure 8.1.9: ^{1}H-^{1}H NOESY NMR Spectrum of OO/67

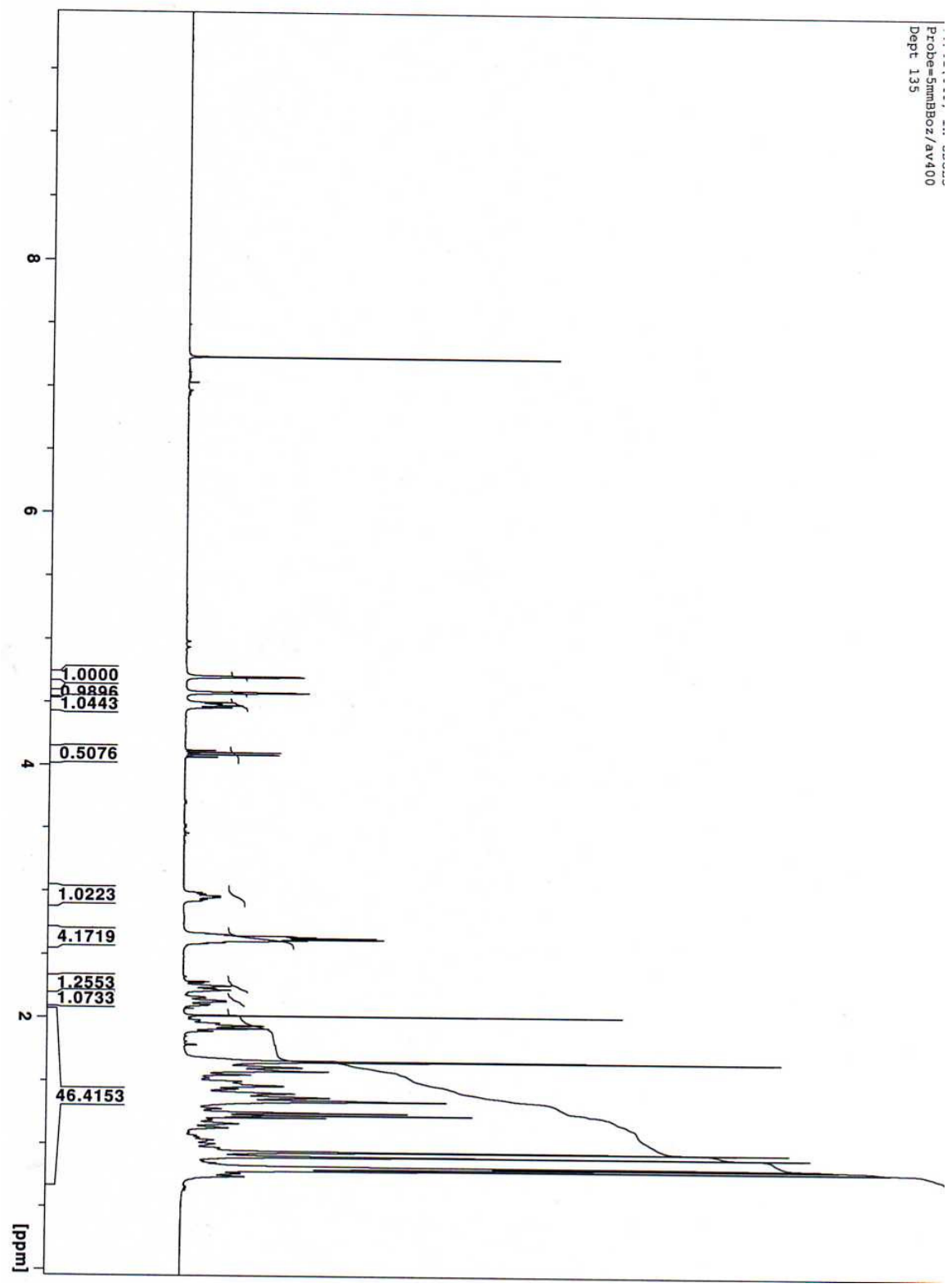


Figure 8.2.1: ^{1}H - NMR Spectrum of OO/61

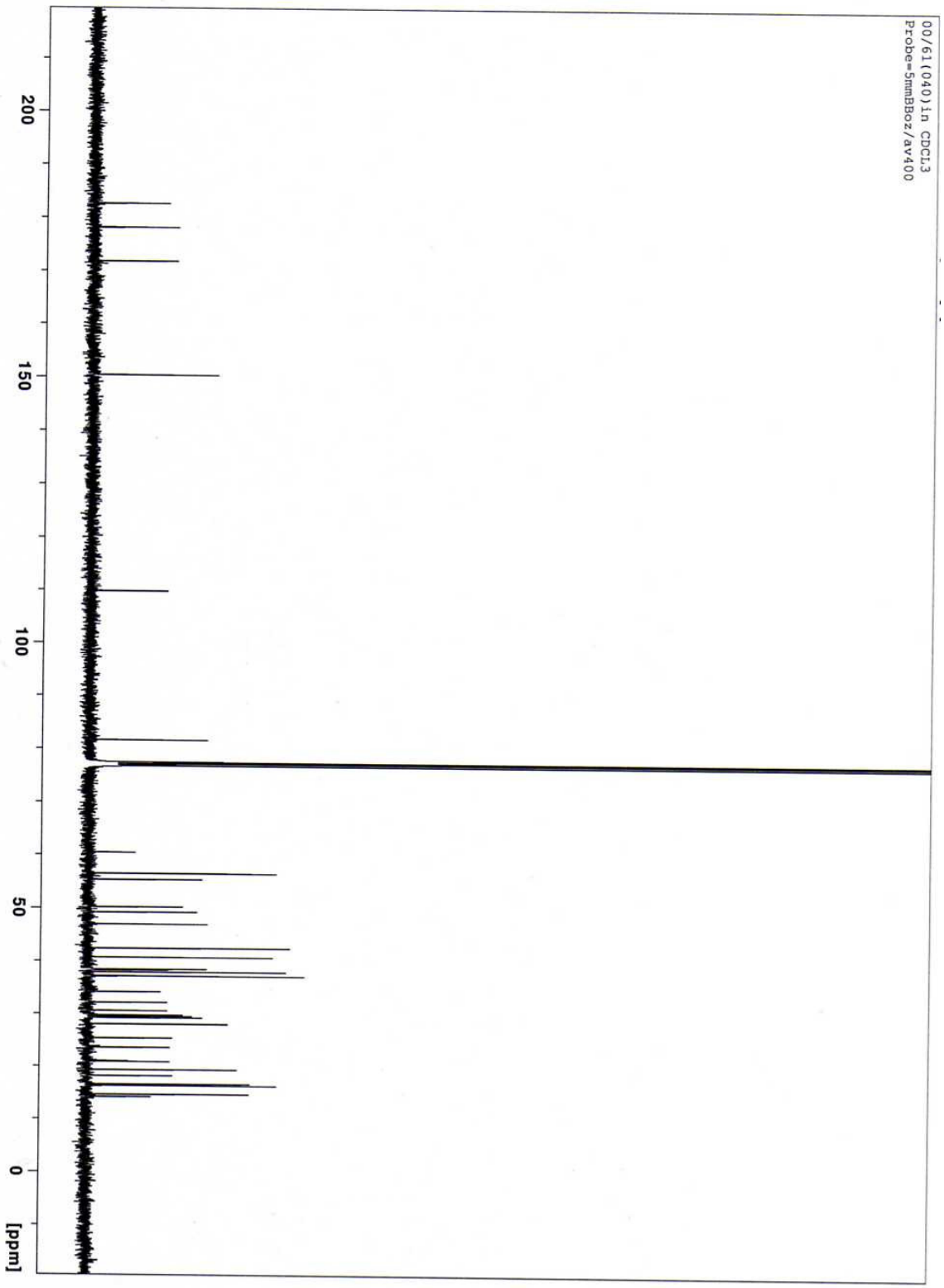


Figure 8.2.2: ^{13}C - NMR Spectrum of OO/61

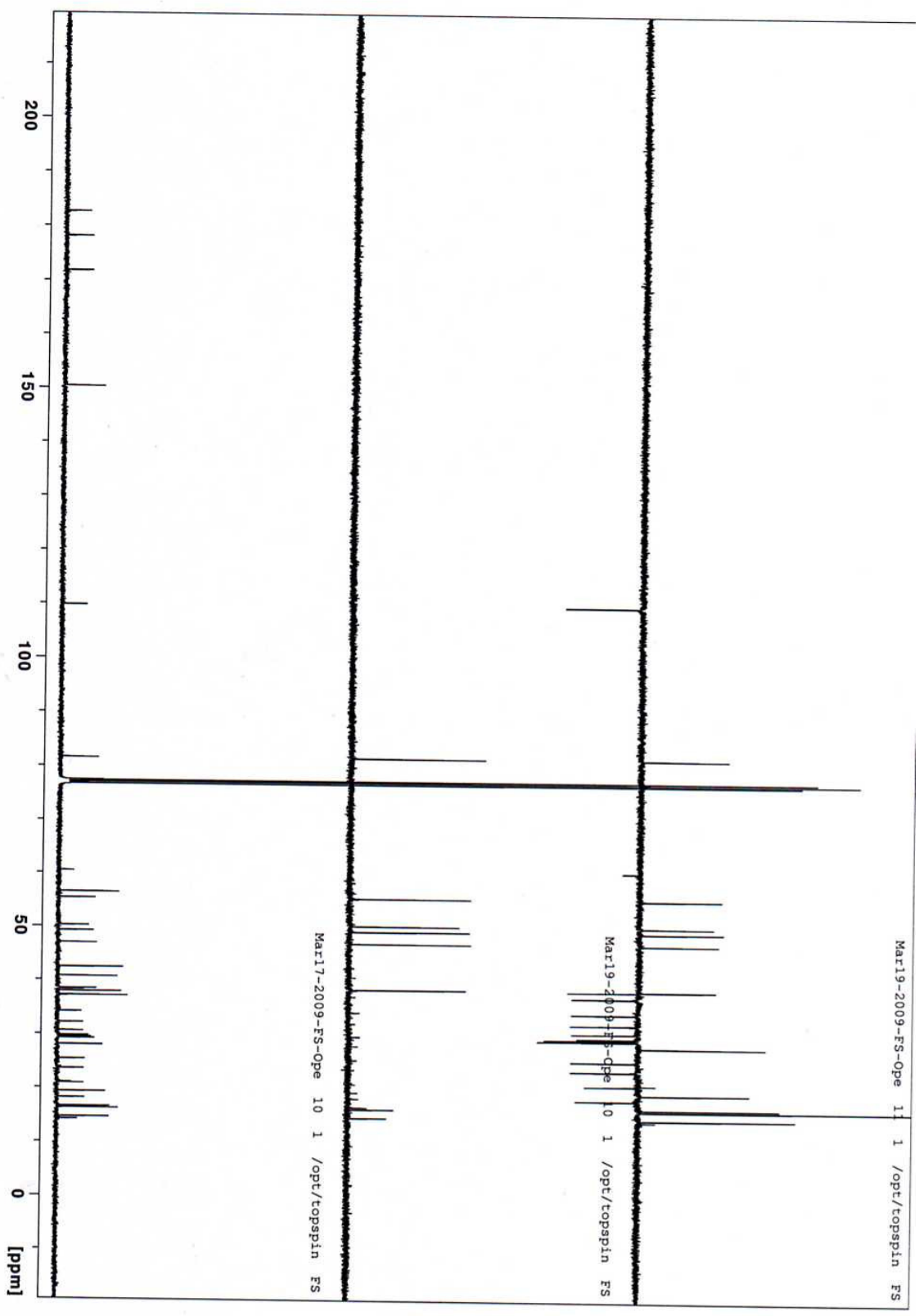


Figure 8.2.3: ^{13}C DEPT - NMR Spectrum of OO/61

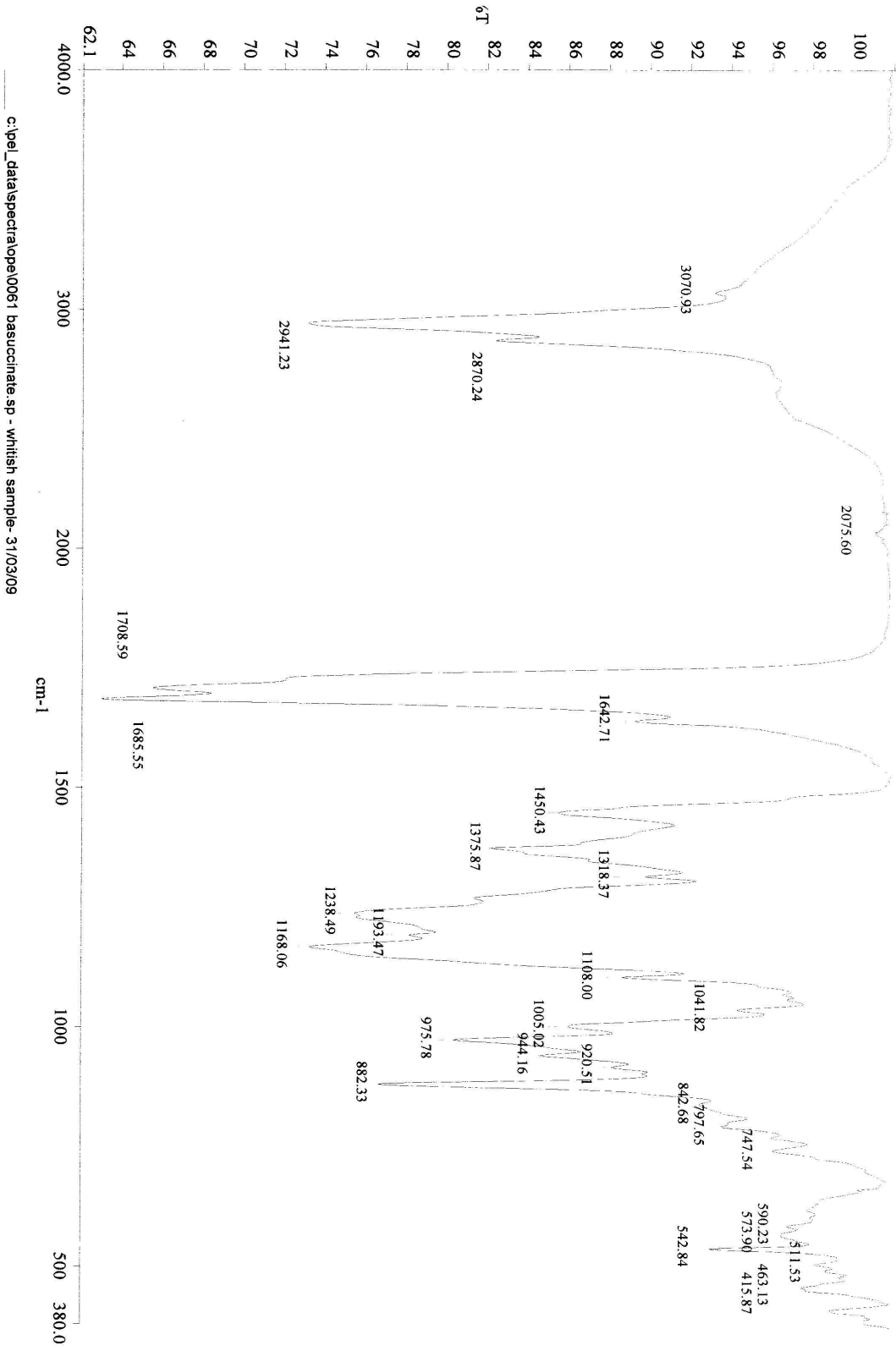


Figure 8.2.4: FTIR Spectrum of OO/61

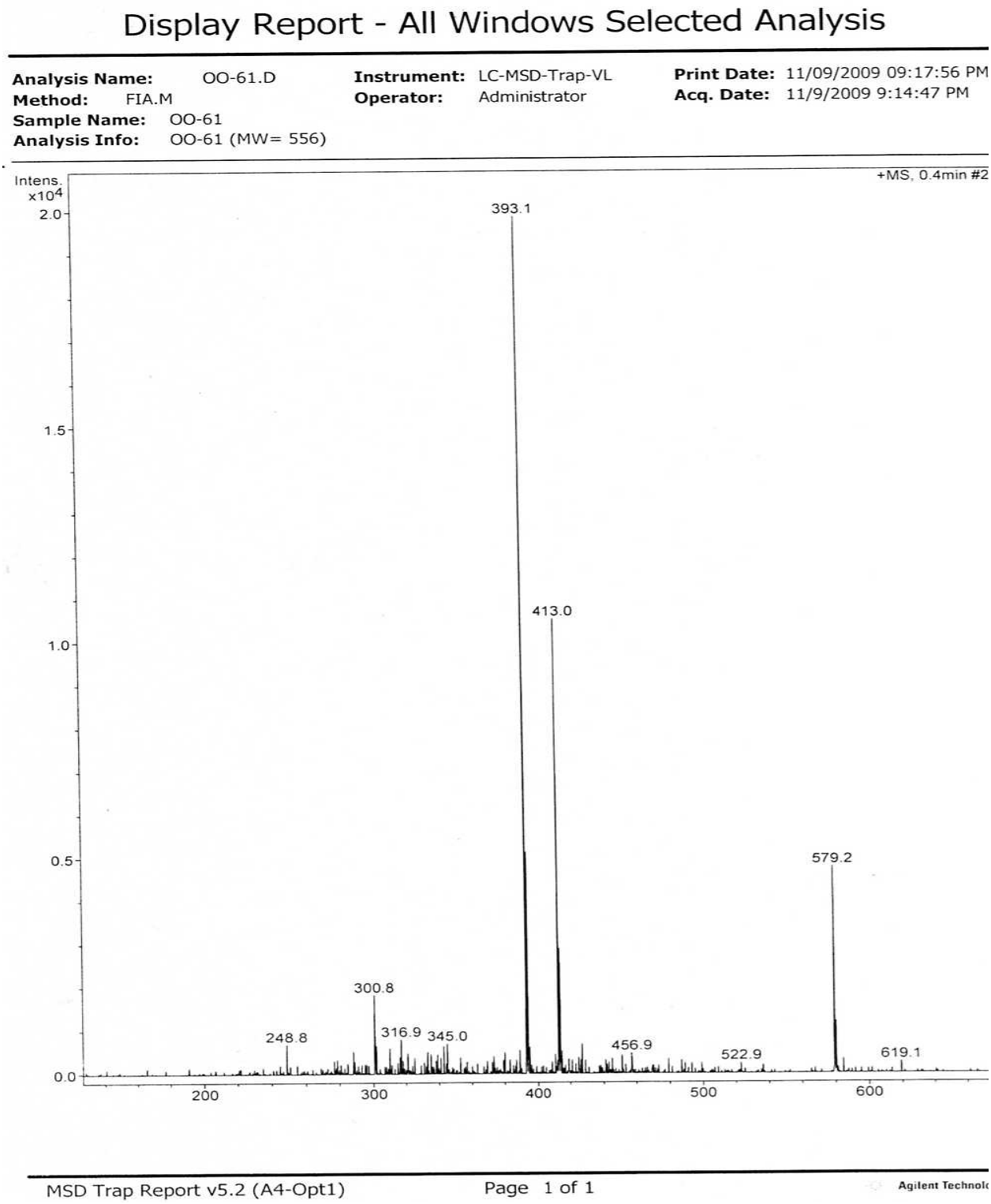


Figure 8.2.5: Mass Spectrum of OO/61

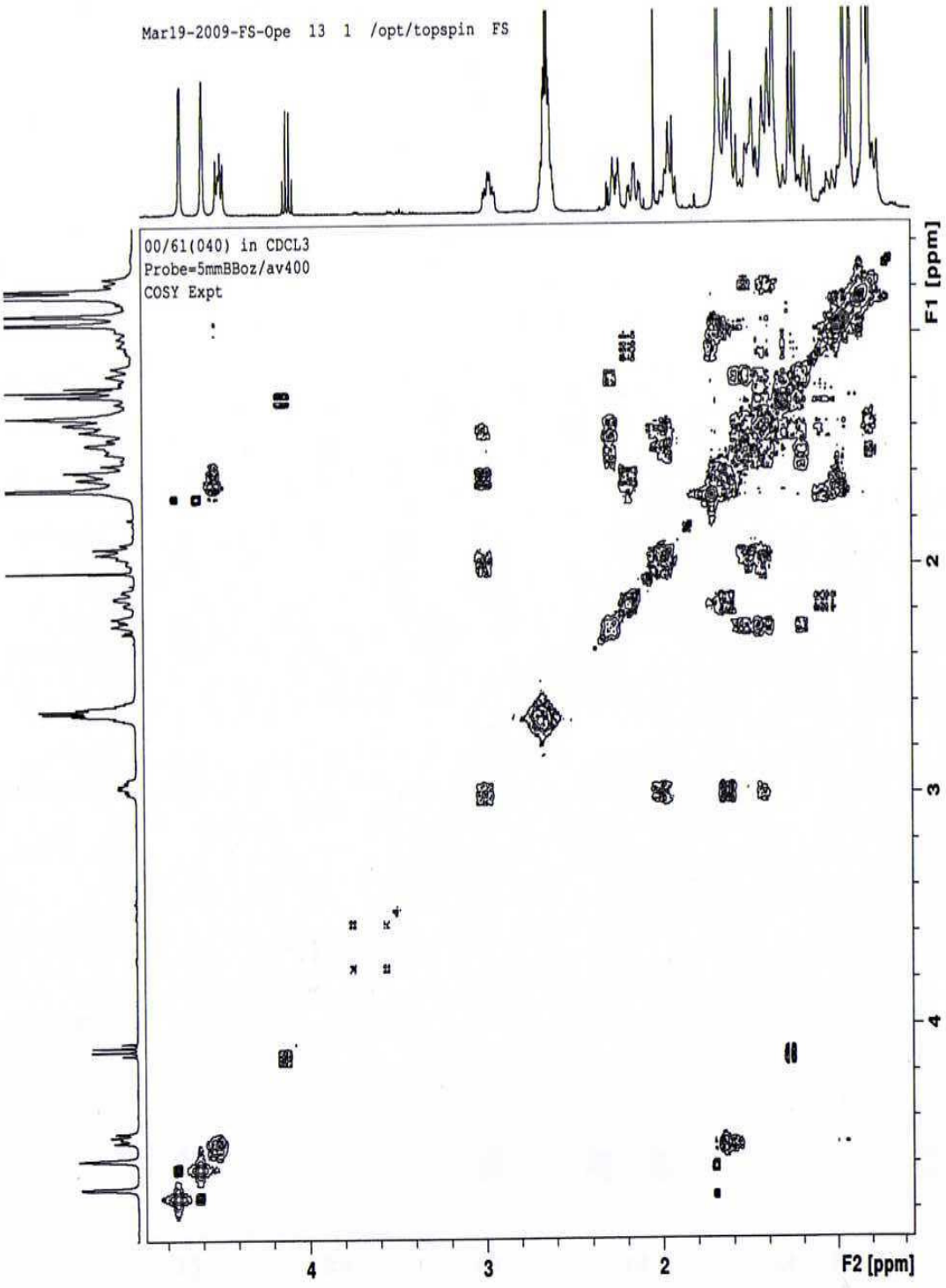


Figure 8.2.6: ^{1}H-^{1}H COSY NMR Spectrum of OO/61

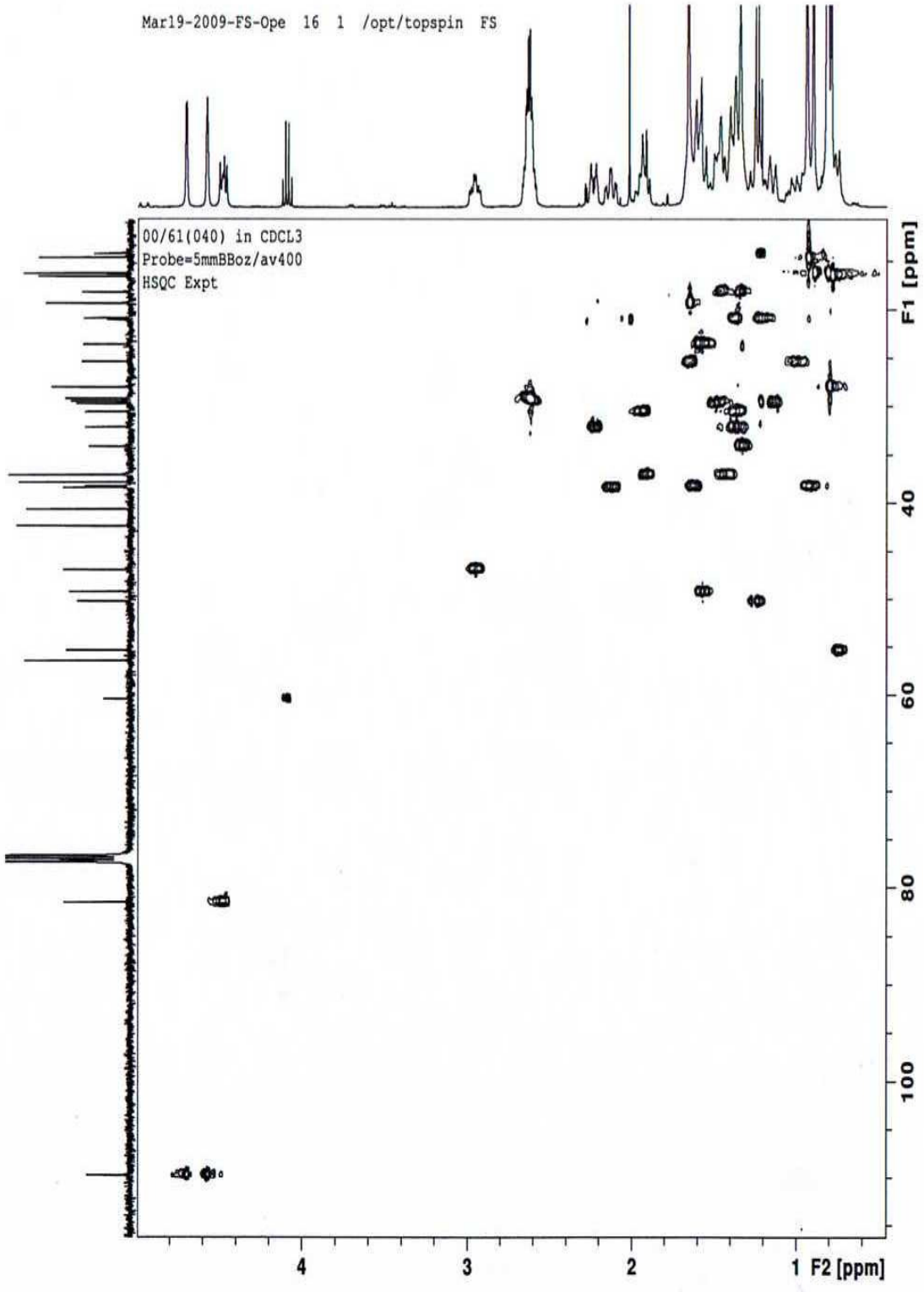


Figure 8.2.7: ^{1}H-^{13}C HSQC NMR Spectrum of OO/61

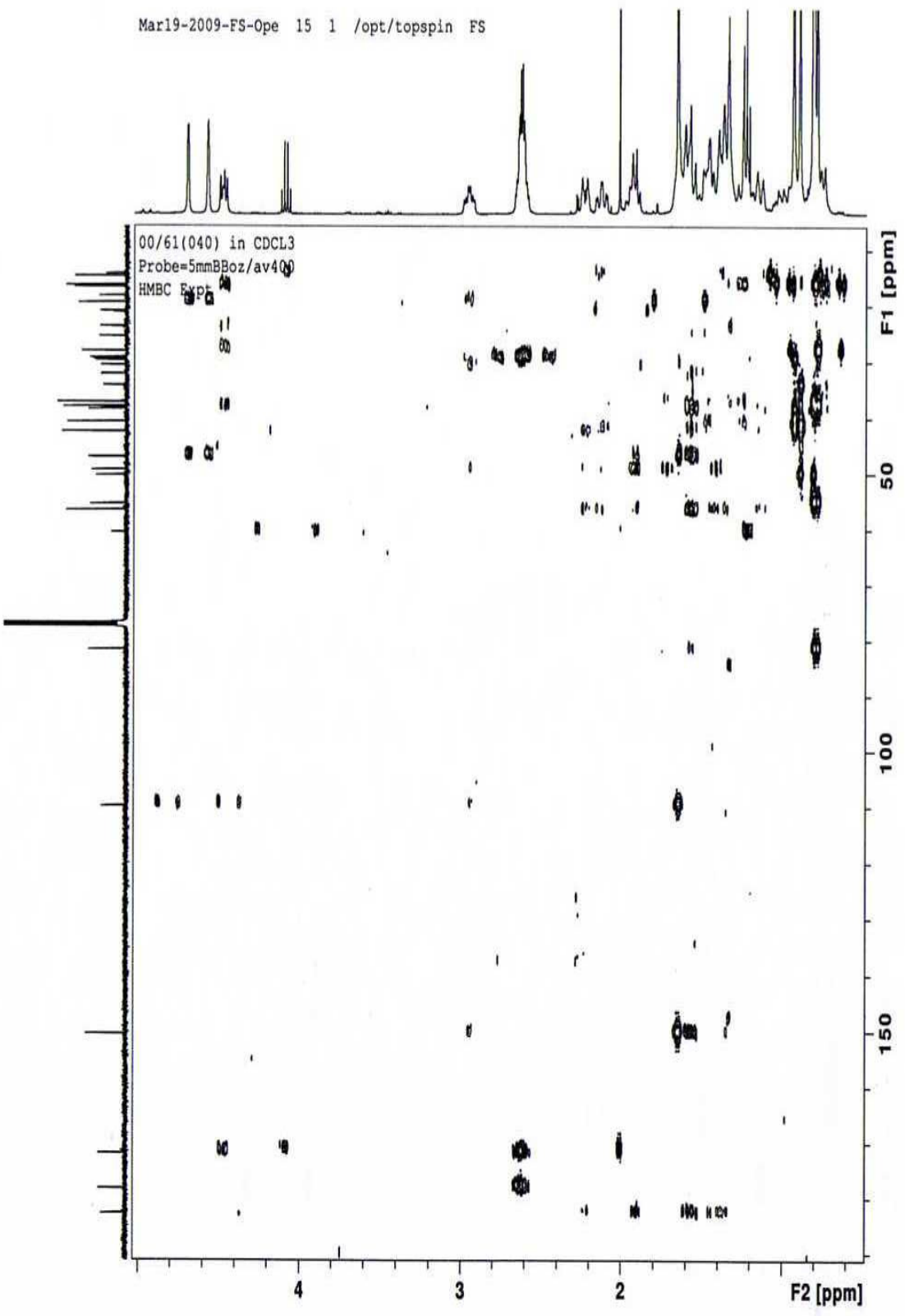


Figure 8.2.8: ^{1}H-^{13}C HMBC NMR Spectrum of OO/61

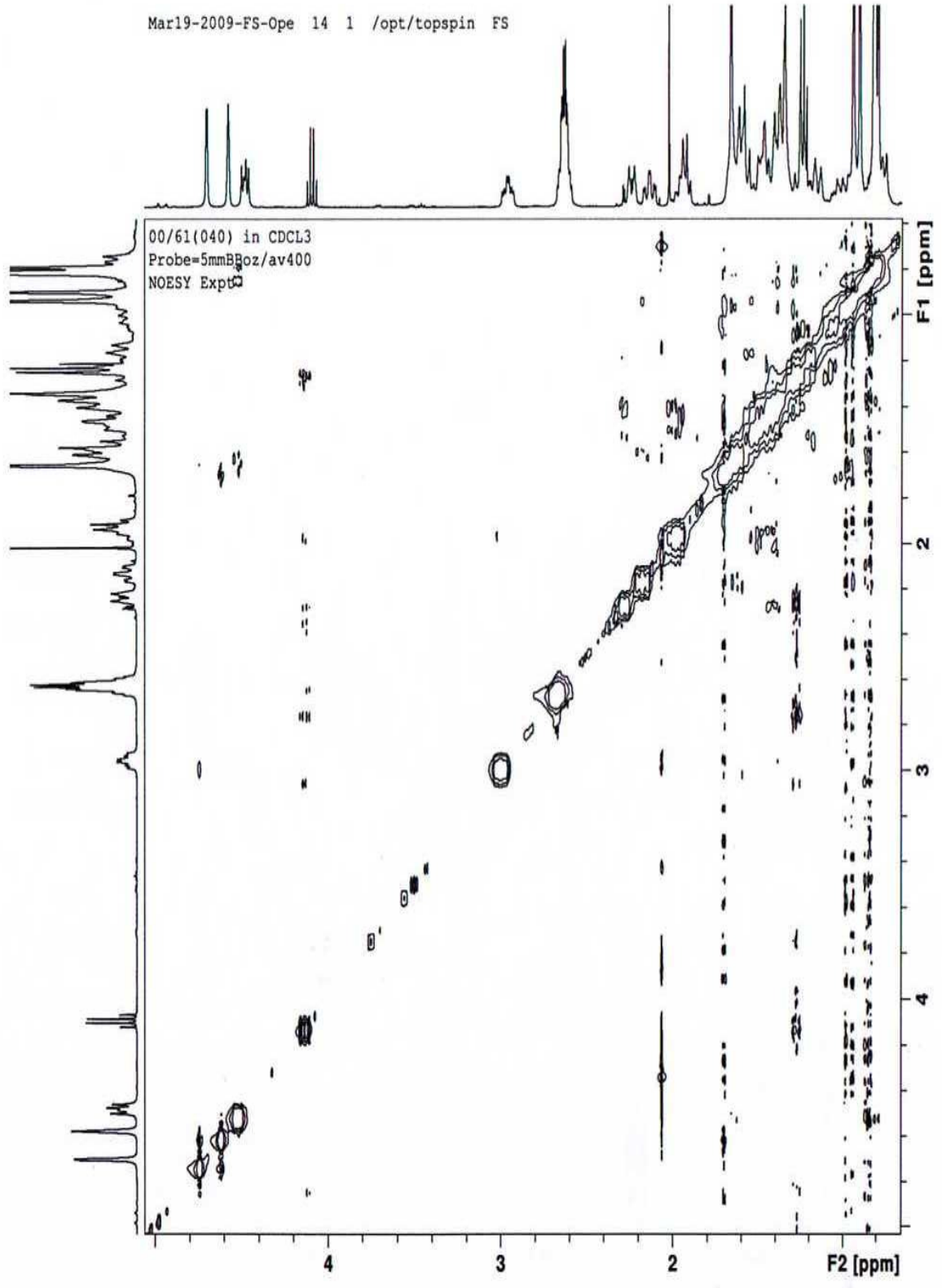


Figure 8.2.9: ^{1}H-^{1}H NOSEY NMR Spectrum of OO/61

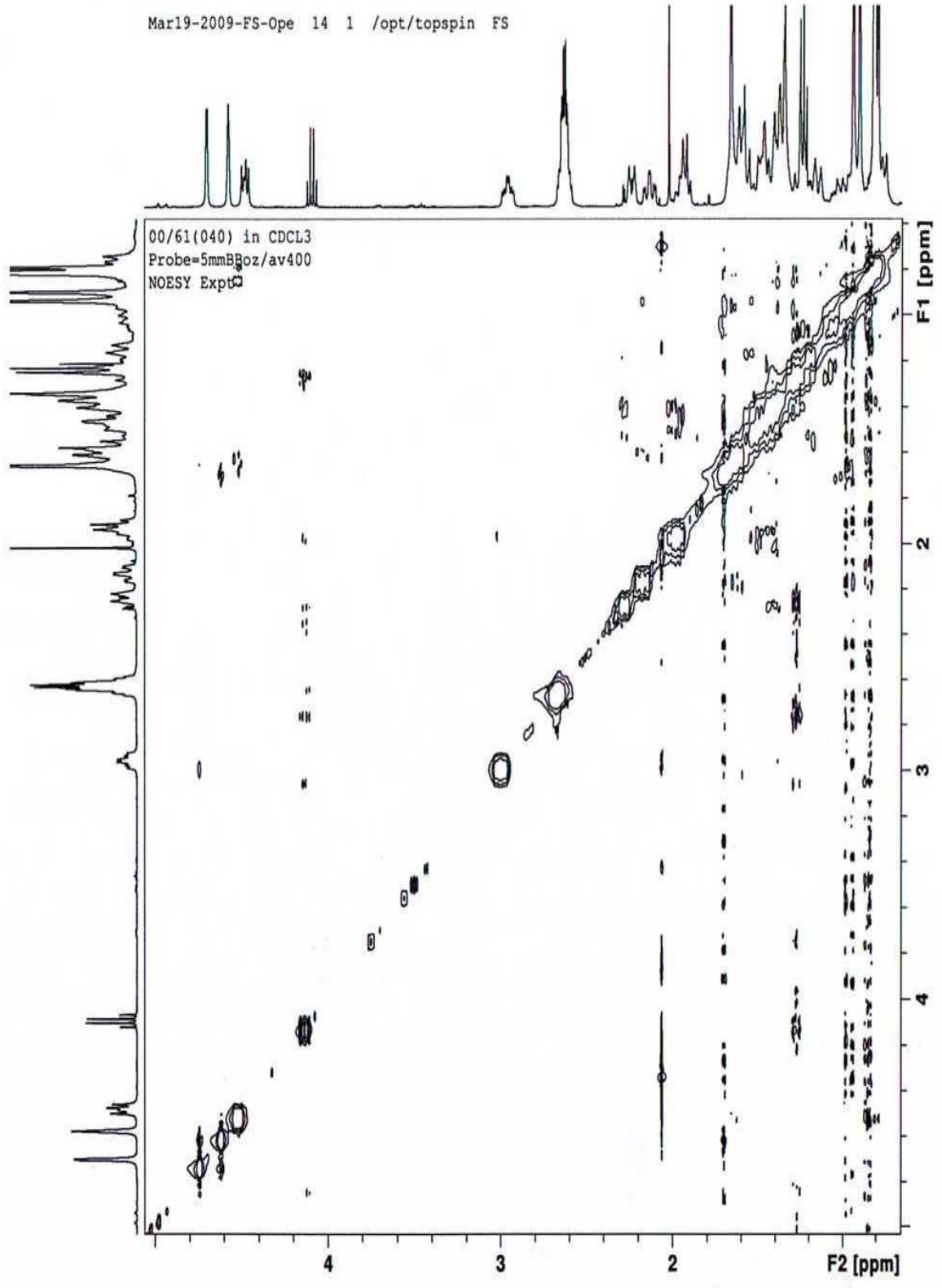


Figure 8.2.9: ^{1}H-^{1}H NOSEY NMR Spectrum of OO/61