BIO-PROSPECTING FOR PHYTOCHEMICALS FOR *ANOPHELES GAMBIAE* LARVAL CONTROL

By

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Declaration

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

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Dedication

This work is dedicated to my father and mother, Mr. and Mrs. Owiti and to my family for their encouragement, spiritual, mental and material support, and for their patience in seeing me through my hard earned educational achievement.

Acknowledgement

I would like to acknowledge my university supervisor, Prof. Ndiege for having introduced me into the field of Natural Products. I greatly acknowledge him for facilitating my attachment to the International Centre of Insect Physiology and Ecology (ICIPE).

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Lastly, I greatly acknowledge the WHO/WORLD BANK/UNDP/TDR/MIM for the financial assistance without which the project would have not been possible.

List of abbreviations

AIDS	Acquired Immune Deficiency Syndrome
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
B.s	*Bacillus sphaericus*
B.t.i	*Bacillus thuringiensis* var. *israelensis*
CC	Column Chromatography
$CDCl_3$	Deuterated chloroform
$(CD_3)_2OD$	Deuterated acetone
CD_3OD	Deuterated ethanol
COSY	Correlation Spectroscopy
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEPT	Distortionless Enhancement by Polarization Transfer
DEET	*N,N*-diethyl-*m*-toluamide
EIR	Entomological Inoculation Rate
EI-MS	Electron Impact Mass Spectrometry
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
HETCOR	Heteronuclear Chemical Shift Correlation
ITN	Insecticide Treated Net
LC	Lethal Concentration
LD	Lethal Dose
HPLC	High Performance Liquid Chromatography
IGR	Insect Growth Regulator
IR	Infra-Red Spectroscopy
mp	Melting Point
MIM	Multilateral Initiative on Malaria
NMK	National Museums of Kenya
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Enhancement
MS	Mass Spectroscopy

OP	Organophosphate
ppm	Parts per million
PTLC	Preparative Thin Layer Chromatography
RBM	Roll Back Malaria
R_f	Retention factor
TB	Tuberculosis
TDE	Dichlorodiphenyldichloroethane
TDR	Tropical Diseases Research
TLC	Thin Layer Chromatography
UNDP	United Nations Development Program
UoN	University of Nairobi
UV	Ultra-Violet Spectroscopy
WHO	World Health Organization

List of Plates, figures and tables

ABSTRACT

Control of disease vectors and pest insects by use of chemical pesticides has generated several problems such as insecticide resistance, environmental pollution, safety risks for humans and domestic animals. The spread in resistance to majority of present synthetic insecticides by several mosquito species, in various geographical areas, calls for multi-pronged vector control programs and the necessity to develop alternative environmentally acceptable and cost-effective compounds. Mosquitoes depend on aquatic habitat for larval development, thus the fundamental means of control lies in eliminating their breeding habitats by drainage (temporary dewatering) or natural control such as removal of aquatic vegetation (protection and food). These approaches rarely achieve complete control and need to be reinforced by other measures such as house screening, insecticide/repellent and larvicide application. Insecticide treatment of larval habitats remains more effective in reducing culicine population than the application of residual insecticides to houses. Plants have provided many useful compounds or templates including drugs, agrochemicals, insecticides and dyes. Thus there is need for research on phytochemistry of plants with potential of inhibiting growth and development of/or larvicidal activity against mosquito larvae.

The current research has undertaken bio-prospecting for phytochemicals for *Anopheles gambiae* larval control. Seventeen (17) plants were assayed against *An. gambiae* larvae. Bio-assay-guided fractionation was performed on crude extracts of *Zanthoxylum gillettii* and *Pittosporum mannii*. A total of thirteen (13) compounds were isolated, four of which were found to exhibit *An. gambiae* larvicidal activity in the range of 3.31–75.72 µg/ml (LD_{50}). Three new oxoaporphine alkaloids were isolated for the first time and identified with other six known compounds by spectroscopic techniques (IR, UV, NMR, MS).

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CHAPTER 1: INTRODUCTION

1.0 Malaria occurrence

Besides the well-known painful bites inflicted by the female mosquitoes, these insects have been shown to be the vectors of four distinct human diseases including malaria, filariasis, yellow and dengue fever. Of these, malaria has been reported to be of greatest economic and medical importance in terms of geographical distribution, incidence, medical costs, days of labour lost and the extent of morbidity and mortality it causes. A single bout of malaria is estimated to cost over ten (10) working days in Africa (Anon, 2000).

Malaria is a disease caused by microscopic organisms that live in the blood, destroying the red corpuscles and causing anemia accompanied by characteristic alternating chills, fever and sweating (Metcalf *et al.*, 1962). Malaria is found in 100 countries (Fig. 1) where over 60% of the world population lives (WHO, 1997).

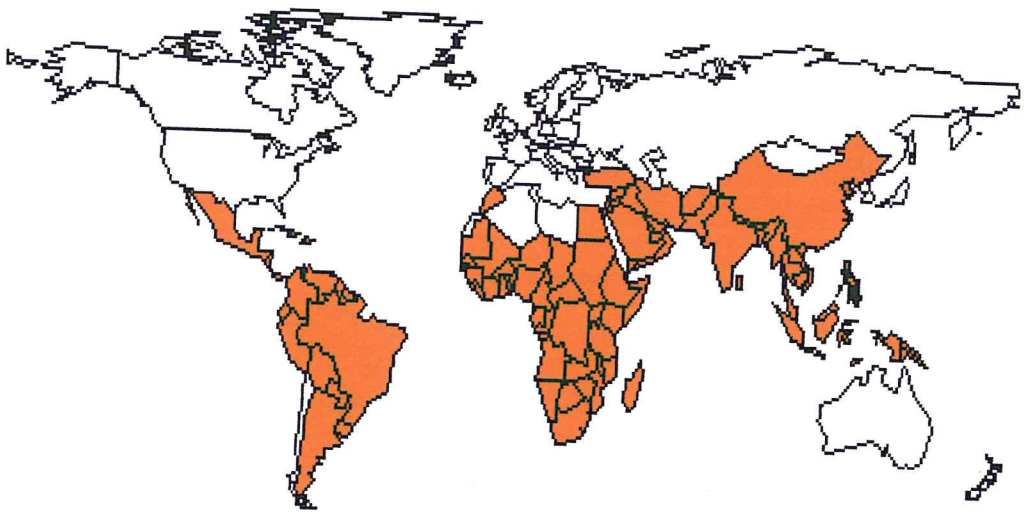


Figure 1. Global malaria distribution (WHO, 1997)

An estimated 500 million clinical cases of malaria are reported each year causing ~ 2.7 million deaths worldwide (Anon, 2001a). Groups at risk include children, pregnant women, refugees, displaced persons, labourers entering endemic areas

and non-immune travelers. Over 90% of these deaths occur in Africa, where 25% of deaths in children below the age of 5 years are attributed to malaria (WHO, 1998). Recent estimates reveal that 74% of all Africans live in malaria endemic areas while 18% live where epidemics are common (Fig. 2). Malaria epidemics affect all age groups and the morbidity and mortality caused overrides the well-known diseases such as AIDS, TB and cancer combined (WHO, 1997). In 1998, five times as many malaria cases were reported as TB, AIDS, measles and leprosy combined (Anon, 2000).

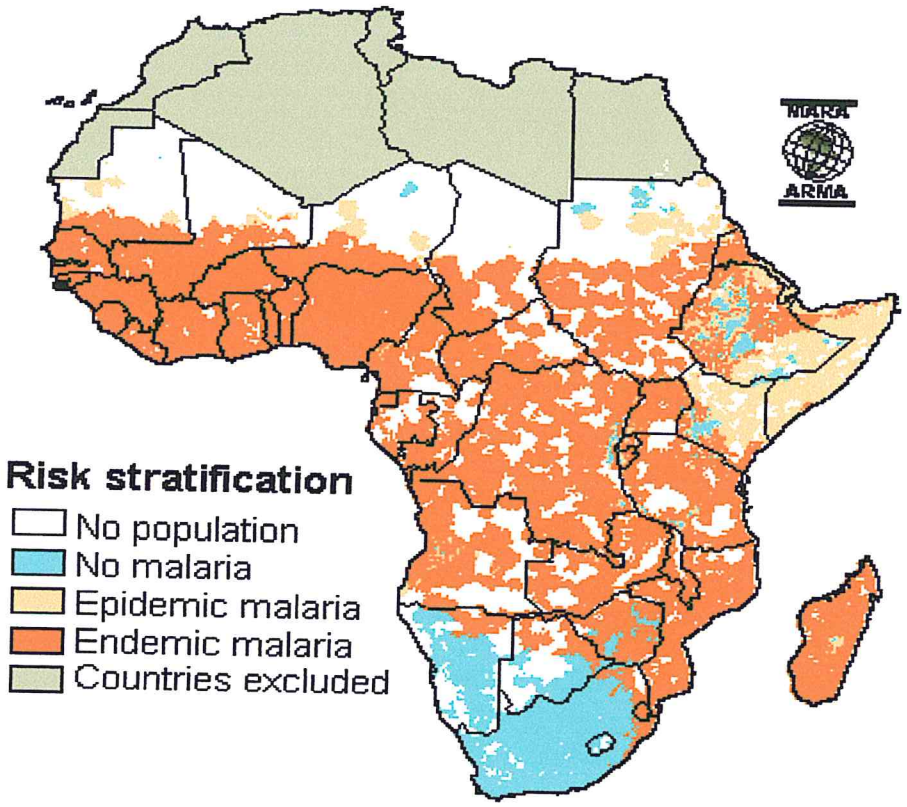


Figure 2. Malaria distribution in Africa (WHO, 1999)

1.1 Malaria prevalence

Malaria epidemics may be attributed to climatic changes such as global warming, *El-Nino* weather phenomenon, and new development projects such as agro-forestry, irrigation, mining, quarrying, logging, road and dam construction amongst others (Palsson, 1999). These conditions provide good breeding

grounds for mosquitoes enabling substantial increase in their densities. Epidemics may also be attributed to the disintegration of national health services, armed conflicts, mass-movement of refugees, vector resistance to insecticides, and emergence of multi-drug resistant strains of the parasite. Malaria attack may depend on; parasite (virulence and cytoadherence); host (immunity, genetics, nutritional status and other infections); vector behaviour like differences in anopheline densities and entomological inoculation rates (EIR) between localities and house holds as well as innate individual differences among people in attracting mosquitoes (De Jong and Knols, 1995; Knols *et al.*, 1995 Lindsay *et al.*, 1993). These factors may affect the rate of inoculation in different locations.

1.2 Malaria parasite

Laveran (1880) discovered that a parasitic micro-organism, *Plasmodium*, causes malaria. Ross (1897) first observed the oocyst and all stages of the parasite (*Plasmodium*) in mosquitoes. Grassi and Felleti (1900) described the developmental cycles of *P. falciparum* and *P. vivax*. They showed that only *Anopheles* mosquitoes transmit human malaria. Malaria in humans has since been shown to be caused by one or more of the four species of *Plasmodium*; *P. vivax, P. ovale, P. malariae* and *P. falciparum*. However, *P. falciparum* is the most virulent species and predominates in the sub-Saharan Africa, Asia, Oceania and Amazons (WHO, 1997).

1.3 Malaria disease

Malaria attack is caused by inoculation of the parasites (*Plasmodium*) by an infected female *Anopheles* mosquito. The parasite undergoes a life cycle constituted of two phases: sexual (sporogony) and asexual (schizogony). Asexual phase occurs in man. Sporozoites inoculated by an infected mosquito develop and multiply in the liver after which they develop into merozoites. The merozoites enter the red blood cells, multiply, rupture the erythrocytes and invade other red blood cells. The incubation period lasts between 6-16 days depending on the parasite species.

The sexual phase of parasite life cycle takes place in female anopheline mosquitoes. A mosquito sucking blood from an infected person picks up the gametocytes that fuse to form a zygote. The zygote penetrates the mosquito's stomach to form an oocyst within which large numbers of slender sporozoites develop. Some of these sporozoites pass through the body cavity while others enter the salivary gland. When an infected mosquito feeds on a new host, it inoculates the infective sporozoites from its salivary gland into the host and the asexual life cycle starts again. Incubation period of the parasites in mosquitoes varies with temperature and the *Plasmodium* species.

1.4 Vectors of malaria

All human malaria vectors belong to the genus *Anopheles* within the family Culicidae that consists of two major sub-families, the Anophelinae and Culicinae. Some 60 species of *Anopheles* are vectors of human malaria and are found in the tropical and sub-tropical regions below 2000 m (Manson Bhar and Bell, 1987). However, there are only three efficient vectors of malaria; *An. gambiae, An. arabiensis* and *An. funestus* (Temu *et al.*, 1998; Palsson, 1999). In sub-Saharan Africa, there are only two important *Anopheles* species, *An. gambiae* and *An. funestus*. These two species are exclusively anthropophilic while *An. arabiensis* also feeds on cattle besides humans. *An. gambiae* (Plate 1) is the most ubiquitous and breeds in quiet water bodies exposed to sunlight whereas *An. funestus* is confined to grassy edges of slow flowing streams (Wigglesworth, 1976).

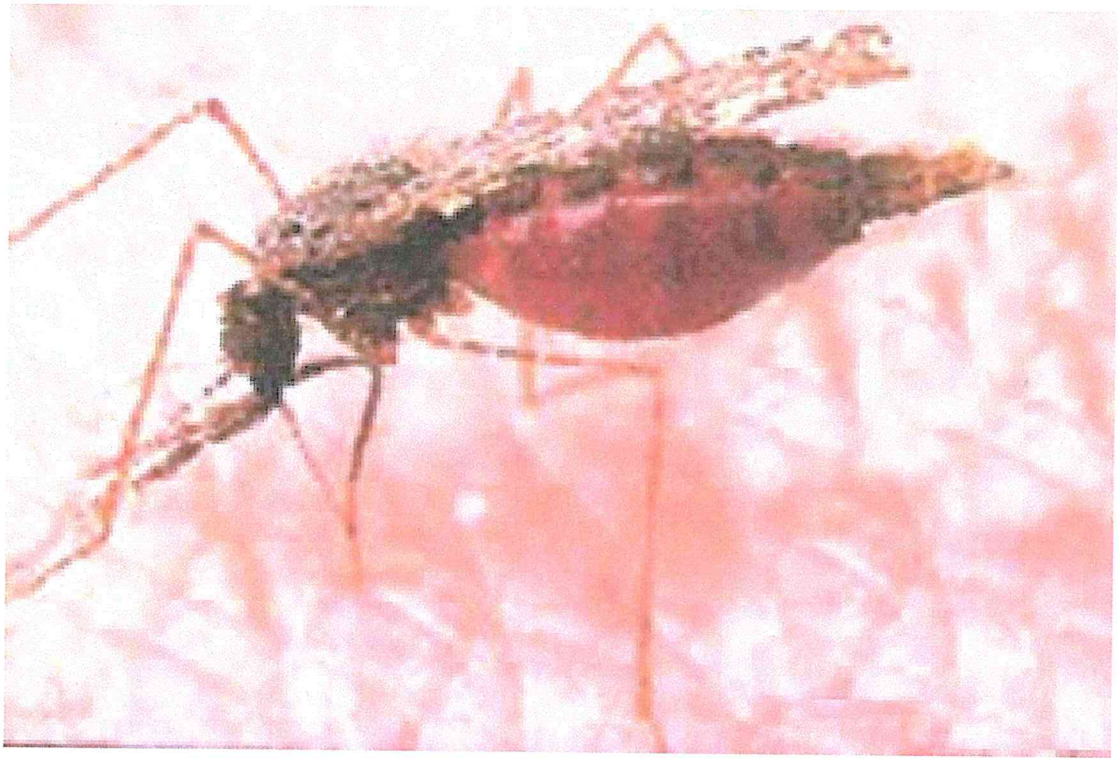


Plate 1. Feeding *An.gambiae*

Anopheline larvae are found in a variety of aquatic habitats such as fresh and salt marshes, lakes, mangrove swamps, rice fields, streams, ponds and even small temporary water bodies like puddles, tree holes, hoof prints, tyre marks, wells and domestic water storage pots. In the tropics, larval development takes 7 days while pupal stage lasts 3 days. Both male and female mosquitoes feed on nectar and other plant fluids, however, female mosquitoes also feed on blood from vertebrates (Clement, 1992). One blood meal is required to produce a batch of 30-150 eggs. In the family Culicidae, *Anopheles* species have the most regular gonotrophic cycle (blood feeding and egg laying), which takes 2-3 days depending on temperature. Different *Anopheles* species have different cycles of activity, some attack at dusk, some during the day, and some at night. Most species are nocturnal. Some species have a strong attraction to man (anthropophilic) and prefer to feed on man (anthropophagic). Zoophilic and zoophagic species are attracted to and feed on animals, respectively. Endophilic and endophagic species normally rest and feed indoor, respectively. Exophilic and

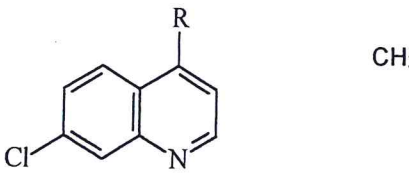
exophagic species rest and feed outdoor. Garret-Jones *et al.* (1980) found that some species may feed indoors on man and fly outside to rest before oviposition in water.

1.5 Malaria control strategies

Various strategies have been adopted towards malaria control. These include chemotherapy (curative and prophylactic), vaccine development, genetically modified (GM) mosquitoes and vector control (personal protection {bed-nets, repellents}, attractants, insecticides and larvicides).

1.5.1 Chemotherapy

Various synthetic anti-malarials are available for curative or prophylactic purposes. Aminoquinolines used to cure malaria include Chloroquine® (**1**), Betaquine®, Amodiaquine®, Mepacrine® (Atebrine™) and Plasmoquine® among others. The amino-quinoline, Primaquine® (**2**) has provided a new agent in the development of prophylactic drugs, it also provides effective cure, but causes severe haemolytic anaemia in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency (Jeffrey, 1998). One of the anti-malarial quinolines, Mefloquine® (**3**) is claimed to be very effective (Clarke, 1996; Croft and Garner, 1997). The structure of Mefloquine® is closer to that of the natural template (Quinine) than chloroquine and analogs. However, parasite resistance to these drugs is on the increase (Brasseur *et al.*, 1992; Edoh *et al.*, 1997; Phillips and Bjorkman, 1990; Serpa *et al.*, 1988; Wongsrichanalai *et al.*, 1992).



$R = HNCH(CH_3)(CH_2)_3N(C_2H_5)_2$

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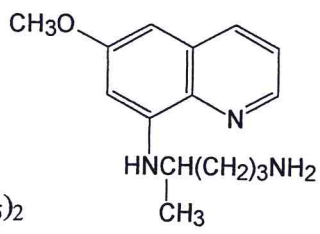

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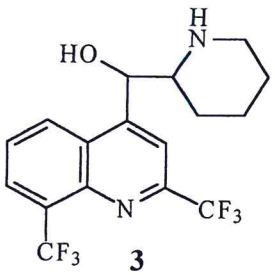

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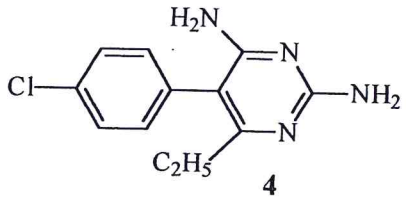
The antifolates, pyrimethamine (**4**) and proguanil (**5**) are the most common prophylactic drugs in malaria control. However, resistance development to this class of drugs has been noted (Edoh *et al.*, 1997).



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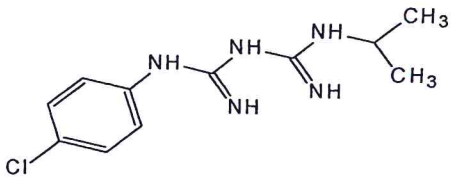

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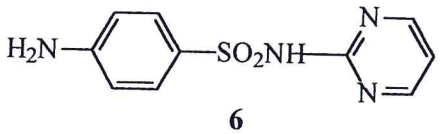
Some of the anti-malarial sulphur-based drugs that have been used to cure malaria include sulfadiazine (**6**), sulfadoxine (**7**) and dapsone (**8**).



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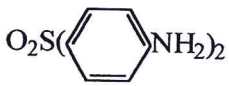

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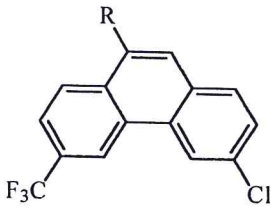
Other anti-malarial candidates purported to be effective against *Plasmodium* include a phenanthrene methanol (**9**) (Halofantrine®).

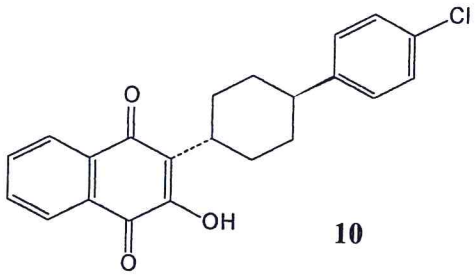


$R = CH(OH)CH_2CH_2N[(CH_2)_3CH_3]_2$

9

Atovaquone (**10**) is a hydroxy-1,4-naphthoquinone analog of ubiquinone which once proved to be a very effective anti-malarial, however, it has recorded very

high therapeutic failures (Dohn *et al.*, 1994; Hughes *et al.*, 1993).



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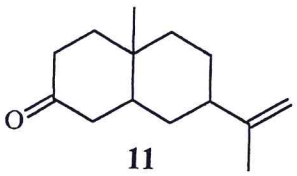
These synthetic anti-malarials have not achieved malaria eradication due to increased cases of drug resistance, high costs and side effects being reported in many parts of the world. Increase in resistance to most of these drugs by *P. falciparum* has been reported. Chloroquine resistance was observed in 1950's along the Thai-Cambodian border (Wongsrichanalai *et al.*, 1992), and in 1960s in Tanzania (first demonstrated in 1982) (Edoh *et al.*, 1997). In Cameroon, resistance was reported in 1986 (Brasseur *et al.*, 1992). In 1987, *P. falciparum* resistance to chloroquine was reported in Nigeria with increased cases of malaria epidemics (Serpa *et al.*, 1988). *P. falciparum* resistance to various alternative anti-malarials had been reported. Resistance to pyrimethamine had been reported in Tanzania as early as 1967 (Edoh *et al.*, 1997) and Amodiaquine® in 1984 (Phillips and Bjorkman, 1990). Mefloquine® resistance had been reported in several places, Tanzania (1983), Nigeria (1987), Thailand (1989) (Phillips and Bjorkman, 1990). Resistance to newer candidate anti-malarials such as Halofantrine® has also been reported (Figgit *et al.*, 1992; Leonardo, 1994).

Several natural products have been tested for *in vitro* anti-plasmodial activity. α-Cyperone (**11**) isolated from *Cyperus rotundus* (Cyperaceae), N-isobutyldeca-2,4-dienamide (**12**), fagaramide (**13**), lupeol (**14**), sesamin (**15**), 4,7,8-trimethoxyfurol[2,3-b]-quinoline (**16**) isolated from *Zanthoxylum gilletii* (Rutaceae), securinine isolated from *Margaritaria discoidea* (Euphorbiaceae) exhibited anti-plasmodial activity (Weenen *et al.*, 1990). Quasinoids, bruceine A,

B, C, D, and brustol isolated from *Brucea javanica* (Simaroubaceae) (O'Neill *et al.*, 1987); rutin and gedunin isolated from *Melia azedarach* (Meliaceae) (Khalid *et al.*, 1986); 16,17-dihydrobrachycalyxolide from *Vernonia brachyclyx* (Asteraceae) (Oketch-Rabah *et al.*, 1998); muzanzagenin, nyasol isolated from *Asparagus africanus* (Liliaceae) (Oketch-Rabah *et al.*, 1997); artemisinin (**17**) from *Artemisia annua* (Asteraceae) and other peroxides from plants in the Asteraceae family have also shown good anti-plasmodial activity. α-Peroxyachfolid from *Achillea millefolium*; 1β-hydroperoxyisonobilin from *Anthemis nobilis*; nardosinon from *Nardostachys chinensis*; rugosal A from *Rosa rugosa* and quinine (**18**) isolated from *Cinchona officinalis* in 1820 (Cragg *et al.*, 1999; Phillipson and Wright, 1991; Rucker *et al.*, 1991), exhibited some good anti-plasmodial activity. Nitidine (**19**) isolated from *Toddalia asiatica* (Rutaceae) (Gakunju *et al.*, 1995), and cryptolepine (**20**) isolated from *Cryptolepis sanguinolenta* (Periplocaceae) (Kanyanga *et al.*, 1997) have also shown interesting results as anti-plasmodial candidates for future development as anti-malarials.



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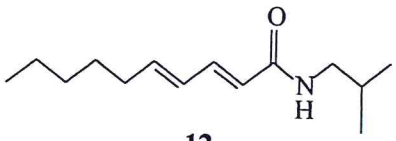

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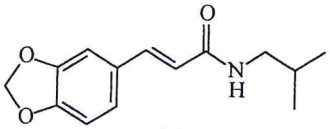

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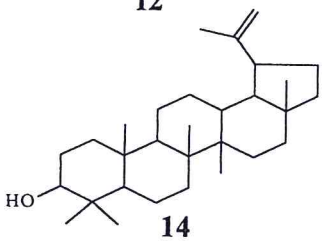

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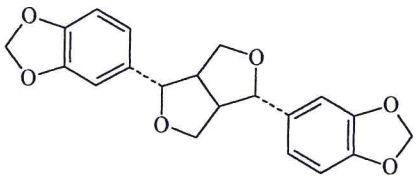

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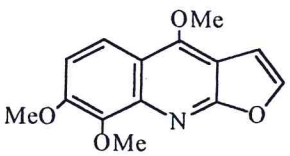

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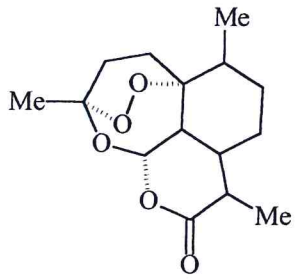

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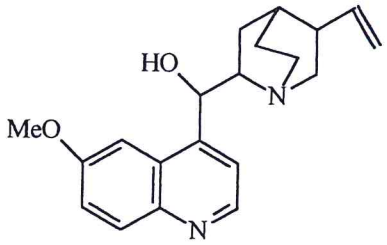

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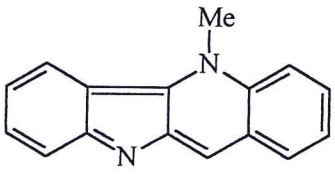

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With the exception of artemisinin (**17**) and quinine (**18**) most plant products have never become clinical agents because they are either not sufficiently active, insufficiently selective, too toxic, or not economically worthwhile (Cragg *et al.*, 1999; Rucker *et al.*, 1991). *P. falciparum* resistance to quinine was reported in 1995 (Jelinek, 1995). Resistance in Kenya is estimated at 80% (Anon. 2001a). Previously, resistance in other African states had been reported; Ghana (1989), Zambia (1988), Malawi (1985), Cameroon and Senegal (1986) (Brasseur *et al.*, 1992; Phillips and Bjorkman, 1990).

Some natural antibiotics, erythromycin, clindamycin, chloramphenical, and tetracycline have also been tested *in vivo* for anti-plasmodial activity against *Plasmodium berghei* with promising results (Kremsner, 1989; Gingras and Jensen, 1993).

Antifolate combination drugs have been attempted in malaria parasite control. Various combinations of dihydrofolate reductase inhibitors (pyrimethamine (**4**), proguanil (**5**) and trimethoprim) and sulfa drugs (sulfadoxine (**7**), dapsone (**8**),

sulfalene and others) have been developed for malaria control. Parasite resistance to these drugs that were once strong anti-malarials when used alone has already been reported. However, when used in combination, these drugs have been reported to produce synergistic effects on the parasite even in the presence of resistance to the individual components. Typical combinations include sulfadoxine/pyrimethamine (Fansidar®), sulfalene-pyrimethamine (Metakelfin®) and sulfamethoxazole-trimethoprim (co-trimoxazole). Lap Dap®, a new antifolate combination of chlorproguanil (**5**) and dapsone (**8**) is currently undergoing trials in Africa (McIntosh and Greenwood, 1998; Watkins, 1997; Wongsrichanalai *et al.*, 1992). This combination had provided promising activity against multiple drug-resistant *P. falciparum* in the Thai-Cambodian border (Shanks *et al.*, 1992). In some areas of south-east Asia, combinations of artemisinins (artesunate, artemether, arteether) and mefloquine® (**3**) offer reliable treatment for uncomplicated malaria due to development of and prevalence of multi-drug resistant falciparum malaria (Prince, 1996; White, 1999).

Malarone® is a new anti-malarial with a combination of atovaquone (**10**) and proguanil chloride (**5**). It is widely accepted by many countries and is extensively used. It has recorded low failure rates in prevention of falciparum malaria in Africa (Lell *et al.*, 1998; Shanks *et al.*, 1998). Resistance status of this drug has not yet been recorded.

The chances of a drug resistant mutant appearing are reduced considerably when combination chemotherapy is used. This is of great importance with new drugs being used, as it is desirable to maintain their efficacy against the multi-drug resistant falciparum parasite for as long as possible. However, development of new drugs is a very costly process, both in time and money. Against all odds, sulfadoxine/pyrimethamine (Fansidar®) resistance was reported in 1981 at the Thai-Cambodian border (Wongsrichanalai *et al.*, 1992).

1.5.2 Vaccine development

Malaria vaccine development was first tried through induction of immunity by sporozoites that had been inactivated by UV light, formalin or mechanical disruption in avian malaria (Mulligan *et al.*, 1941). Vaccination against the exoerythrocytic (EE) cycle of development in mammalian malaria was successfully achieved by use of irradiated sporozoites obtained by dissection of infected mosquito salivary glands or inoculated through the bite of infected, irradiated mosquito (Vanderberg *et al.*, 1969). However, Bray (1976) reported that when children were inoculated on two occasions with *P. falciparum* sporozoites dissected from irradiated infected *An. gambiae*, no immunity was induced against the natural malaria infection. A vaccine, SPf66, has undergone trials in children between ages of 1-15 years. SPf66's protective efficacy was found to be 30% in Tanzania and 9% in Thailand in contrast to its lack of protection in Gambia. This vaccine has however been found to cause mild erythema, induration and tenderness at site of injection (Alonso *et al.*, 1994; D'Alessandro *et al.*, 1995; Migasena *et al.* 1997, Nosten *et al.*, 1996). Most recently developed vaccine that has been tried on humans with promising results is the RTS,S/AS02 which showed 47% protective efficacy in Gambia. Although its protection is incomplete and short-lived, further tests of this vaccine are continuing in Mozambique (Bojang *et al.*, 2001). Some European and South African scientists have reported a breakthrough in development of a vaccine G25, this has only been tried on monkeys and not human subjects (Wengelnik, 2002). Malaria vaccine development has experienced problems such as the mode of antigen presentation and isolation of antigens capable of inducing protective responses against exoerythrocytic forms within the hepatic parenchyma. Other drawbacks in vaccine development involve the short intravascular life of the sporozoites since they do not protect against the subsequent erythrocytic cycle of development, and the complexity of the parasite's life cycle and different faces that it presents to the immune system. The difficulty encountered with attempts to produce *P. falciparum* in large scale employing the currently available culture techniques has also not been overcome. There is need for better methods of merozoite collection with reduced contamination of host-cell components (Bojang *et al.*, 2001; Cohen

et al., 1980), since prolonged cultivation of *P. falciparum* can lead to loss of some of its characteristics suitable for host antigen production. There is still a need for more effective means of controlling malaria attacks besides the use of vaccines that may sometimes fail to work as a result of the factors explained above.

1.5.3 Genetic modification

A new breed of genetically modified (GM) mosquito that will not spread malaria parasites has been created by some scientists. It is hoped that GM mosquito will mate with the normal one when released in the wild. The resulting offspring of a natural male and a GM female mosquito is a GM offspring. However, when a GM male mosquito mates with a natural female, no offspring will result. Either way, malaria control will be achieved. However, survival of the GM mosquito in the wild still poses a great challenge to the scientist. Even if the GM mosquito survives in the wild, mass-production may pose a major challenge (Anon, 2001b; Batler, 1999).

1.5.4 Vector control

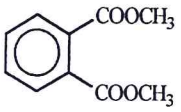
This involves the use of methods of controlling mosquito population at larval or adult stages of their life cycle. Vector control has turned to be the most effective method for malaria control since the lower the anopheline densities, the lower the malaria cases. It is easier to control mosquito populations within a given geographical area than giving vaccines for protection or administration of drugs to individual persons.

1.5.4.1 Personal protection

One suggested effective control is by sleeping under mosquito nets (Christopher, 1939). For a long time, it has been assumed that bed nets could reduce the chances of contracting malaria. This is due to the fact that most *Anopheles* species bite at night.

Repellents have been used in the control of insect bites. These are substances that protect animals, plants or products from insect attack by making food or

living conditions unattractive or offensive. Repellents may be mildly poisonous. Synthetic repellents such as dimethyl phthalate (**21**), 2-ethyl-2-butyl-1,3-propanediol (**22**), *o*-chloro-N,N-diethylbenzamide (**23**), 2-ethyl-1,3-haxanediol (**24**), *n*-propyl-N,N-diethyl-succinamate (**25**) and N,N-diethyl-*m*-toluamide (DEET) (**26**) have not provided a great impact in controlling the rate of inoculation and transmission of malaria parasite. They are volatile and only provide short-lived protection against the vector.



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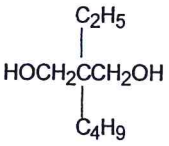

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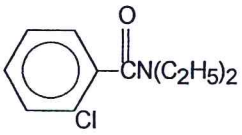

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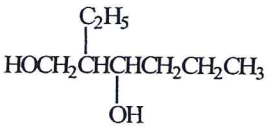

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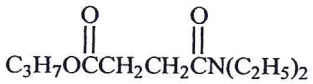

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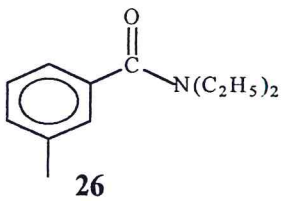
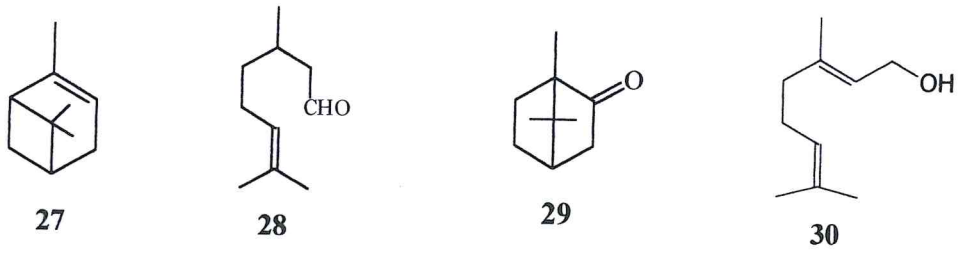


26

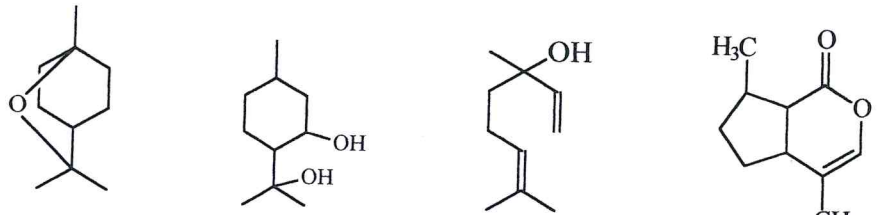
Some of the well-known synthetic commercial repellents like DEET (**26**) have been reported to be ineffective against anopheline mosquitoes. DEET is an irritant, and may initiate skin allergy in certain individuals (Carl *et al.*, 1991).

Traditionally, use was made of plants such as *Ocimum spp* (Labiatae) (Chogo and Crank, 1981; White, 1973), *Azadirachta indica* (Meliaceae), *Hyptis suaveolens* (Lamiaceae), *Daniellia olivera* (Caesalpiniaceae), *Lantana spp* (Verbenaceae), *Ajuga remota*, and *Nepeta cataria* (Labiatae) among others to repel mosquitoes from houses (Anon 2001c; Eisner 1964; Palsson, 1999; Sharma *et al.*, 1993). These plants were smoldered to produce smoke carrying chemical compounds that would repel mosquitoes away from the house. This reduced mosquito numbers indoor and rates of inoculation (White, 1973; Palsson, 1999; Sharma *et al.*, 1993).

Lately, use is being made of essential oils extracted from some of these plants. These include α-pinene (**27**), citronellal (**28**), camphor (**29**), geraniol (**30**), 1,8-cineole (**31**), *p*-menthane-3,8-diol (**32**) linalool (**33**) and nepetalactone (**34**). (Curtis *et al.*, 1987; Dethier, 1947; Granett, 1940).



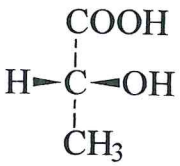
27 **28** **29** **30**



31 **32** **33** **34**

1.5.4.2 Attractants

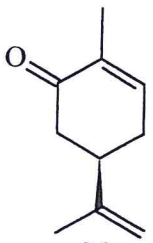
Research has shown that there are innate individual differences among people in attracting mosquito. The blend of attractants from human body is not well understood. However, carbon dioxide (CO_2) and L-lactic acid (**35**) have been extensively studied and shown to be among the major attractants (Kline *et al.* 1990; 1991ab).



35

Some chemicals such as D-carvone (**36**) and 1-octen-3-ol (**37**) have been tested and found to be good mosquito attractants. Consequently D-carvone has found use as bait enhancer in the application of acute bio-toxins in mosquito control

(Jerry *et al.*, 1998). Products such as Detur® and Flowtro® octenol have been developed from 1-octen-3-ol (Canyon and Hii, 1997).



36

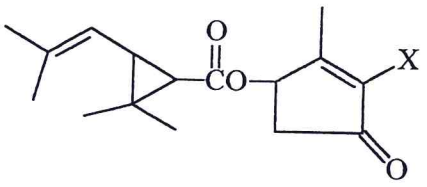
$H_3C(CH_2)_4C(OH)CH{=}CH_2$

37

From the knowledge gathered on mosquito attractants, instruments such as "mosQUITo™ trap" and Mosquito Eradicator® have been developed. Mosquito Eradicator® makes use of CO_2 (breath mimic), lactic acid (sweat mimic) and spectrum light as an added attractant. The "mosQUITo™ trap" uses colourless, odourless standard CO_2 to attract mosquitoes and other blood-sucking insects from a distance. A combination of lactic acid and octenol acts as a medium range attractant and a guide towards the unit, whereas heat and moisture in the unit lures the mosquitoes and other blood-sucking insects into the trap entrance (close range attractant). Once in the trap, UV light attracts the flying insects ensuring that they do not escape. A small fan in the unit blows the trapped insects into the trap attractant solution tray, where they drown (Anon, 1999).

1.5.4.3 Insecticides

Insecticides (natural or synthetic chemical compounds that kill a target insect) have been used in vector control for about 100 years. Various classes of synthetic insecticides have been used in control of mosquito larvae and adults. These include inorganic (arsenates, fluorates, fluorides, cuprates), organic (organochlorides such as DDT, DDE, TDE, methoxychlor; organophospates such as parathion, fenthion, temephos; carbamates such as propoxur, bendiocarb; synthetic pyrethroids such as allethrine (**38**), cyclethrine (**39**), dimethrine, permethrine, biopermethrine, phenothrin, fenvalerate, resmethrin, bioresmethrin, and decamethrin) among others (Brown, 1986; Kirk and Orthmer, 1981; Metcalf *et al.*, 1962).

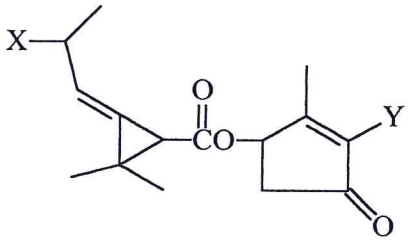


X = C_3H_5 **38**

X =  **39**

Development of resistance by mosquitoes to compounds used against them as adulticides was first observed in 1947, when salt-marsh mosquitoes, *Aedes taeniorhynchus* and *Ae. sollicitans* began to show resistance to DDT in Florida. Since then, populations that have developed resistance to organochlorines (DDT and/dieldrin) have been known in 109 mosquito species throughout the world. Some 58 species had developed resistance to organophosphate (OP) insecticides, of which 4 had not been reported as organochlorine resistant. Also among these species, 17 have now shown adult resistance to carbamates propoxur or bendiocarb, and 10 have shown either resistance or cross-resistance to certain pyrethriods. Multiple resistances to all 4 of the above-mentioned chemical groups in the same population of mosquito species have been reported in certain areas (Brown, 1986). Resistance to these insecticides had been reported in different parts of the world. OP resistance was reported in Pakistan in 1986 (Scott and Georghiou, 1996), in the USA in 1990 (Mekuria *et al.*, 1994). Other reports of resistance to chlorpyrifos, dichlorvos and pirimiphos methyl had been documented as matters of concern (Georgiou *et al.*, 1987; Wirth and Georghiou, 1996).

The best-known examples of botanical insecticides are from the plants *Chrysanthemum* (Asteraceae), *Tephrosia*, *Derris* (Leguminosae), and *Nicotiana* (Solanaceae). These plants have given effective insecticidal compounds. *Chrysanthemum* extracts have been used extensively in mosquito control. The activity of these extracts is attributed to the synergistic effect of a mixture of pyrethrins; pyrethrin I (**40**) and II (**41**), cinerin I (**42**) and II (**43**), and jasmolin I (**44**) and II (**45**) (Kirk and Orthmer, 1981).

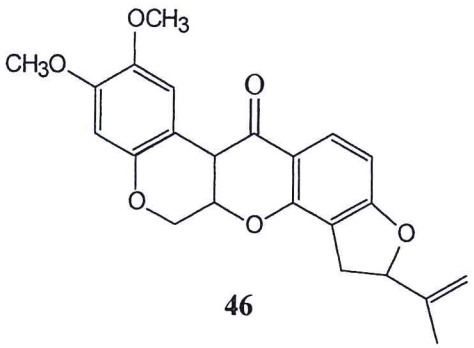


X	Y	
CH_3	C_3H_7	**40**
CH_3OCO	C_5H_7	**41**
CH_3	C_4H_7	**42**
CH_3OCO	C_4H_7	**43**
CH_3	C_5H_9	**44**
COOMe	C_5H_9	**45**

High cost of isolation of natural pyrethrins makes them expensive and uneconomical. This encouraged the development of synthetic derivatives modeled on the natural compound. The synthetic pyrethroids that have been used in mosquito control are allethrine (**38**), cyclethrine (**39**), dimethrine, permethrine, biopermethrine, phenothrin, fenvalerate, resmethrin, bioresmethrin, and decamethrin (Kirk and Orthmer, 1981; Metcalf *et al.*, 1962).

Although these synthetic pyrethroids have lower mammalian toxicity, they have high degree of environmental persistence than the natural pyrethrins, high toxicity to fish and beneficial insects like bees (Kirk and Orthmer, 1981).

Roots of *Derris elliptica*, *D. malaccensis*, *Lonchocarpus utilis*, *L. urucu* and *Tephrosia virginiania* have yielded rotenone (**46**). This compound has been known to be a good mosquito larvicide. Its toxicity to various insects has been known since 1884 leading to its isolation in 1902. The toxicity of rotenoids to fish and other beneficial insects has prevented their development into commercial insecticides (Kirk and Orthmer, 1981; Metcalf *et al.*, 1962).



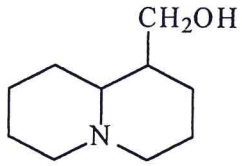
46

Nicotiana tobacum and *N. rustica* (Solanaceae) have yielded two well-known insecticides, nicotine (**47**) and anabasine (**48**). Nicotine has been used for a long time in mosquito larvae control. The toxicity of these compounds to other organisms including mammals has prevented their wide-scale application (Campbell and Sullivan, 1933).

47

48

The quinolizidine alkaloid lupinine (**49**) had also been reported to have larvicidal activity against *Culex pipiens*, *C. territans* and *C. quinquefasciatus*. Quinolizidine alkaloids are known to have non-specific insecticidal activity (Campbell and Sullivan, 1933).

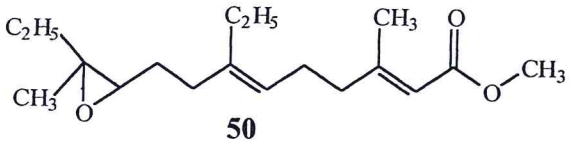


49

With the exception of pyrethrins, most of these compounds never found wide application against insect vectors because of their toxicity to non-target organisms such as fish (Campbell and Sullivan, 1933; Kirk and Orthmer, 1981; Metcalf *et al.*, 1962).

Insect Growth Regulators (IGRs) have been used to control mosquito densities. IGRs are chemicals which inhibit/disrupt insect growth. These chemicals mimic the action of insect growth regulatory hormones such as ecdysone and juvenile hormone. They prevent normal maturation of insect larvae. IGRs that interfere with biochemical and physiochemical processes that are unique to the arthropods,

such as moulting, ecdysis and formation of the chitinous exoskeleton, are much more selective insecticides. Most of these compounds have been grouped as juvenile hormone mimics or chitin synthetase inhibitors. Some known juvenoids are neonetin (**50**), R-20458 (**51**), hydropene (**52**), and methoprene (**53**) (Kirk and Orthmer, 1981).



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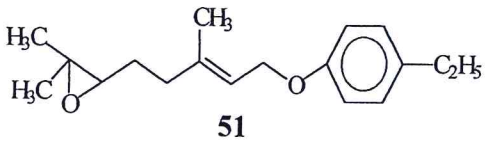

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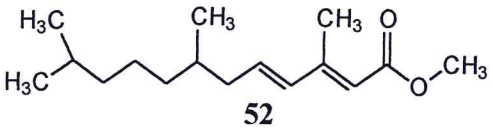

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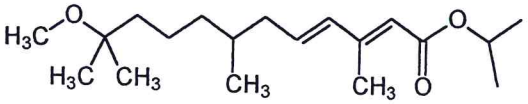


53

The best-known synthetic chitin synthesis inhibitor is diflubenzuron (**54**) (Kirk and Othmer, 1981).

54

Successful laboratory tests had been done with IGR's such as S-31183 [1-(4'-phenoxyphenoxy)-2-(2'-pyridoxy)propane] and S-21149 [*o*-(2-4'-phenoxy-phenoxy)ethyl propionaldoxime]. A carbamate, fenoxycarb (RO13-5223) [Ethyl-p-phenoxyphenoxy-ethylcarbamate] has also been tested with interesting results (Mulla *et al.*, 1986). Currently, the most widely used IGR in Europe is Altosid® that contains methoprene as the active ingredient. Methoprene is claimed to have low toxicity to fish and birds. Altosid®, a methoprene based product is applied to ditches, ponds, marshes or flooded areas (Knepper *et al.*, 1992). It is only applied to non-drinking water sources.

IGRs are presumed to have little or no toxicity to other non-target organisms. But long-term cumulative effect of the synthetic analogs may not be absolutely ruled out in non-target organisms (Mulla *et al.*, 1986). IGRs are relatively specific to the insect and are primarily active against the immature stages of mosquitoes, flies and other insects. Some IGRs induce sterility while others induce reproductive anomalies in the adult stage (WHO, 1996b).

1.5.4.4 Larval control

Mosquito larval control has proved to be the most effective means of controlling anopheline densities. The mosquito life cycle is cut before the emergence of adults, which bite and transmit malaria. Besides, habitats of adult mosquitoes are likely to be less accessible than larval breeding sites (Jamieson *et al.*, 1994; Rattanarithikul *et al.*, 1995).

Use of classical larvicides such as kerosene to control mosquito population in small pools was rampant before the discovery of other synthetic larvicides (Wigglesworth, 1976). This method as is popularly believed does not suffocate the larvae by preventing their breathing at water surface. Some of the oil enters the tracheal system and act as poisons that presumably affect the nervous system (Wigglesworth, 1976). *Anopheles* larvae below such film at 24^0C should all be dead in two to three hours. This has since been replaced by use of synthetic compounds such as inorganic (arsenates, fluorates, fluorides, cuprates), organochlorines (DDT, DDE, TDE, methoxychlor), organophospates (parathion, fenthion, temephos), and carbamates (propoxur, bendiocarb) among others.

Paris green $\{Cu(C_2H_3O_2)_2.3Cu(AsO_2)_2\}$ dust has been used to control mosquito larvae at 1 to 2 pounds per acre. Cupric metarsenite $\{Cu(AsO_2)_2\}$ has also been used to a limited scale. Most of these inorganic larvicides are however highly toxic to most aquatic organisms and plants because of relatively large amounts of water-soluble arsenic in them. Cuprous cyanide (CuCN) and zinc phosphide (Zn_3P_2) have also been used as stomach poisons for mosquito larvae.

Synthetic organic chemicals (organochlorines, organophosphates and carbamates) have been used in mosquito larval control.

Various organochlorine representatives have been used to control larval populations. Use of emulsions or granular formulations of DDT, TDE, DDE, methoxychlor, dieldrin, chlordane, heptachlor, or lindane at 0.05 to 1.0 pounds per acre of water body has been attempted.

Organophosphorus (OP) insecticides have been widely used where strains resistant to organochlorines have been encountered. Fenthion, fenitrithion, malathion, dichlorvos, dicapthon, chlorthion, ronnel, parathion and temephos have been used in mosquito larvae control. The OP insecticides are good larvicides as they are persistent, hence long-term control can be achieved.

Carbamates such as propoxur have been used to control household insects. They have also found use as potential mosquito larvicides. Carbamates are bio-degradable, have low soil persistence and toxicity to fish.

Environmental management in mosquito control may involve covering all open water surfaces, removal of non-essential water containers from around houses, such as jars, storage pots and tins. This protects breeding places and prevents adult mosquitoes from laying eggs in these places. Draining the breeding sites into the sea has controlled mosquito larvae. "Flushing" has been used to control stream-breeding mosquitoes (MacDonald, 1939). It is believed that larvae migrating to the margins of stream are stranded as the flow subsides and die. However, this method requires more study.

Larvae control has been achieved through shading. This method was successfully used by Ramsay (1930) in Assam and northern Bengal against *An. minimus.* It was found that when suitable bushes were planted along the streams so that they are densely shaded, *An. minimus* disappeared. The cause for disappearance is still a matter of speculation. Contrary to the general belief, the stream breeding

larvae have weak powers of resistance to flowing water. *An. minimus* larvae cannot remain anchored if the rate of flow exceeds 0.29 ft/s. They normally find the still water necessary for their security in the grassy margins and remain there to avoid moving water, light and to find shade.

Biological control implies the use of predators, parasites or entomo-pathogens, against a given vector or parasite. This form of control has taken three major dimensions; larvivorous fish, bacteria, and nematodes, algae and fungi.

Attempts have been made to control mosquito larvae by these means. Larvivorous fish such as *Gambusia affinis* (Meisch, 1985) have been used. Previously, other fishes such as Amargosa pupfish (*Cyprinoden nevadensis amargosae*) and Guppies (*Poecillia reticulata*) were used (Moyle, 1976). These fish species feed on the larvae and thus control their levels at the points of introduction. Predatory activity of *Fundulus zebrinus*, a North American indigenous fish, has also been studied extensively and found to be comparable to the activity of the mosquito-fish, *Gambusia affinis* (Nelson and Keenan, 1992). Laboratory experiments also showed that a cyprinodontid fish, *Aphanius mento*, could be used to control *Culex pipiens* (Blaustein and Byard, 1993). It had recently been reported that in Namanjalal sub-location in Kitale, *Tilapia* species are bred to feed on mosquito larvae with great success (Anon, 2001a).

Although, use of mosquito feeding fish has succeeded experimentally, large-scale application in the field has experienced a number of setbacks. During rainy seasons, which are also peak of malaria transmission periods, there are uncountable rainwater pools that furnish mosquitoes with adequate and favourable breeding sites, which are less favourable for fish survival. Besides, some of these fish (*G. affinis*) are unable to survive in flood muddy-water (Mahmoud, 1985). *G. affinis* give birth to live fingerlings and therefore have small fecundity. Difficulties in their mass rearing represent a major obstacle in increasing their effectiveness (Joseph *et al.*, 1987). Agricultural practices have contributed to fish kills, thus requiring continuous need for restocking. To date,

there is no mosquito-feeding fish production adequate for stocking on a large operational scale (James *et al.*, 1985). These fishes may have a negative effect on other fish species when introduced in areas outside their normal habitat (Nelson and Keenan, 1992).

Toxins produced by certain *Bacillus* species have shown excellent results in attempts to control mosquito larvae. The *Bacillus* species that have been used include *Bacillus thuringiensis H-4* and *Bacillus sphaericus* (WHO, 1996a). Commercially available formulations of *Bacillus thuringiensis* var. *israelensis* iclude Bactimos®, Vectobac® and Tenkar®. Major isolates of *Bacillus sphaericus* are 1593 (IF-119) and 2362 (IF-118). These formulations and isolates were tried against *An. gambiae* larvae in Ougadougou (Giancarlo *et al.*, 1987). These bacteria species have high molecular weight proteins; this leads to their sinking at the bottom. Since mosquito larvae are surface feeders, efficacy of these bacterial formulations is questionable, unless a good base is used to suspend them at the surface. Studies have shown that *Bacillus sphaericus* die quickly in hot sun, and have short persistence thus requiring regular application. As a result of this, high levels of resistance have been reported in a field population of *Culex quinquefasciatus*. This is likely to spread among other mosquito species due to continued exposure (Barbazan *et al.*, 1997; Rao *et al.*, 1995).

Laboratory tests of a fungus, *Tolypocladium clindrosporum*, against *Aedes agypti*, *Ae. vexans* and *Ae. triseriatus*, gave interesting results. Field application has not been undertaken hence stability of this fungus under normal environmental conditions has not been established (Goettel, 1987; Nadeau and Boisvert, 1994). Suspension and supernatant of algae, *Chlorella ellipsoide*, were found to be toxic to *Culex quinquefasciatus* under laboratory conditions. This indicates the potential candidacy of this alga for use in mosquito control (Dhillon and Mulla, 1981). In the rice fields where mosquitoes are a great menace, use has been made of a mermithid nematode (*Romanomermis culicivorax*) and fungus (*Lagenidium giganteum*). The mermithid nematode and entomo-pathogenic fungi have demonstrated little or no adverse effects on populations of non-target

vertebrate and invertebrate organisms (Lawrence and Cynthia, 1990). The potentials of this method in large-scale mosquito larvae control are yet to be realized.

Use has also been made of botanical derivatives such as pyrethrins that are highly toxic to the mosquito larvae, environmentally friendly and less toxic to man (Kirk and Othmer, 1981; Metcalf *et al.*, 1962).

Extracts from some plants have exhibited insecticidal and IGR properties. Most of these plants have not been studied chemically. Their study may unveil environmentally friendly and target specific compounds for mosquito control (Supavarn *et al.*, 1974).

1.6 Justification

Malaria has turned out to be a very important vector-borne disease. A lot of research money is being spent on means and ways of reducing malaria occurrence and the mortality it causes. This money could otherwise be channeled to other sectors such as education and agricultural research that need it badly.

Bed-nets as a means of containing the disease has not been put into good and effective practice, as insecticide treated nets (ITN) are expensive to most people. Besides, some mosquitoes still find their way into the nets hence their limitation as a control tool if used alone. World Health Organization (WHO), through Roll Back Malaria (RBM) program in Africa, has focused on intensifying the use of insecticide treated bed nets (INT). This is intended to prevent mosquito bites at night. However, the current use of only one class of insecticide (pyrethroids) to treat nets has led to the development of resistance by anopheline mosquitoes.

Insecticide sprays and methods of dispersing mosquitoes have also been attempted. However, increased cases of resistance to these insecticides by adult mosquitoes, and their migration to other places that have not been sprayed have further rendered this practice inefficient.

Synthetic insecticides have been used as effective larvicides. However, these compounds are expensive, carcinogenic, accumulate in the biotic chain, toxic to beneficial insects (bees), aquatic organisms (fish) and mammals, and have prolonged persistence in the environment (pollution). Resistance to most of these insecticides has been reported. Still, control of mosquito larvae before emergence of adults remains the most efficient and economical means of controlling malaria epidemics besides chemotherapy, vaccination, use of insecticides and repellents as supplements.

The classical method that has been used to kill mosquito larvae involves the application of oil on water surface. However, what is the fate of other aquatic organisms? This question arises since an oil film on water surface is likely to prevent free exchange of oxygen between water surface and the free air and can lead to suffocation of other organisms in the water. This factor has prompted the employment of other means of control of mosquito larvae.

Environmental management as control tool for mosquito larvae may be achieved by reduction of number of larval habitats. This is done to a point whereby the density of emerging adult populations would be too low for transmission of disease to occur. However, the huge costs involved make this approach impractical in large-scale operations.

Widespread drug resistance has been reported in nearly all classes of drugs. More so, development of new drugs is a costly process both in time and money.

To date, no effective vaccine has been developed against malaria. This process is equally expensive and it will take a long time to develop a vaccine that will be protective and safe to humans.

1.7 Objectives

The general objective of this research project was to examine the larvicidal activity and/or growth inhibition of extracts from various plants against *An. gambiae* larvae.

The specific objectives were to collect and extract plants within the families with potential mosquito larvicidal activity, and screen their extracts for activity against *An. gambiae*. We also intended to carry out detailed phytochemical investigation of plants showing good performance as larvicides, isolate and determine the chemical structures of the active principles.

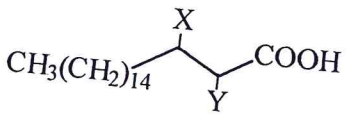
CHAPTER 2: LITERATURE REVIEW

2.0 Isolated phytochemical larvicides

Many plants have been extracted and tested for mosquito larvicidal activity (Saxena *et al.*, 1993; Sukumar *et al.*, 1991; Minjas and Sarda, 1986). Some of these plants have been investigated and the isolated compounds show potent activity. However, many remain un-investigated and the chances of finding potent larvicides remain high. In as much as this approach has not been used widely, its potential in mosquito control is high.

Several natural organic chemicals have been extracted from some of these plants and assayed for efficacy against mosquito larvae. It has been shown that some limonoids, alkaloids, flavonoids, thiophenes, quinones, butyl-amides, fatty acids, chromenes, terpenoids, curcuminoids, tumerones, trialcohols, and phenyl propanoids, extracted from plants show high degree of larvicidal activity against mosquito larvae.

Some branched chain fatty acids, 2-ethyloctadecanoic acid (**55**), 3-methyloctadecanoic acid (**56**), and 2,3-dimethyloctadecanoic acid (**57**), have also displayed good larvicidal activity against *Culex pipiens* and *C. quinquefasciatus* (Ikeshoji and Mulla, 1974).



X	Y	
H	CH_3CH_2	**55**
CH_3	H	**56**
CH_3	CH_3	**57**

1,2,4-Trialcohols of long chain hydrocarbons isolated from unripe avocado fruit, *Persea americana* (Lauraceae), showed larvicidal activity against *Aedes aegypti*. These include 1,2,4-trihydroxynonadecane (**58**), 1,2,4-trihydroxyhepta-dec-16-ene (**59**), and 1,2,4-trihydroxyheptadec-16-yne (**60**) (Oberlies *et al.*, 1998).

$CH_3(CH_2)_{14}CH(OH)CH_2CH(OH)CH_2OH$

58

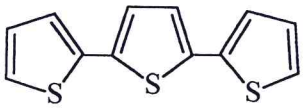
$CH_2CH(CH_2)_{11}CH(OH)CH_2CH(OH)CH_2OH$

59

$HCC(CH_2)_{11}CH(OH)CH_2CH(OH)CH_2OH$

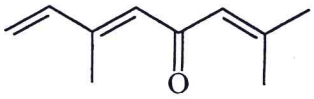
60

α-terthienyl (**61**), a naturally occurring thiophene isolated from *Ajuga remota* (Lamiaceae), has been reported to exhibit high phototoxic larvicidal activity against mosquito larvae (Arnason *et al.*, 1981; Kagan *et al.*, 1987).



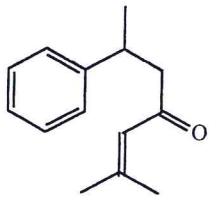
61

The larvicidal activity of a terpenoid, 5(*E*)-ocimenone (**62**), isolated from *Tagetes minuta* (Compositae) has been reported against *Aedes aegyti* (Maradufu *et al.*, 1978).

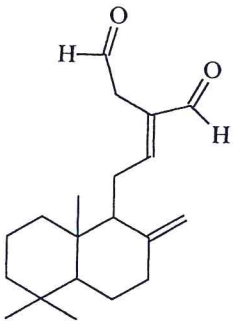


62

Larvicidal activity of *ar*-tumerone (**63**), fractionated from the volatile oil of rhizomes of *Curcuma longa* (Zingiberaceae) against *Aedes aegypti* has also been reported (Roth *et al.*, 1998). The leaves also yielded λ-8(17),12-diene-15,16-dial (**64**) with larvicidal activity against the same species (Roth *et al.*, 1998).

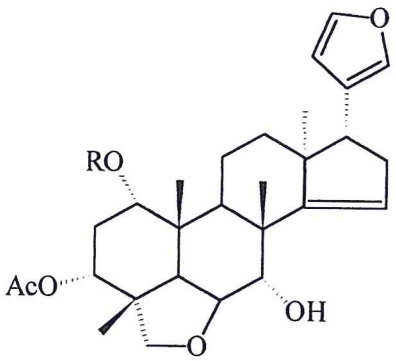


63

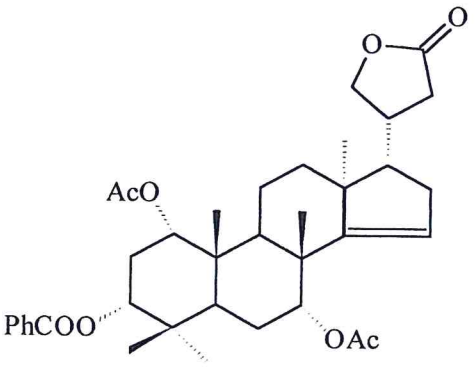


64

Several triterpenoids have also been reported to exhibit larvicidal activity. Meliavolkinin (**65**), 1,3-diacetylvilasinin (**66**) and melianin B (**67**) have been reported to posses mosquito larvicidal activity against *Aedes aegypti*. These compounds were isolated from *Melia volkensii* (Meliaceae) (Rogers *et al.*, 1998).

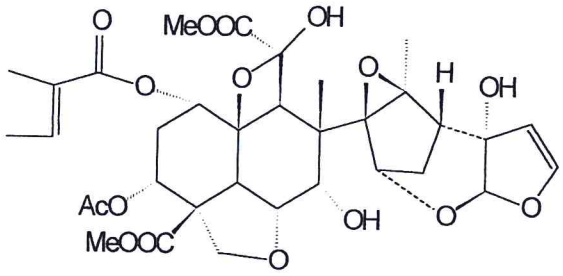


X = PhCO **65**
X = Ac **66**



67

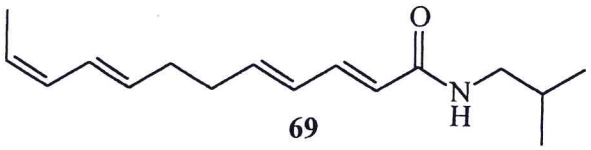
Azadirachtin (**68**), a limonoid isolated from *Azadirachta indica* (Meliaceae) was reported to have larvicidal activity against various mosquito species (Zebitz, 1984; 1986).



68

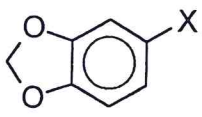
Larvicidal activity of some long chain fatty acid amides had been reported. Pellitorine [N-(2-methylpropyl)-(*E,E*)-2,4-decadienamide] (**12**) isolated from *Fagara macrophylla* (Rutaceae), was found to be active against *Culex pipiens* larvae (Kubo *et al.*, 1984). The same compound was isolated from *Achillea millefolium* (Asteraceae) and found to be active against *Aedes triseriatus* (Lalonde *et al.*, 1980).

N-isobutyl-2*E*,4*E*,8*E*,10*Z*-dodeca-2,4,8,10-tetraenamide (**69**) isolated from *Spilanthes mauritiana* (Asteraceae) was also reported to have larvicidal activity against *Ae. aegypti* (Jondiko *et al.*, 1986)



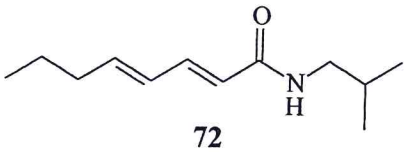
69

Other isobutyl-amides isolated from *Fagara macrophylla* (Rutaceae), piperlongumine (**70**), fagaramide (**13**), 4,5-dihydropiperlongumine (**71**) and N-isobutyl-2*E*,4*E*-octadienamide (**72**), have also demonstrated good activity against *Culex pipiens* larvae (Kubo *et al.*, 1984).



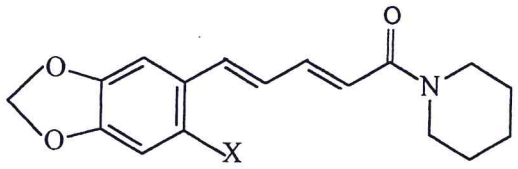
$X = (CH)_4CONHCH_2CH(CH_3)_2$ **70**

$X = (CH_2)_2(CH)_2CONHCH_2CH(CH_3)_2$ **71**



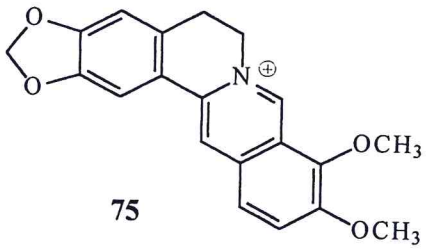
72

Piperine (**73**) and wisanine (**74**) are piperidine alkaloids that were isolated from *Piper guineense* (Piperaceae) and found to be effective on *Aedes aegypti* larvae (Addae-Mensah and Achieng, 1986).



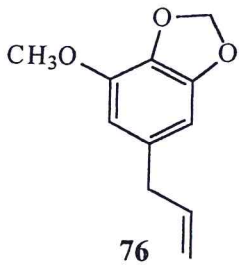
X = H, Piperirne **73**
X = OCH_3, Wisanine **74**

Berberine (**75**), a naturally occurring isoquinoline alkaloid, is present in at least nine botanical families; Annonaceae, Berberidaceae, Jungladaceae, Magnoliaceae, Menispermaceae, Papaveraceae, Ranunculaceae, Rubiaceae and Rutaceae. Its activity against *Aedes atropalpus* in the presence of near UV has been reported. (Philogéne *et al.*, 1984).



75

Phenylpropanoids, myristicin (**76**), elemicin (**77**), and *trans*-isoelemicin (**78**), together with a furanocoumarin, oxypeucedanin (**79**), isolated from the leaves of *Diplolophium buchanani* (Umbelliferae), exhibited larvicidal activity against *Aedes aegypti* (Marston *et al.*, 1995).



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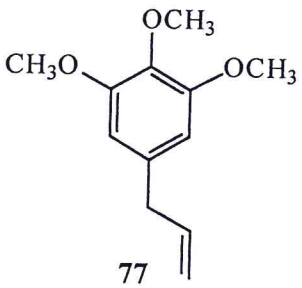

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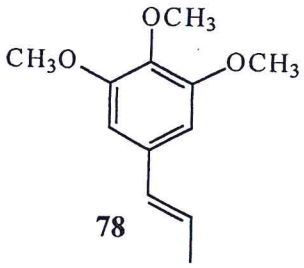

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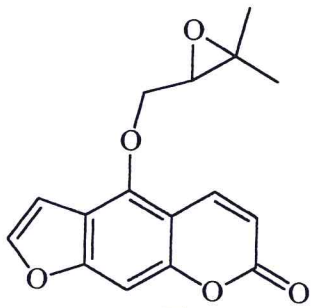
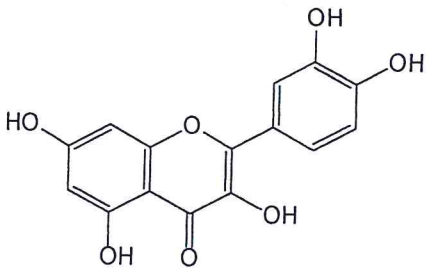


79

Flavonoids such as quercetin (**80**) and 2′,6′-dihydroxy-4′-methoxydihydrochalcone (**81**) isolated from *Polygonum senegalense* (Polygonaceae) have been reported to posses larvicidal activity on *Aedes aegypti* larvae (Gikonyo *et al.*, 1998).



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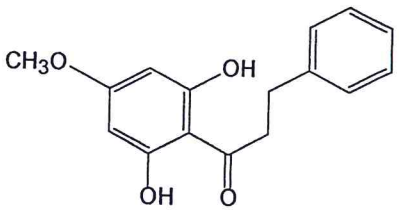
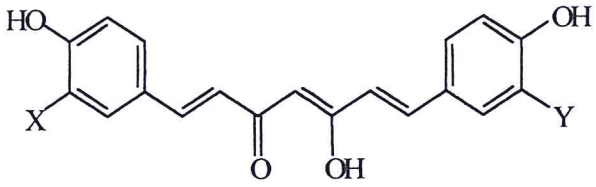


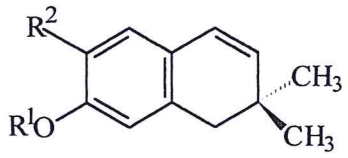
81

Curcuminoids isolated from the rhizomes of *Curcuma longa* (Zingiberaceae), exhibited larvicidal activity against *Aedes aegypti*. These include curcumin I (**82**), curcumin II (**83**), and curcumin III (**84**) (Roth *et al.*, 1998).



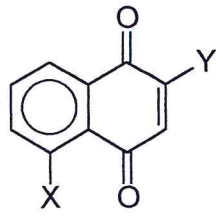
X	Y	
OCH_3	OCH_3	**82**
H	OCH_3	**83**
H	H	**84**

Chromenes isolated from the volatile oil of *Hemizonia fitchii* (Asteraceae), have shown larvicidal activity against *Culex pipiens*. These include encecalin (**85**), chromene (desmethyl-encecalin) (**86**) and 6-vinyl-7-methoxy-2,2-dimethylchromene (**87**) (Klocke *et al.*, 1985).

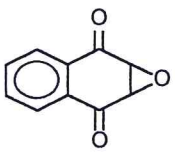


R^1	R^2	
CH_3	Ac	**85**
H	Ac	**86**
CH_3	$-CH_2CH_2-$	**87**

Some quinones isolated from *Plumbago zeylenica* (Plumbaginaceae) displayed effective larvicidal activity against *Aedes aegypti*, these include plumbagin (**88**), juglone (**89**), 2-methyl-1,4-naphthoquinone (**90**), 1,4-naphthoquinone (**91**), 2-hydroxy-1,4-naphthoquinone (**92**), 2,3-epoxy-1,4-naphthoquinone (**93**), 1,4-benzoquinone (**94**), and 1,2-naphthoquinone (**95**) (Hassanali and Lwande, 1989).

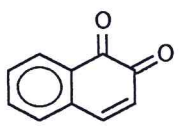


X	Y	
OH	CH_3	**88**
OH	H	**89**
H	CH_3	**90**
H	H	**91**
H	OH	**92**



93

94



95

There is a wide diversity in structures of compounds showing larvicidal activity and by no means is this list exhaustive. In as much as this approach has not been used against the major malaria vectors, *Anopheles gambiae*, *An. arabiensis* and *An. funestus*, the probability of containing malaria in any region of the world is very high. As long as a pure isolated compound, or extract mixture is stable under various environmental conditions, there is no limitation to application of this method in stamping out malaria.

In our bio-prospecting survey, two plants *Zanthoxylum gillettii* and *Pittosporum mannii* showed good larvicidal activity.

2.1 *Zanthoxylum gillettii* (De. Wild.) (Syn *Fagara macrophylla*)

Z. gillettii (De Wild.) Waterm. (Plate 2), formerly referred to as *Fagara macrophylla* (Oliv.) Engl., belongs to the family Rutaceae. It is also referred to as African satinwood. Different communities have varied names for this plant. Nandi and Kipsigis refer to it as Sagawoita, Kikuyu as Muchagatha, Luhya as Shikhoma, Ndorobo as Kikomit, Luo as Sogo-Maitha, and Sukuma as Mfwakumbi.

Plate 2. A young *Z. gillettii* plant.

The wood is scented, hard, tough, used for carpentry and boat building but not durable in the ground. The plant has varied medicinal uses in alleviation of several ailments depending on the community. Ndorobo use the bark as cough medicine. Bark decoction is used by the Luhyas to cure malaria and stomachache. The bark is also chewed to alleviate toothache. Bark decoction is used by the Luo to treat constipation, snakebite and rheumatism (Beentje, 1994; Kokwaro and Timothy, 1998; Kokwaro, 1993).

Some compounds with anti-plasmodial activity had been isolated from the root bark of this plant. These included N-isobutyldeca-2,4-dienamide (**12**), fagaramide (**13**), lupeol (**14**), sesamin (**15**), and 4,7,8-trimethoxyfurol-[2,3-b]-quinoline (**16**) (Weenen *et al.*, 1990). Isobutyl-amides, N-isobutyldeca-2,4-dienamide (**12**), fagaramide (**13**), piperlongumine (**70**), 4,5-dihydropiper-longumine (**71**) and N-isobutyl-2*E*,4*E*-octadienamide (**72**), isolated from this plant, have been reported to have mosquito larvicidal activity against *Ae. aegypti* (Kubo *et al.*, 1984). However, activity of this plant against *An. gambiae* has never been reported.

2.2 *Pittosporum mannii* (Hook)

P. mannii Hook. F. (Plate 3) belongs to the family Pittosporaceae. It is a shrub or tree with grey bark. It is also referred to as achak (Luo) and Lalondet (Tugen). The wood is hard. It is used for making clubs, building poles and as a source of fuel (firewood) to the rural community. The plant has white fragrant flowers. The fruits are black, containing red seeds in gelatinous pulp (Beentje, 1994; Kokwaro and Timothy, 1998; Kokwaro, 1993).

Plate 3. *P. mannii* plant.

Specific medicinal use of this plant is not well documented. However medicinal use of other *Pittosporum* species is well documented. *P. lanatum* (*P. abyssinicum* Del.) has different names depending on different communities, it is referred to as musumara (Kikuyu), chemaroriet (Kipsigis), olengeraiyan (Maasai), chemnosa (Marakwet), and segersa (Nandi). Bark infusion of this plant is used as an emetic, purgative and even as anti-malarial by the Kipsigis. *P. viridiflorum* Sims. has varied names depending on various communities, munyamati (Kikuyu), kaluma (Kamba), olengararia (Maasai), kipkeriet, chemngariot (Kipsigis), chemnoa (Marakwet), munati (Meru), sitot (Nandi), tonguteut, tabonet (Ndorobo), cheluro (Pokot), lukeruki (Samburu), segersa, chemwororia (Sebei), kijulu (Taita) and yerkua (Tugen). The bark is used as an emetic, remedy for chest complaints and as an anti-malarial, the wood is used for building poles and root fibers for basketry by the Taita (Beentje, 1994; Kokwaro, 1993).

CHAPTER 3: BIO-EVALUATION OF PHYTOCHEMICAL LARVICIDES

3.0 Plants and families

Previous research has shown that some plants used to treat different ailments (medicinal plants) also show potency as mosquito larvicides or repellents (Ranaweera, 1996; Sukumar *et al.*, 1991; Suparvarn *et al.*, 1974). A total of seventeen medicinal plants, within ten (10) families, were screened for larvicidal activity against *An. gambiae* larvae. The plants screened belong to the families; Rutaceae, Mimosaceae, Lamiaceae, Euphorbiaceae, Rosaceae, Piperaceae, Meliaceae, Solanaceae, Asteraceae and Pittosporaceae. Different plant parts were sequentially extracted with n-hexane, chloroform, ethyl acetate and methanol; the extracts were then assayed for larvicidal activity against *Anopheles gambiae* according to WHO (1996b) protocol.

Plants screened are *Zanthoxylum gillettii* (De. Wild) Waterm, *Z. mildbraedii* (Engl.) Waterm, *Teclea simplicifolia* (Engl.) Verdoorn, *Toddalia asiatica* (L.) Lam, *Clausena anisata* (Willd) Benth (Rutaceae), *Albizia coriaria* (Oliv.) (Mimosaceae), *Tetradenia riparia* (Hochst.) Codd {Syn. *Iboza multiflora* (Benth) E. A. Bruce} (Lamiaceae), *Neoboutonia macrocalyx* (Pax.) (Euphorbiaceae), *Hagenia abyssinica* (Bruce) J. F. Gmel (Rosaceae), *Piper guineense* (Schum. & Thonn.), *P. umbellatum* L., *P. capense* L. (Piperaceae), *Melia azedarach* L. (Meliaceae), *Solanum aculeastrum* Dunal., *S. mauritianum* Scop. (Solanaceae), *Bidens pilosa* L. (Asteraceae) and *Pittosporum mannii* Hook. F. (Pittosporaceae).

Percentage yields for most of the extracts, especially those of *n*-hexane, chloroform and ethyl acetate were fairly low as compared to the yields of methanol extracts (Table 1).

Table 1. % Yield of extracts.

PLANT	PART COLLECTED	% YIELD			
		C_6H_{14}	$CHCl_3$	EtOAc	MeOH
Bidens pilosa	WP	0.25	0.29	0.31	0.58
Neoboutonia macrocalyx	B	0.80	1.20	0.95	0.97
Tetradenia riparia	B	0.60	0.60	0.45	1.01
Melia azedarach	F	0.74	0.81	1.31	1.66
Melia azedarach	L	0.62	0.65	0.80	0.97
Albizia coriaria	L	1.10	0.89	1.30	1.45
Albizia coriaria	B	1.60	1.70	2.00	5.23
Piper capense	L	0.90	1.09	1.30	1.56
Piper guineense	L	1.00	1.30	1.21	1.42
Piper umbellatum	L	0.86	1.20	1.60	1.81
Pittosporum mannii	B	1.50	1.54	1.61	1.78
Hagenia abyssinica	B	0.70	0.65	0.85	1.10
Clausena anisata	B	0.61	0.64	0.72	0.77
Teclea simplicifolia	B	0.45	0.69	0.70	0.88
Toddalia asiatica	R	0.54	0.52	0.67	0.81
Zanthoxylum gillettii	B	1.90	2.10	1.02	3.60
Zanthoxylum gillettii	L	0.65	0.90	1.04	1.45
Zanthoxylum mildbraedii	B	1.47	1.82	1.85	1.89
Solanum aculeastrum	F	1.45	1.61	1.73	5.47
Solanum aculeastrum	R	1.21	1.24	2.04	4.85
Solanum mauritianum	L	1.31	1.44	1.57	2.76

L = Leaves, B = Bark, F = Fruit, R = Roots, WP = Whole plant.

3.1 Preliminary bio-assay

A standard concentration (100 µg/ml) of each extract was used in the preliminary assays using 3rd instar larvae of *Anopheles gambiae*. A total of 25 insects were used (5 insects per vial) per set of the experiment. Three sets of each test were done in five replicates. Mortality was observed for 24, 48 and 72 hours. Mean % mortality and standard error (SE) were calculated.

The preliminary assay of *n*-hexane extracts (Table 2) revealed that *Zanthoxylum gillettii* (bark), *Piper guineense* (leaves), *Toddalia asiatica* (roots) and *Pittosporum mannii* (bark) had the highest performance as *An. gambiae* larvicides. *Piper capense* (leaves), *P. umbellatum* (leaves), *Melia azedarach* (fruits and leaves), *Solanum aculeastrum* (fruits), *S. mauritianum* (leaves) and *Clausena anisata*

(bark) showed improved performance with time reaching a maximum after 72 hours. This may imply the presence of slow acting larvicide or IGRs.

Table 2. Larvicidal assay of *n*-hexane extracts.

PLANT	MEAN % MORTALITY ± SE		
	24 h	48 h	72 h
Albizia coriaria (L)	-	8.33 ± 1.20	8.33 ± 1.20
Albizia coriaria (B)	-	-	8.00 ± 0.58
Bidens pilosa (WP)	24.33 ± 1.33	44.33 ± 1.20	60.00 ± 1.00
Clausena anisata (B)	16.00 ± 1.00	39.67 ± 0.67	72.00 ± 0.58
Hagenia abyssinica (B)	-	12.33 ± 0.33	24.33 ± 0.88
Tetradenia riparia (B)	4.33 ± 0.33	11.67 ± 1.45	24.33 ± 0.33
Melia azedarach (F)	36.33 ± 0.33	63.67 ± 0.882	80.00 ± 0.58
Melia azedarach (L)	32.33 ± 1.76	48.33 ± 0.33	99.67 ± 0.33
Neoboutonia macrocalyx (B)	-	8.33 ± 0.33	15.66 ± 0.88
Piper capense (L)	31.67 ± 0.33	60.33 ± 0.33	80.33 ± 0.67
Piper guineense (L)	99.67 ± 0.33	-	-
Piper umbellatum (L)	16.00 ± 1.16	40.33 ± 0.33	56.33 ± 1.20
Pittosporum mannii (B)	88.33 ± 0.88	99.67 ± 0.33	-
Solanum aculeastrum (F)	27.67 ± 1.20	32.00 ± 0.58	68.00 ± 1.53
Solanum aculeastrum (R)	20.00 ± 1.16	48.33 ± 1.20	64.33 ± 0.33
Solanum mauritianum (L)	12.33 ± 1.45	44.33 ± 1.45	68.00 ± 0.58
Teclea simplicifolia (B)	-	-	8.33 ± 0.88
Toddalia asiatica (R)	100.00	-	-
Zanthoxylum gillettii (B)	100.00	-	-
Zanthoxylum gillettii (L)	16.33 ± 0.88	32.33 ± 0.33	56.33 ± 0.33
Zanthoxylum mildbraedii (B)	11.67 ± 1.33	40.33 ± 0.67	64.33 ± 1.45

L = Leaves, B = Bark, R = Roots, F = Fruits, - = No data, WP = Whole plant.

Bio-assay of chloroform extracts (Table 3) revealed the best performing extracts as *Piper guineense* (leaves), *Toddalia asiatica* (roots) and *Zanthoxylum gillettii* (bark). *Melia azedarach* (fruits and leaves), *Piper capense* (leaves), *Solanum mauritianum* (leaves) and *Bidens pilosa* (Whole plant) showed improving performance with time reaching a maximum after 72 hours. This suggests the presence of insect growth regulators (IGR) or slow-acting larvicides in the chloroform extract.

Table 3. Larvicidal assay of chloroform extracts.

PLANT	MEAN % MORTALITY ± SE		
	24 h	48 h	72 h
Albizia coriaria (L)	-	7.67 ± 0.67	24.33 ± 0.88
Albizia coriaria (B)	12.33 ± 0.33	19.67 ± 1.20	24.33 ± 1.45
Bidens pilosa (WP)	32.33 ± 0.33	51.67 ± 0.88	71.67 ± 0.88
Clausena anisata (B)	7.67 ± 0.88	27.00 ± 0.58	39.67 ± 0.88
Hagenia abyssinica (B)	6.67 ± 0.88	9.33 ± 0.67	11.33 ± 0.88
Tetradenia riparia (B)	24.00 ± 1.53	28.33 ± 0.33	51.67 ± 0.88
Melia azedarach (F)	55.67 ± 0.88	76.33 ± 1.20	87.67 ± 0.88
Melia azedarach (L)	11.67 ± 0.88	56.33 ± 0.33	60.33 ± 0.88
Neoboutonia macrocalyx (B)	19.67 ± 0.88	31.67 ± 0.88	31.67 ± 0.88
Piper capense (L)	27.67 ± 0.88	43.67 ± 0.88	67.67 ± 0.88
Piper guineense (L)	100.00	-	-
Piper umbellatum (L)	8.33 ± 0.33	32.33 ± 0.88	52.33 ± 0.33
Pittosporum mannii (B)	28.33 ± 1.45	48.00 ± 1.16	52.33 ± 1.20
Solanum aculeastrum (F)	-	7.67 ± 0.88	31.67 ± 0.88
Solanum aculeastrum (R)	27.67 ± 0.88	40.33 ± 0.67	47.67 ± 0.33
Solanum mauritianum (L)	23.67 ± 0.88	35.67 ± 0.88	60.33 ± 0.33
Teclea simplicifolia (B)	-	19.67 ± 0.67	51.67 ± 0.33
Toddalia asiatica (R)	100.00	-	-
Zanthoxylum gillettii (B)	99.67 ± 0.33	-	-
Zanthoxylum gillettii (L)	3.67 ± 0.88	23.67 ± 0.88	44.33 ± 0.33
Zanthoxylum mildbraedii (B)	28.33 ± 0.33	36.33 ± 0.33	52.33 ± 0.33

L = Leaves, B = Bark, R = Roots, F = Fruits, - = No data, WP = Whole plant.

The assay of ethyl acetate extracts of the 17 plants (Table 4) showed that *Zanthoxylum gillettii* (bark), *Toddalia asiatica* (roots), *Melia azedarach* (fruits) and *Piper guineense* (leaves), had reasonable larvicidal activity after 24 hours. The activity of *Clausena anisata* (bark), *Piper capense* (leaves) and *Solanum mauritianum* (leaves) improved with time reaching a maximum at 72 hours after application. Whereas *Z. gillettii*, *T. asiatica*, and *P. guineense* have fast acting larvicides in the ethyl acetate extracts; *M. azedarach*, *P. capense*, *C. anisata* and *S. mauritianum* show the presence of slow acting larvicides or insect growth inhibitory compounds in them.

Table 4. Larvicidal assay of ethyl acetate extracts.

PLANT	MEAN % MORTALITY ± SE		
	24 h	48 h	72 h
Albizia coriaria (L)	8.33 ± 0.33	11.67 ± 0.88	24.33 ± 0.33
Albizia coriaria (B)	4.33 ± 0.33	16.00 ± 1.53	39.67 ± 0.88
Bidens pilosa (WP)	3.67 ± 0.33	20.33 ± 0.33	43.67 ± 2.03
Clausena anisata (B)	11.67 ± 0.88	39.67 ± 1.20	68.33 ± 0.88
Hagenia abyssinica (B)	3.67 ± 1.20	11.67 ± 1.67	19.67 ± 0.33
Tetradenia riparia (B)	16.33 ± 0.33	16.33 ± 0.33	27.67 ± 0.88
Melia azedarach (F)	60.33 ± 1.20	84.33 ± 0.88	95.67 ± 1.20
Melia azedarach (L)	8.33 ± 1.20	32.33 ± 0.33	47.67 ± 1.20
Neoboutonia macrocalyx (B)	12.33 ± 1.45	39.67 ± 0.88	44.33 ± 0.88
Piper capense (L)	16.00 ± 0.58	40.33 ± 0.33	59.67 ± 0.88
Piper guineense (L)	64.33 ± 0.33	99.67 ± 0.33	-
Piper umbellatum (L)	4.00 ± 0.19	20.33 ± 1.33	39.67 ± 1.20
Pittosporum mannii (B)	-	4.33 ± 0.33	4.33 ± 0.33
Solanum aculeastrum (F)	3.67 ± 0.67	11.67 ± 0.67	31.67 ± 1.67
Solanum aculeastrum (R)	27.67 ± 0.88	36.33 ± 0.33	48.33 ± 0.88
Solanum mauritianum (L)	19.67 ± 0.88	36.33 ± 1.20	64.33 ± 0.33
Teclea simplicifolia (B)	-	3.67 ± 0.88	19.67 ± 0.33
Toddalia asiatica (R)	99.67 ± 0.33	-	-
Zanthoxylum gillettii (B)	75.67 ± 0.88	100.00	-
Zanthoxylum gillettii (L)	-	-	16.33 ± 0.88
Zanthoxylum mildbraedii (B)	20.33 ± 1.45	31.67 ± 0.88	55.67 ± 0.88

L = Leaves, B = Bark, R = Roots, F = Fruits, - = No data, WP = Whole plant.

For the methanol extracts, the bio-assay revealed that *Zanthoxylum gillettii* (bark) and *Melia azedarach* (fruits and leaves) had some activity, though much less than in all the other extracts (Table 5). Again improved activity was observed for *Solanum aculeastrum* (fruits and roots).

Table 5. Larvicidal assay of methanol extracts.

PLANT	MEAN % MORTALITY ± SE		
	24 h	48 h	72 h
Albizia coriaria (L)	-	-	-
Albizia coriaria (B)	-	-	7.67 ± 0.33
Bidens pilosa (WP)	-	12.00 ± 0.14	28.33 ± 0.33
Clausena anisata (B)	-	-	19.67 ± 0.88
Hagenia abyssinica (B)	7.67 ± 0.33	11.67 ± 0.88	24.33 ± 0.33
Tetradenia riparia (B)	3.67 ± 0.33	3.67 ± 0.33	7.67 ± 0.88
Melia azedarach (F)	68.00 ± 0.58	99.67 ± 0.33	-
Melia azedarach (L)	63.67 ± 0.82	83.67 ± 0.67	100.00
Neoboutonia macrocalyx (B)	-	-	7.67 ± 0.67
Piper capense (L)	-	35.67 ± 0.33	48.00 ± 1.16
Piper guineense (L)	19.67 ± 1.45	-	-
Piper umbellatum (L)	-	12.33 ± 1.45	31.67 ± 0.88
Pittosporum mannii (B)	-	-	-
Solanum aculeastrum (F)	24.00 ± 0.58	51.67 ± 0.88	75.33 ± 0.67
Solanum aculeastrum (R)	11.67 ± 0.67	32.33 ± 0.33	51.67 ± 0.33
Solanum mauritianum (L)	12.33 ± 1.45	19.67 ± 1.33	40.33 ± 0.67
Teclea simplicifolia (B)	-	-	-
Toddalia asiatica (R)	52.33 ± 0.33	60.33 ± 0.33	-
Zanthoxylum gillettii (B)	75.67 ± 2.03	100.00	-
Zanthoxylum gillettii (L)	-	-	16.35 ± 1.20
Zanthoxylum mildbraedii (B)	-	12.33 ± 1.45	36.00 ± 0.33

L = Leaves, B = Bark, R = Roots, F = Fruits, - = No data, WP = Whole plant.

From the preliminary data, four best performing plants were selected for detailed phytochemical investigation. These included *n*-hexane and chloroform extracts of *Zanthoxylum gillettii* (Rutaceae) and *Piper guineense* (Piperaceae), *n*-hexane extracts of *Toddalia asiatica* (Rutaceae) and *Pittosporum mannii* (Pittosporaceae).

3.2 Detailed bio-assay

The crude extracts of the four selected plants were assayed using two concentration ranges, (5, 10, 20, 30, 40 µg/ml) and (1, 2, 4, 6, 8, 10 µg/ml). In each experiment, 20 third instar *An. gambiae* larvae were used. The assays were replicated five times for each concentration.

The percentage mortality observed was calculated using Abbott's formula (Busvine, 1971) in the form;

$$P_T = \frac{P_O - P_C}{100 - P_C} \times 100$$

Where, P_T = corrected mortality, P_O = observed mortality and P_C = control mortality (all %'s).

Table 6. *Zanthoxylum gillettii*, *n*-hexane extract.

Dose (µg/ml)	Corrected % mortality	Log (+3) dose	Empirical probit	Expected probit	Working probit
10	100	2.000	-	6.6	7.10
8	93 ± 0.50	1.903	6.48	6.3	6.46
6	80 ± 1.00	1.778	5.84	6.0	5.82
4	65 ± 0.50	1.602	5.39	5.5	5.39
2	42 ± 0.30	1.301	4.80	4.7	4.80
1	12 ± 0.33	1.000	3.82	3.8	3.83

Regression equation; y = 2.7443x + 1.1035

Table 7. *Zanthoxylum gillettii*, chloroform extract.

Dose (µg/ml)	Corrected % mortality	Log (+3) dose	Empirical probit	Expected probit	Working probit
10	100 ± 0.33	2.000	-	6.3	6.87
8	87 ± 0.67	1.903	6.13	6.0	6.11
6	72 ± 1.76	1.778	5.58	5.7	5.57
4	46 ± 0.58	1.602	4.90	5.1	4.90
2	34 ± 0.33	1.301	4.59	4.3	4.62
1	4 ± 0.33	1.000	3.25	3.4	3.27

Regression equation; y = 2.9209x + 0.4596

Table 8. *Piper guineense*, *n*-hexane extract.

Dose (µg/ml)	Corrected % mortality	Log (+3) dose	Empirical probit	Expected probit	Working probit
10	100 ± 0.33	2.000	-	6.1	6.72
8	85 ± 0.33	1.903	6.04	5.8	6.01
6	62 ± 1.76	1.778	5.31	5.5	5.30
4	47 ± 1.20	1.602	4.92	5.0	4.93
2	25 ± 0.33	1.301	4.33	4.2	4.33

Regression equation; y = 2.6555x + 0.7791

Table 9. *Piper guineense*, chloroform extract.

Dose (µg/ml)	Corrected % mortality	Log (+3) dose	Empirical probit	Expected probit	Working probit
10	100	2.000	-	6.0	6.65
8	81 ± 0.33	1.903	5.88	5.7	5.86
6	53 ± 0.67	1.778	5.08	5.3	5.07
4	39 ± 0.33	1.602	4.72	4.7	4.72
2	12 ± 1.20	1.301	3.82	3.8	3.83

Regression equation; y = 3.2095x + 0.4079

Table 10. *Pittosporum mannii*, *n*-hexane extract.

Dose (µg/ml)	Corrected % mortality	Log (+3) dose	Empirical probit	Expected probit	Working probit
40	72 ± 1.76	2.602	5.58	5.8	5.56
30	60 ± 1.20	2.477	5.25	5.4	5.25
20	52 ± 1.20	2.301	5.05	4.9	5.05
10	48 ± 0.57	2.000	4.95	4.1	5.21
5	1 ± 0.33	1.699	2.67	3.3	2.94

Regression equation; y = 2.8024x – 1.5095

Table 11. *Toddalia asiatica*, *n*-hexane extract

Dose (µg/ml)	Corrected % mortality	Log (+3) dose	Empirical probit	Expected probit	Working probit
40	79 ± 1.20	2.602	5.81	5.9	5.80
30	64 ± 0.33	2.477	5.36	5.6	5.34
20	58 ± 0.02	2.301	5.20	5.1	5.20
10	51 ± 2.00	2.000	5.03	4.3	5.16
5	2 ± 0.01	1.699	2.95	3.5	3.13

Regression equation; y = 2.7436x – 1.2093

Probit analysis was performed on the results obtained from each of the four plants. From the regression analysis and probit transformations, LC_{25}, LC_{50}, LC_{75}, and LC_{90} values were calculated (Table 12).

Table 12. LC values of crude plant extracts.

Plant/extract	Lethal concentration (LC) in µg/ml			
	25	50	75	90
Zg/C_6H_{14}	1.499	2.629	4.613	7.696
Zg/$CHCl_3$	2.114	3.585	6.080	9.833
Pg/C_6H_{14}	2.174	3.886	6.947	11.790
Pg/$CHCl_3$	2.994	4.841	7.829	12.127
Pm/C_6H_{14}	12.127	21.030	36.468	60.198
Ta/C_6H_{14}	10.447	18.331	32.166	53.670

Zg = *Zanthozylum gillettii*, Pg = *Piper guineense*, Pm = *Pittosporum mannii*, Ta = *Toddalia asiatica*.

The analysis revealed LC_{50} values of 2.629, 3.585, 3.886, 4.841, 21.030 and 18.331 µg/ml for *Zanthoxylum gillettii* and *Piper guineense* (*n*-hexane and chloroform extracts), *Pittosporum mannii* and *Toddalia asiatica* (*n*-hexane extracts) respectively.

Results in Table 12 clearly indicate that *Zanthozylum gillettii* showed the best performance of the four plants investigated. The larvicidal principles of *Piper guineense* against *Aedes aegypti* have been investigated. Piperine (**73**) and wisanine (**74**) were isolated and shown to be responsible for the observed activity (Addae-Mensah and Achieng, 1986). The plant is currently being assessed under field conditions in Kilifi District, Kenya, for mosquito control. Pellitorine (**12**), fagaramide (**13**), piperlongumine (**70**), 4,5-dihydropiperlongumine (**71**) and N-isobutyl-2*E*,4*E*-octadienamide (**72**) isolated from *Z. gillettii* displayed appreciable larvicidal activity against *Culex pipiens* and *Aedes triseriatus* (Kubo *et al.*, 1984; Lalonde *et al.*, 1980). However, no work on this plant has been reported on *Anophleles* species. *Toddalia asiatica* belongs to the same taxonomic family as *Zanthoxylum gillettii* and was therefore left out on that basis. *Z. gillettii* and *P. mannii* were chosen for further investigations on the compounds responsible for their larvicidal activity against *Anopheles gambiae*.

3.3 *Zanthoxylum gillettii*

TLC analysis (SiO_2) of both hexane and chloroform extracts of this plant gave seven spots each (R_f 0.14, 0.22, 0.29, 0.48, 0.64, 0.73 and 0.85). The two extracts were co-spotted and developed with 1:3 ethyl acetate:hexane mixture using silica gel plates (5 x 10 cm) with fluorescence indicator. This was a clear indication that the hexane and chloroform extracts had same compounds, but probably in varying concentrations, hence the differences in larvicidal activity.

Therefore, 7 g of hexane and 3 g of chloroform extracts were combined to give a total of 10 g of crude extract. The crude material was subjected to column chromatography on silica gel and eluted with different mixtures of hexane and ethyl acetate in increasing polarity. A total of 203 (30 ml) fractions were collected and pooled into 13 fractions ($\mathbf{F_1}$-$\mathbf{F_{13}}$) based on the R_f values. $\mathbf{F_1}$ (1.6 g), $\mathbf{F_2}$ (4.1 g), $\mathbf{F_3}$ (0.9 g), $\mathbf{F_9}$ (0.0062 g) and $\mathbf{F_{10}}$ (0.0064 g) were available in sufficient quantities for larvicidal assays whereas $\mathbf{F_5}$ (0.0038 g), $\mathbf{F_6}$ (0.0032 g), $\mathbf{F_7}$ (0.0025 g) and $\mathbf{F_8}$ (0.003 g) were only enough for spectroscopic analysis. $\mathbf{F_{11}}$ (0.0002 g), $\mathbf{F_{12}}$ (0.0001 g) and $\mathbf{F_{13}}$ (0.0001 g) were so minimal even for spectroscopic analysis. $\mathbf{F_4}$ (0.11 g) could not dissolve in acetone or ethanol for bio-assay, hence its activity could not be determined.

Detailed larvicidal bio-assay of the column fractions $\mathbf{F_1}$ (un-crystallized), $\mathbf{F_1}$ (crystallized), $\mathbf{F_2}$, $\mathbf{F_3}$ and $\mathbf{F_9}$ were performed with 3rd instar *An. gambiae*. The data was examined by probit analysis and regression equations obtained (Tables 13-16).

Table 13a. Probit analysis of $\mathbf{F_1}$ (un-crystallized) bio-assay data (24 hrs)

Dose (µg/ml)	Corrected % mortality	Log (+3) dose	Empirical probit	Expected probit	Working probit
10	97 ± 0.33	2.000	6.88	6.8	6.88
8	92 ± 1.20	1.903	6.41	6.5	6.39
6	86 ± 1.20	1.778	6.08	6.0	6.07
4	59 ± 0.88	1.602	5.23	5.3	5.23
2	21 ± 0.33	1.301	4.19	4.2	4.19

Regression equation; $y = 3.8448x - 0.8424$

Table 13b. Probit analysis of F_1 (crystallized) bio-assay data (24 hrs)

Dose (µg/ml)	Corrected % mortality	Log (+3) dose	Empirical probit	Expected probit	Working probit
100	78 ± 1.00	3.000	5.77	5.7	5.77
80	67 ± 0.67	2.903	5.44	5.4	5.44
60	45 ± 0.33	2.778	4.87	5.1	4.87
40	34 ± 0.88	2.602	4.59	4.6	4.58
20	13 ± 0.33	2.301	3.87	3.8	3.88

Regression equation; y = 2.6550x – 2.3052

Table 13c. Probit analysis of F_1 (crystallized) bio-assay data (48 hrs)

Dose (µg/ml)	Corrected % mortality	Log (+3) dose	Empirical probit	Expected probit	Working probit
100	95 ± 0.33	3.000	6.55	6.6	6.65
80	94 ± 1.33	2.903	6.64	6.3	6.52
60	71 ± 1.20	2.778	5.55	5.8	5.53
40	53 ± 1.00	2.602	5.08	5.2	5.08
20	25 ± 0.33	2.301	4.33	4.2	4.33

Regression equation; y = 3.4662x – 3.7871

Table 14. Probit analysis of F_2 bio-assay data (72 hrs)

Dose (µg/ml)	Corrected % mortality	Log (+3) dose	Empirical probit	Expected probit	Working probit
100	74 ± 0.88	3.000	5.64	5.7	5.64
80	53 ± 0.33	2.903	5.08	5.1	5.08
60	38 ± 1.53	2.778	4.69	4.4	4.72
40	15 ± 0.58	2.602	3.96	3.9	3.97
20	3 ± 0.88	2.301	3.12	3.3	3.15

Regression equation; y = 6.0867x – 12.523

Table 15. Probit analysis of F_3 bio-assay data (24 hrs)

Dose (µg/ml)	Corrected % mortality	Log (+3) dose	Empirical probit	Expected probit	Working probit
50	95 ± 0.88	2.699	6.64	6.7	6.64
40	89 ± 0.58	2.602	6.23	6.4	6.21
30	83 ± 0.33	2.477	5.95	5.9	5.95
20	73 ± 0.33	2.301	5.61	5.4	5.61
10	52 ± 0.58	2.000	5.05	4.4	5.14
5	2 ± 0.88	1.699	2.95	3.4	3.09

Regression equation; y = 3.3029x – 2.1795

Table 16. Probit analysis of F_9 bio-assay data (24 hrs)

Dose (µg/ml)	Corrected % mortality	Log (+3) dose	Empirical probit	Expected probit	Working probit
10	99 ± 0.67	2.000	7.33	6.8	7.13
8	88 ± 0.67	1.903	6.18	6.4	6.14
6	73 ± 0.33	1.778	5.61	5.9	5.57
4	37 ± 1.16	1.602	4.67	5.1	4.67
2	24 ± 0.67	1.301	4.29	3.9	4.38

Regression equation; $y = 4.1026x - 1.4273$

From the regression equations, LC values were calculated at 25, 50, 75 and 90 %. (Table 17).

Table 17. LC values of column fractions.

Compound (C)	Duration (Hrs)	Lethal concentration (LC) in µg/ml			
		25	50	75	90
F_1 (un-crystallized)	24	2.215	3.308	4.941	7.120
F_1 (crystallized)	24	31.560	56.427	100.889	171.237
F_1 (crystallized)	48	21.968	34.283	53.502	80.235
F_2	72	58.725	75.724	97.494	122.799
F_3	24	9.351	14.917	23.798	36.411
F_9	24	2.531	3.687	5.370	7.562

The LC_{50} values of 3.308, 56.427, 34.283, 75.724, 14.917 and 3.687 (µg/ml) were realized for F_1 (un-crystallized), F_1 (crystallized) (24 hr), F_1 (crystallized) (48 hr), F_2, F_3 and F_9, respectively. It is important to note that the activity of F_1 reduced considerably on recrystallization. From the LC values, it is evident that F_9 was the most active in the *n*-hexane/chloroform extract of *Z. gillettii*.

3.4 *Pittosporum mannii*

TLC analysis of n-hexane extract of *P. mannii* gave five spots (R_f 0.92, 0.72, 0.55, 0.49, 0.35) upon development in 3:7 ethyl acetate:hexane mixture using silica gel plates (5 x 10 cm) with fluorescence indicator. The crude material (3.2 g) was subjected to column chromatography on silica gel and eluted with different mixtures of hexane and ethyl acetate in increasing polarity. A total of 120 (20 ml) fractions were collected and pooled into 6 fractions (G_1-G_6) based on the R_f

values. Upon re-crystallization, the R_f values of **G_1** (0.0071 g), **G_2** (0.0072 g), **G_3** (0.0073 g), **G_4** (0.0033 g), **G_5** (0.0052 g) and **G_6** (0.0048 g) could not be determined since they were not visible on the TLC plate on visualization under UV, spraying with 1% methanolic acid, 10% phosphomolybdic acid in absolute ethanol and on exposure to iodine vapor. Thus, mass spectroscopy was used to determine the purity of these compounds based on ions viewed from the chromatogram obtained. Several NMR solvents were attempted to dissolve these compounds with little success.

Activity of these compounds could not be determined since they could not dissolve in acetone, ethanol and water that are used to set up the larvicidal assays.

CHAPTER 4: STRUCTURE DETERMINATION

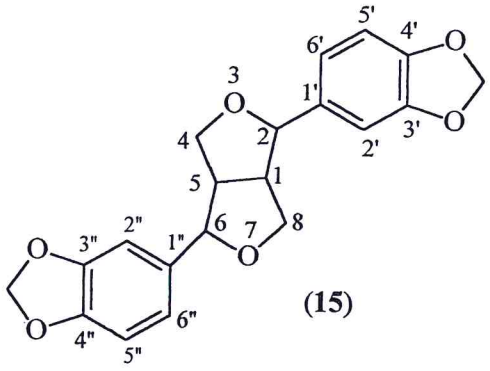
4.0 Compounds isolated from *Z. gillettii*

F_1

From the proton nmr (^{1}H NMR) spectrum of **F_1**, eight signals were observed, 3 aromatic protons at δ 6.83 (1H, s) and 6.77 (2H, s). A methylene dioxide proton signal observed δ 5.94 (2H, d) confirmed the presence of a 1,3-benzodioxole moiety in this compound. Other signals were observed at δ 4.69 (1H, d), 4.21 (1H, t), 3.85 (2H, dd) and 3.03 (1H, t). The signals at δ 3.85 and 4.21 suggested oxygenation at those carbons while the one at δ 3.03, a tertiary centre or presence of nitrogen. The ^{1}H-^{1}H COSY NMR spectrum revealed coupling between the signals at δ 4.69 and 4.21 to the one at 3.03. This suggested that the signal at δ 3.03 was in between the ones at δ 4.69 and 3.85.

^{13}C NMR showed 10 signals, six aromatic carbons at 147.90, 147.04, 134.92, 119.35, 108.15 and 106.45 ppm. From DEPT analysis three aromatic carbons 147.90, 147.04 and 134.92 ppm were shown to be quaternary. The presence of the peaks at 147.94 and 147.04 ppm suggested *ortho* oxygenation confirming the 1,3-benzodioxole group. In addition to the aromatic carbons, a methylenedioxy at 101.05, two methine carbons at 54.24, 85.73, and a methylene carbon at 71.65 ppm were also observed. The signals at 71.65 and 85.73 suggested oxygenation on these carbons while the one at 54.24 ppm suggested a tertiary carbon. IR revealed the methylenedioxy group at 1036 and 935 cm^{-1} while the UV spectrum of **F_1** showed intense absorption bands at 231, 233 and 283 nm. At this juncture a methylenedioxyphenylpropanoid skeleton was suggested.

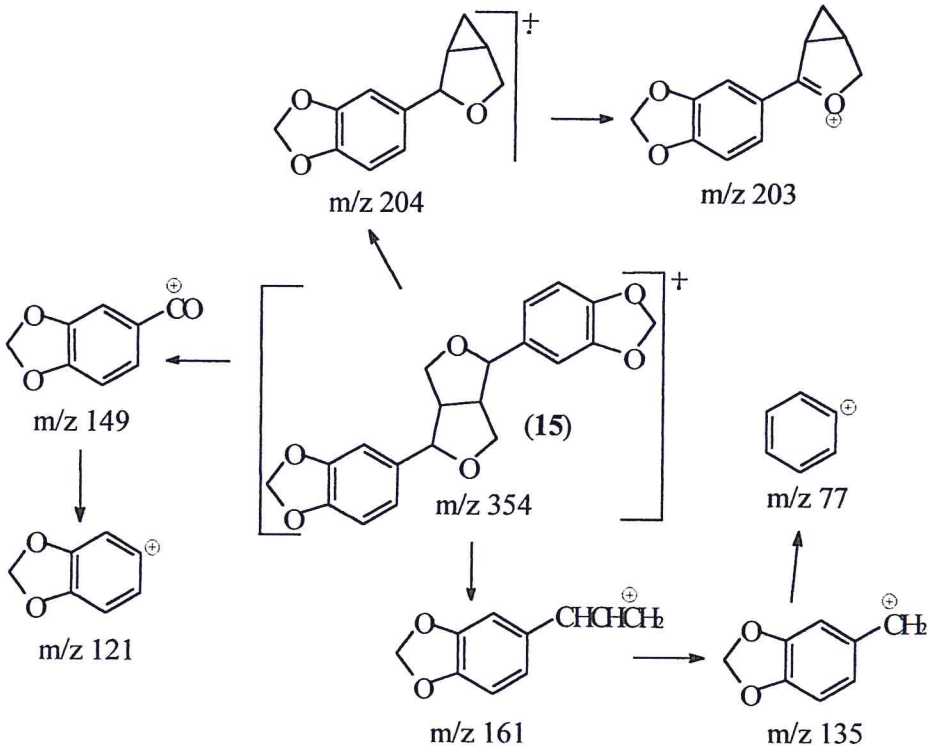
EI-MS revealed molecular ion peak $[M]^+$ at m/z 354 (34%) which is consistent with the molecular formula $C_{20}H_{18}O_6$. This suggested some kind of dimerization of the methylenedioxyphenylpropanoid skeleton and the presence of symmetry. Sesamin (**15**) was proposed.



(15)

The base peak at m/z 149 (100%) was attributed to the ion $[C_8H_5O_3]^+$. Other peaks were observed at m/z 204 (9%) $[C_{12}H_{12}O_3]^+$, 203 (28%) $[C_{12}H_{11}O_3]^+$, 161 (60%) $[C_{10}H_9O_2]^+$, 135 (90%) $[C_8H_7O^2]^+$, 121 (41%) $[C_7H_5O_2]^+$ and 77 (25%) $[C_6H_5]^+$. The ions can be accounted for by the fragmentation pattern in scheme 1.

Scheme 1. Fragmentation pattern of sesamin (**15**)

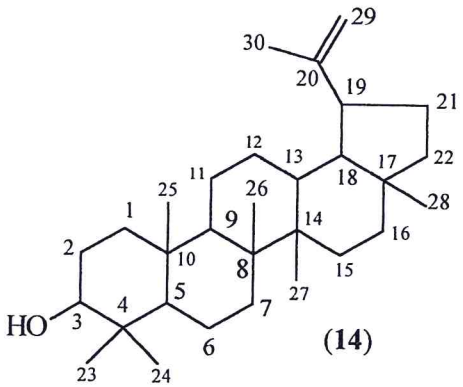


F_1 was hence identified as sesamin, the ^1H and ^{13}C NMR data was comparable to that published by Pelter *et al.* (1976) and Anjaneulu *et al.* (1977).

F_2

The ^1H NMR spectrum of F_2 showed eleven clear signals with geminal olefinic protons at δ 4.68 (1H, s) and 4.46 (1H, s), a proton on carbon attached to an oxygen δ 3.19 (1H, dd), allylic proton δ 2.38 (1H, dt) and seven methyl groups at δ 1.68 (3H, s), 1.02 (3H, s), 0.94 (3H, s), 0.92 (3H, s), 0.82 (3H, s), 0.78 (3H, s), 0.76 (3H, s). The rest of the protons were in a complex continuous multiplet between δ 2.10 and 1.00.

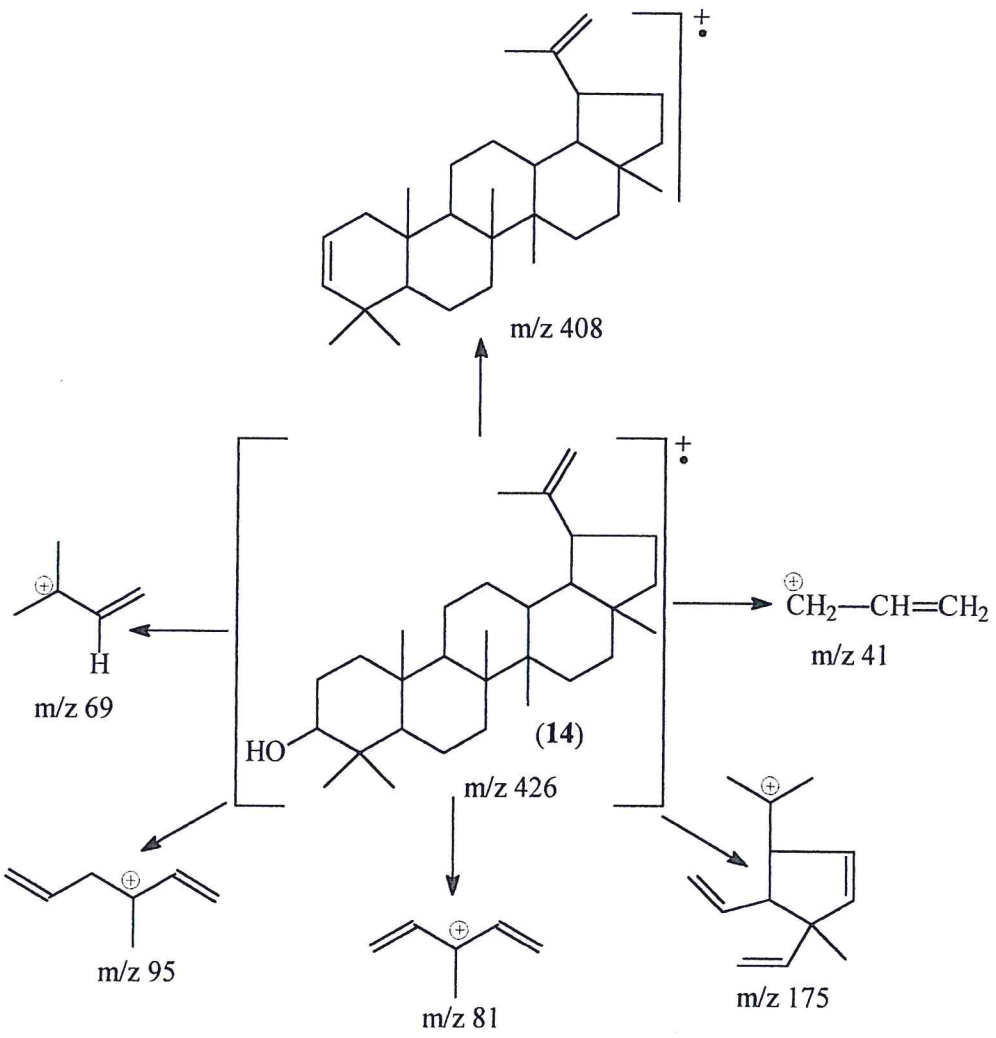
^{13}C NMR showed 29 signals. The signal at 27.62 ppm was most intense and was assumed to arise from two carbons. From DEPT analysis, seven methyl groups at 14.77, 15.62, 16.20, 16.36, 18.23, 19.53, 28.21 ppm were confirmed. Others were; 11 methylene carbons at 18.54, 38.91, 21.14, 27.62, 25.33, 30.05, 35.80, 38.25, 34.48 and 109.57; 5 methine carbons at 79.24, 50.64, 48.21, 48.50, 55.49; 6 quaternary carbons at 43.23, 40.23, 43.05, 39.09, 151.25, 41.03 ppm. The IR revealed OH group at 3335 and a C-O stretch at 1045 cm^{-1}. UV spectrum of F_2 showed intense absorption band at 204 nm. At this juncture a triterpene alcohol was proposed. The presence of 7 methyl groups and a terminal disubstituted alkene suggested a lupeol (**14**) type of compound.



(14)

EI-MS revealed $[M]^+$ at m/z 426 (12%), a value consistent with the molecular formula $C_{30}H_{50}O$. The base peak at m/z 95 (100%) was attributed to $C_7H_{11}^+$. Other peaks were observed at m/z 408 (2%) $[M-H_2O]^+$, 175 (34%) $[C_{13}H_{19}]^+$, 135 (77%) $[C_{10}H_{15}]^+$, 121 (78%) $[C_9H_{12}]^+$, 107 (79%) $[C_8H_{11}]^+$, 81 (80%) $[C_6H_9]^+$, 69 (61%) $[C_5H_9]^+$, 55 (62%) $[C_4H_7]^+$, 41 (57%) $[C_3H_5]^+$. The MS could be accounted for by the fragmentation in the scheme 2.

Scheme 2. Fragmentation pattern of lupeol (**14**)



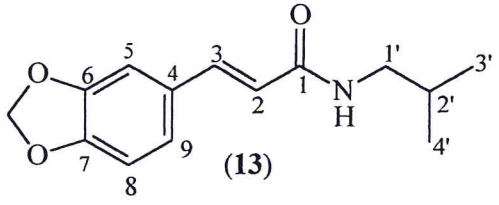
The NMR data was in agreement with that published by Lee *et al.* (2001), Marques *et al.* (1998) and Souza *et al.* (2001).

F_3

From ^1H NMR, 9 signals were observed. Three signals due to aromatic protons were observed at δ 6.99 (1H, s), 6.97 (1H, d) and 6.78 (1H, d). Two olefinic signals due to coupled protons at δ 7.53 (1H, d) and 6.26 (1H, d), a methylenedioxy proton at 5.98 (2H, s), amide proton at δ 5.88 (1H, N-H, br.), methylene protons on carbon attached to a nitrogen atom at δ 3.21 (2H, t), methine proton at δ 1.83 (1H, m) coupled to two methyl protons at δ 0.94 (6H, d) and the methylene protons at δ 3.21.

^{13}C NMR showed 13 signals. Six aromatic carbons 129.45, 108.74, 149.22, 148.41, 106.52 and 118.94 ppm was observed. From DEPT analysis 4 carbons at 166.47, 148.41, 149.22 and 118.94 ppm were found to be quaternary. The presence of 148.41 and 149.22 ppm suggested *ortho* oxygen substituents on the ring as supported by the methylene dioxy group in ^1H NMR. The signal at 20.42 ppm was almost twice as intense as the next intense line and was assigned to the two equivalent methyl groups. Other carbons observed were; two olefinic carbons at 124.12 and 140.97, a methylene carbon attached to nitrogen at 47.34 and a methine carbon 28.88 ppm. A methylenedioxy carbon signal was observed at 101.66 ppm. From the signals at 166.47, 140.97 and 124.12 ppm, an α,β-unsaturated carbonyl was proposed (Silverstein *et al.*, 1991; Williams and Fleming, 1973). The presence of signals at 47.34, 28.88 and 20.42 ppm suggested an isobutyl amide group. IR data revealed an NH stretch at 3296, a methylenedioxy group at 2790 and C=O stretch due to amide I at 1651 cm^{-1}. The UV spectrum of **F_3** showed intense absorption bands at 217, 274 and 309 nm. The structure was proposed to be fagaramide (**13**).

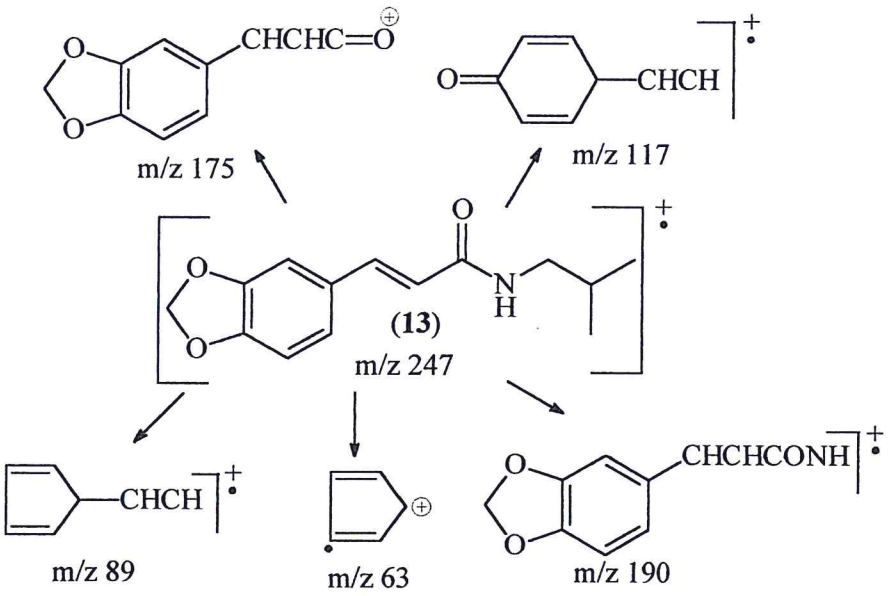
The NMR data was comparable to that published by Shobert *et al.* (2001).



(13)

EI-MS revealed $[M]^+$ at m/z 247 (48%), a value consistent with the molecular formula $C_{14}H_{17}O_3N$. The base peak at m/z 175 (100%) is attributed to $[C_{10}H_7O_3]^+$. Other peaks at m/z 190 (88%) $[C_{10}H_8NO_3]^+$, 147 (21%) $[C_9H_7O_2]^+$ and 145 (95%) $[C_9H_5O_2]^+$. The MS could be rationalised based on the fragmentation pattern in scheme 3.

Scheme 3. Fragmentation pattern of fagaramide (**13**)



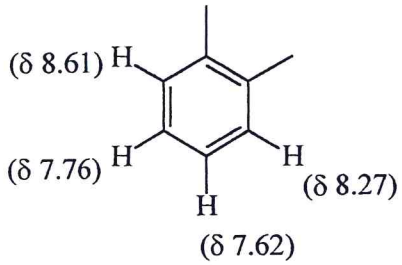
F_4

The ^1H NMR and ^{13}C NMR of **F_4** could not be obtained for structure elucidation since it could not dissolve in most of the common solvents used in NMR.

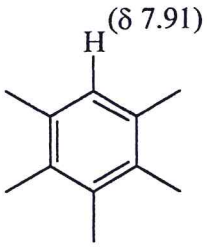
F_5

The ^1H NMR of **F_5** showed eight signals. Seven aromatic proton peaks were observed at δ 8.72 (1H, d), 8.61 (1H, d), 8.27 (1H, d), 8.07 (1H, d), 7.91 (1H, s), 7.76 (1H, dd), 7.62 (1H, dd). A peak appearing as a broad singlet at δ 7.30 (1H, s) was suggested to be arising from a proton attached to nitrogen (-NH-) while that at δ 4.08 (3H) attributed to a methoxy group (OCH_3). The coupling constant of 4.6 Hz between the peaks at δ 8.72 and 8.07 suggested the presence of an

isoquinoline skeleton; this system is comparable to that of liriodenine and fissiceine (Chang *et al.*, 2000; Lo *et al.*, 2000). In the ^{1}H-^{1}H COSY spectrum of **F_5**, coupling was observed between H-4 (δ 8.07) and H-5 (δ 8.72), H-8 (δ 8.27) and H-9 (δ 7.62), H-9 (δ 7.62) and H-10 (δ 7.76), H-10 (δ 7.76) and H-11 (δ 8.61). The coupling pattern between 8.61, 7.76, 7.62, and 8.27 suggested the presence of *ortho* disubstituted benzene skeleton (**96**). This system was comparable to that of liriodenine and lysicamine (Chang *et al.*, 2000; Guinaudeu *et al.*, 1994; Hsieh *et al.*, 1999). The singlet at 7.91 suggested the presence of a pentasubstituted benzene skeleton (**97**).



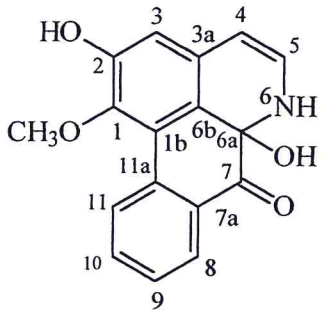
96



97

NOE experiments showed interactions between the signals at δ 4.08 (OCH_3) and 6.52 (OH), but no interactions between δ 4.08 (OCH_3) and 7.91 (H), hence a 2-hydroxy-1-methoxy system was suggested (**98**).

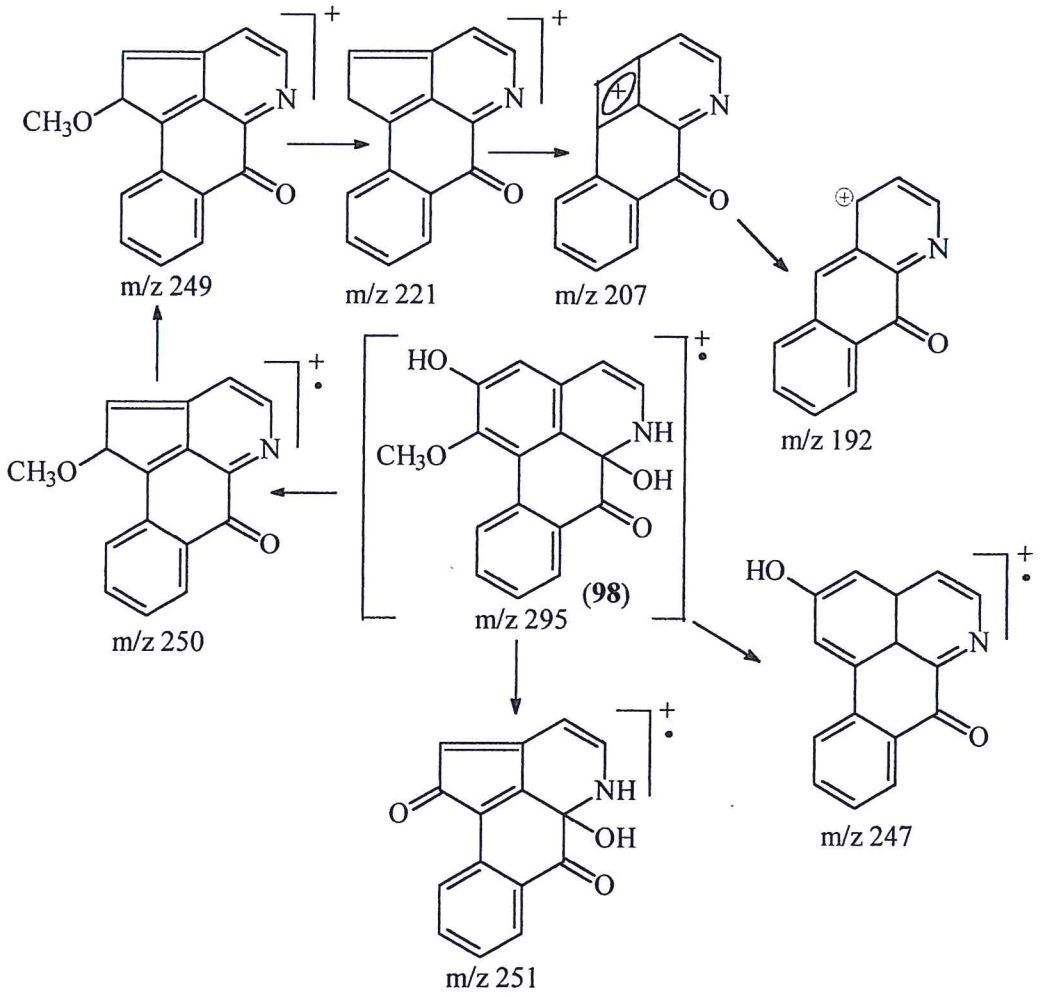
The ^{13}C NMR showed 8 signals, mainly due to protonated carbons. Seven aromatic carbons were observed at 146.38, 130.91, 126.30, 123.69, 117.34, 114.52 and 110.53 ppm. The carbon signal at 56.99 ppm suggested the presence of a methoxy (OCH_3) moiety. The IR spectrum revealed the presence of a methoxy group at 2849 and a carbonyl system at 1672 cm^{-1}. UV spectrum of **F_5** showed intense absorption bands at 240, 258, 289, 335, 351 and 369 nm. At this juncture, 2,6a-dihydroxy-1-methoxyoxoaporphine (**98**) was proposed.



98

The molecular formula of **F5** was deduced to be $C_{17}H_{13}O_4N$. EI-MS revealed the $[M]^+$ at m/z 295 (1%) a value consistent with the molecular formula $[C_{17}H_{13}O_4N]^+$, other peaks were observed at m/z 251 (16%) $[C_{15}H_9O_3N]^+$, 250 (88%) $[C_{16}H_{12}O_2N]^+$, 249 (48%) $[C_{16}H_{11}O_2N]^+$, 247 (9%) $[C_{16}H_9O_2N]^+$, 221 (100%) $[C_{15}H_{11}ON]^+$, 207 (36%) $[C_{14}H_9ON]^+$, 192 (48) $[C_{13}H_6ON]^+$. The fragmentation pattern could be accounted for by scheme 4.

Scheme 4. Fragmentation pattern of 2,6a-dihydroxy-1-methoxyoxoaporphine (**98**).

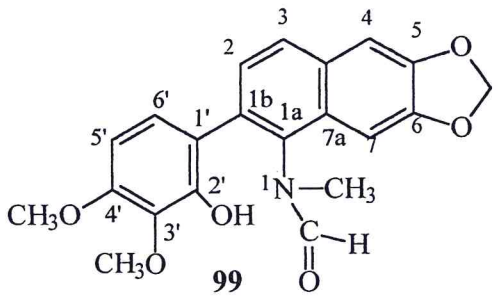


F6

The ^{1}H NMR of **F6** showed 14 signals. Six aromatic protons were observed at δ 7.74 (1H, d), 7.32 (1H, d), 7.20 (1H, s), 7.08 (1H, s), 6.81 (1H, d) and 6.54 (1H, d). The singlet at δ 8.16 (1H, s) confirmed presence of an aldehyde type proton. The peak at δ 6.09 (2H, s) suggested the presence of methylenedioxy group (OCH_2O) whereas that at 5.99 (1H, br, s) the presence of a phenolic proton. Proton signals at δ 3.92 (3H, s) and 3.90 (3H, s) indicated the presence to two methoxy groups in this compound, whereas a singlet peak at δ 3.00 (3H, s)

indicated the presence of a methyl group attached to nitrogen (NCH_3). In the ^1H-^1H COSY spectrum of **F_6**, coupling was observed between signals at δ 7.74 and 7.32; 6.81 and 6.54. NOE experiments showed the interactions between the N-CH_3 (δ 3.00) with the signal at 7.08 (1H) and 3.93 (OCH_3).

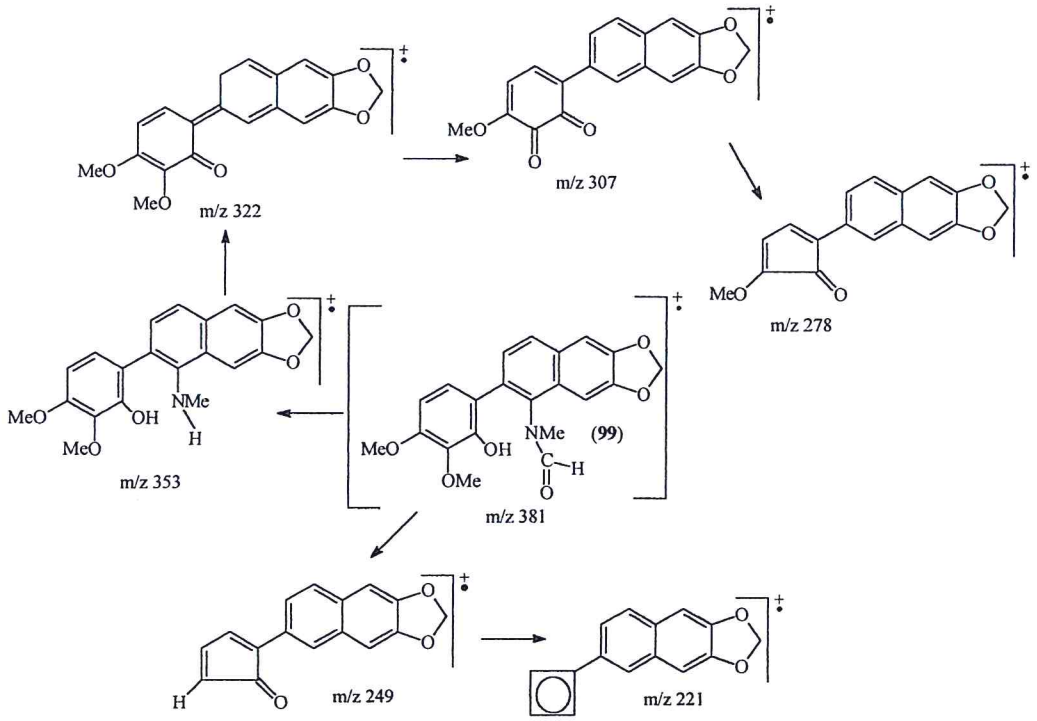
The ^{13}C NMR showed 21 signals. Sixteen aromatic carbons were observed and assigned at 152.02, 149.29, 148.09, 146.64, 135.72, 135.48, 133.31, 131.19, 128.67, 127.45, 127.35, 125.10, 118.51, 104.32, 103.85 and 99.24. The peak at 164.61 suggested the presence of an amide carbonyl in **F_6**. From DEPT, a methylenedioxy carbon (OCH_2O) was confirmed at 101.48 ppm. Two methoxy groups resonated at 55.80 and 61.17 where as a methyl carbon attached to nitrogen (NCH_3) resonated at 33.02 ppm. IR spectrum revealed the presence of a methoxy group at 2837, a carbonyl at 1659 and a methylenedioxy at 1055 and 934 cm^{-1}. The UV spectrum of **F_6** showed intense absorption bands at 213, 234, 273, 311, 332 nm. At this juncture the structure was proposed to be that of arnottianamide (**99**).



99

EI-MS revealed $[M]^+$ at m/z 381 (100) a value consistent with the molecular formula $[C_{21}H_{19}O_6N]^+$. Other peaks were observed at 353 (31%) $[C_{20}H_{19}O_5N]^+$, 322 (54%) $[C_{19}H_{14}O_5]^+$, 307 (42%) $[C_{18}H_{11}O_5]^+$, 278 (15) $[C_{17}H_{10}O_4]^+$, 249 (9) $[C_{16}H_9O_3]^+$, 221 (12) $[C_{15}H_9O_2]^+$. The MS could be accounted for by the fragmentation pattern in scheme 5.

Scheme 5. Fragmentation pattern of arnottianamide (**99**)

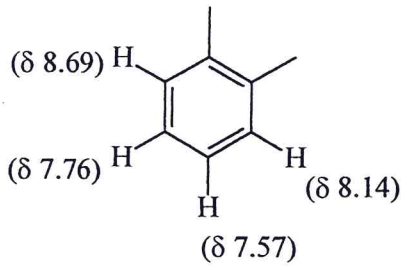


The ^{1}H NMR data for **F$_6$** was consistent with that reported for arnottianamide by Ishii and Ishikawa (1976), Krane *et al.*, (1984) and Sharma *et al.*, (1982).

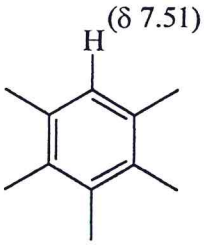
F$_7$

The ^{1}H NMR of **F$_7$** showed ten signals. Seven aromatic proton peaks were observed at δ 8.78 (1H, d), 8.69 (1H, d), 8.14 (1H, d), 8.04 (1H, d), 7.76 (1H, dd), 7.57 (1H, dd), 7.52 (1H, s). Two phenolic proton peaks appeared as broad singlets at δ 7.51 (1H) and 7.27 (1H). The proton peak at δ 3.20 (3H) was suggested to be arising from an N-methyl group (N-CH_3). The coupling constant of 5.0 Hz between the peaks at δ 8.78 and 8.04 suggested the presence of an isoquinoline skeleton, this system was compared to that of liriodenine and fissiceine (Chang *et al.*, 2000; Lo *et al.*, 2000). In the ^{1}H-^{1}H COSY spectrum of **F$_7$**, coupling was observed between δ 8.69 and 7.76; 7.76 and 7.57; 7.57 and 8.14. The coupling pattern between the protons whose signals appeared at δ 8.69, 7.76, 7.57 and 8.14 suggested the presence of *ortho* disubstituted benzene

skeleton (**100**) system as seen in liriodenine and lysicamine (Chang *et al.*, 2000; Guinaudeu *et al.*, 1994; Hsieh *et al.*, 1999). The singlet at δ 7.52 suggested the presence of a pentasubstituted benzene skeleton (**101**). NOE experiments showed interactions between the signals at δ 3.20 (NCH_3) and δ 7.48 (O-H).

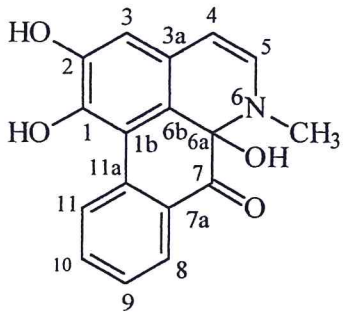


100



101

The ^{13}C NMR showed 13 signals. Eleven signals due to aromatic carbons were observed at 157.91, 157.54, 145.45, 139.39, 131.48, 130.90, 126.13, 125.35, 124.49, 122.95, 117.50, and 117.40 ppm. The signals at 157.91, 157.54, 139.39, 130.90 and 124.49 ppm indicated the presence of quaternary carbons. The signals at 157.91 and 157.54 ppm suggested the presence of *ortho* oxygenated aromatic carbons. The carbon signal at 42.00 ppm suggested the presence of an N-methyl (N-CH_3) moiety (Silverstein *et al.*, 1991; Williams and Fleming, 1973). Two olefinic carbons resonated at 145.45 and 122.95 ppm. The IR spectrum of **F_7** revealed the presence of a hydroxyl group at 3391 and a carbonyl at 1720 cm^{-1} while the UV spectrum showed intense absorption bands at 239, 247, 256 and 336 nm. At this juncture, N-methyl-1,2,6a-trihydroxyoxoaporphine (**102**) was proposed.

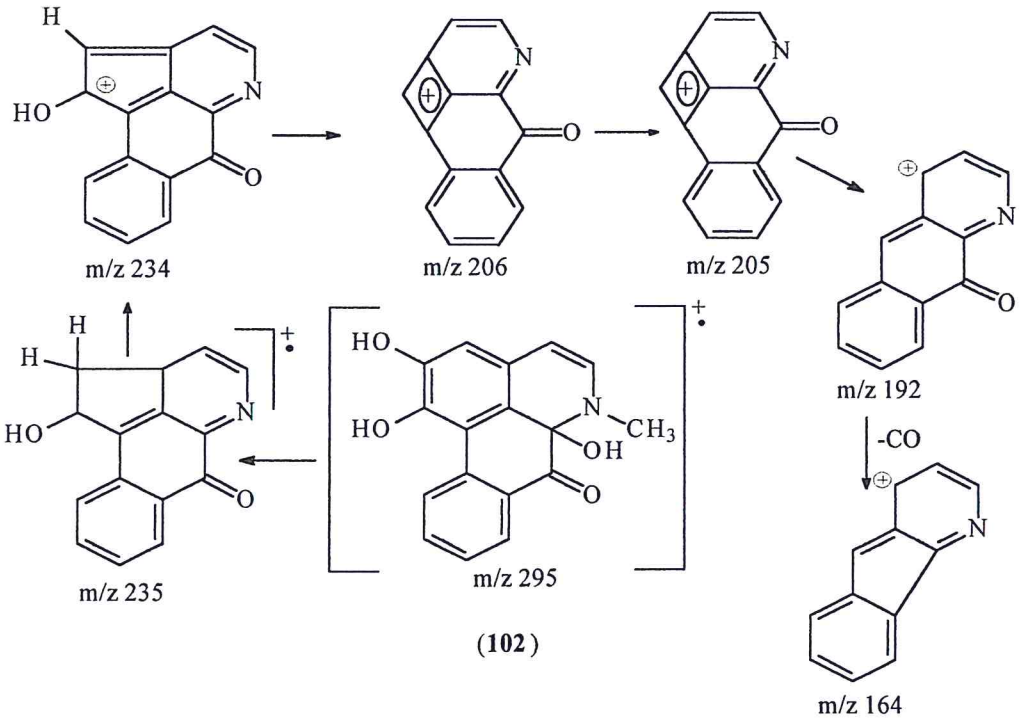


102

Assignment of the protonated aromatic carbons of **F_7** were further confirmed from the HMQC spectrum by direct correlation of the protons to the carbons. From HMBC spectrum, cross peaks were observed between δ_H 8.78 with C-3a and C-4; δ_H 8.14 with C-10 and C-11a; δ_H 8.04 with C-3a and C-5; δ_H 7.76 with C-8 and C-11a; δ_H 7.57 with C-7a and C-11; δ_H 7.52 with C-1. This enabled the assignment of quaternary aromatic carbons.

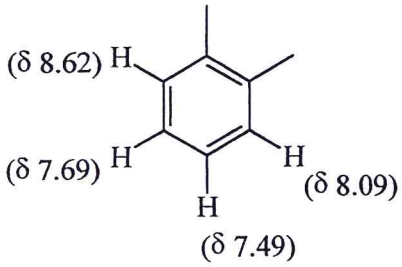
The molecular formula of **F_7** was deduced to be $C_{17}H_{13}O_4N$. EI-MS revealed $[M]^+$ at m/z 295 (2%) a value consistent with the molecular formula $[C_{17}H_{13}O_4N]^+$, other peaks were observed at m/z 235 (29) $[C_{15}H_9O_2N]^+$, 234 (100) $[C_{15}H_8O_2N]^+$, 206 (29) $[C_{14}H_8ON]^+$, 205 (14) $[C_{14}H_7ON]^+$, 192 (95) $[C_{13}H_6ON]^+$, 164 (31) $[C_{12}H_6N]^+$. The fragmentation pattern could be accounted for by scheme 6.

Scheme 6. Fragmentation pattern of N-methyl-1,2,6a-trihydroxyoxoaporphine (**102**).

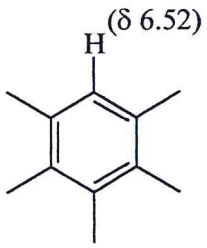


F_8

The ^1H NMR of **F_8** showed nine signals. Seven aromatic proton peaks were observed at δ 8.79 (1H, d), 8.62 (1H, d), 8.09 (1H, d), 7.99 (1H, d), 7.69 (1H, t), 7.49 (1H, t), 6.52 (1H, s). The proton peak observed at δ 2.58 (3H) was suggested to be arising from an N-methyl (N-CH_3) moiety. In the ^1H-^1H COSY spectrum of **F_8**, coupling was observed between δ 7.99 and 8.79; 7.69 and 8.62; 7.49 and 7.69; 7.49 and 8.09. The coupling constant between the signals at 7.99 and 8.79 was found to be 5.0 Hz suggesting the presence of a benzoisoquinoline system as observed in **F_7**. The coupling pattern observed for signals at δ 8.62, 7.69, 7.49, and 8.09 suggested the presence of ortho disubstituted benzene (**103**) system as seen in liriodenine and lysicamine (Chang *et al.*, 2000; Guinaudeu *et al.*, 1994; Hsieh *et al.*, 1999). The presence of an aromatic singlet signal at δ 6.52 suggested the presence of a pentasubstituted benzene skeleton (**104**). NOE experiments showed interactions between the signals at δ 2.58 (NCH_3) and δ 6.52 (H).



103

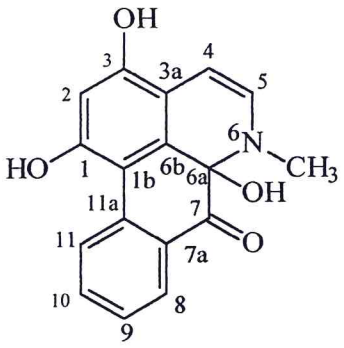


104

The ^{13}C NMR showed 13 signals, of which 12 aromatic carbons were observed at 158.39, 155.18, 144.90, 139.45, 135.14, 130.99, 130.82, 125.33, 122.71, 117.44, 117.12 and 117.03 ppm. The signals at 158.39, 155.18, 139.45, 135.14 and 130.82 ppm indicated the presence of quaternary carbons.

IR spectrum of **F_8** revealed C-H stretch at 2925 and 2854 due to N-CH_3 while a carbonyl group function was revealed at 1664 cm^{-1}. The UV spectrum of **F_8** showed intense absorption bands at 242, 259, 278 and 340 nm. Due to the

similarity of the ^{1}H NMR spectrum of this compound to that of **F_7**, N-methyl-1,3,6a-trihydroyoxoaporphine (**105**) was proposed. The ^{13}C signals at δ 158.39 and 155.18 indicated the presence of a *meta* oxygenation pattern.

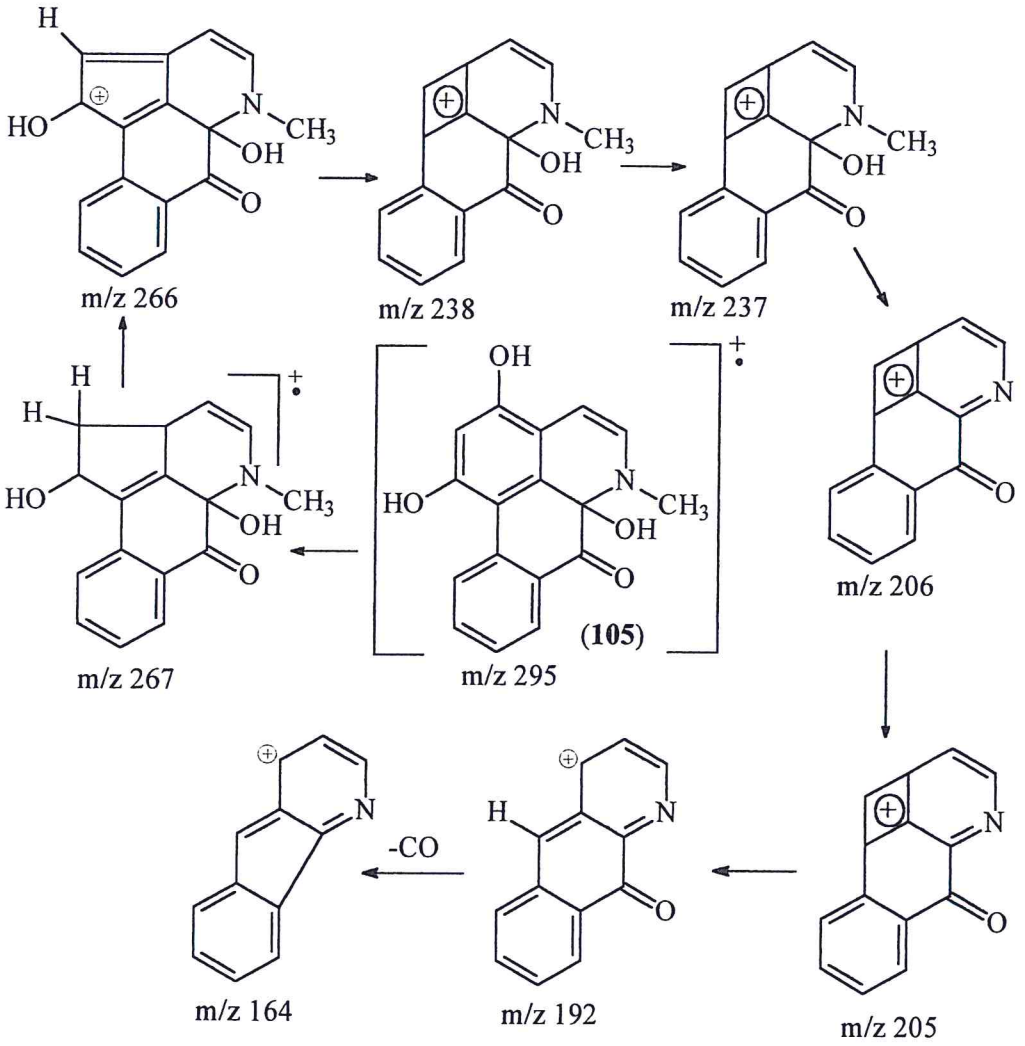


105

Assignment of the protonated aromatic carbons of **F_8** was confirmed from the HSQC spectrum by direct correlation of the protons to the carbons. From HMBC spectrum, cross peaks were observed between δ_H 8.79 with C-3a; δ_H 8.62 with C-7a; δ_H 8.09 with C-10 and C-11a; δ_H 7.99 with C-3a, C-5 and C-6b; δ_H 7.69 with C-8 and C-11a; δ_H 7.49 with C-7a and C-11; δ_H 6.52 with 1a; δ_H 2.58 with C-5 and 6a.

The molecular formula of **F_8** was deduced to be $C_{17}H_{13}O_4N$. EI-MS revealed $[M]^+$ at m/z 295 (2%) a value consistent with the molecular formula $[C_{17}H_{13}O_4N]^+$. Other peaks were observed at m/z 267 (22) $[C_{16}H_{13}O_3N]^+$, 266 (100%) $[C_{16}H_{12}O_3N]^+$, 238 (24) $[C_{15}H_{12}O_2N]^+$, 237 (28) $[C_{15}H_{11}O_2N]^+$, 206 (18) $[C_{14}H_8ON]^+$, 205 (31) $[C_{14}H_7ON]^+$, 192 (54) $[C_{13}H_6ON]^+$, 164 (21) $[C_{12}H_6N]^+$. The fragmentation pattern could be accounted for by scheme 7.

Scheme 7. Fragmentation pattern of N-methyl-1,3,6a-trihydroxyoxoaporphine (**105**).



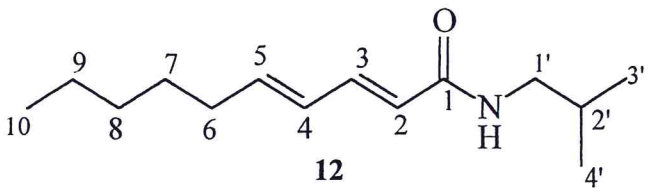
F_9

Proton NMR of **F_9** showed thirteen signals of which four olefinic proton resonances were observed at δ 7.14 (1H, dd), 6.14 (1H, m), 6.04 (1H, m), and 5.70 (1H, d). A broad peak attributed to an exchangeable proton was observed at δ 5.51 (1H, br). A methylene proton peak on a carbon attached to nitrogen resonated at 3.18 (2H, br) while signals due to two allylic protons were observed at δ 2.07 (2H, m). A methine proton at δ 1.73 (1H, m), 6 methylene protons at δ 1.35-1.19 (6H, m), and nine methyl protons were observed in the range δ 0.95-

0.89 (9H, m). The ^{1}H-^{1}H COSY spectrum of **F_9** showed coupling between protons resonating at δ 6.04 and 2.07, 7.14 and 6.14, 1.21 and 0.89 respectively.

^{13}C NMR showed 13 signals. The presence of a signal at 166.44 ppm suggested an α,β-unsaturated system (Silverstein *et al.*, 1991; Williams and Fleming, 1973). The signal at 20.12 ppm that was almost twice as intense as next intense line was assigned to the two equivalent methyl groups. Another methyl signal was observed at δ 14.01. Four olefinic carbon signals appeared at δ 121.56, 141.45, 128.14 and 143.38 and one carbonyl carbon at δ 166.44 suggesting an α,β,γ,δ-unsaturated carbonyl. The presence of a methylene attached to a nitrogen (δ 46.91), a methine group (δ 28.59) and signals due to two methyl groups at δ 20.12 suggested the presence of an isobutyl group. The remaining four methylenes signals appeared at δ 32.91, 31.34, 28.44, 22.45 and a methyl group at δ 14.01 suggested the presence of an *n*-pentyl group. The IR spectrum revealed an NH stretch at 3250 and a carbonyl group at 1660 cm^{-1}. The UV spectrum of **F_9** showed intense absorption bands at 218, 236 and 258 nm. At this juncture the structure was proposed to be that of pelletorine (**12**).

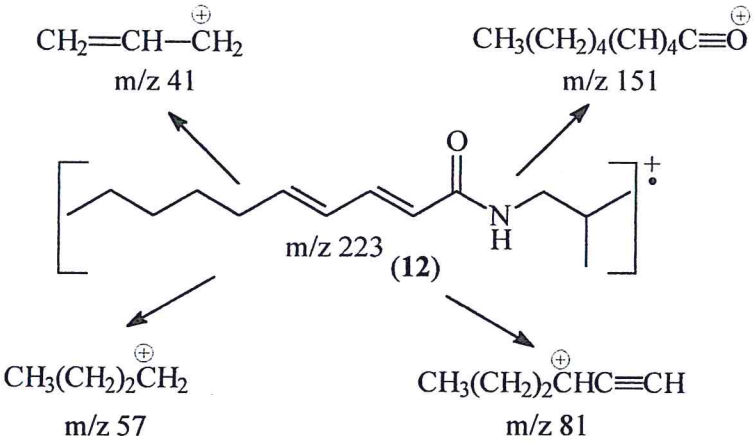
The NMR data is in agreement with that published by Chen *et al.* (1999) and Lalonde *et al.* (1980).



12

EI-MS revealed the molecular ion peak $[M]^+$ at m/z 223 (19%), a value consistent with the molecular formula $C_{14}H_{25}NO$. The peak at m/z 151 (100%) was attributed to $[C_9H_{15}CO]^+$; 96 (51%) $[C_7H_{12}]^+$; 81 (88%) $[C_6H_9]^+$; 57 (49%) $[C_4H_9]^+$; 41 (98%) $[C_3H_5]^+$. The MS could be accounted for by the fragmentation pattern in scheme 8.

Scheme 8. Fragmentation pattern of pellitorine (**12**)

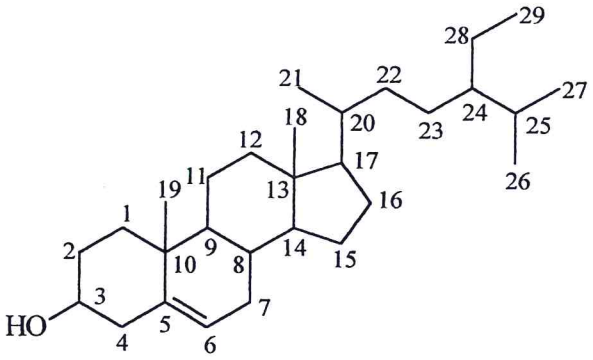


F_{10}

The ^{1}H NMR spectrum of **F_{10}** showed nine signals. The doublet at δ 5.34 integrating to one proton was assigned to an olefinic proton. The signal at δ 3.50 (1H, m) suggested the presence of an alpha proton typical of sterols hydroxylated at C-3. Two allylic protons signals were observed at δ 2.25 (2H, d) and 1.99 (2H, d) indicating the presence of a double bond in a two-ring system, whereas the signal at δ 1.83 (2H, d) indicated the presence of a methine moiety **F_{10}**. Five methyl proton signals were observed at δ 0.99 (3H, s), 0.84 (3H, d), 0.81 (6H, d), 0.66 (3H, s). The rest of the protons were in a complex continuous multiplet between δ 1.67 and 0.86.

^{13}C NMR showed 26 signals. However, from DEPT analysis, six methyl groups at 20.08, 19.65, 19.24, 19.01, 12.53, 12.10 ppm, eleven methylene carbons at 42.50, 39.97, 37.46, 31.87, 34.12, 21.30, 28.49, 24.53, 26.19, 33.26 ppm, nine methine carbons at δ 121.98, 72.04, 32.13, 50.31, 56.96, 56.22, 36.38, 46.00, 29.29 were confirmed. The IR spectrum of **F_{10}** revealed the presence of a hydroxyl group at 3415, a C=O stretch at 1052 and C-H stretch at 2933 cm^{-1}. The UV spectrum of **F_{10}** showed intense absorption band at 213 nm. At this juncture, stigmast-5-en-3-ol (**106**) was proposed.

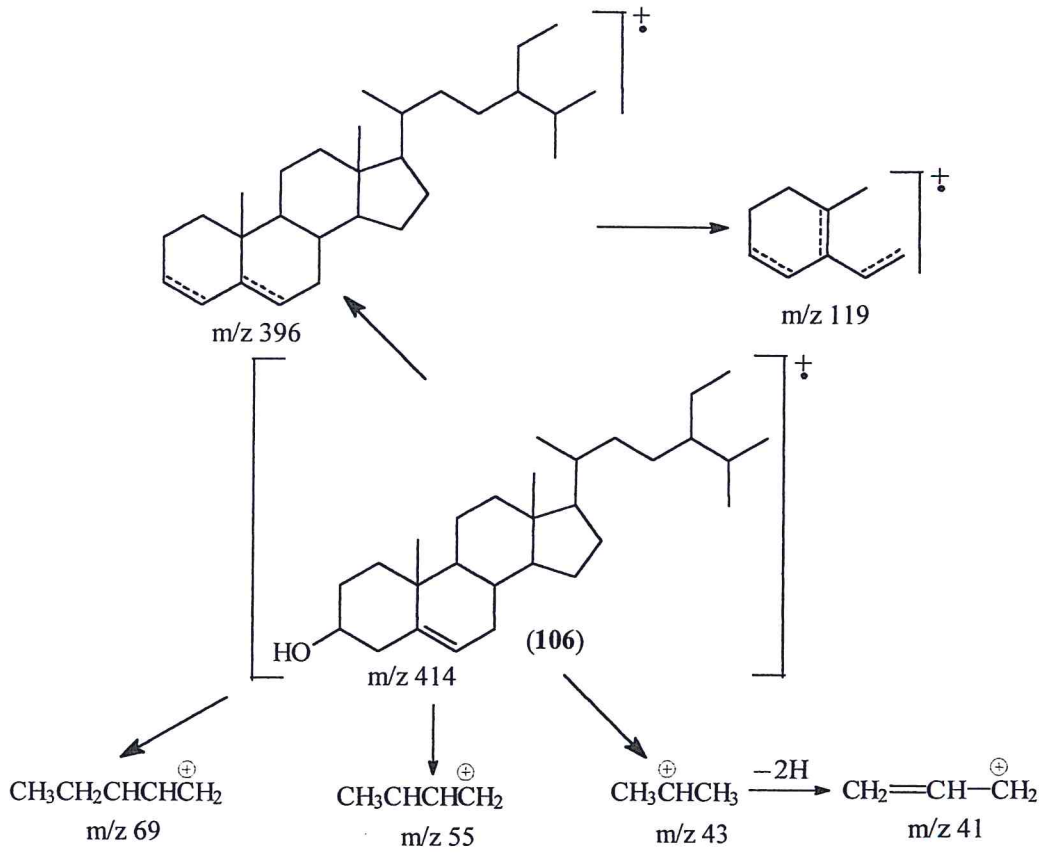
The NMR spectral data of **106** is in agreement with that published by Gupta *et al.* (1992), Greca *et al.* (1990), Lee *et al.* (2001).



106

EI-MS revealed molecular ion peak $[M]^+$ at m/z 414 (7%), a value that is consistent with the molecular formula $[C_{29}H_{50}O]^+$. Other peaks were observed at m/z 396 (8%) $[C_{29}H_{48}]^+$, 119 (25%) $[C_9H_{12}]^+$, 69 (44%) $[C_5H_9]^+$, 55 (79%) $[C_4H_9]^+$, 43 (100%) $[C_3H_7]^+$, 41 (53) $[C_3H_5]^+$. The MS could be accounted for by the fragmentation pattern in scheme 9.

Scheme 9. Fragmentation pattern of stigmast-5-en-3-ol (**106**)



The other compounds (F_{11}-F_{13}) were in minor quantities hence could not be identified spectroscopically.

CHAPTER 5: CONCLUSIONS

5.0 Conclusions

Phytochemicals may provide a good source of environmentally friendly insecticides or IGRs that can be used for effective control of the *Anophelene* density, hence control of malaria vector and eventual control of the disease can be achieved.

Of the plants examined, the trunk and root barks contain the most active phytochemicals against *Anopheles gambiae* larvae.

From the preliminary data, it was apparent that the *n*-hexane and chloroform extracts showed the best larvicidal performance for most of the plants assayed. Beside *Melia azedarach*, methanol extracts gave the poorest performance as *Anopheles gambiae* larvicides.

Plant sterols may not be fast acting *An. gambiae* larvicides. This was based on the low activity lupeol (**F_2**) and lack of activity in stigmast-5-en-3-ol (**F_{10}**). However, slow activity of lupeol may be due to its IGR activity.

Of all the isolated compounds assayed, isobutyl amides displayed the best mosquito larvicidal activity against *An. gambiae*. This could be seen from the LC_{50} values of 14.92 and 3.69 µg/ml for fagaramide (**F_3**) and pellitorine (**F_9**) against 56.43 and 75.72 µg/m for sesamin (**F_1**) and lupeol (**F_2**), respectively. The fatty acid amides seem to be better larvicides than the aromatic ones (cf pellitorine and fagaramide).

Among the three mosquito genera, *Anopheles, Culex* and *Aedes*, the *Anopheles* is more susceptible to the amides than the remaining two genera.

Three oxoaporphine alkaloids (**F_5, F_7, F_8**) were isolated for the first time from *Z. gillettii*.

5.1 Recommendations

There is need for further quantitative isolation of the three oxoaporphines in substantial amounts and to determine their mosquito larvicidal activity.

Further efforts to avail the other compounds (F_5, F_6, F_{11}, F_{12} and F_{13}) in adequate amounts should be made to enable their mosquito larvicidal activities to be determined.

The IGR properties of the slow acting extracts should be investigated and any bio-active principles isolated. Their glycosides should also be investigated to find out if this may increase the larvicidal activity.

Toxicological studies and semi-field trials of: *n*-hexane extracts of (*Zanthoxylum gillettii* (bark), *Toddalia asiatica* (roots), *Pittosporum manii* (bark); chloroform extracts of *Piper guineense* (leaves), *Toddalia asiatica* (roots), *Zanthoxylum gillettii* (bark); ethyl acetate extracts of *Zanthoxylum gillettii* (bark), *Toddalia asiatica* (roots), *Melia azedarach* (fruits), *Piper guineense* (leaves); methanol extracts of *Zanthoxylum gillettii* (bark), *Melia azedarach* (fruits) *Clausena anisata* (bark) should be done to evaluate their potentials as larvicides. These could provide future bio-pesticide candidates in malaria vector management.

CHAPTER 6: EXPERIMENTAL

6.0 General procedures

All solvents used were analytical grade (E. Merck, D-6100 Darmstadt, F. R. Germany).

Melting points of the isolated crystallised compounds were determined on Sanyo Gallenkamp electronic melting point apparatus and are uncorrected.

Proton NMR (^{1}H NMR) spectra were run on Varian Gemini 200 MHz in $CDCl_3$, CD_3OD or $(CD_3)_2CO$. Proton noise decoupled carbon-13 NMR (^{13}C NMR) spectra were determined on Varian Gemini 200 MHz at 50 MHz in $CDCl_3$, CD_3OD or $(CD_3)_2CO$. HMBC, HMQC and HSQC were run on Varian Unity Inova 400 MHz machine. The multiplicities were determined from DEPT analysis and are recorded as s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet. The chemical shift values (δ) were recorded in parts per million (ppm).

Infrared (IR) spectra were obtained using KBr pellets from Shimadzu Fourier Transform (FT) Spectrophotometer. Absorption bands were recorded in wave numbers cm^{-1} ($\bar{\nu}$) and (%) transmittance (T).

Electron impact mass spectra (EI-MS) were obtained from a Fissions VG Platform 11 MS at 70 eV.

Analytical thin layer chromatography was performed on 60 F_{254} plates (5 x 10 cm, thickness 0.20 mm) with fluorescence indicator. The spots were viewed using multi-brand UV-254/366 nm lamp (UV GL-58). The TLC plates were then sprayed with 1% methanolic acid and kept in the oven at 110 ^{0}C until the spots appeared. Other plates were viewed using iodine vapour in a small TLC tank. Visualisation was also done using 10% phosphomolybdic acid in absolute ethanol, as well as acetic acid and *p*-anisaldehyde in sulphuric acid and ethanol. Preparative thin layer chromatography (TLC) was done on silica gel 60 F_{254} plates (20 x 20 cm, thickness 0.25 mm) with fluorescence indicator.

6.1 Collection and preparation of plant materials

Different plants were collected based on their ethnobotanical, chemotaxonomic and phytochemical information. The plants were collected from various parts of Central, Nairobi, Nyanza, Rift Valley and Western Provinces (Table 18). The plants were identified at the University of Nairobi (UoN), Botany Department and The National Museums of Kenya (NMK). Specimens were deposited at the East African Herbarium at the National Museums of Kenya and University of Nairobi, Botany Department. Parts collected include roots, barks, leaves and fruits. The plant materials were dried under shade for 14 days and ground into powder form in preparation for extraction.

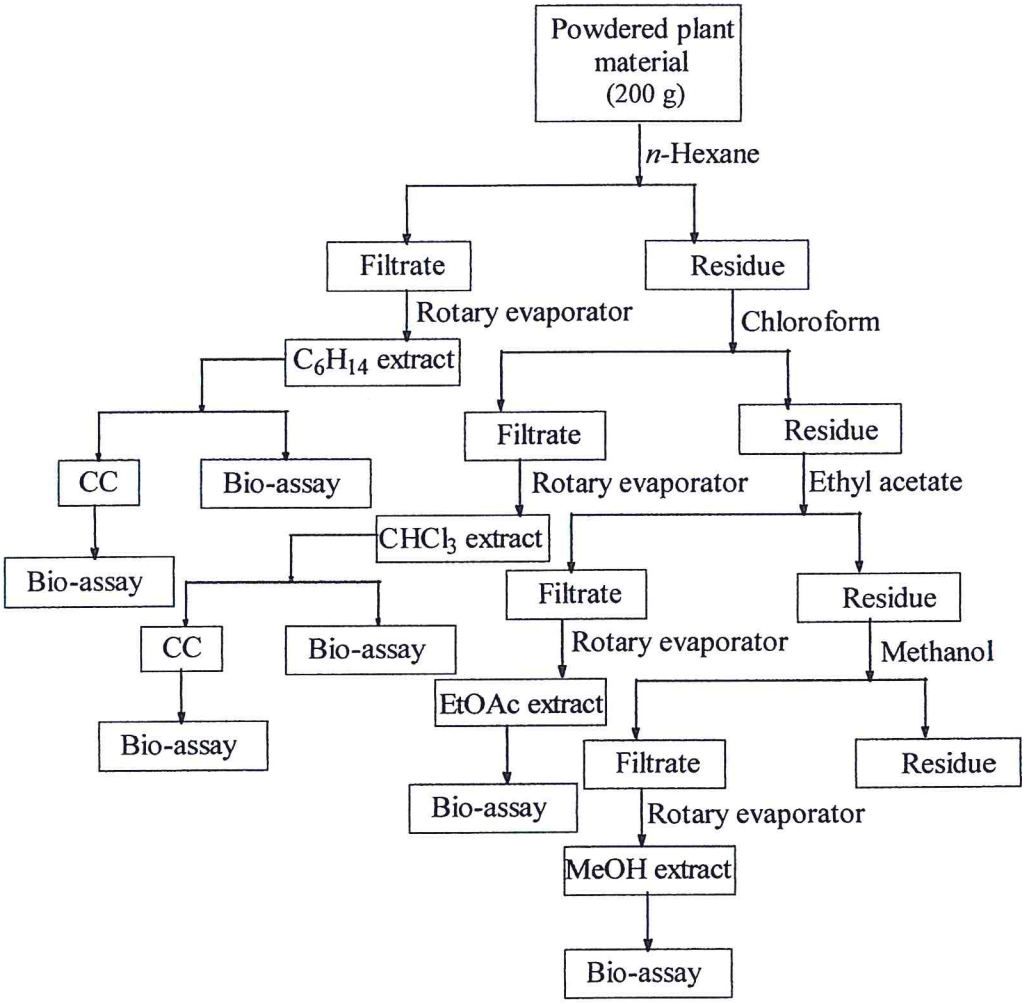
Table 18. Location of plant collection.

Plant	Location	Plant	Location
Neoboutonia macrocalyx	Gakoe	*Solanum aculeastrum*	Limuru
Bidens pilosa	Nairobi	*Piper capense*	Kakamega forest
Clausena anisata	Ngong forest	*Piper guineense*	" "
Teclea simplicifolia	" "	*Pittosporum mannii*	" "
Albizia coriaria	Got Ramogi	*Piper umbellatum*	" "
Melia azedarach	Ogango	*Solanum mauritianum*	" "
Hagenia abyssinica	Kinale forest	*Zanthoxylum gillettii*	" "
Iboza multiflora	Naivasha	*Zanthoxylum midelbraedii*	" "
Toddalia asiatica	Kapenguria		

6.2 Extraction

Cold extraction method was employed on the powdered plant material. The powder (200 g) was soaked in 1000 ml of the extraction solvent for 3 days with occasional shaking. The solvent extracts were filtered and concentrated at low temperature and reduced pressure in a rotary evaporator. Sequential solvent extraction was done with *n*-hexane, chloroform, ethyl acetate and methanol in this order to obtain different extracts (Scheme 10). The extraction was set up with hexane for 3 days, the extract filtered out under suction and chloroform added to the residue. This process was repeated for ethyl acetate and methanol, respectively. Each solvent extract was concentrated as described above to obtain different plant extracts.

Scheme 10. Extraction process



6.3 Larvicidal assay

Different solvents were used to dissolve and disperse the crude extracts in water. Solvents investigated include 'Tween' 80, dioxan, acetone and ethanol. 'Tween' 80 is a surfactant; it killed the larvae and hence could not be used. The sample dissolved in dioxan but precipitated on addition of water. Acetone was found to be the best solvent for hexane, chloroform and ethyl acetate extracts. Methanol extracts could not dissolve in acetone and therefore absolute ethanol was used in the preparation of the solutions for bio-assay.

The crude extract (100 mg) was dissolved in 10.0 ml of acetone to make a 1%

solution (for hexane, chloroform and ethyl acetate extracts). For methanol crude extracts, 100 mg of the extract was dissolved in 10.0 ml of absolute ethanol to make a 1% stock solution. The stock solutions were serially diluted with acetone or ethanol (as appropriate) to achieve 0.1% and 0.01% concentrations.

6.4 Preliminary assay

From the stock solutions (0.1 and 0.01%), 1.0 ml was drawn and added to 99.0 ml of distilled water in a beaker (250 ml) to give 0.0001 and 0.00001% solutions, respectively. The resulting solutions were divided into five equal portions of 20.0 ml and put in vials (30 ml). Five (5) late 3rd or early 4th instar *An. gambiae* larvae were introduced into each vial and fed with fish food. Control experiments were prepared by adding 1.0 ml of acetone (for hexane, chloroform and ethyl acetate extracts) or absolute ethanol (for methanol extract) to 99.0 ml of distilled water in a beaker (250 ml). The resulting solution was treated as detailed above.

Mortality was monitored for 24, 48, 72 hr until emergence of adults in the test and control solutions was observed. Larvicidal activity of the various extracts was determined on the basis of the % mortality of the larvae introduced into the test vials. Mortality in test vials was weighted against that of the control vials and % mortality calculated using the formula;

$$\%\text{Mortality} = \left\lfloor {}^{Y}\!/_{X} \right\rfloor \times 100$$

Where, Y is the mean death count defined by the difference between the mean test deaths and mean control deaths, and X is the initial larvae population (WHO, 1996b). The results are summarised in tables 2-5.

6.5 Detailed larvicidal assay

Detailed assays were performed on the most active crude extracts (Tables 6-11) and the fractions obtained from the column chromatography (Tables 13-16) of *Z. gillettii* hexane and chloroform extracts. In this case, twenty (20) late 3rd or early

4th instar larvae of *An. gambiae* were used in 250 ml beakers. Control experiments were set up as earlier described but with 20 *An. gambiae* larvae. However, assays of *P. mannii* column fractions could not be performed since none of the fractions obtained could dissolve in any of the solvents used to carry out the assays.

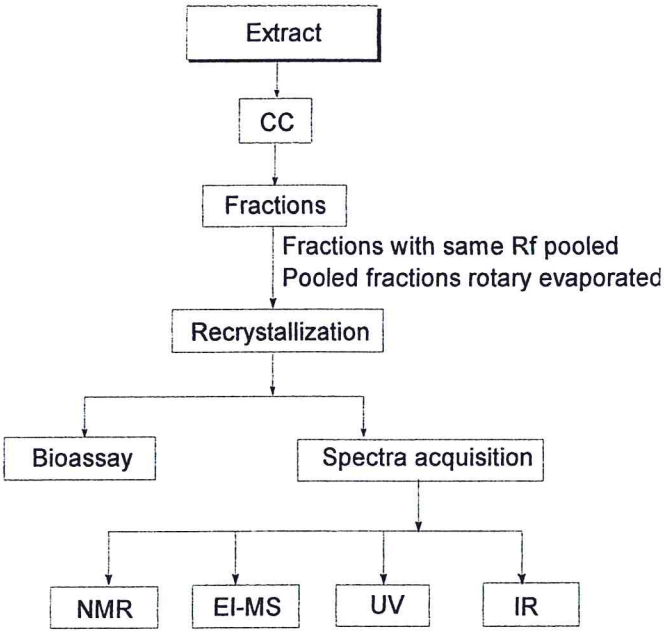
Different concentrations ranges (1, 2, 4, 6, 8, 10 and 5, 10, 20, 40, 60, 80, 100 µg/ml) were used. Each concentration range was assayed in five replicates in three experiments. Each experiment was done with a different batch of larvae. Levels of activity of the assays were determined as detailed above.

Probit analysis was done to determine the regression equations. This involved log dose and probit transformations of dose and percentage response (mortality), employing probit plane model by Busvine (1971). Probit transformations were plotted against log (+3) dose in a graph using excel program in a computer. A regression equation was then obtained from this plot and used to calculate the expected probits and the lethal doses (LD) (Tables 12 and 17).

6.6 Isolation of larvicidal compounds from *Z. gillettii*

Z. gillettii bark (600 g) was extracted with 3 l of hexane to give a yield of 11.7 g of crude extract. A mixture of the *n*-hexane (7 g) and chloroform (3 g) extract was fractionated by column chromatography (CC) on silica gel 60 G (Merck grade), particle size 0.040-0.063 mm (230-400 mesh ASTM) and eluted with varying concentrations of *n*-hexane/ethyl acetate mixture. The fractions bearing compounds with same R_f values were pooled together. Bio-assays of the fractions obtained were done as detailed above and those showing larvicidal activity targeted for further chemical investigation. Recrystalization of purified compounds in bio-active as well as inactive fractions was done. Some fractions were further purified by preparative thin layer chromatography (PTLC) using precoated TLC plates. The pure isolated compounds were subjected once again to larvicidal assays as described above (Scheme 11).

Scheme 11. Fractionation and Identification.



From *Z. gillettii*, thirteen (**F_1-F_{13}**) compounds were isolated. Only nine compounds (**F_1, F_2, F_3, F_5, F_6, F_7, F_8, F_9, F_{10}**) could be identified through spectroscopic analysis.

Sesamin (F_1) (15)

The compound was isolated in 1.6 g, R_f 0.38 (1:3 ethyl acetate:hexane). Found: mp 123-5 ^{0}C (lit. 123-4 ^{0}C; Anjaneulu *et al.*, 1977; Pelter *et al.*, 1976); ^{13}C NMR (200 MHz, $CDCl_3$), δ 147.90 (s, C-4′, C-4″), 147.04 (s, C-3′, C-3″), 134.92 (s, C-1′, C-1″), 119.35 (d, C-6′, C-6″), 108.15(d, C-2′, C-2″), 106.45 (d, C-5′, C-5″), 101.05 (t, -OCH_2O-), 85.73 (d, C-2, C-6), 71.65 (d, C-4, C-8), 54.24 (t, C-1, C-5) ppm; ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.83 (d, 2H, J = 11.4, H-2′, H-2″), 6.77 (s, 4H, H-5′, H-5″, H-6′, H-6″), 5.94 (d, 4H, J = 0.6 Hz, -OCH_2O-), 4.69 (d, 2H, J = 3.6 Hz, H-2, H-6), 4.21 (t, 2H, J = 6.2 Hz, H-4, H-8), 3.85 (dd, 2H, J = 3.0, 3.0 Hz, H-4, H-8), 3.03 (t, 2H, J = 1.2 Hz, H-1, H-5); ^{1}H-^{1}H COSY revealed coupling between H-2, H-6 (δ 4.69); H-4, H-8 (δ 4.21) to H-1, H-5 (δ 3.03); IR ν_{max} (KBr) 3074, 2964, 1501, 1036, 935 cm^{-1}; UV λ_{max} 213, 233, 283 nm; EI-MS m/z 354 (34) $[M]^+$, 204 (9), 203 (28), 161 (60), 149 (100), 135 (90), 121 (41), 77 (25) (Anjaneulu *et al.*,

1977; Pelter *et al.*, 1976).

Lupeol (F_2) (14)

The compound was isolated in 4.1 g, R_f 0.52 (1:3 ethyl acetate:hexane). Found: mp 215-7 ^{0}C (lit. 211-4, 215-6 ^{0}C; Lee *et al.*, 2001; Marques *et al.*, 1998); ^{13}C NMR (200 MHz, $CDCl_3$), δ 151.25 (s, C-20), 109.57 (t, C-29), 79.24 (d, C-3), 55.49 (d, C-19), 50.64 (d, C-5), 48.50 (d, C-13), 48.21 (d, C-9), 43.23 (s, C-4), 43.05 (s, C-10), 41.03 (s, C-28), 40.23 (t, C-8), 39.09 (t, C-14), 38.91 (t, C-2), 38.25 (d, C-21), 37.38 (s, C-17), 35.80 (t, C-16), 34.48 (t, C-22), 30.05 (t, C-12), 28.21 (q, C-30), 27.62 (t, C-7, C-15), 25.33 (t, C-11), 21.14 (t, C-6), 19.54 (q, C-26), 18.54 (t, C-1), 18.23 (q, C-25), 16.36 (q, C-24), 16.20 (q, C-23), 15.62 (q, C-27), 14.77 (q, C-28) ppm; ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.68 (s, 1H, H-29), 4.56 (s, 1H, H-29), 3.19 (dd, 1H, J = 5.6, 5.4 Hz, H-3), 2.38 (dt, 1H, J = 10.6, 11.4 Hz, H-19), 1.68 (s, 3H, H-30), 1.02 (s, 3H, H-23), 0.94 (s, 3H, H-25), O.92 (s, 3H, H-24), 0.82 (s, 3H, H-26), 0.78 (s, 3H, H-27), 0.76 (s, 3H, H-28); IR ν_{max} (KBr) 3335, 3067, 2943, 2872, 1638, 1188, 983 cm^{-1}; UV λ_{max} 204 nm; EI-MS m/z 426 (12) $[M]^+$, 408 (2), 175 (34), 135 (77), 121 (78), 107 (79), 95 (100), 81 (80), 69 (61), 55 (62), 41 (57) (Lee *et al.*, 2001; Marques *et al.*, 1998; Souza *et al.*, 2001).

Fagaramide (F_3) (13)

The compound was isolated in 0.9 g, R_f 0.11 (1:3 ethyl acetate:hexane). Found: mp 119-21 ^{0}C (lit. 115-6 ^{0}C; Shobert *et al.*, 2001); ^{13}C NMR (200 MHz, $CDCl_3$), δ 166.47 (s, C-1), 149.22 (s, C-6), 148.41 (s, C-7), 140.97 (d, C-3), 129.45 (s, C-4), 124.12 (d, C-2), 118.94 (d, C-9), 108.74 (d, C-5), 106.52 (d, C-8), 101.66 (t, -OCH_2O-), 47.34 (t, C-1′), 28.88 (d, C-2′), 20.42, 20.42 (q, C-3′, C-4′) ppm; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.53 (d, 1H, J = 15.4 Hz, H-3), 6.99 (s, H-5), 6.97 (d, 1H, J = 8.2 Hz, H-8), 6.78 (d, 1H, J = 8.0 Hz, H-9), 6.26 (d, 1H, J = 15.6 Hz, H-2), 5.98 (s, 2H, -OCH_2O-), 5.88 (s, br, 1H, H-N), 3.21 (t, 1H, J = 6.3 Hz, H-1′), 1.83 (m, 1H, H-2′), 0.94 (d, 6H, J = 6.6 Hz, H-3′, H-4′); ^{1}H-^{1}H COSY revealed coupling between H-3′, H-4′ (δ 0.94) with H-2′ (δ 1.83), H-2′ (δ 1.83) with H-1′ (δ 3.21), H-2 (δ 6.26) with H-3 (δ 7.53); IR ν_{max} (KBr) 3296, 2790, 1651 1493, 1448, 1254 cm^{-1}; UV λ_{max} 217, 274, 309 nm; EI-MS m/z 247 (48) $[M]^+$, 204 (5), 190

(89), 175 (100), 147 (28), 145 (95), 135 (29) (Adesina, 1986; Adesina and Reisch, 1988; Shobert *et al.*, 2001).

2,6a-dihydroxy-1-methoxyoxoaporphine (F_5) (98)

The compound was isolated in 0.0018 g, R_f 0.12 (1:3 ethyl acetate:hexane). Found: mp 224-6 ^{0}C; ^{13}C NMR (200 MHz, $(CD_3)_2CO$) 146.38 (d, C-5), 130.91 (d, C-11), 126.30 (d, C-8), 123.69 (d, C-4), 117.34 (d, C-10), 114.52 (d, C-9), 110.53 (d, C-3), 56.99 (q, OCH_3) ppm; ^{1}H NMR (200 MHz, CD_3OD) δ 8.72 (d, 1H, J = 4.6 Hz, H-5), 8.61 (d, 1H, J = 8.0 Hz, H-11), 8.27 (d, 1H, J = 7.6 Hz, H-8), 8.07 (d, 1H, J = 4.6, H-4), 7.91 (s, 1H, H-3), 7.76 (dd, 1H, J = 7.6, 8.0 Hz, H-10), 7.60 (dd, 1H, J = 7.6, 7.6 Hz, H-9), 7.30 (s, 1H, N-H), 6.52 (s, 1H, O-H), 4.08 (s, 3H, OCH_3); ^{1}H-^{1}H COSY revealed coupling between H-4 (δ 8.07) and H-5 (δ 8.72), H-8 (δ 8.27) and H-9 (δ 7.62), H-9 (δ 7.62) and H-10 (δ 7.76), H-10 (δ 7.76) and H-11 (δ 8.61); NOE interactions were observed between OCH_3 (δ 4.08) and OH (δ 6.52); IR ν_{max} (KBr) 2916, 2849, 1672, 1020 cm^{-1}; UV λ_{max} 240, 258, 289, 335, 351, 369 nm; EI-MS m/z 295 (1) $[M]^+$, 251 (16), 250 (88), 249 (48), 247 (9), 221 (100), 207 (36), 192 (48).

Arnottianamide (F_6) (93)

The compound was isolated in 0.032 g, R_f 0.14 (1:3 ethyl acetate:hexane). Found: mp 265-6 ^{0}C (lit. 264-7, 267-70, 271-3 ^{0}C; Krane *et al.*, 1994; Sharma *et al.*, 1982); ^{13}C NMR (200 MHz, $CDCl_3$), δ 164.61 (s, CHO), 152.02 (s, C-1b), 149.29 (s, C-5), 148.29 (s, C-6), 146.64 (s, C-2′), 135.72 (s, C-7a), 135.48 (s, C-3a), 133.31 (s, C-3′), 131.19 (d, C-1a), 128.67 (s, C-4′), 127.45 (d, C-6′), 127.35 (d, C-2), 125.10 (d, C-3), 118.51 (d, C-1′), 104.32 (d, C-7), 103.85 (d, C-4), 101.48 (t, $-OCH_2O-$), 99.24 (d, C-5′), 61.17 (q, OCH_3), 55.80 (q, OCH_3), 33.02 ($N-CH_3$) ppm; ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.16 (s, 1H, CHO), 7.74 (d, 1H, J = 8.2 Hz, H-2), 7.32 (d, 1H, J = 8.2 Hz, H-3), 7.20 (s, 1H, H-4), 7.08 (s, 1H, H-7), 6.81 (d, 1H, J = 8.7 Hz, H-5′), 6.54 (d, 1H, J = 8.7 Hz, H-6′), 6.09 (s, 2H, $-OCH_2O-$), 5.99 (s, br, 1H, OH) 3.92 (s, 3H, OCH_3) and 3.90 (s, 3H, OCH_3), 3.00 (s, 3H, NCH_3); ^{1}H-^{1}H COSY revealed coupling between H-2 (δ 7.74) and H-3 (δ 7.32), H-5′ (δ 6.81) and H-6′ (δ 6.54); NOE interactions were observed between $N-CH_3$ (δ

3.00) with H-7 (δ 7.08) and CH_3O on C-7 (δ 3.92); IR ν_{max} (KBr) 2837, 1659, 1055, 934 cm^{-1}; UV λ_{max} 213, 234, 273, 311, 332 nm; EI-MS m/z 381 (100) $[M]^+$, 382 (26), 353 (31), 322 (54), 307 (42) (Adesina and Reisch (1988); Ishii and Ishikawa, 1976; Krane *et al.*, 1984; Sharma *et al.*, 1982).

N-methyl-1,2,6a-trihydroxyoxoaporphine (F_7) (102)

The compound was isolated in 0.0025 g, R_f 0.01 (1:3 ethyl acetate:hexane). Found: mp 235-8 ^0C; ^{13}C NMR (200 MHz, $CDCl_3$) 157.91 (s, C-2), 157.54 (s, C-1), 191.00 (s, C-7), 145.45 (d, C-5), 139.39 (s, C-11a), 131.48 (d, C-10), 130.90 (s, C-3a), 126.13 (d, C-9), 125.35 (d, C-3), 124.49 (s, C-7a), 122.95 (d, C-8), 117.50 (d, C-4), 117.40 (d, C-11), 114.00 (s, 6a), 42.05 (q, NCH_3); ^1H NMR (200 MHz, $CDCl_3$); 8.78 (d, 1H, J = 5.0 Hz, H-5), 8.69 (d, 1H, J = 8.0 Hz, H-11), 8.14 (d, 1H, J = 7.2 Hz, H-8), 8.04 (d, 1H, J = 5.0 Hz, H-4), 7.76 (dd, 1H, J = 8.0, 7.8 Hz, H-10), 7.57 (dd, 1H, J = 7.8, 7.2 Hz, H-9), 7.52 (s, 1H, H-3), 7.51 (s, 1H, O-H), 7.27 (s, 1H, O-H), 3.20 (s, 3H, N-CH_3); ^1H-^1H COSY revealed coupling between H-4 (δ 8.04) and H-5 (δ 8.78), H-8 (δ 8.14) and H-9 (δ 7.57), H-9 (δ 7.57) and H-10 (δ 7.76), H-10 (δ 7.76) and H-11 (δ 8.69); NOE interactions were observed between NCH_3 (δ 3.20) and OH (δ 7.48); IR ν_{max} (KBr) 3391, 2924, 1720, 1443, 1038 cm^{-1}; UV λ_{max} 239, 247, 256, 336 nm; EI-MS m/z 295 (2) $[M]^+$, 235 (29), 234 (100%), 206 (29), 205 (14), 192 (95), 164 (31).

N-methyl-1,3,6a-trihydroxyoxoaporphine (F_8) (105)

The compound was isolated in 0.003 g, R_f 0.17 (1:3 ethyl acetate:hexane). Found: mp 252-3 ^0C; ^{13}C NMR (200 MHz, $CDCl_3$); 158.39 (s, C-3), 155.18 (s, C-1), 144.90 (d, C-5), 139.45 (s, C-11a), 135.14 (s, C-1a), 130.99 (d, C-10), 130.82 (s, C-3a), 125.33 (d, C-9), 124.50 (s, C-6b), 124.25 (s, C-7a), 123.00 (s, C-6a), 122.71 (d, C-8), 117.44 (d, C-4), 117.12 (d, C-11), 117.03 (d, C-2), 13.90 (q, N-CH_3); ^1H NMR (200 MHz, $CDCl_3$); 8.79 (d, 1H, J = 5.0 Hz, H-5), 8.62 (d, 1H, J = 8.4 Hz, H-11), 8.09 (d, 1H, J = 7.6 Hz, H-8), 7.99 (d, 1H, J = 5.0 Hz, H-4), 7.69 (dd, 1H, J = 8.4, 7.6 Hz, H-10), 7.49 (dd, 1H, J = 7.6, 7.6 Hz, H-9), 6.52 (s, 1H, H-2), 2.58 (s, 3H, CH_3); ^1H-^1H COSY revealed coupling between H-4 (δ 7.99) and H-5 (δ 8.79), H-8 (δ 8.09) and H-9 (δ 7.49), H-9 (δ 7.49) and H-10 (δ 7.69), H-10

(δ 7.69) and H-11 (δ 8.62); NOE interactions were observed between NCH_3 (δ 2.58) and H-3 (δ 6.52); IR ν_{max} (KBr) 2925, 2854, 1664 cm^{-1}; UV λ_{max} 242, 259, 278, 340 nm; EI-MS m/z 295 (2%) $[M]^+$, 267 (22), 266 (100), 238 (24), 237 (28), 206 (18), 205 (31), 192 (54), 164 (21).

Pellitorine (F_9) (12)

The compound was isolated in 0.0062 g, R_f 0.19 (1:3 ethyl acetate:hexane). Found: mp 91-2 ^0C (lit. 90-3 ^0C; Chen *et al.*, 1999; Lalonde *et al.*, 1980); ^{13}C NMR (200 MHz, $CDCl_3$) 166.44 (s, C-1), 143.38 (d, C-5), 141.45 (d, C-3), 128.14 (d, C-4), 121.56 (d, C-2), 46.91 (t, C-1'), 32.91 (t, C-6), 31.34 (t, C-7), 28.59 (d, C-2'), 28.44 (t, C-8), 22.45 (t, C-9), 20.12, 20.12, 14.01 (q, C-3', C-4', C-10) ppm; ^1H NMR (200 MHz, $CDCl_3$) δ 7.14 (dd, 1H, J = 9.4, 14.2 Hz, H-3), 6.14 (m, 1H, H-4), 6.04 (m, 1H, H-5), 5.70 (d, 1H, J = 14.2 Hz, H-2), 5.51 (br, 1H, H-N), 3.18 (br, 2H, H-1'), 2.07 (m, 2H, H-6), 1.73 (m, 1H, H-2'), 1.35-1.19 (m, 6H, H-7, H-8, H-9), 0.95-0.89 (m, 9H, H-3', H-4', H-10); ^1H-^1H COSY revealed coupling between H-5 (δ 6.04) and H-6 (δ 2.07), H-3 (δ 7.14) and H-4 (δ 6.14), H-9 (δ 1.21) and H-10 (δ 0.89); IR ν_{max} (KBr) 3250, 1660, 1000 cm^{-1}; UV λ_{max} 218, 236, 258 nm; EI-MS m/z 223 (19) $[M]^+$, 151 (100), 96 (51), 81 (88), 57 (49), 41 (98) (Adesina, 1986; Adesina and Reisch, 1988; Chen *et al.*, 1999; Lalonde *et al.*, 1980).

Stigmast-5-en-3-ol (F_{10}) (106)

The compound was isolated in 0.0044 g, R_f 0.36 (1:3 ethyl acetate:hexane). Found: mp 133-6 ^0C (lit. 136-7 ^0C; Lee *et al.*, 2001); ^{13}C NMR (200 MHz, $CDCl_3$) 140.72 (s, C-5), 121.98 (d, C-6), 72.04 (d, C-3) 56.96 (d, C-14), 56.22 (d, C-17), 50.31 (d, C-9), 46.00 (d, C-24), 42.50 (t, C-4), 39.97 (t, 16), 37.46 (t, C-1), 36.38 (d, C-20), 34.12 (t, C-7), 32.13 (d, C-8), 31.87 (t, C-2), 29.29 (d, C-25), 28.49 (t, C-12), 26.19 (t, C-23), 24.53 (t, C-15), 23.26 (t, C-28), 21.30 (t, C-11), 20.08 (q, C-21), 19.65 (q, C-27), 19.24 (q, C-26), 19.01 (q, C-19), 12.53 (q, C-29), 12.10 (q, C-18) ppm; ^1H NMR (200 MHz, $CDCl_3$) δ 5.34 (d, 1H, J = 4.0 Hz, H-6), 3.50 (m, 1H, H-3), 2.25 (d, 2H, J = 10.0 Hz, H-4), 1.99 (d, 2H, J = 8.0 Hz, H-7), 1.83 (d, 2H, J = 8.0 Hz, H-2), 0.99 (s, 3H, H-19), 0.84 (d, 3H, J = 1.4 Hz, H-21), 0.81 (d, 6H, J = 2.2 Hz, H-26, H-27), 0.66 (s, 3H, H-18); IR ν_{max} (KBr) 3415, 2933,

1052 cm^{-1}; UV λ_{max} 213 nm; EI-MS m/z 414 (7) $[M]^+$, 396 (8), 119 (25), 69 (44), 55 (79), 43 (100), 41 (53) (Gupta *et al.*, 1992; Greca *et al.*, 1990; Lee *et al.*, 2001).

6.7 Isolation of compounds from *P. mannii*

P. mannii bark (600 g) was extracted with 3 l of hexane to give 3.4 g of the crude extract. The extract (3.0 g) was fractionated by column chromatography (CC) on silica gel 60 G (Merck grade), particle size 0.040-0.063 mm (230-400 mesh ASTM) and eluted with varying concentrations of *n*-hexane/ethyl acetate mixture. The fractions bearing compounds with same R_f values were pooled together. The process in scheme 10 above was adopted. However, bio-assay of the fractions obtained could not be performed because after re-crystallisation, the purified compounds could not dissolve in any of the solvents used in the bio-assay.

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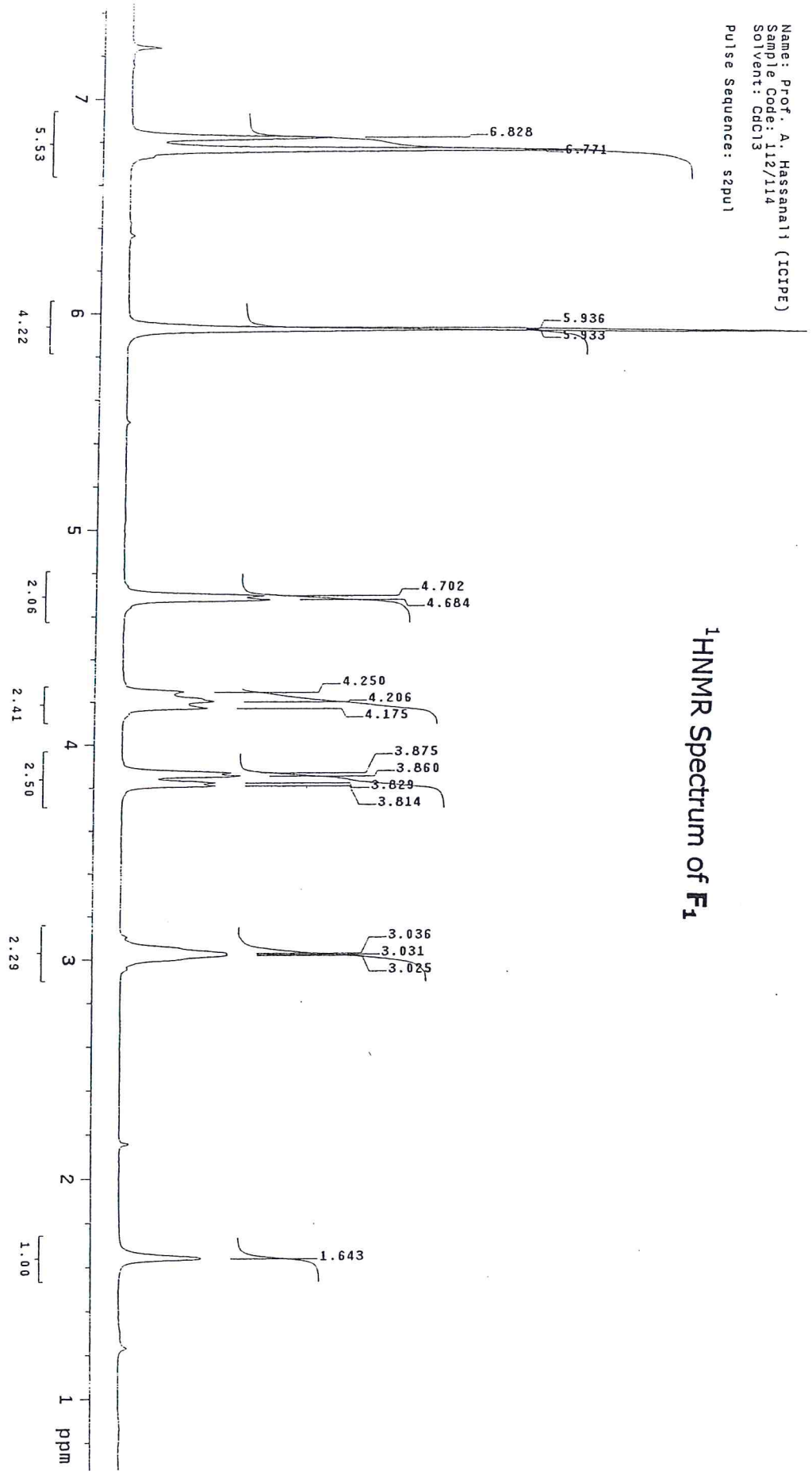
APPENDICES



^{1}HNMR Spectrum of **F_1**

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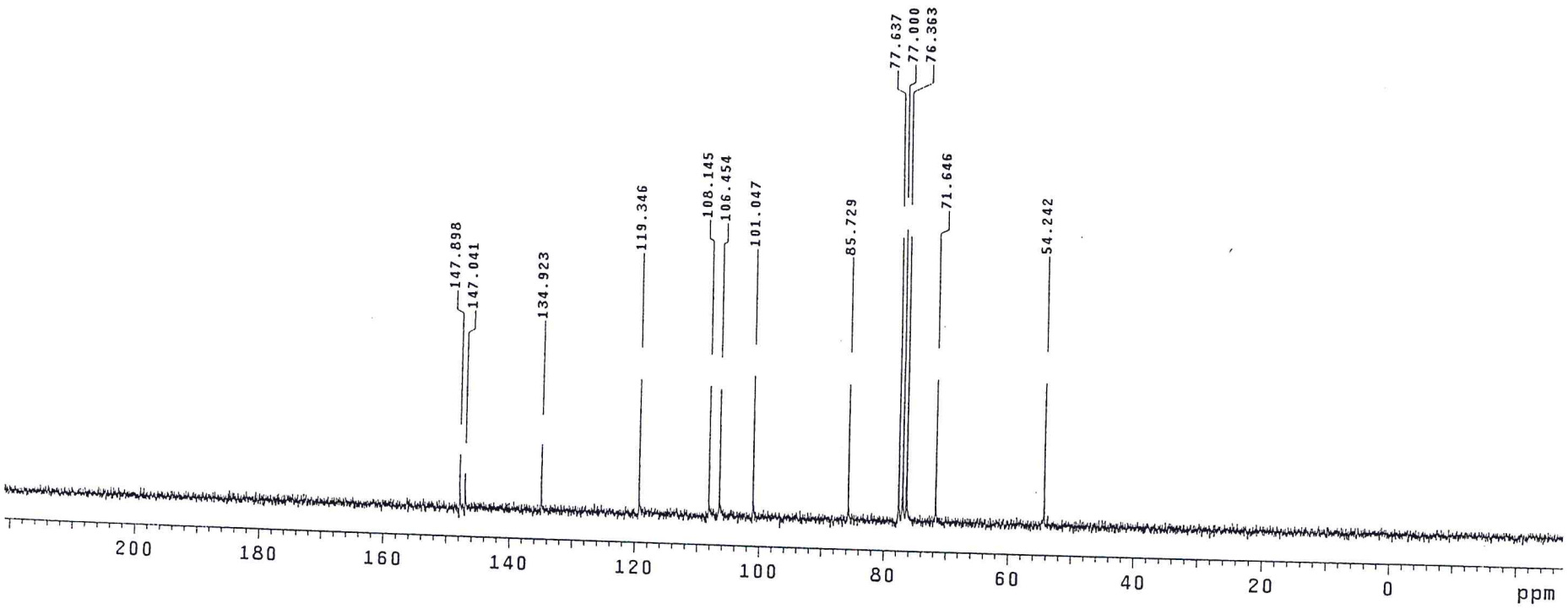
Sample code: 112/114
Solvent: CdCl3

Pulse Sequence: s2pul

^{13}CNMR Spectrum of $\mathbf{F_1}$



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Pulse Sequence: dept

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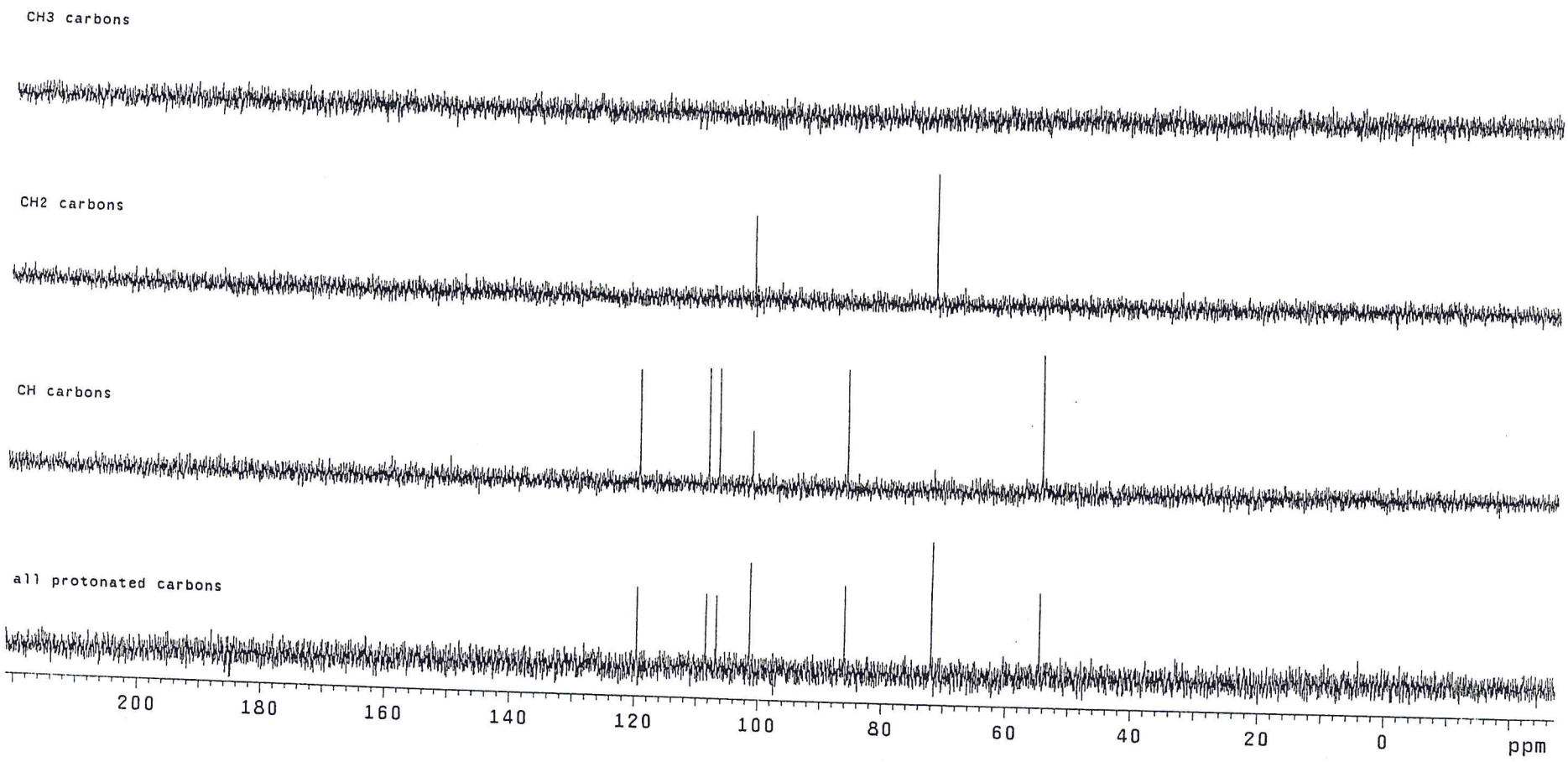


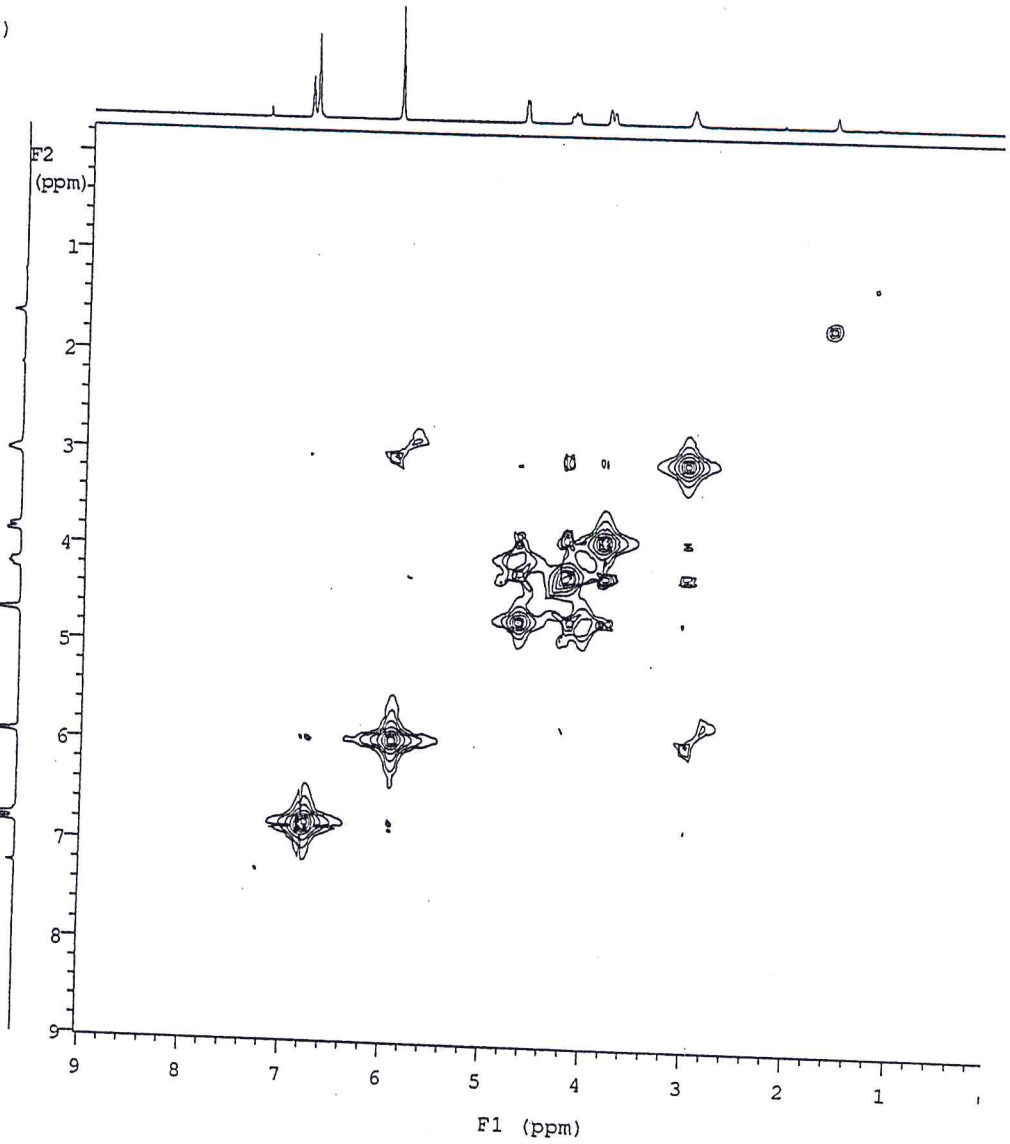
COSY Spectrum of F_1

Name: Prof. A. Hassanali (ICIPE)
Sample code: 112/114
Solvent: CdCl3

Pulse Sequence: relayh

Solvent: CDCl3
Ambient temperature
Mercury-200 "uonnmr200"

PULSE SEQUENCE: relayh
Relax. delay 1.000 sec
COSY 90-90
Acq. time 0.277 sec
Width 1851.2 Hz
2D Width 1851.2 Hz
2 repetitions
84 increments
OBSERVE H1, 200.0557687 MHz
DATA PROCESSING
Sine bell 0.260 sec
Shifted by -0.262 sec
F1 DATA PROCESSING
Line broadening 0.3 Hz
Sq. sine bell 0.060 sec
Shifted by -0.065 sec
FT size 1024 x 1024
Total time 4 min, 12 sec



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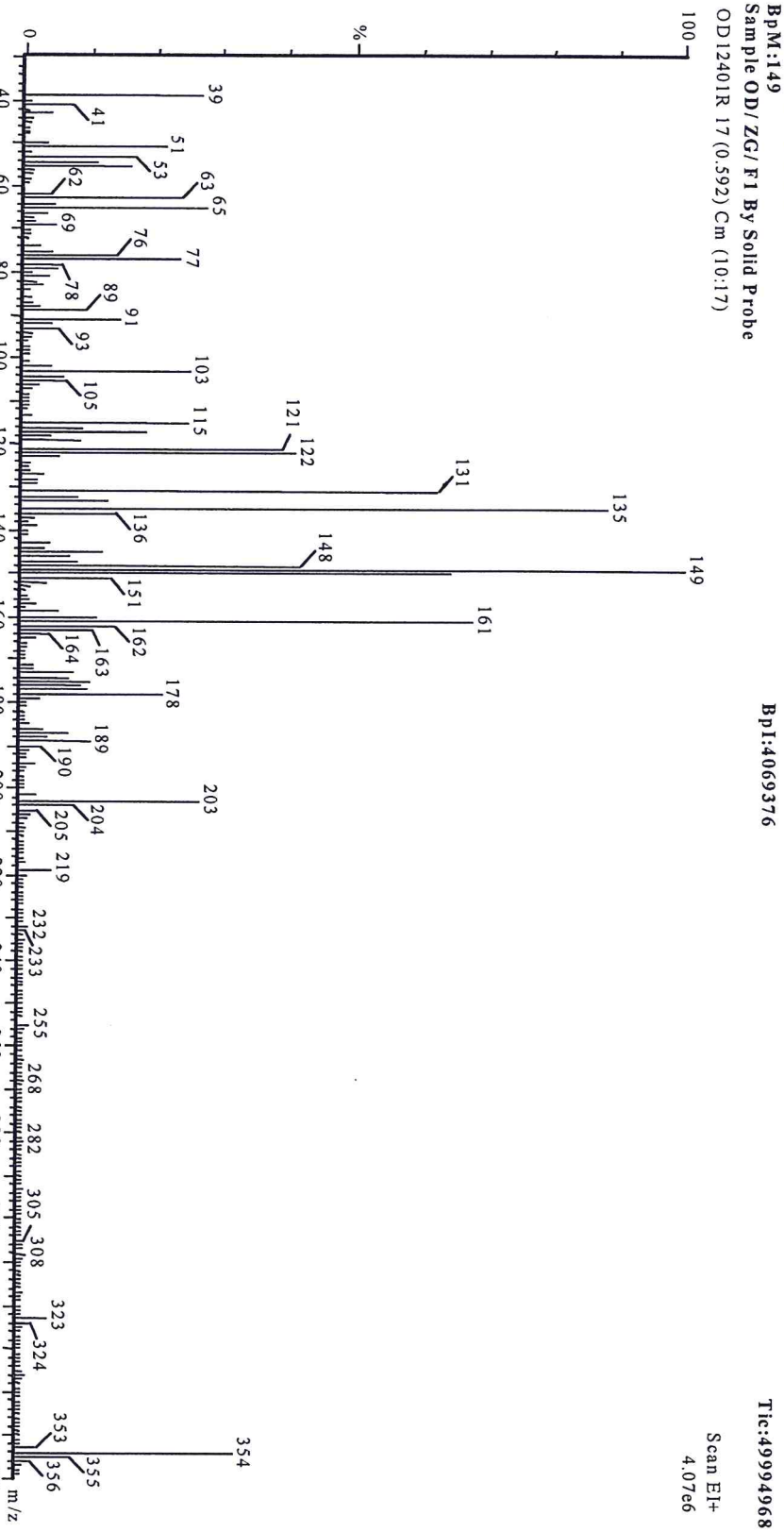
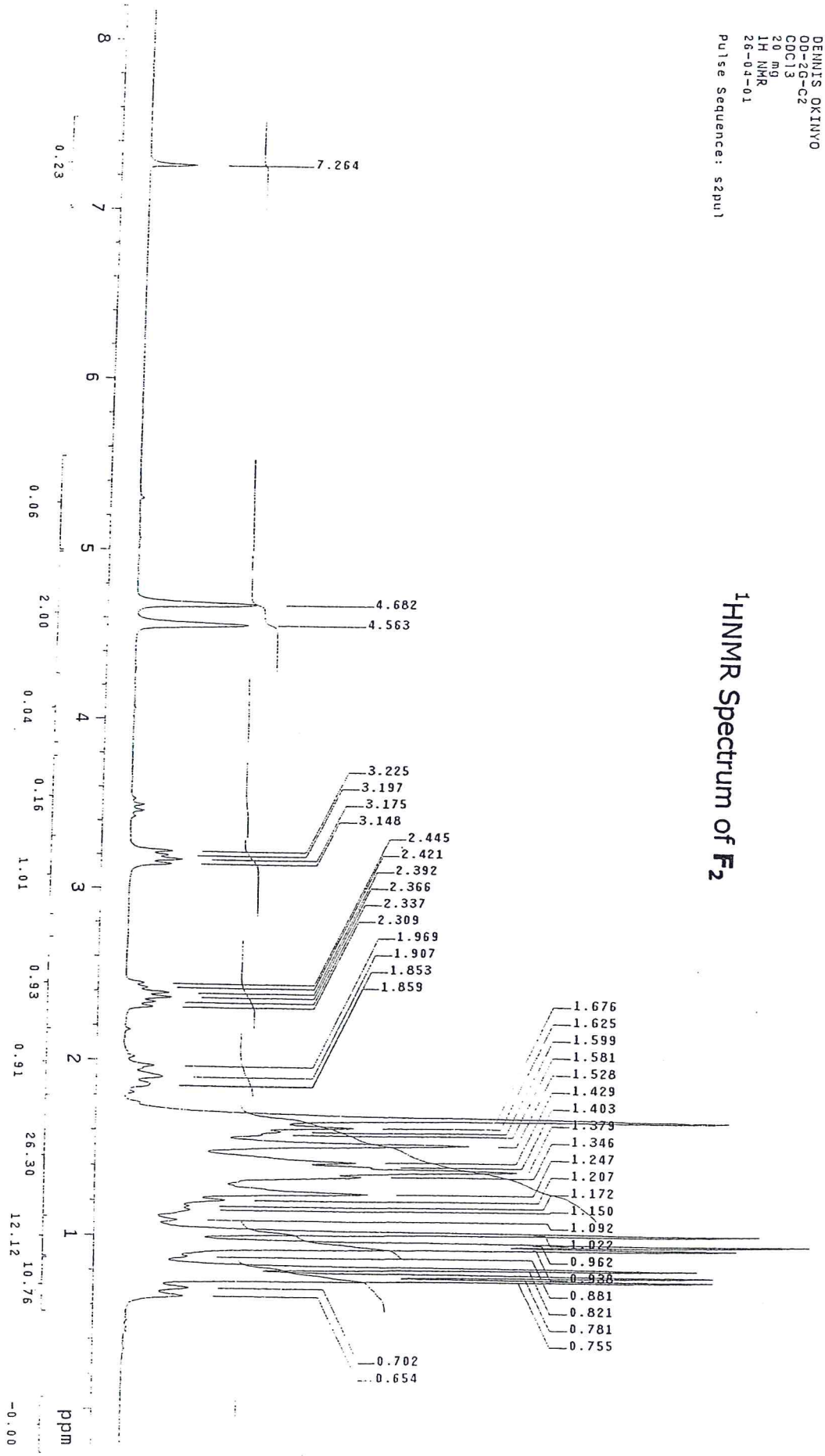
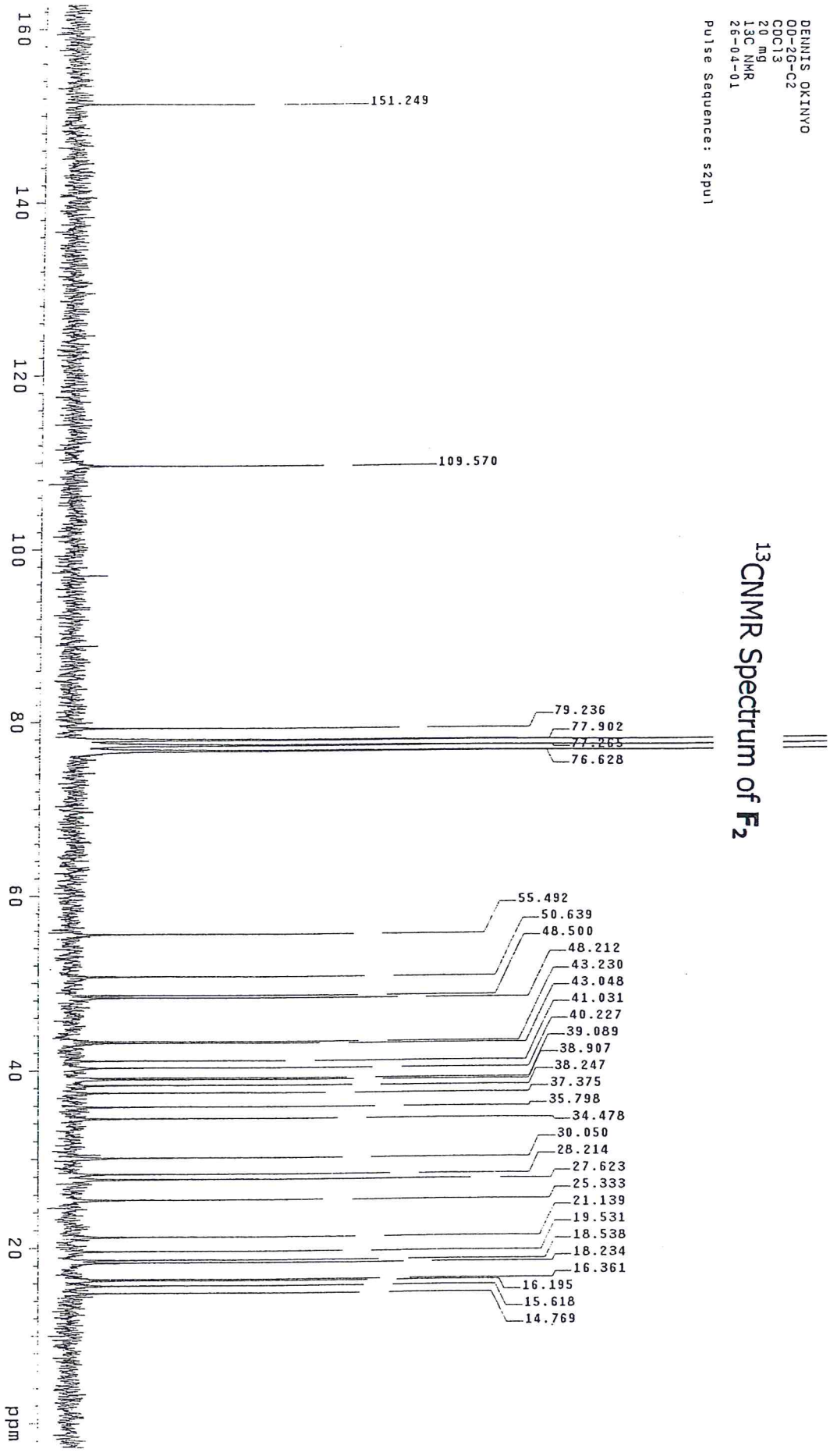




^{1}HNMR Spectrum of $\mathbf{F_2}$



^{13}CNMR Spectrum of $\mathbf{F_2}$

DENNIS OKINYO
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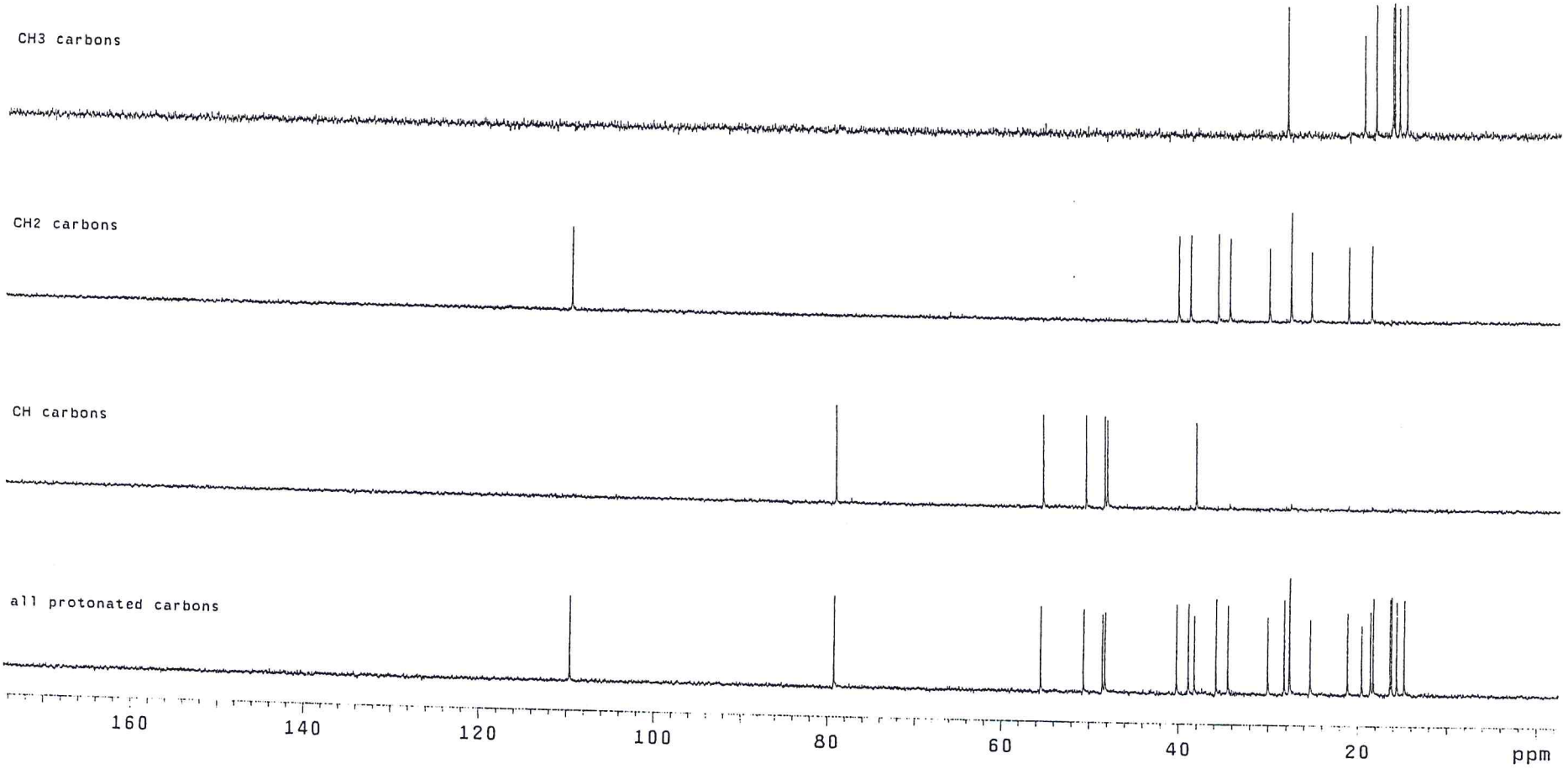
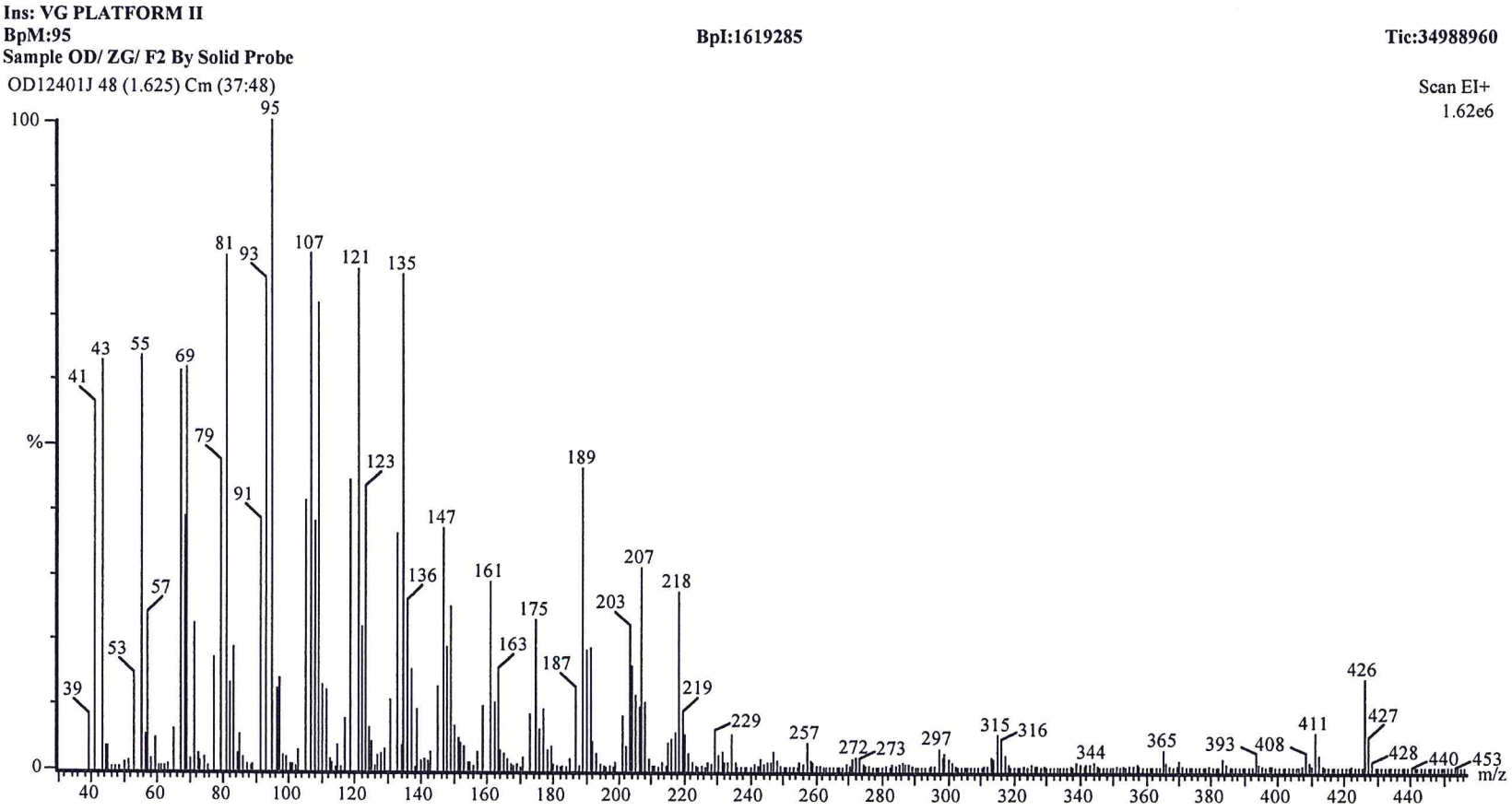
DEPT
27-04-01

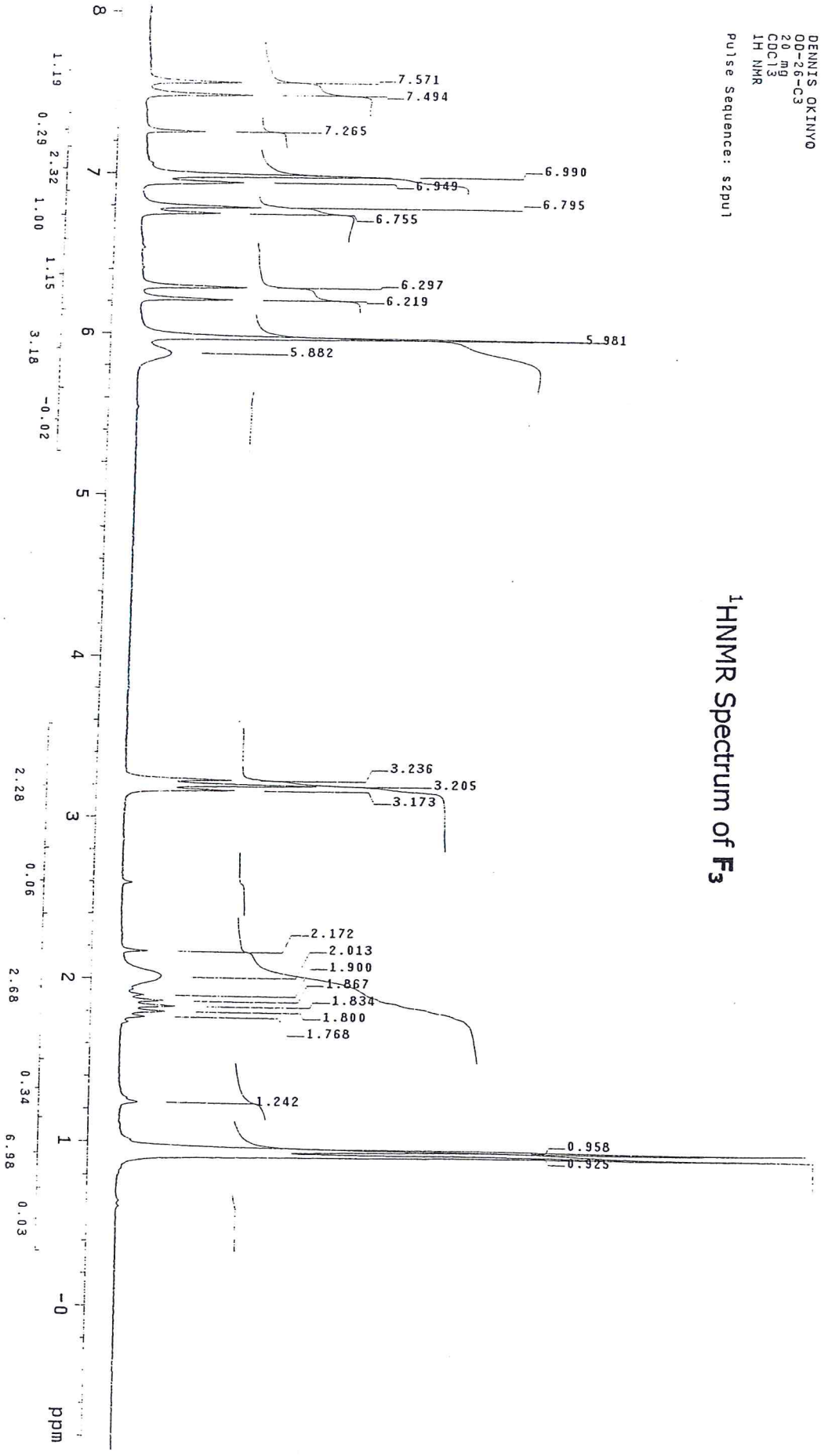
Pulse Sequence: dept

DEPT Spectrum of F_2



Mass spectrum of F_2



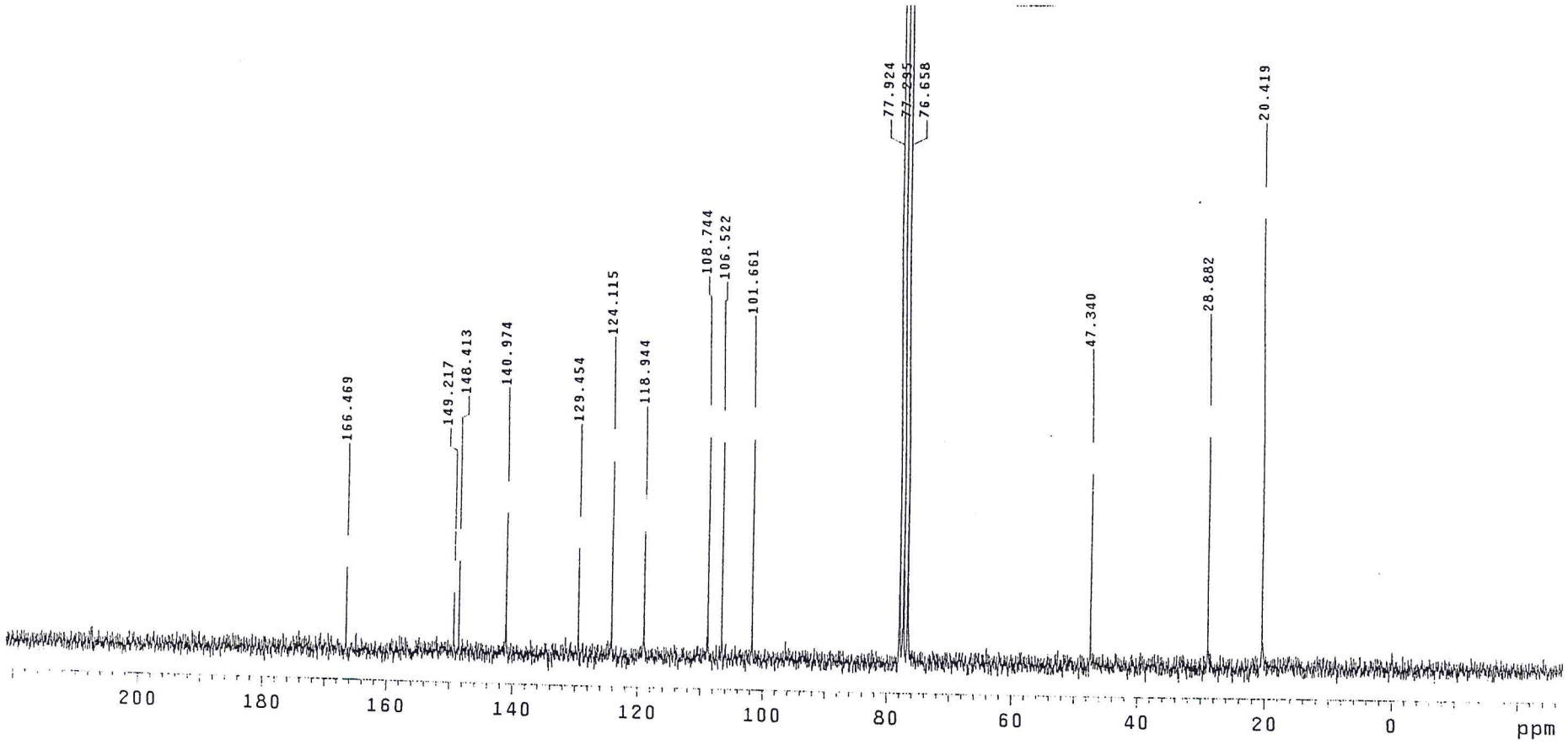


^{1}HNMR Spectrum of **F$_3$**

DENNIS OKINYO
OD-26-C3
20 mg
CDCl3
13C NMR

Pulse Sequence: s2pul

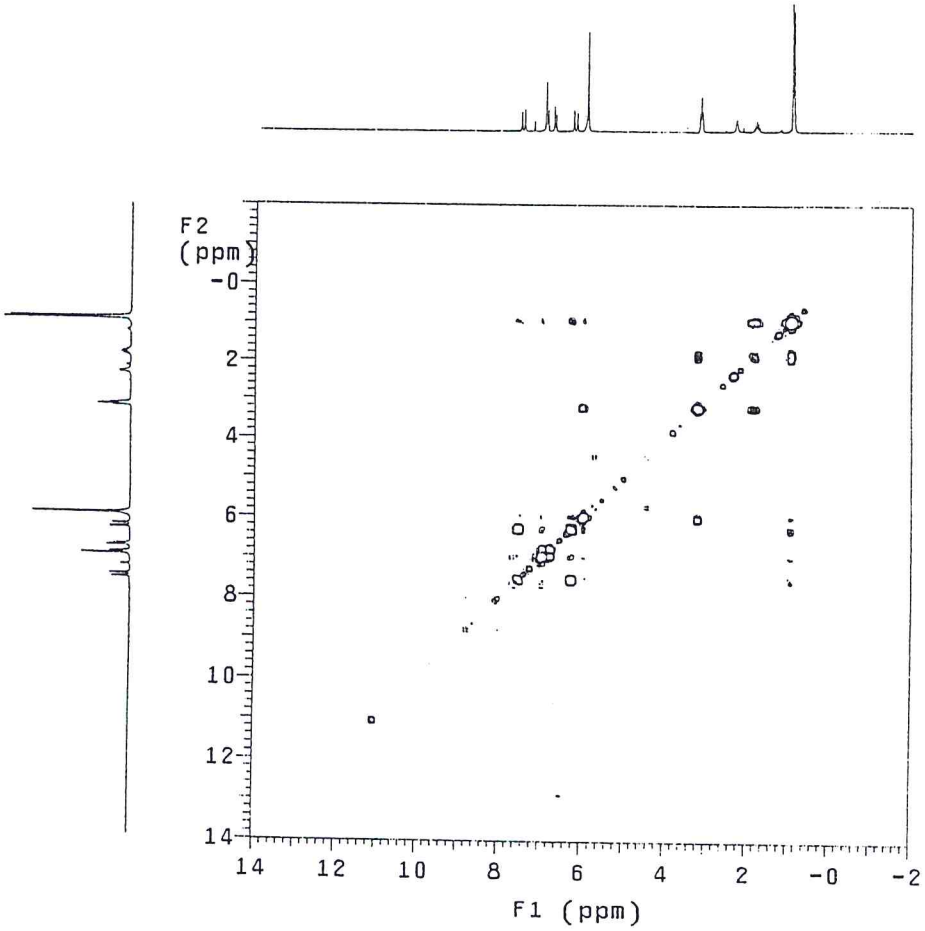
^{13}CNMR Spectrum of F_3



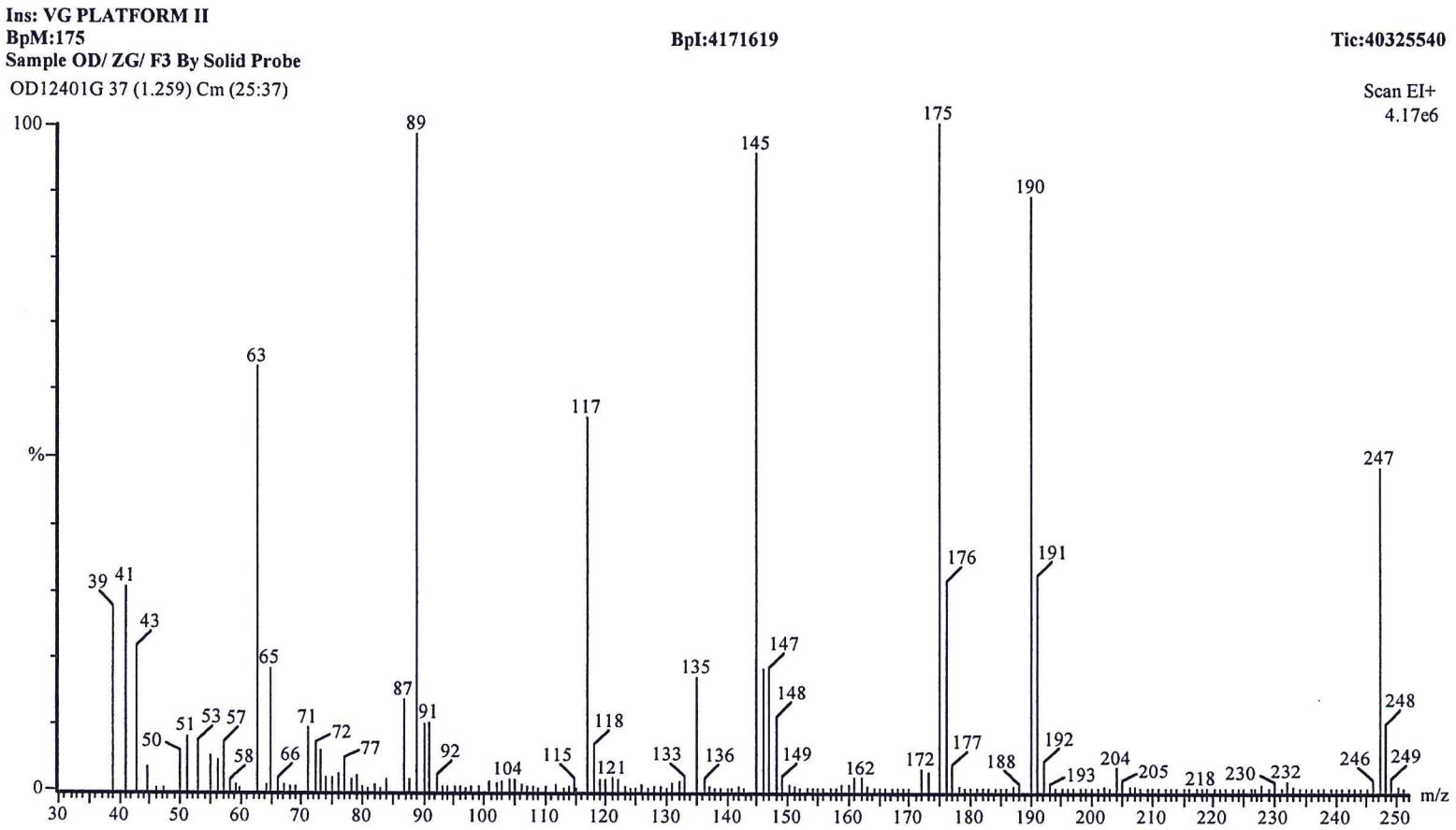
DENNIS OKINYO
OD-26-C3
CDCl3
COSY

Pulse Sequence: COSY
Solvent: CDCl3
Ambient temperature
Mercury-200 "uonnmr200"
PULSE SEQUENCE: COSY
Relax. delay 1.000 sec
Acq. time 0.160 sec
Width 3200.9 Hz
2D Width 3200.9 Hz
2 repetitions
128 increments
OBSERVE H1, 200.0557687 MHz
DATA PROCESSING
Sq. sine bell 0.080 sec
F1 DATA PROCESSING
Sq. sine bell 0.040 sec
FT size 1024 x 1024
Total time 6 min, 36 sec

COSY Spectrum of F_3



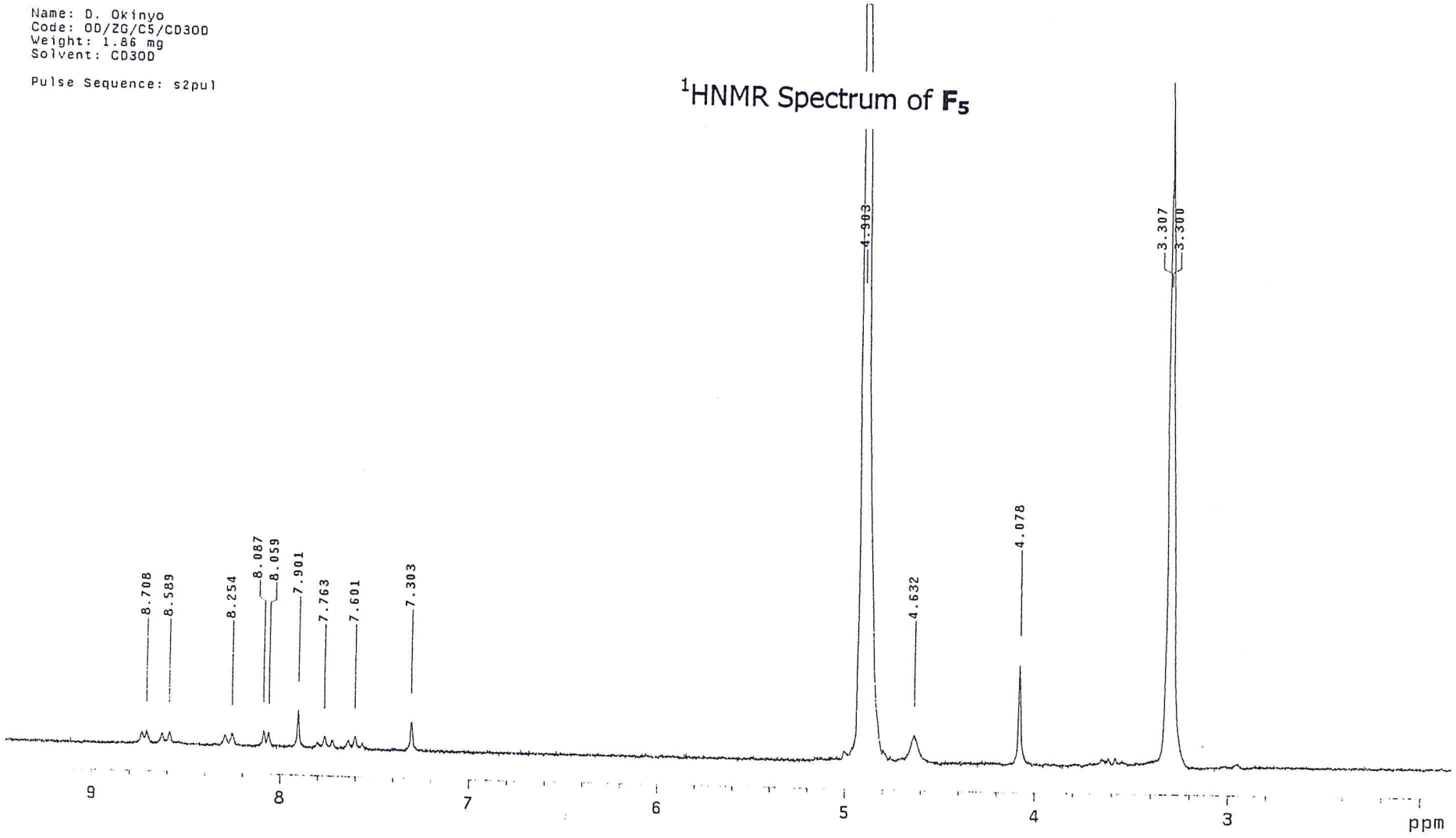
Mass spectrum of F_3



Name: D. Okinyo
Code: OD/ZG/C5/CD3OD
Weight: 1.86 mg
Solvent: CD3OD

Pulse Sequence: s2pul

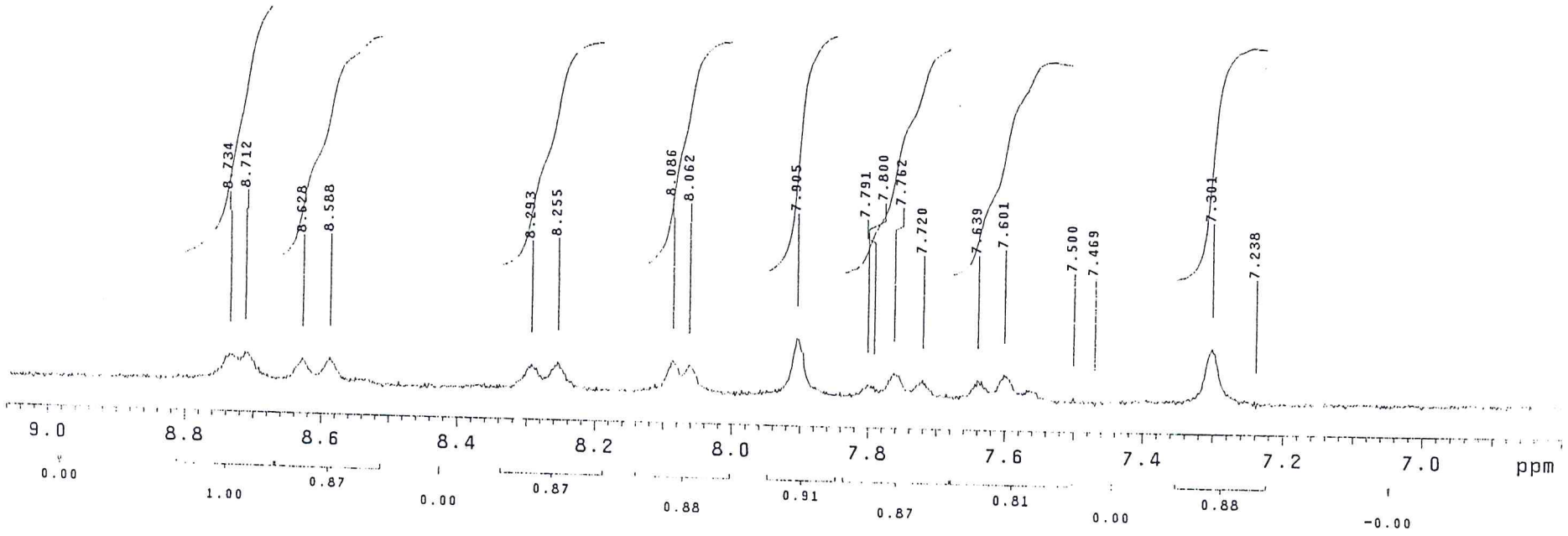
^{1}HNMR Spectrum of F_5

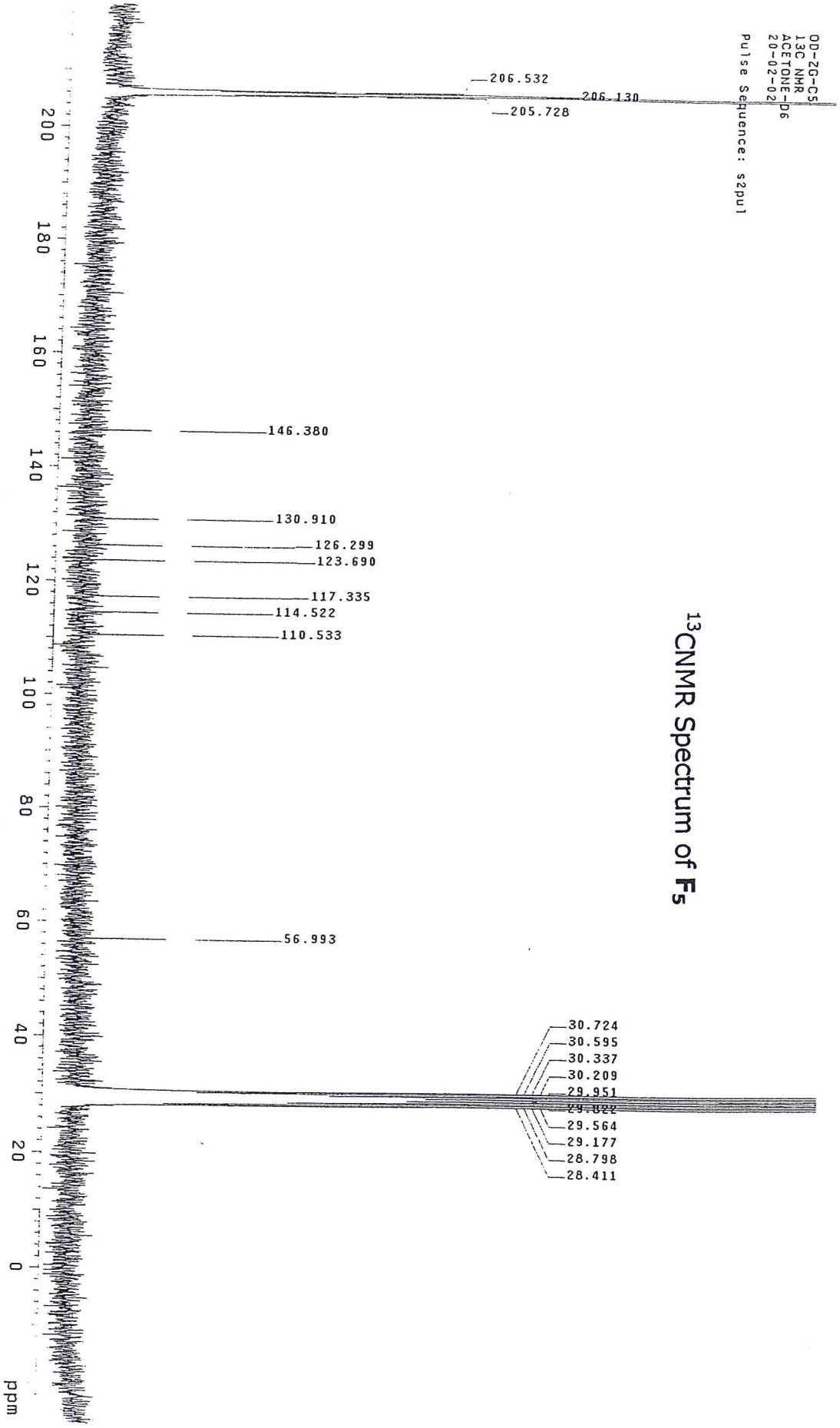


OD/ZG/C5-CD3OD

Pulse Sequence: s2pul

^{1}HNMR Spectrum of **F_5**



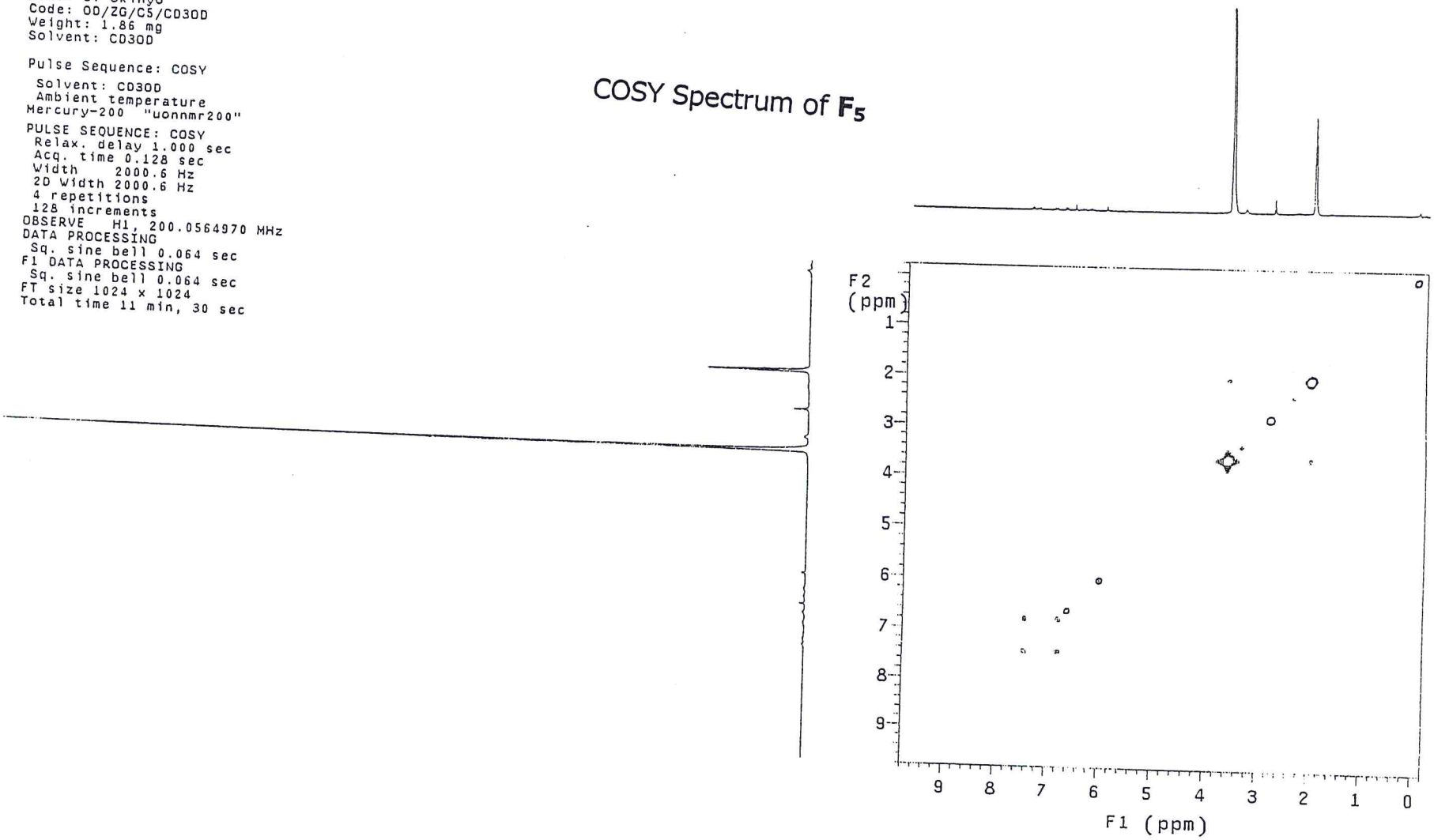


^{13}CNMR Spectrum of **F5**

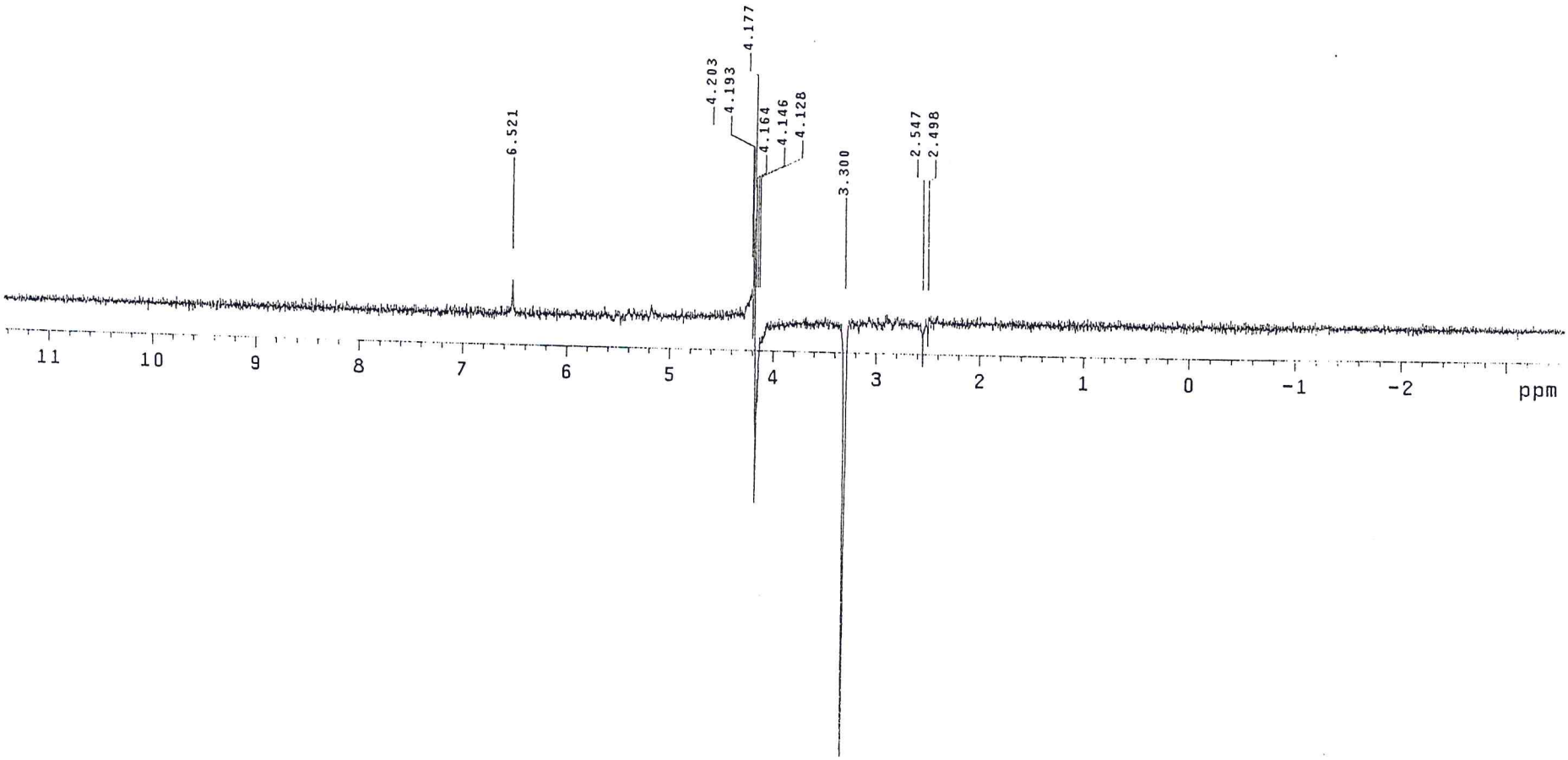
Name: D. Okinyo
Code: OD/ZG/C5/CD3OD
Weight: 1.86 mg
Solvent: CD3OD

Pulse Sequence: COSY
Solvent: CD3OD
Ambient temperature
Mercury-200 "uonnmr200"
PULSE SEQUENCE: COSY
Relax. delay 1.000 sec
Acq. time 0.128 sec
Width 2000.6 Hz
2D Width 2000.6 Hz
4 repetitions
128 increments
OBSERVE H1, 200.0564970 MHz
DATA PROCESSING
Sq. sine bell 0.064 sec
F1 DATA PROCESSING
Sq. sine bell 0.064 sec
FT size 1024 x 1024
Total time 11 min, 30 sec

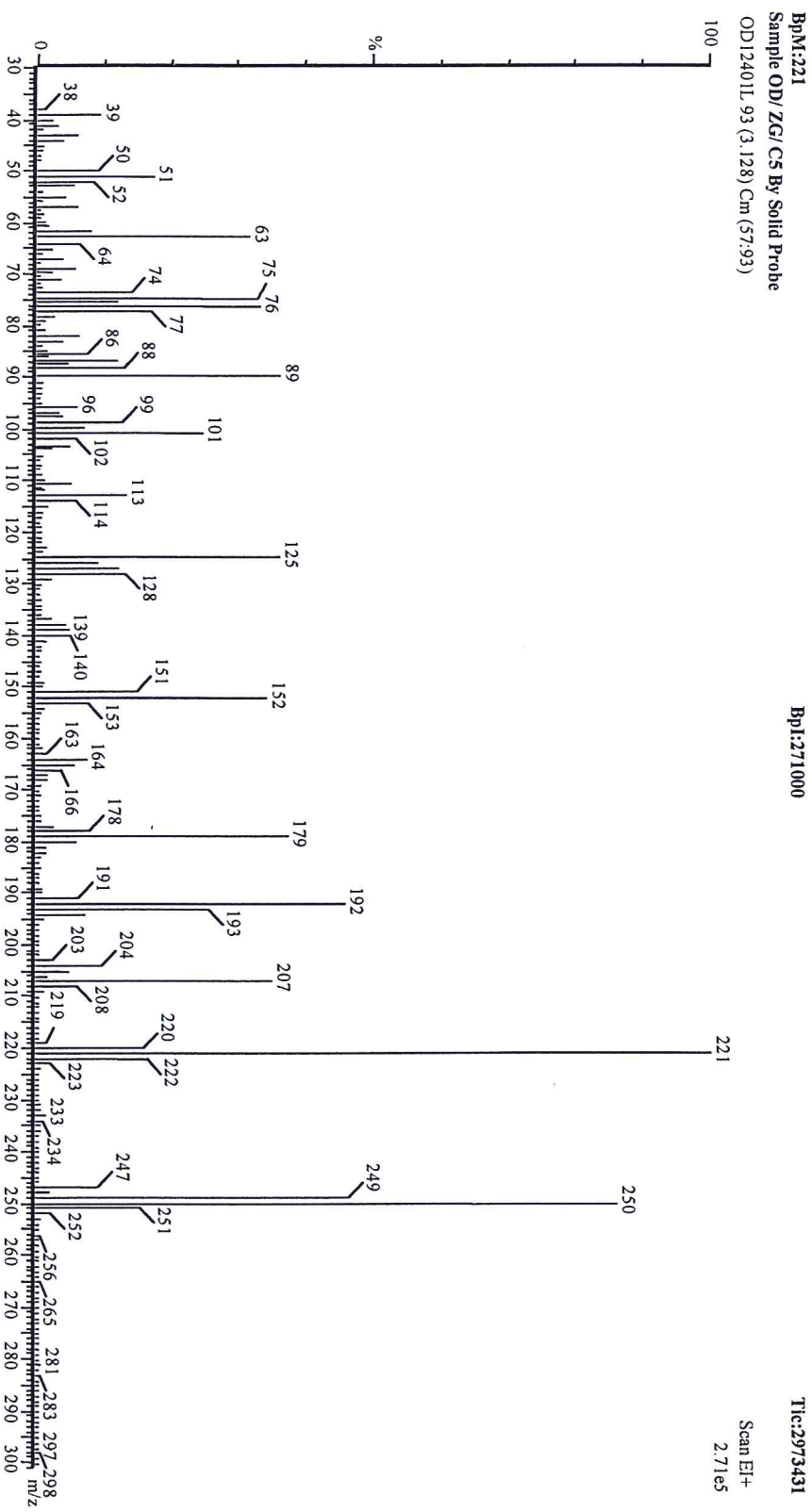
COSY Spectrum of **F_5**

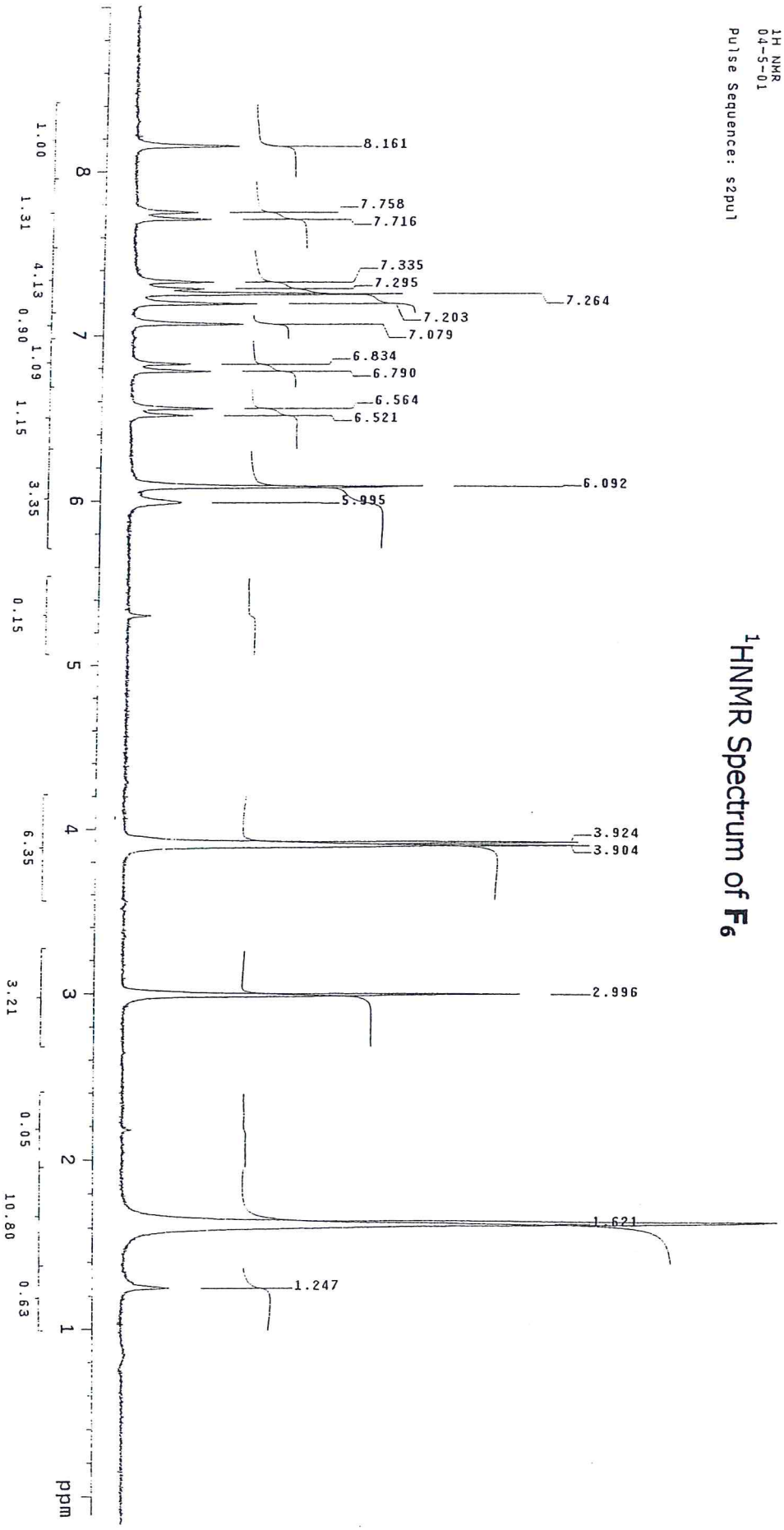


NOE Spectrum of **F_5**



Mass spectrum of F_5



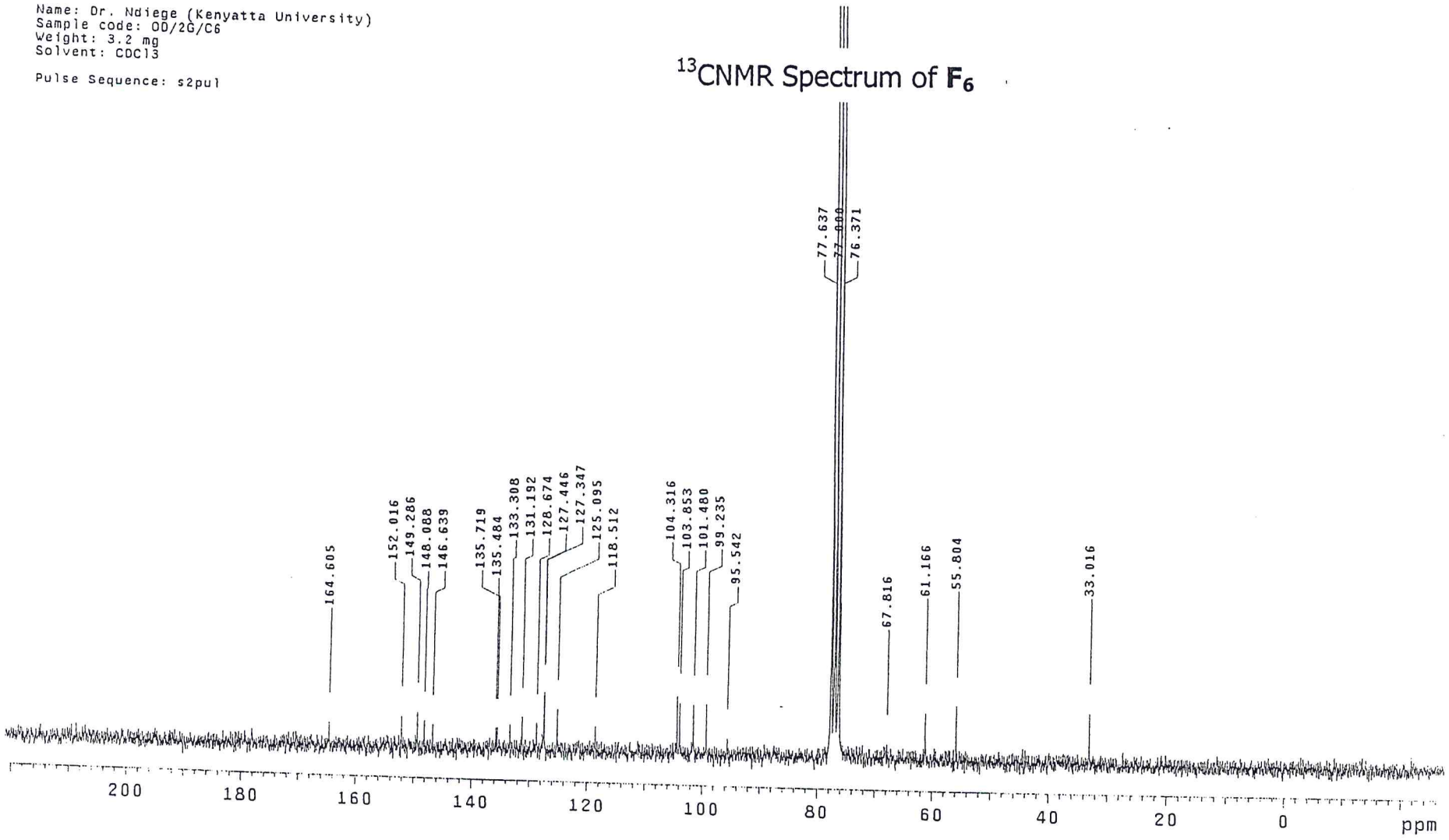


^{1}HNMR Spectrum of **F$_6$**

Name: Dr. Ndiege (Kenyatta University)
Sample code: OD/2G/C6
Weight: 3.2 mg
Solvent: CDCl3

Pulse Sequence: s2pul

^{13}CNMR Spectrum of **F$_6$**



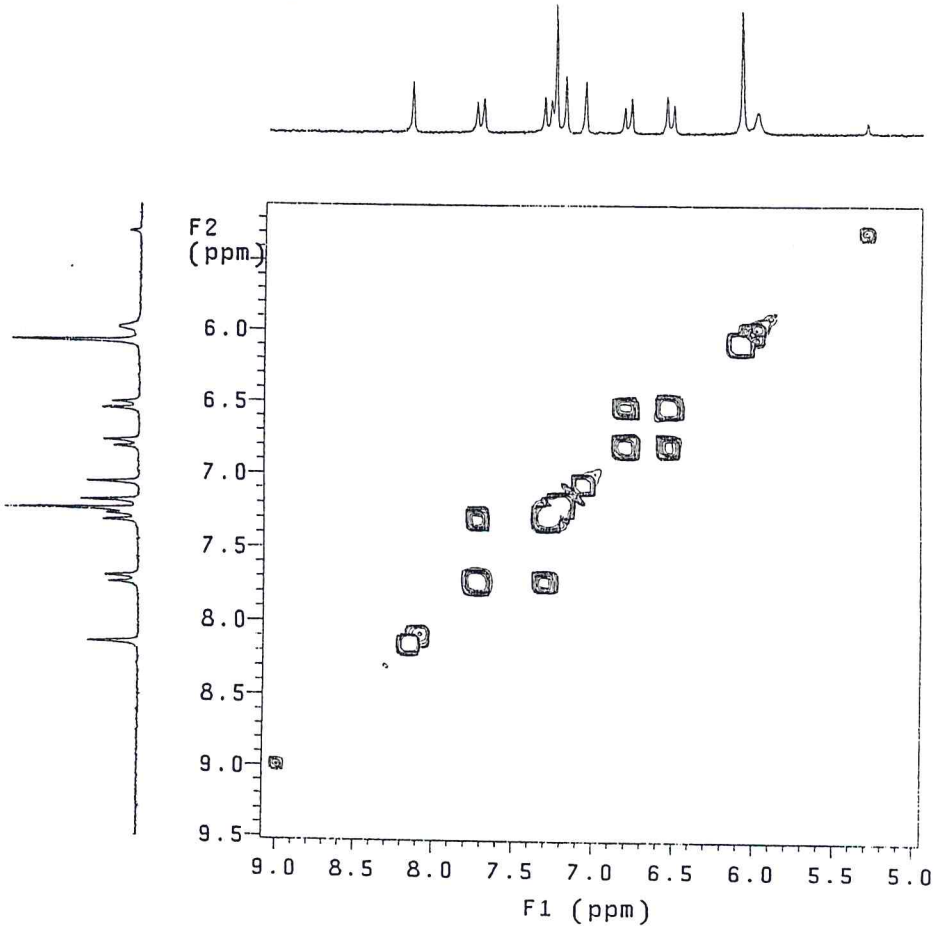
DENNIS OKINYO
OD-2G-C6
3 MG
04-5-01

expl COSY

SAMPLE	
date	May 4 2001
solvent	CDCl3
sample	OD-2G-C6-CO~
	SY_04May2001
ACQUISITION	
sw	3200.9
at	0.160
np	1024
fb	1800
ss	32
d1	1.000
nt	2
2D ACQUISITION	
sw1	3200.9
ni	128
TRANSMITTER	
tn	H1
sfrq	200.057
tof	255.7
tpwr	53
pw	20.000
PRESATURATION	
satmode	n
satpwr	0
satdly	0
satfrq	0
DECOUPLER	
dn	C13
dm	nnn
FLAGS	
hs	nn
sspul	n

SPECIAL	
temp	not used
gain	32
spin	0
GRADIENTS	
PFGflg	n
hsglvl	2000
hsgt	0.005000
F2 PROCESSING	
sb	-0.080
sbs	not used
fn	1024
F1 PROCESSING	
sb1	-0.040
sbs1	not used
proc1	lp
fn1	1024
DISPLAY	
sp	1021.8
wp	883.2
sp1	990.5
wp1	826.8
rf1	400.1
rfp	0
rfl1	400.1
rfp1	0
PLOT	
wc	107.6
sc	10.0
wc2	107.6
sc2	0
vs	146
th	7
ai cdc av	

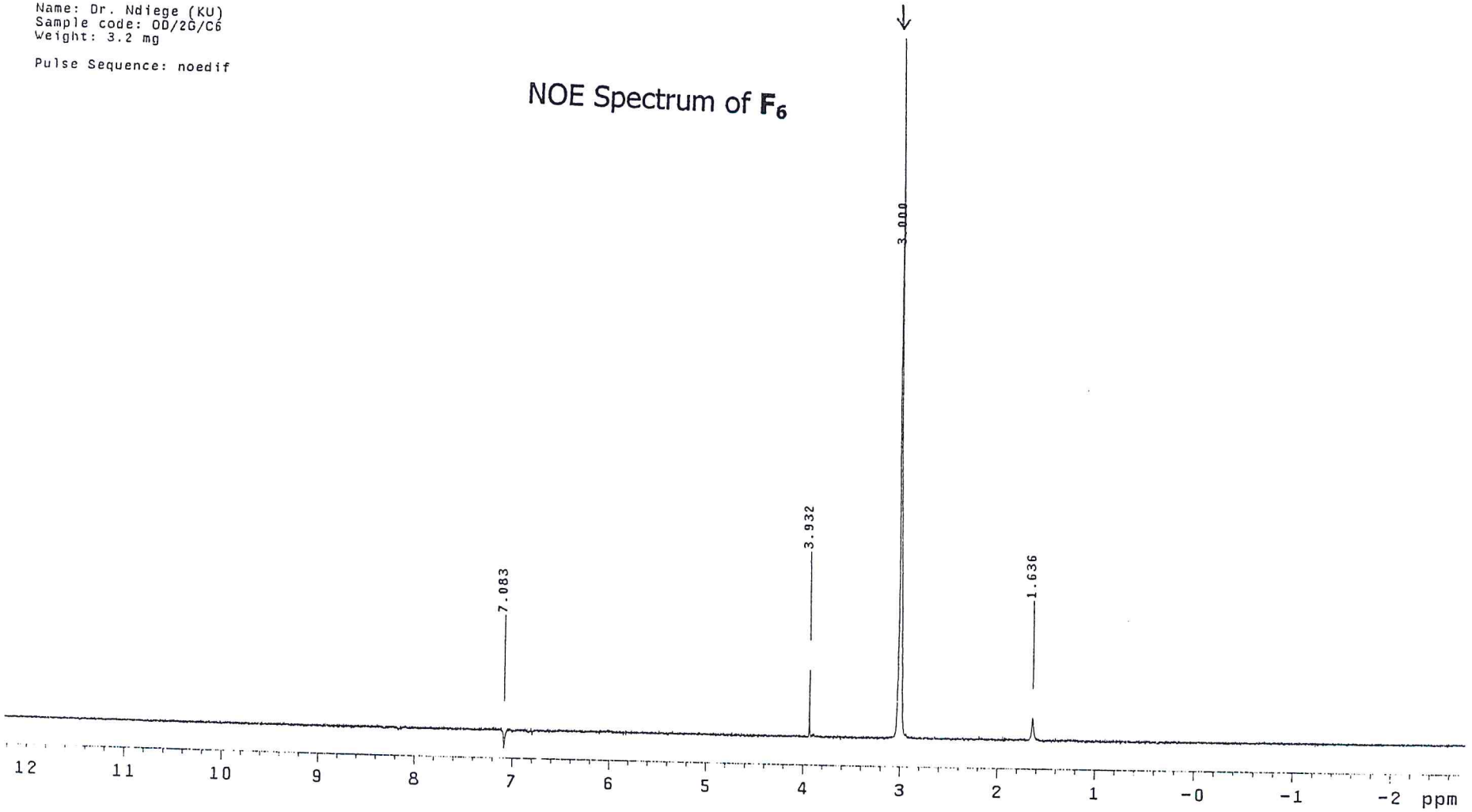
COSY Spectrum of F_6

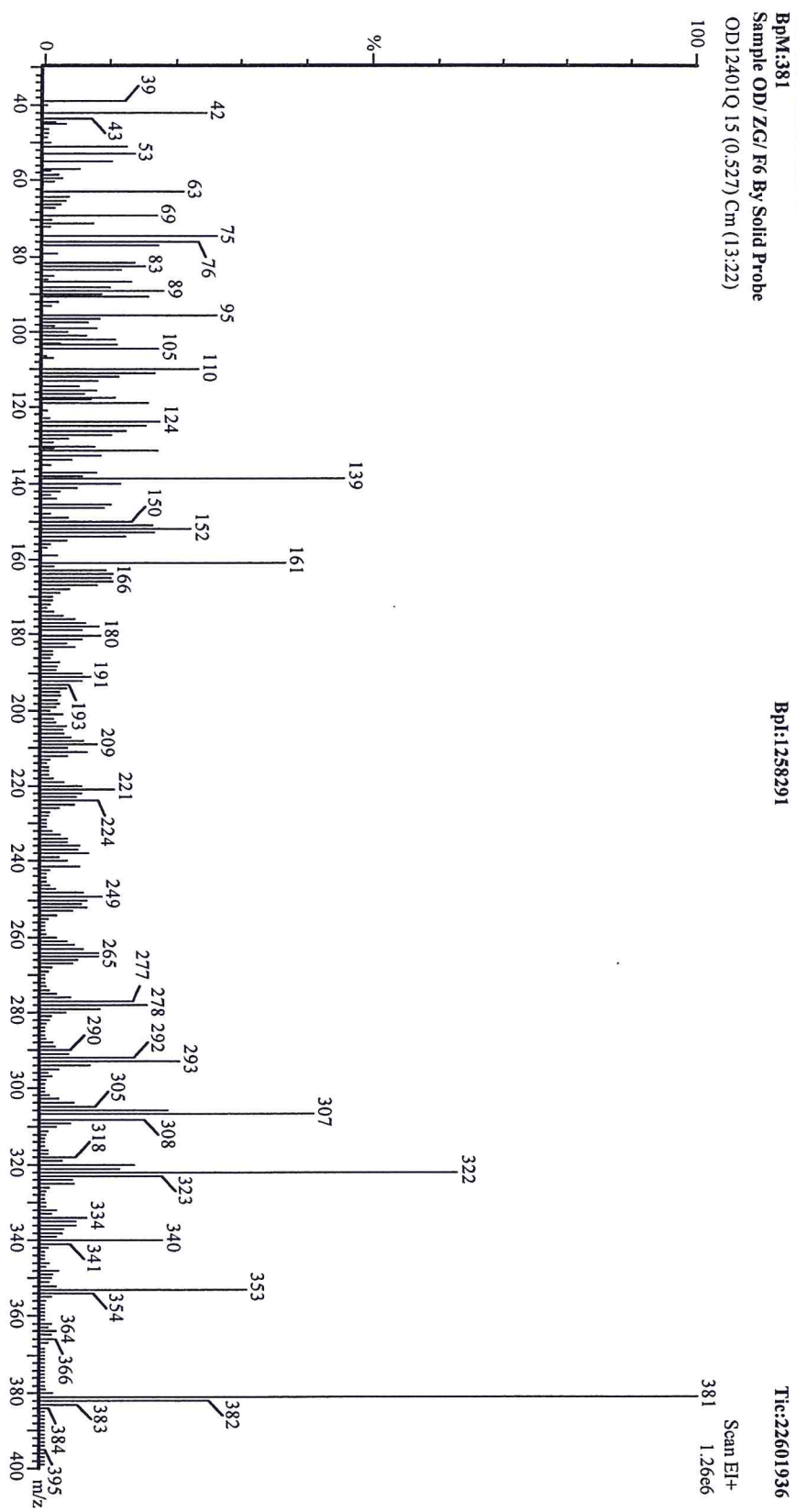


Name: Dr. Ndiege (KU)
Sample code: OD/2G/C6
Weight: 3.2 mg

Pulse Sequence: noedif

NOE Spectrum of F_6



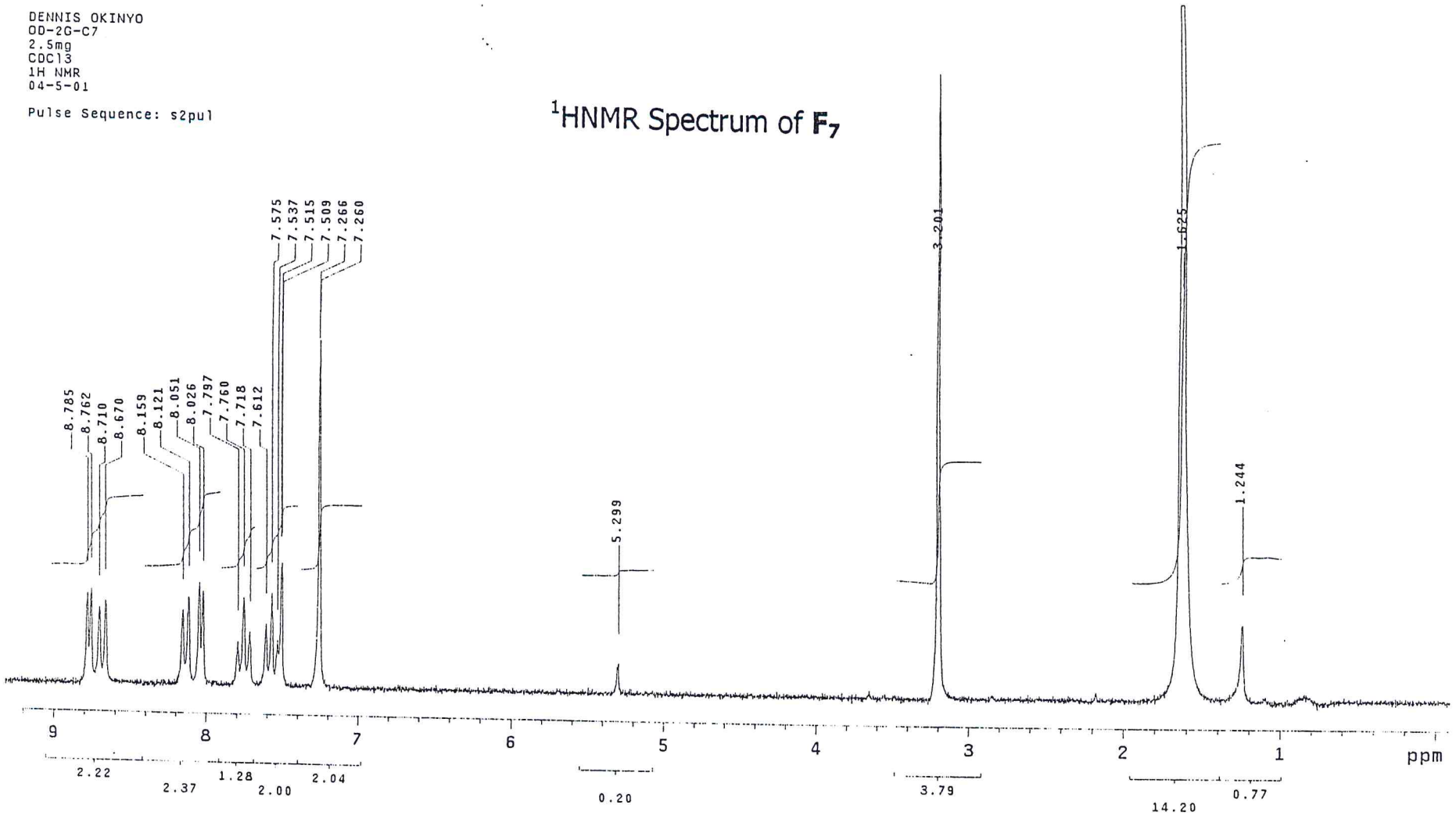


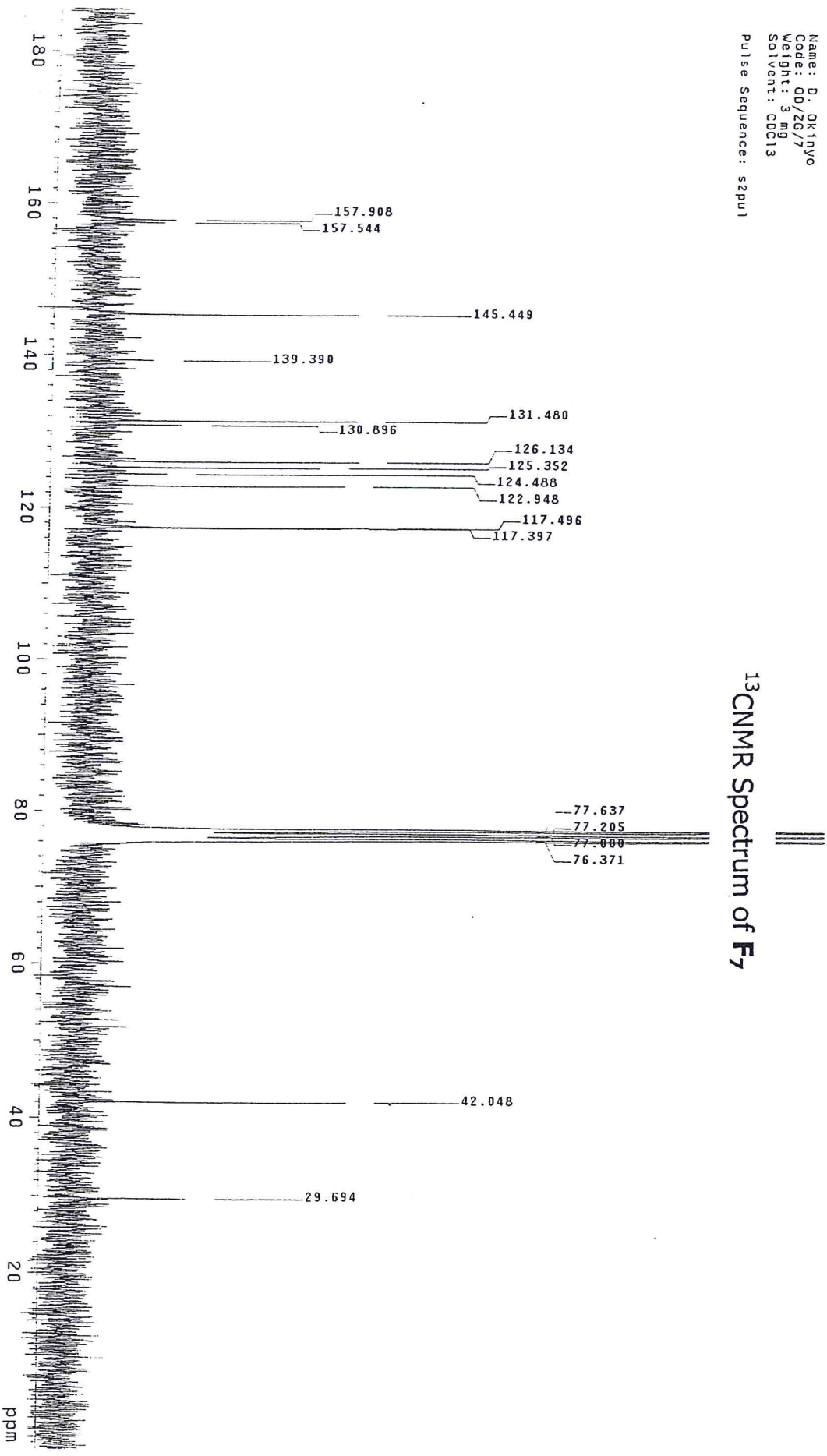
Mass spectrum of F_6

DENNIS OKINYO
OD-2G-C7
2.5mg
CDCl3
1H NMR
04-5-01

Pulse Sequence: s2pul

^{1}HNMR Spectrum of F_7





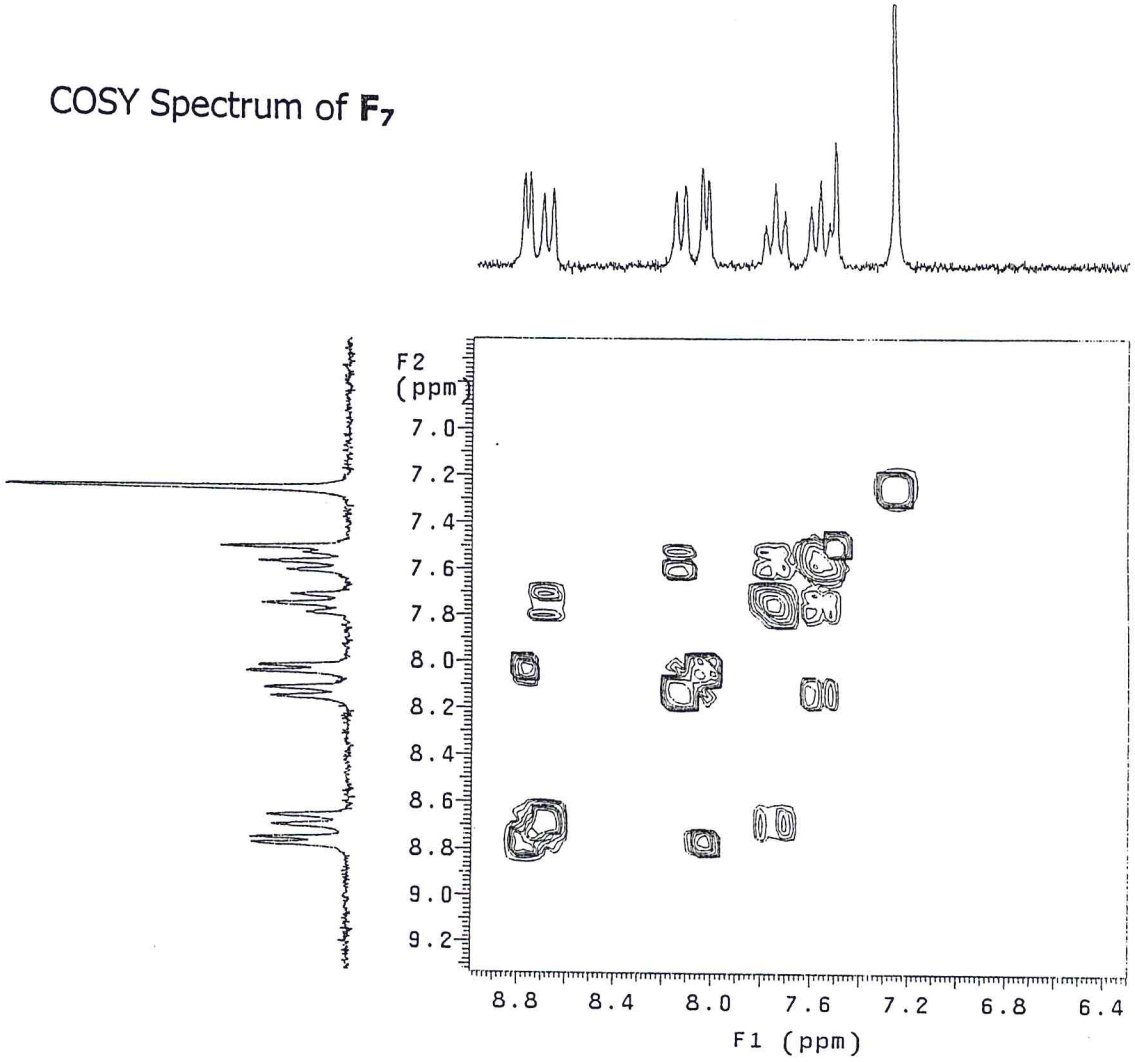
^{13}CNMR Spectrum of **F$_7$**

DENNIS OKINYO
OD-2G-C7
2. 5MG
04-5-01

expl COSY

SAMPLE		SPECIAL	
date	May 4 2001	temp	not used
solvent	CDCl3	gain	36
sample	OD-2G0C7-CO~	spin	0
	SY_04May2001	GRADIENTS	
ACQUISITION		PFGflg	n
sw	3200.9	hsglvl	2000
at	0.160	hsgt	0.005000
np	1024	F2 PROCESSING	
fb	1800	sb	-0.080
ss	32	sbs	not used
d1	1.000	fn	1024
nt	2	F1 PROCESSING	
2D ACQUISITION		sb1	-0.040
sw1	3200.9	sbs1	not used
ni	128	proc1	lp
TRANSMITTER		fn1	1024
tn	H1	DISPLAY	
sfrq	200.057	sp	1322.5
tof	255.7	wp	545.0
tpwr	53	sp1	1259.8
pw	20.000	wp1	538.7
PRESATURATION		rf1	400.1
satmode	n	rfp	0
satpwr	0	rfl1	400.1
satdly	0	rfp1	0
satfrq	0	PLOT	
DECOUPLER		wc	107.6
dn	C13	sc	10.0
dm	nnn	wc2	107.6
FLAGS		sc2	0
hs	nn	vs	74
sspul	n	th	6
		ai cdc av	

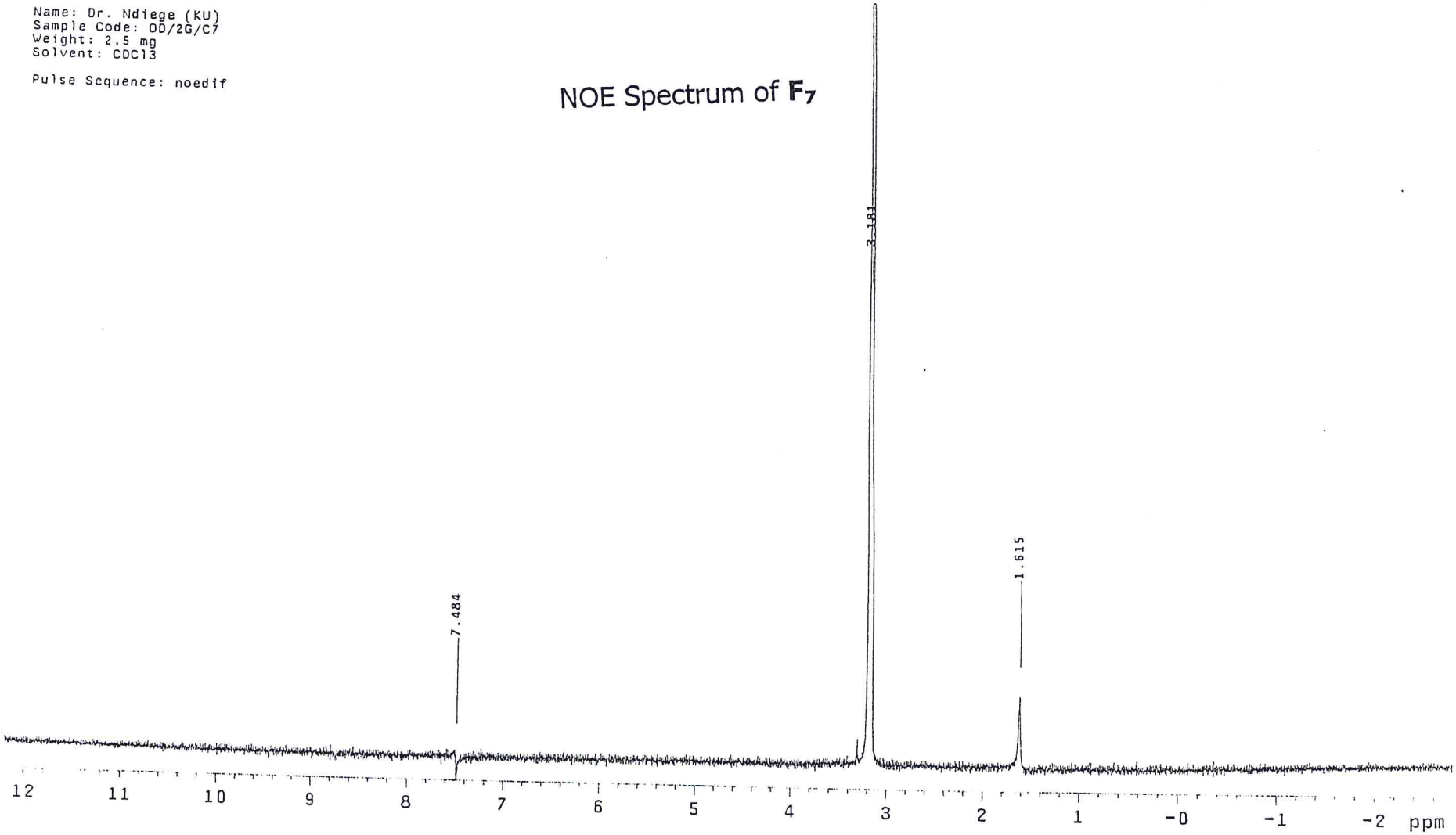
COSY Spectrum of F_7



Name: Dr. Ndiege (KU)
Sample Code: OD/2G/C7
Weight: 2.5 mg
Solvent: CDCl3

Pulse Sequence: noedif

NOE Spectrum of F_7



STANDARD 1H OBSERVE

Archive directory: /i400/jdw/vnmrsys/data
Sample directory:
File: PROTON

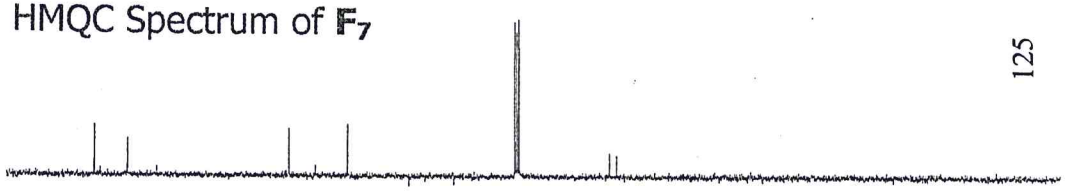
08/24/07

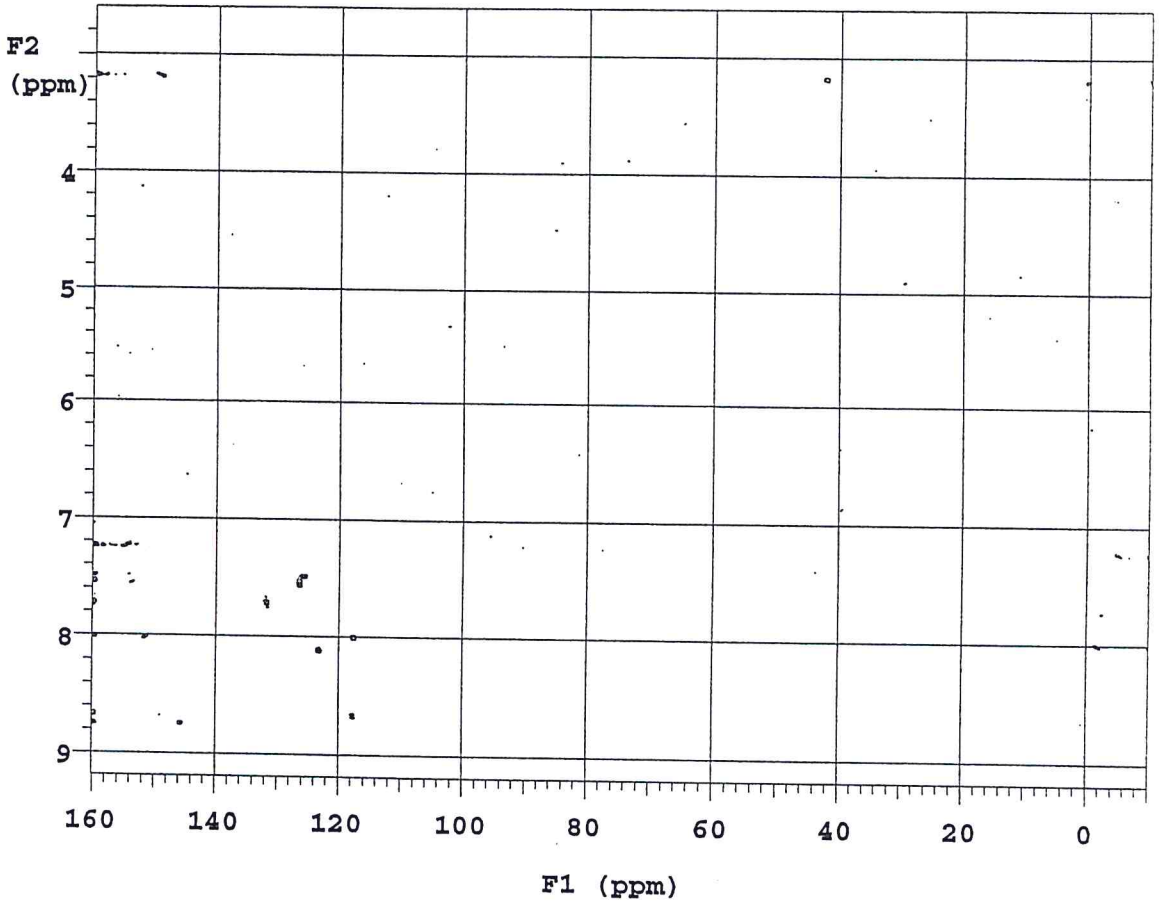
Pulse Sequence: HMQC

Solvent: CDCl3
Ambient temperature
INOVA-400 "narnia"

Relax. delay 1.000 sec
Acq. time 0.195 sec
Width 2631.6 Hz
2D Width 17105.0 Hz
24 repetitions
2 x 128 increments
OBSERVE H1, 400.1063260 MHz
DECOUPLE C13, 100.6143372 MHz
Power 38 dB
on during acquisition
off during delay
GARP-1 modulated
DATA PROCESSING
Gauss apodization 0.090 sec
F1 DATA PROCESSING
Gauss apodization 0.014 sec
FT size 1024 x 2048
Total time 2 hr, 6 min, 37 sec

HMQC Spectrum of F_7





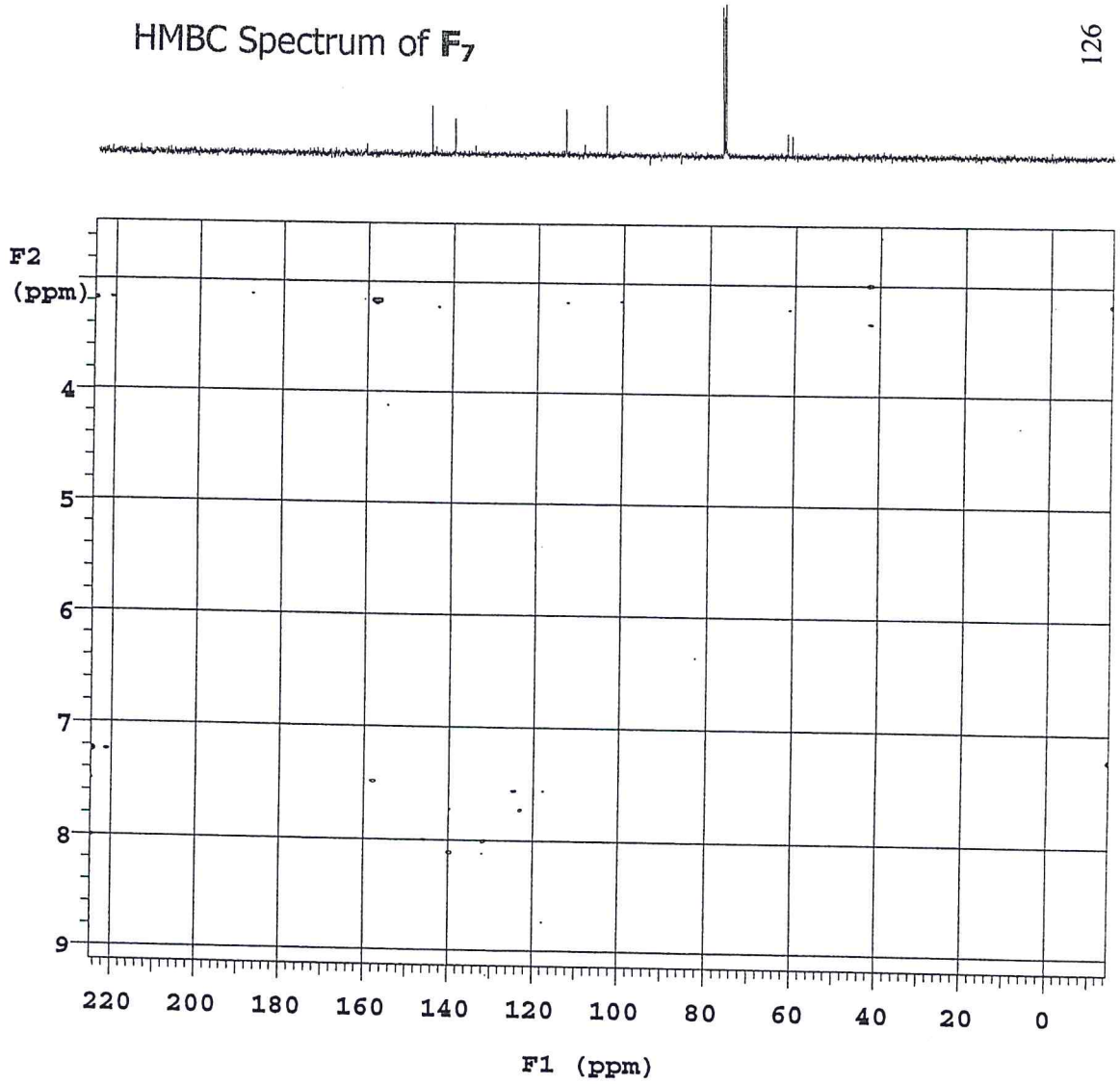
sem649ci, OD/ZG/C7

Archive directory: /i400/jdw/vnmrsys/data
Sample directory:

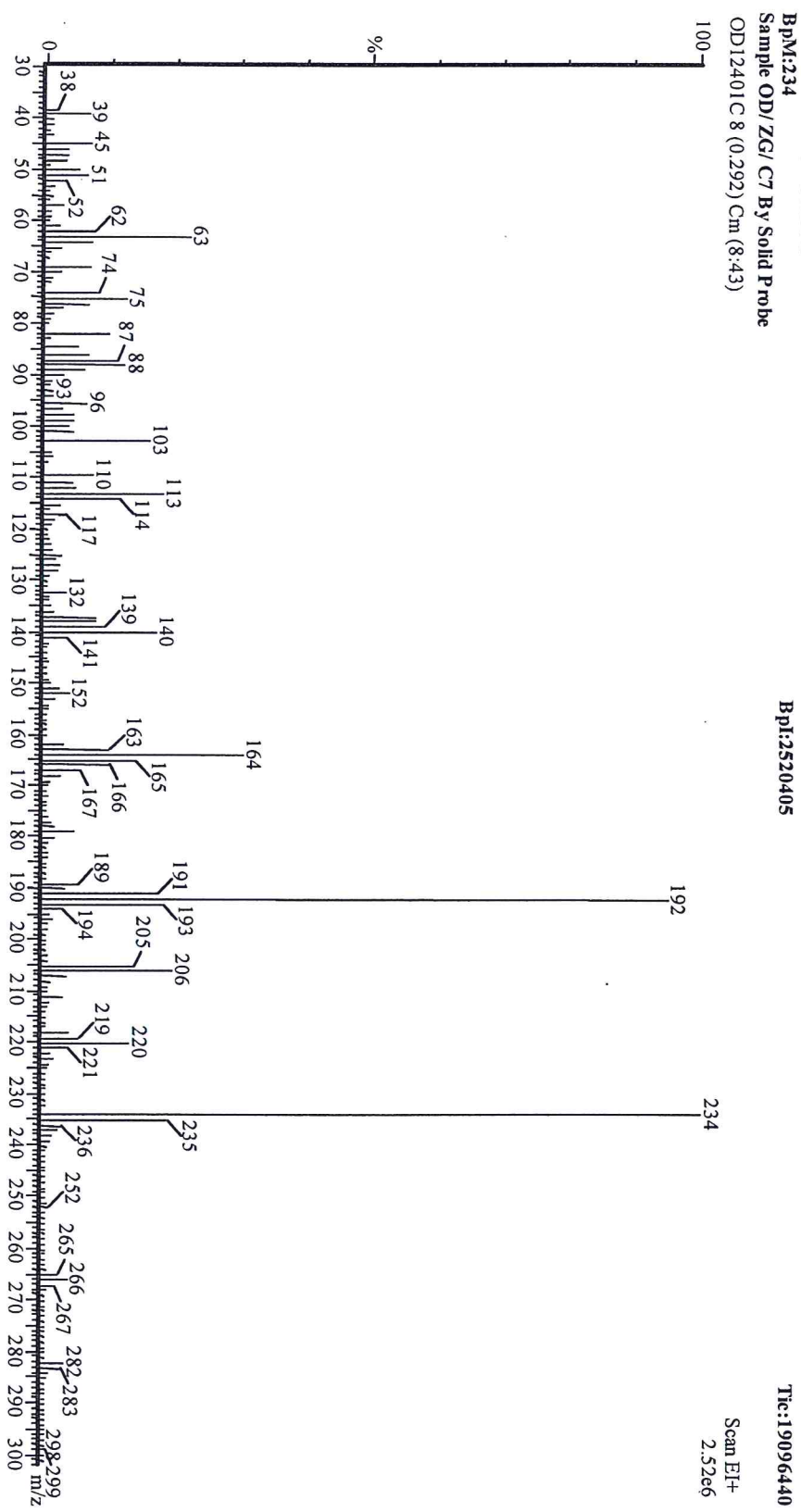
Pulse Sequence: HMBC

Solvent: CDCl3
Ambient temperature
File: sem649ci
INOVA-400 "narnia"

Relax. delay 1.000 sec
Acq. time 0.164 sec
Width 3112.5 Hz
2D Width 24147.3 Hz
8 repetitions
2 x 256 increments
OBSERVE H1, 400.1063260 MHz
DATA PROCESSING
Sine bell 0.082 sec
F1 DATA PROCESSING
Sine bell 0.005 sec
FT size 1024 x 2048
Total time 1 hr, 25 min, 59 sec



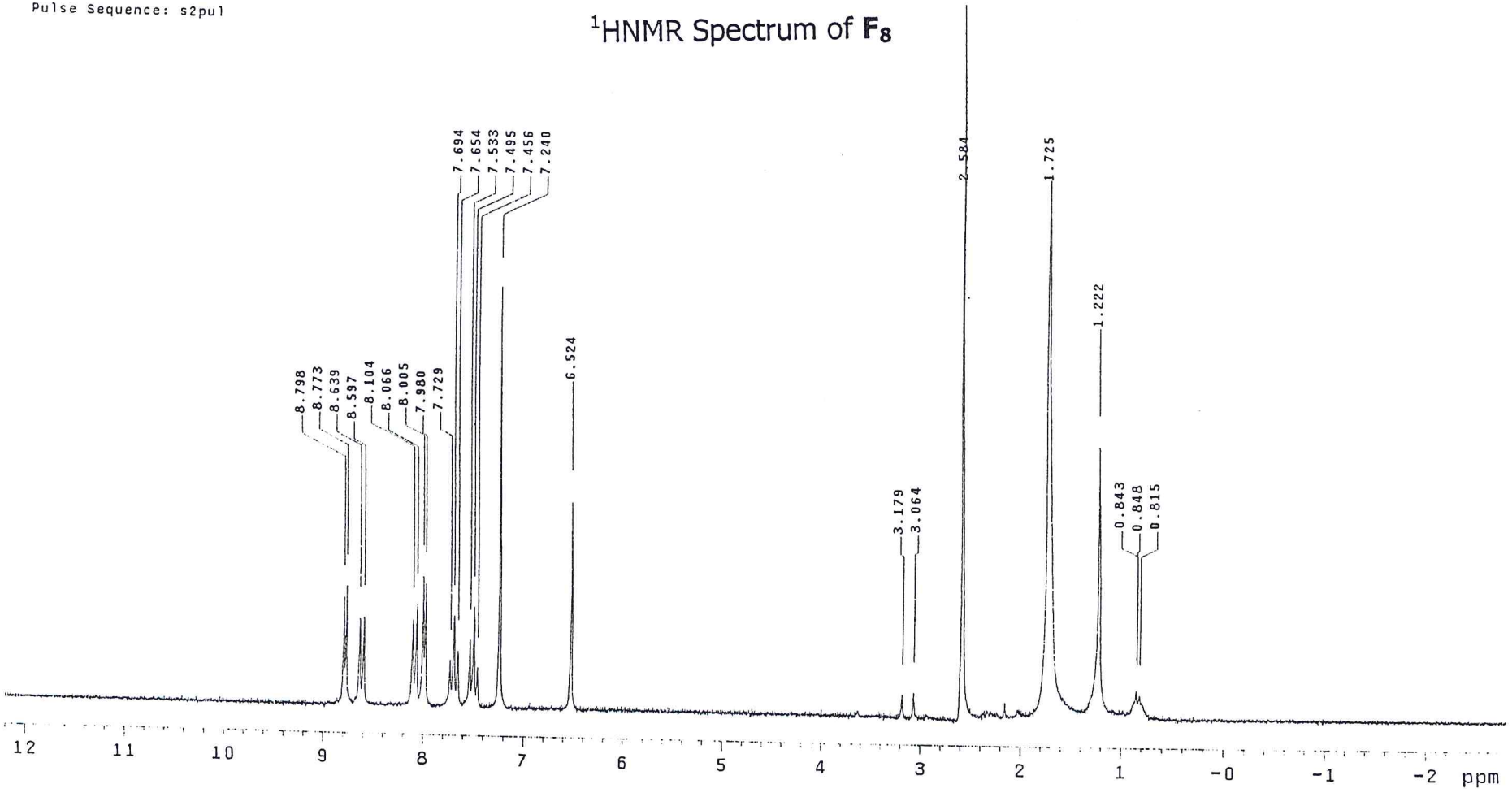
Mass spectrum of F_7

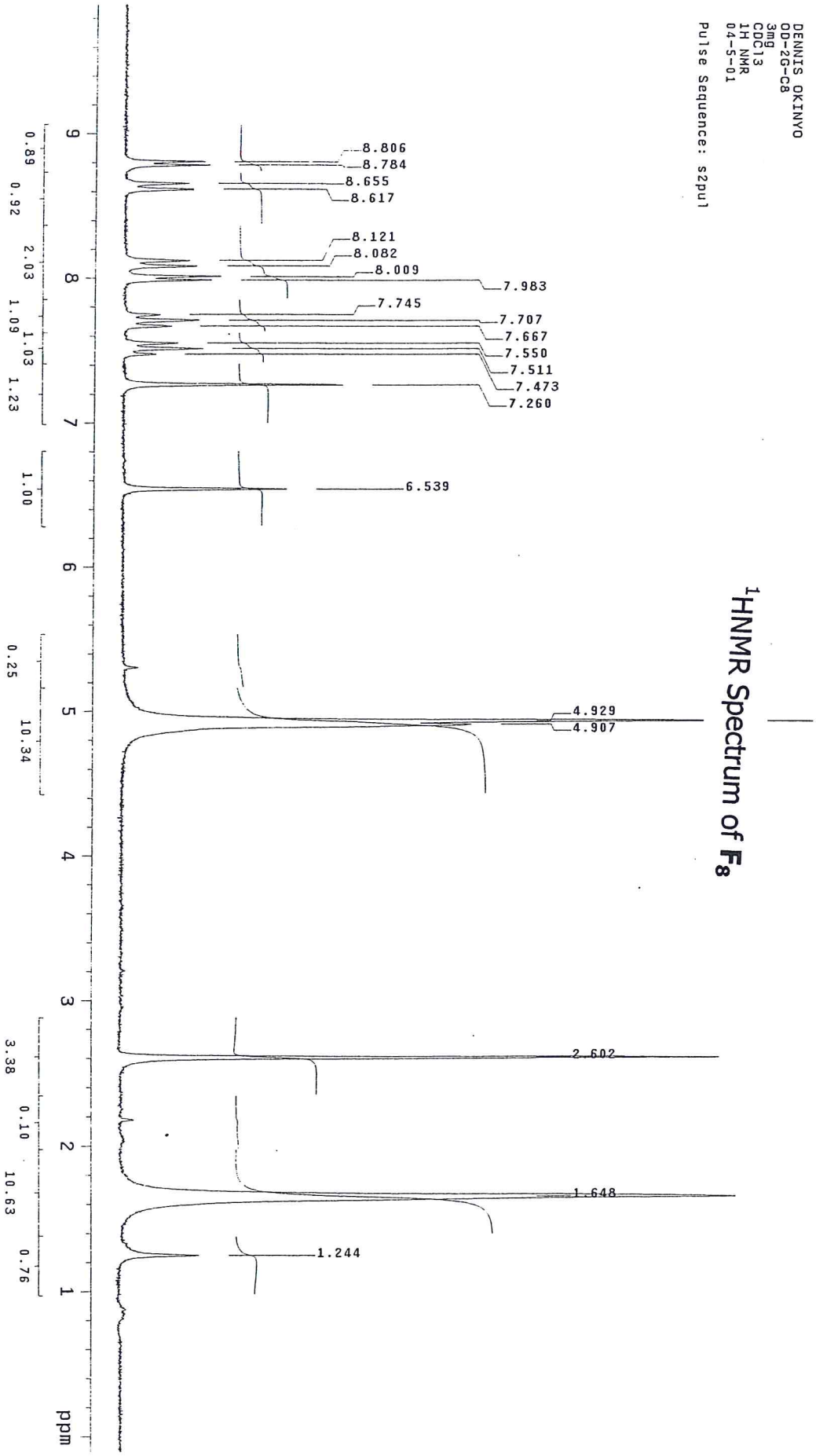


Name: Dr. Ndiege (KU)
Sample Code: OD/2G/C8
Weight: 3 mg
Solvent: CDCl3

Pulse Sequence: s2pul

^{1}HNMR Spectrum of **F8**



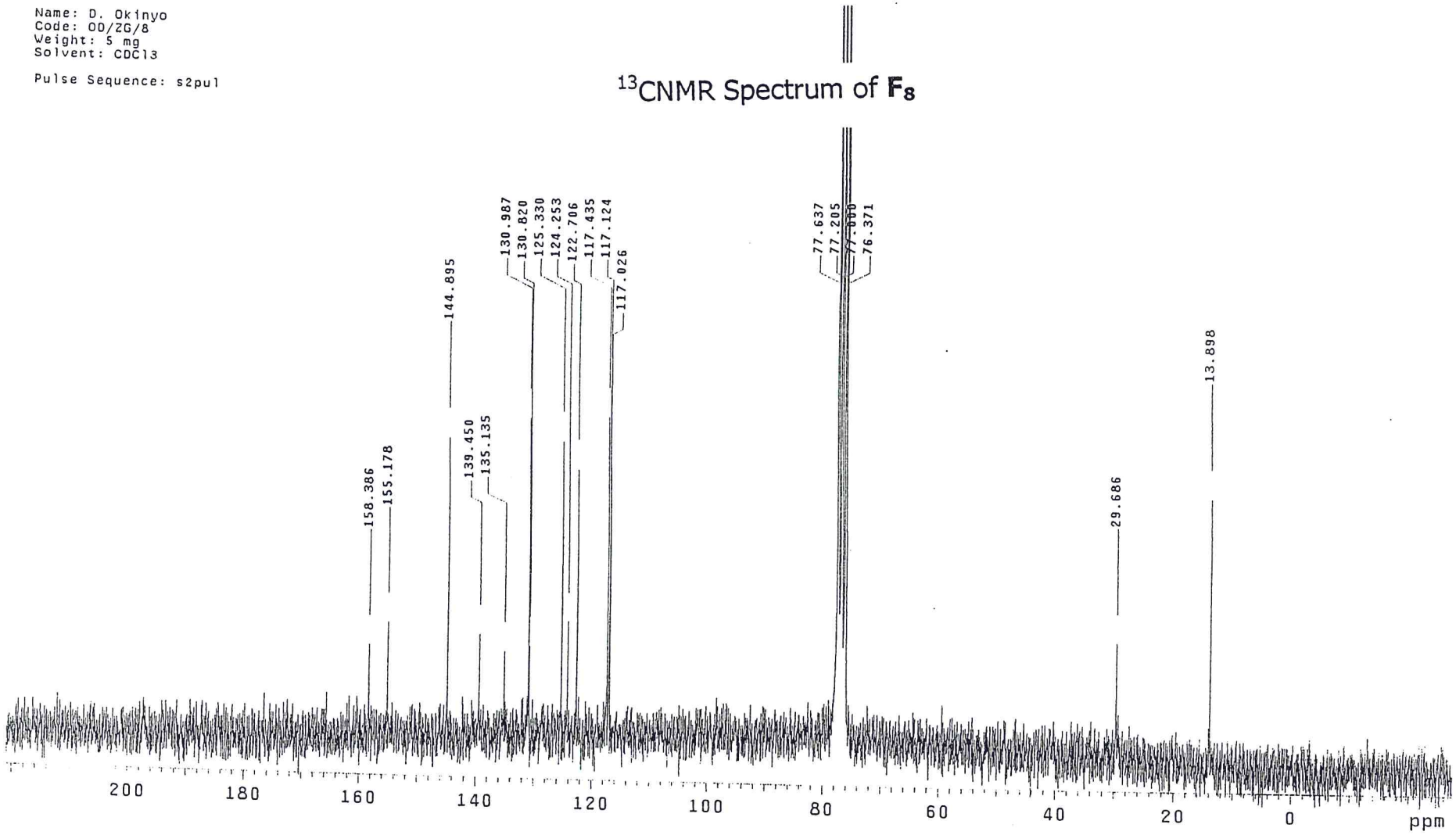


^{1}HNMR Spectrum of **F$_8$**

Name: D. Okinyo
Code: OD/ZG/8
Weight: 5 mg
Solvent: CDCl3

Pulse Sequence: s2pul

^{13}CNMR Spectrum of **F8**

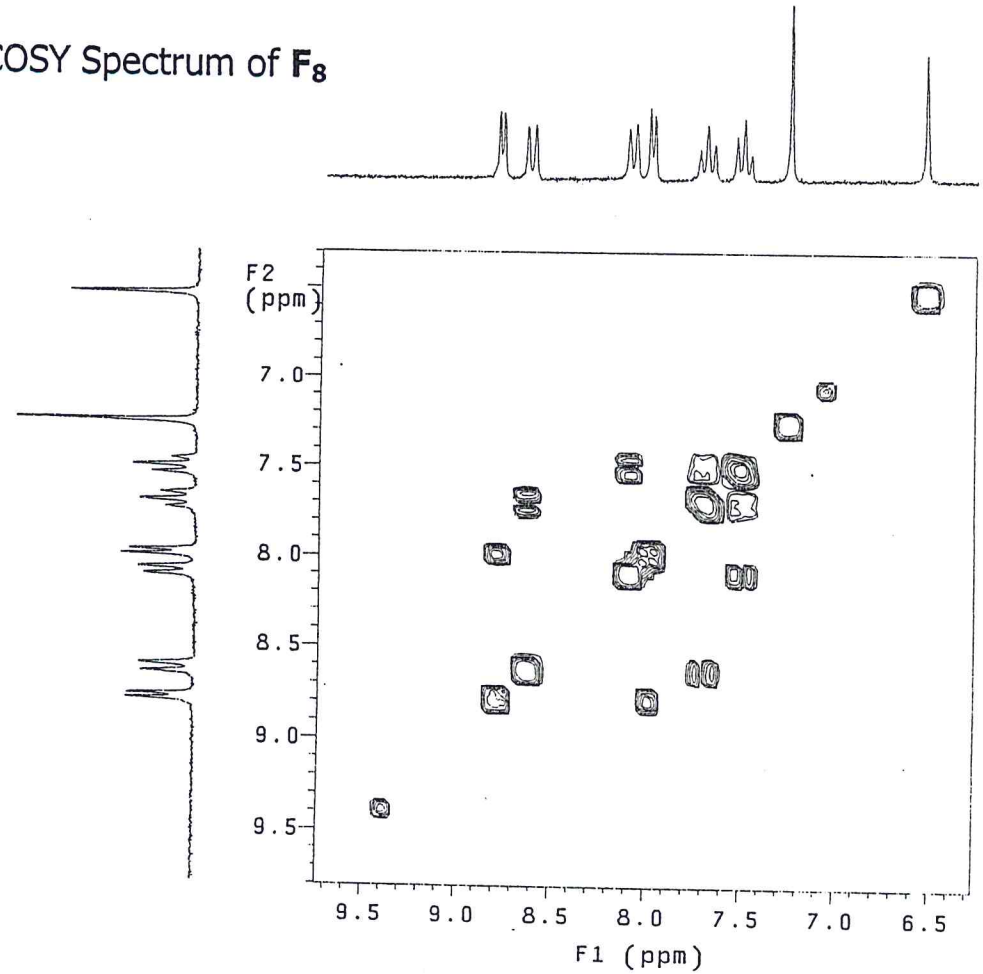


DENNIS OKINYO
OD-2G-C8
3 MG
04-5-01

expl COSY

SAMPLE		SPECIAL	
date	May 4 2001	temp	not used
solvent	CDCl3	gain	26
sample	OD-2G-C8-CO~	spin	0
	SY_04May2001	GRADIENTS	
ACQUISITION		PFGflg	n
sw	3200.9	hsglvl	2000
at	0.160	hsgt	0.005000
np	1024	F2 PROCESSING	
fb	1800	sb	-0.080
ss	32	sbs	not used
d1	1.000	fn	1024
nt	2	F1 PROCESSING	
2D ACQUISITION		sb1	-0.040
sw1	3200.9	sbs1	not used
ni	128	proc1	lp
TRANSMITTER		fn1	1024
tn	H1	DISPLAY	
sfrq	200.057	sp	1259.8
tof	255.7	wp	701.6
tpwr	53	sp1	1253.6
pw	20.000	wp1	695.3
PRESATURATION		rfl	400.1
satmode	n	rfp	0
satpwr	0	rfl1	400.1
satdly	0	rfp1	0
satfrq	0	PLOT	
DECOUPLER		wc	107.6
dn	C13	sc	10.0
dm	nnn	wc2	107.6
FLAGS		sc2	0
hs	nn	vs	263
sspul	n	th	7
		ai cdc av	

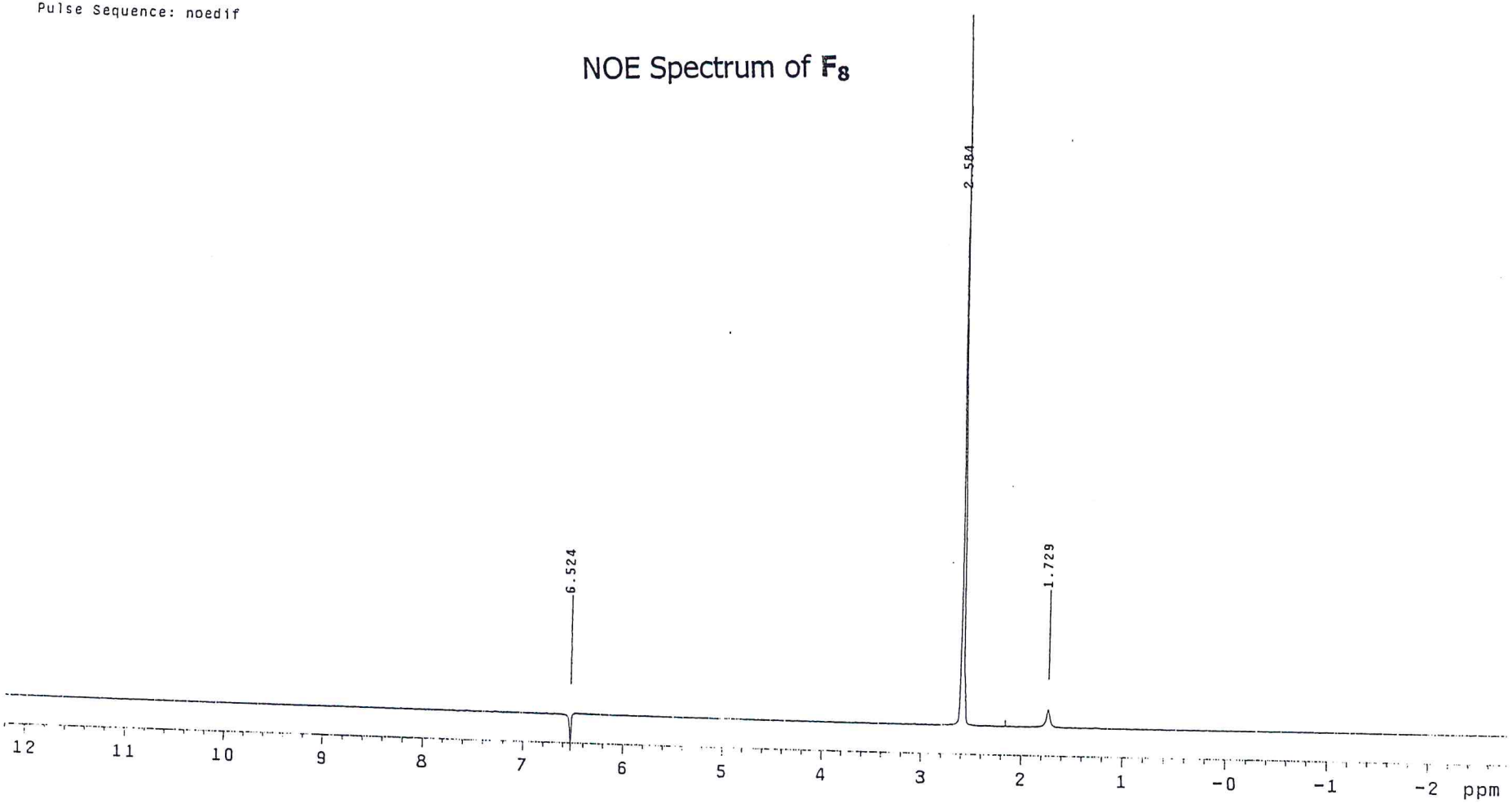
COSY Spectrum of F_8



Name: Dr. Ndiege (KU)
Sample Code: OD/2G/C8
Weight: 3 mg
Solvent: CDCl3

Pulse Sequence: noedif

NOE Spectrum of F_8



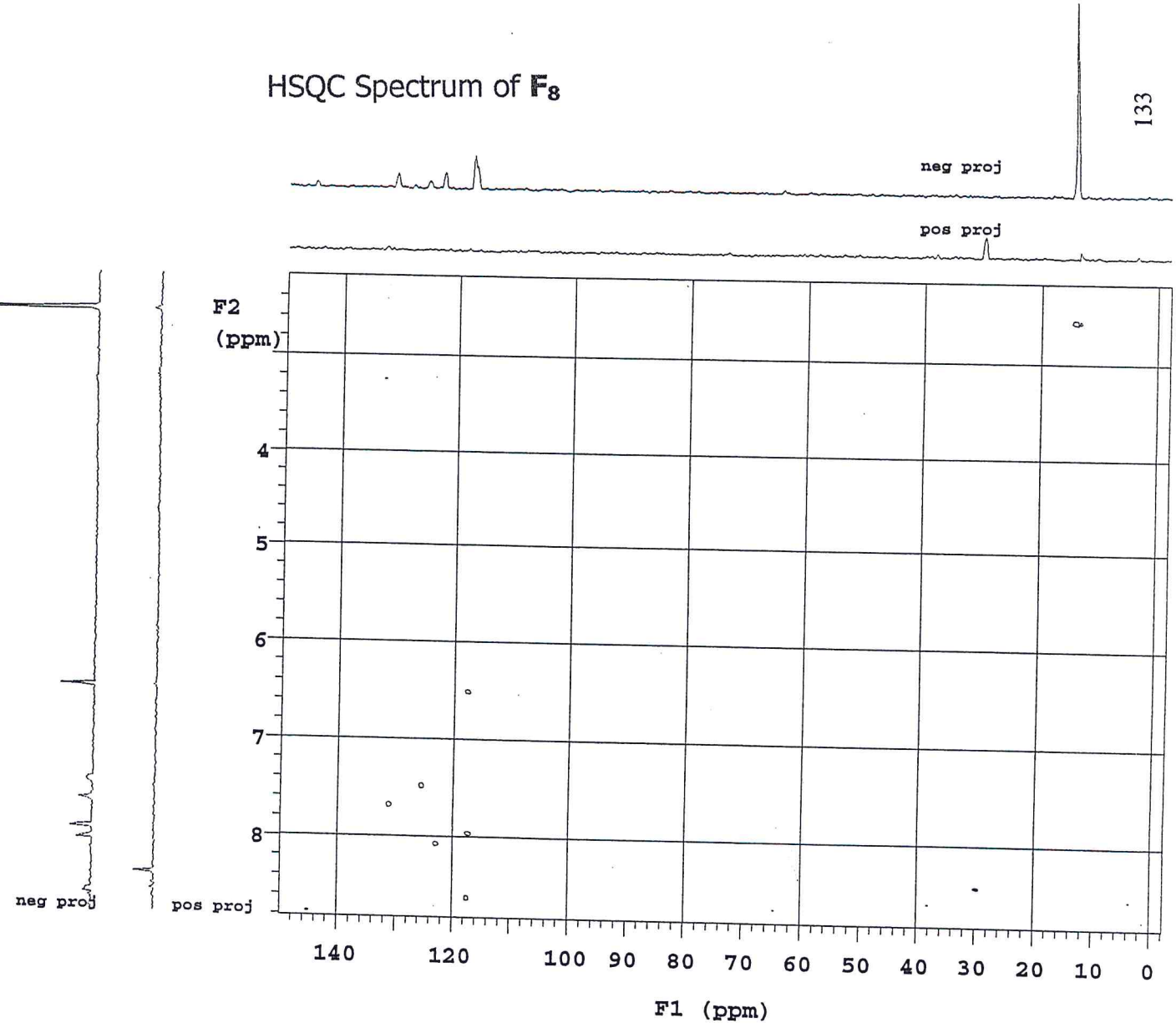
sem648di, OD/ZG/C8

Pulse Sequence: HSQC

Solvent: CDCl3
Ambient temperature
File: sem648ei
INOVA-400 "narnia"

Relax. delay 1.000 sec
Acq. time 0.178 sec
Width 2875.7 Hz
2D Width 17105.0 Hz
16 repetitions
2 x 128 increments
OBSERVE H1, 400.1063260 MHz
DECOUPLE C13, 100.6143372 MHz
Power 38 dB
on during acquisition
off during delay
GARP-1 modulated
DATA PROCESSING
Gauss apodization 0.082 sec
F1 DATA PROCESSING
Gauss apodization 0.014 sec
FT size 1024 x 2048
Total time 1 hr, 24 min, 39 sec

HSQC Spectrum of F_8



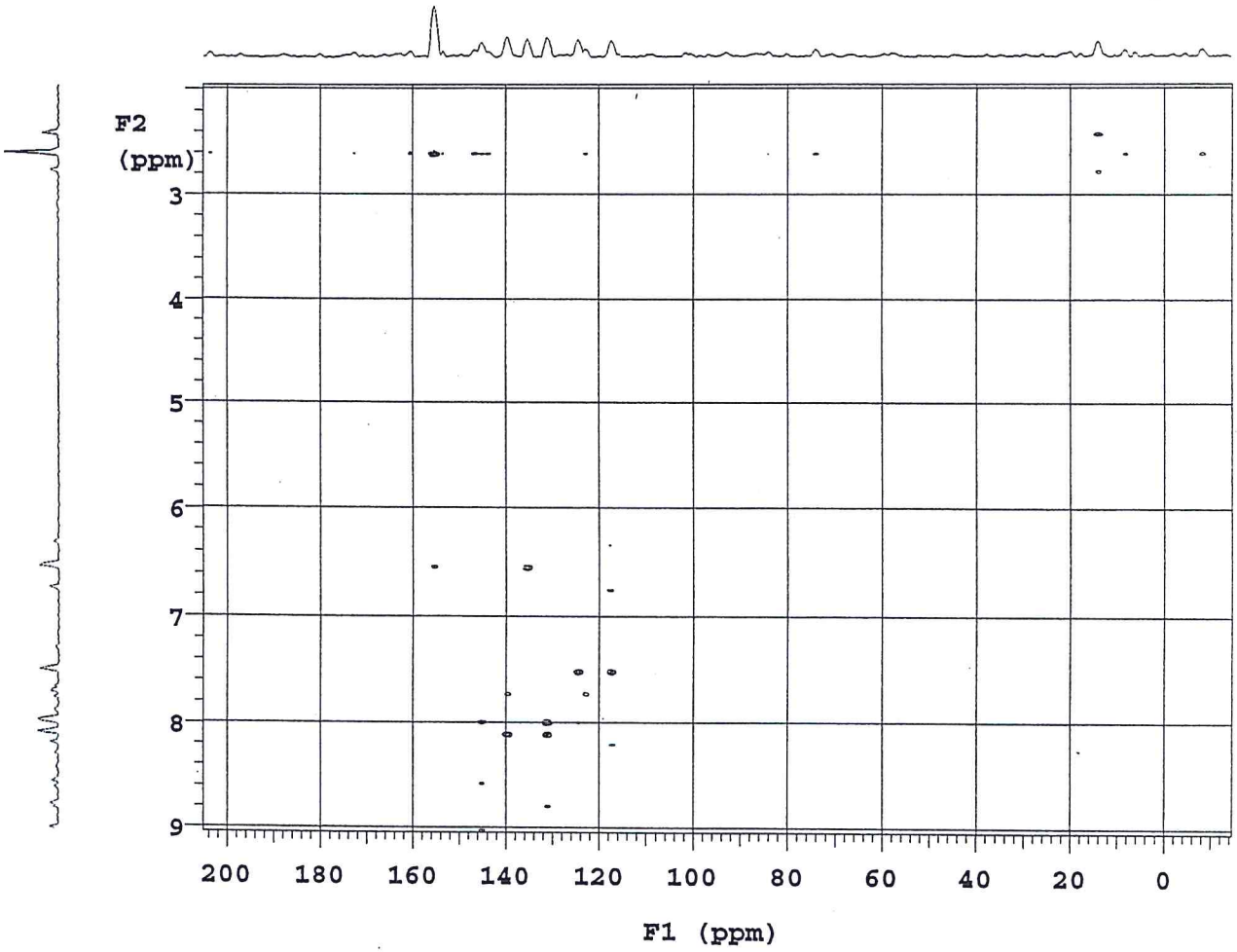
sem648di, OD/ZG/C8

Pulse Sequence: HMBC

Solvent: CDCl3
Ambient temperature
File: sem648di
INOVA-400 "narnia"

Relax. delay 1.000 sec
Acq. time 0.153 sec
Width 3349.1 Hz
2D Width 24147.3 Hz
24 repetitions
2 x 256 increments
OBSERVE H1, 400.1063121 MHz
DATA PROCESSING
Sine bell 0.076 sec
F1 DATA PROCESSING
Sine bell 0.005 sec
FT size 1024 x 2048
Total time 4 hr, 14 min, 16 sec

HMBC Spectrum of F_8



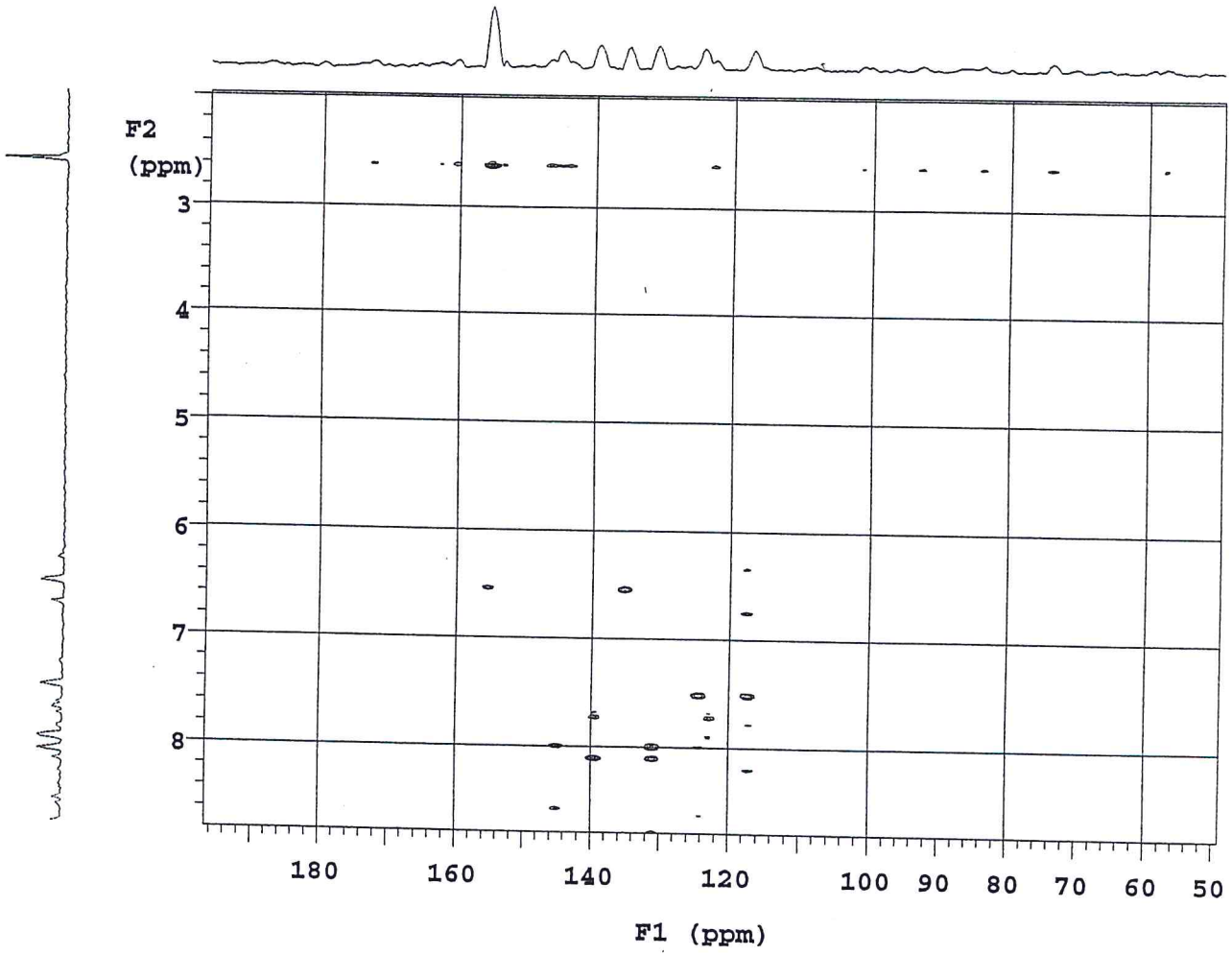
sem648di, OD/ZG/C8

Pulse Sequence: HMBC

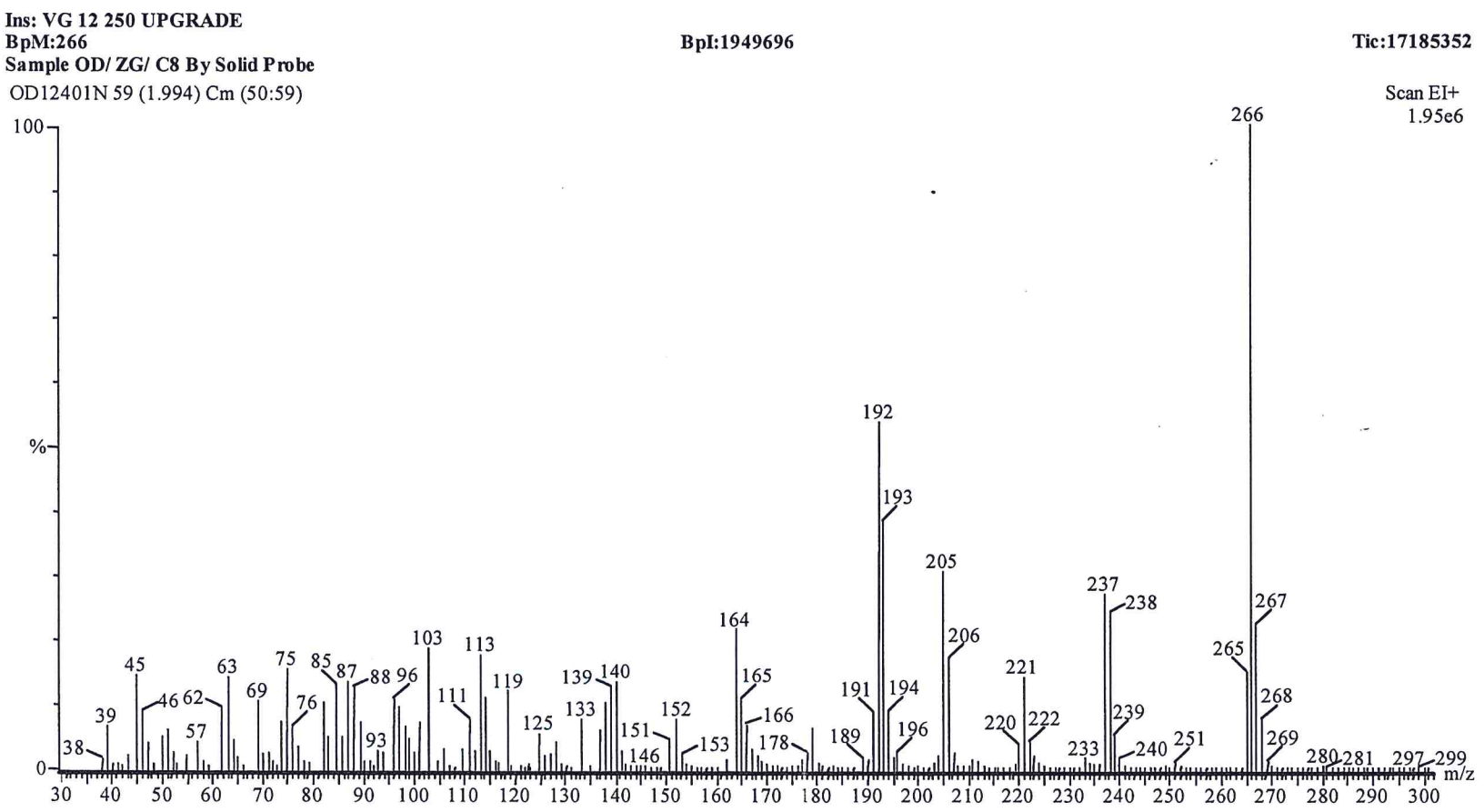
Solvent: CDCl3
Ambient temperature
File: sem648di
INOVA-400 "narnia"

Relax. delay 1.000 sec
Acq. time 0.153 sec
Width 3349.1 Hz
2D Width 24147.3 Hz
24 repetitions
2 x 256 increments
OBSERVE H1, 400.1063121 MHz
DATA PROCESSING
Sine bell 0.076 sec
F1 DATA PROCESSING
Sine bell 0.005 sec
FT size 1024 x 2048
Total time 4 hr, 14 min, 16 sec

HMBC Spectrum of F_8



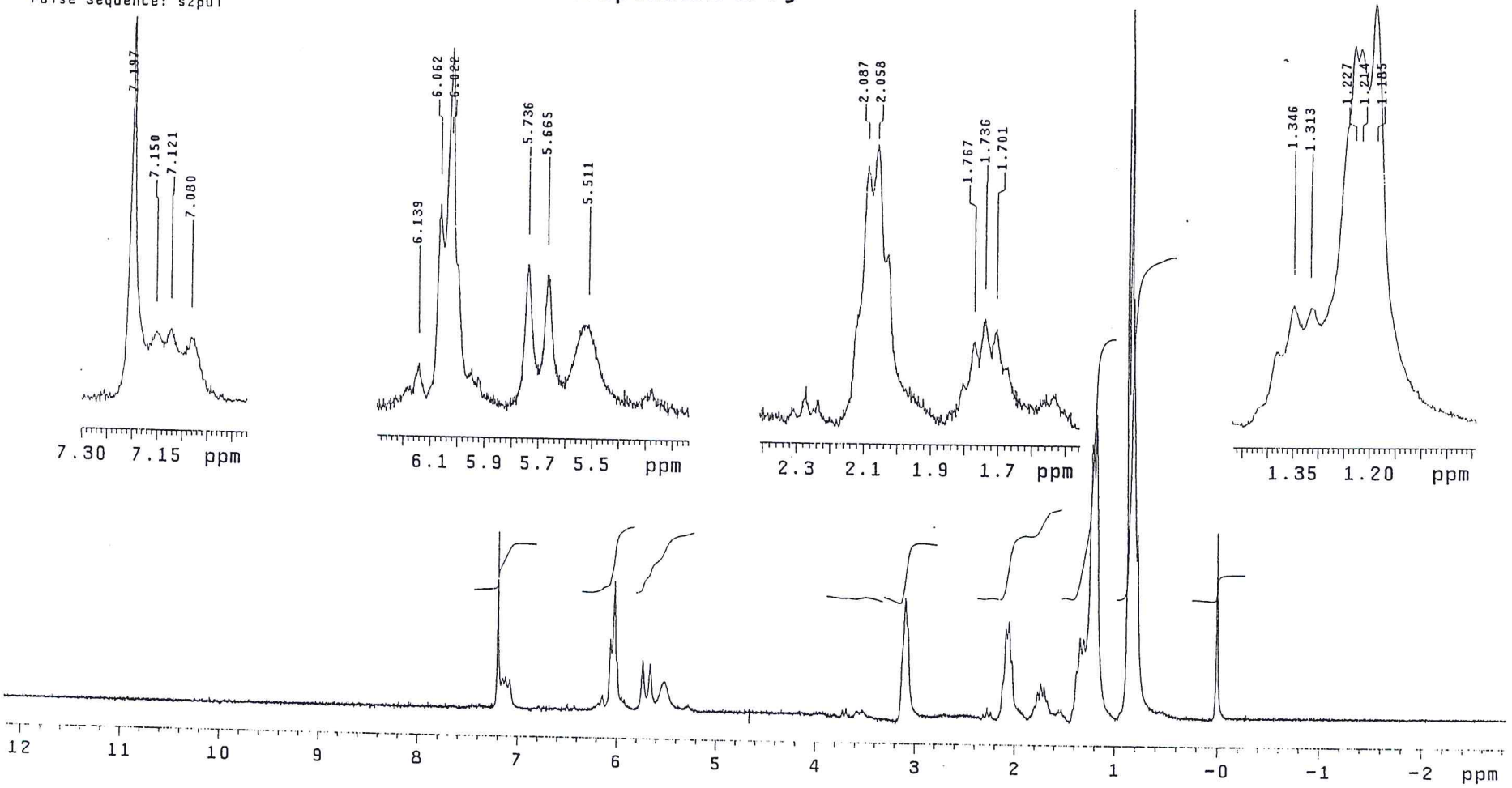
Mass spectrum of F_8



Name: Dennis Okinyo (Kenyatta University)
Sample code: 160/168
Solvent: CdCl3

Pulse Sequence: s2pul

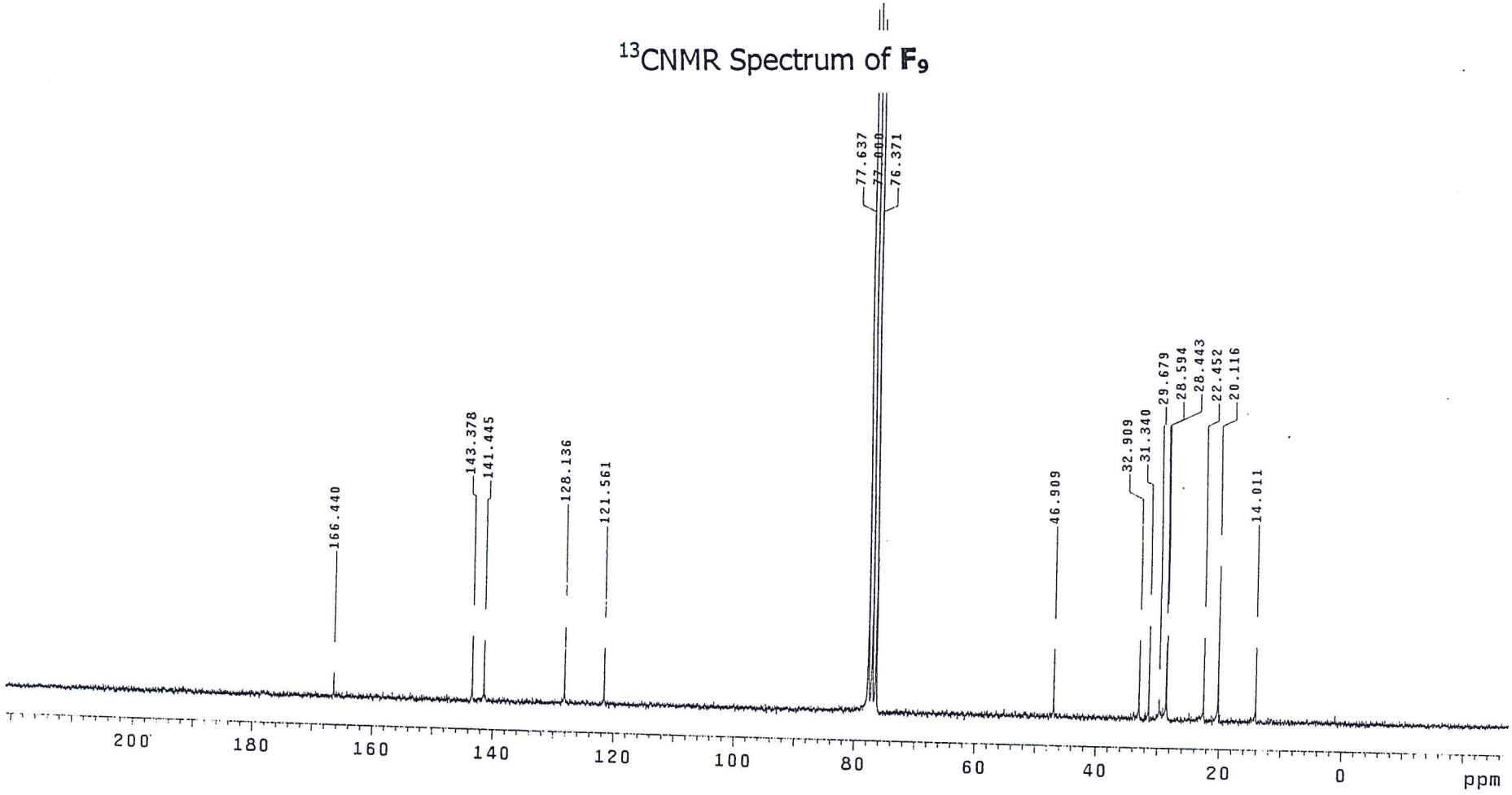
^{1}HNMR Spectrum of **F$_9$**



Name: Dennis Okinyo (Kenyatta University)
Sample code: 160/168
Solvent: CdCl3

Pulse Sequence: s2pul

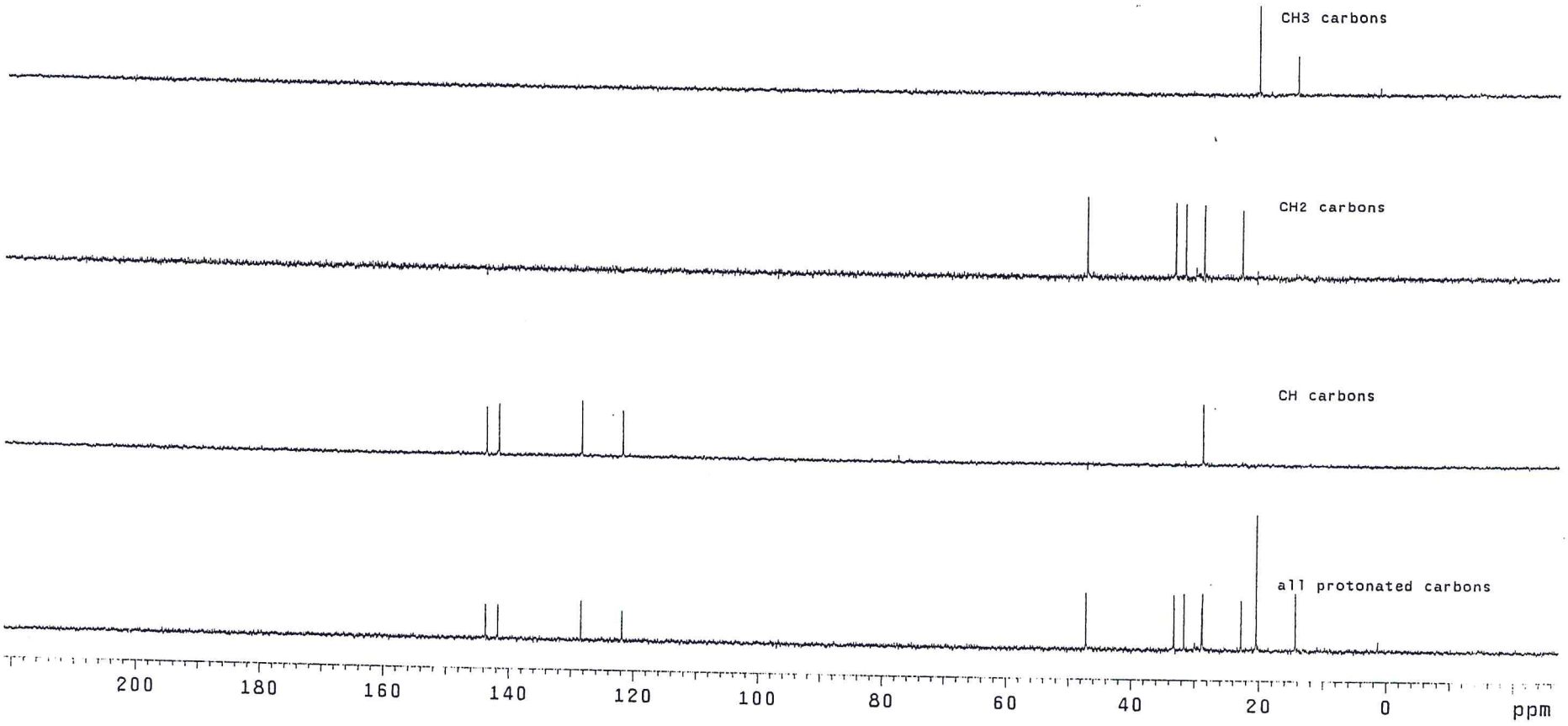
^{13}CNMR Spectrum of **F$_9$**



Name: Dennis Okinyo (Kenyatta University)
Sample code: 160/168
Solvent: CdCl3

Pulse Sequence: dept

DEPT Spectrum of F_9



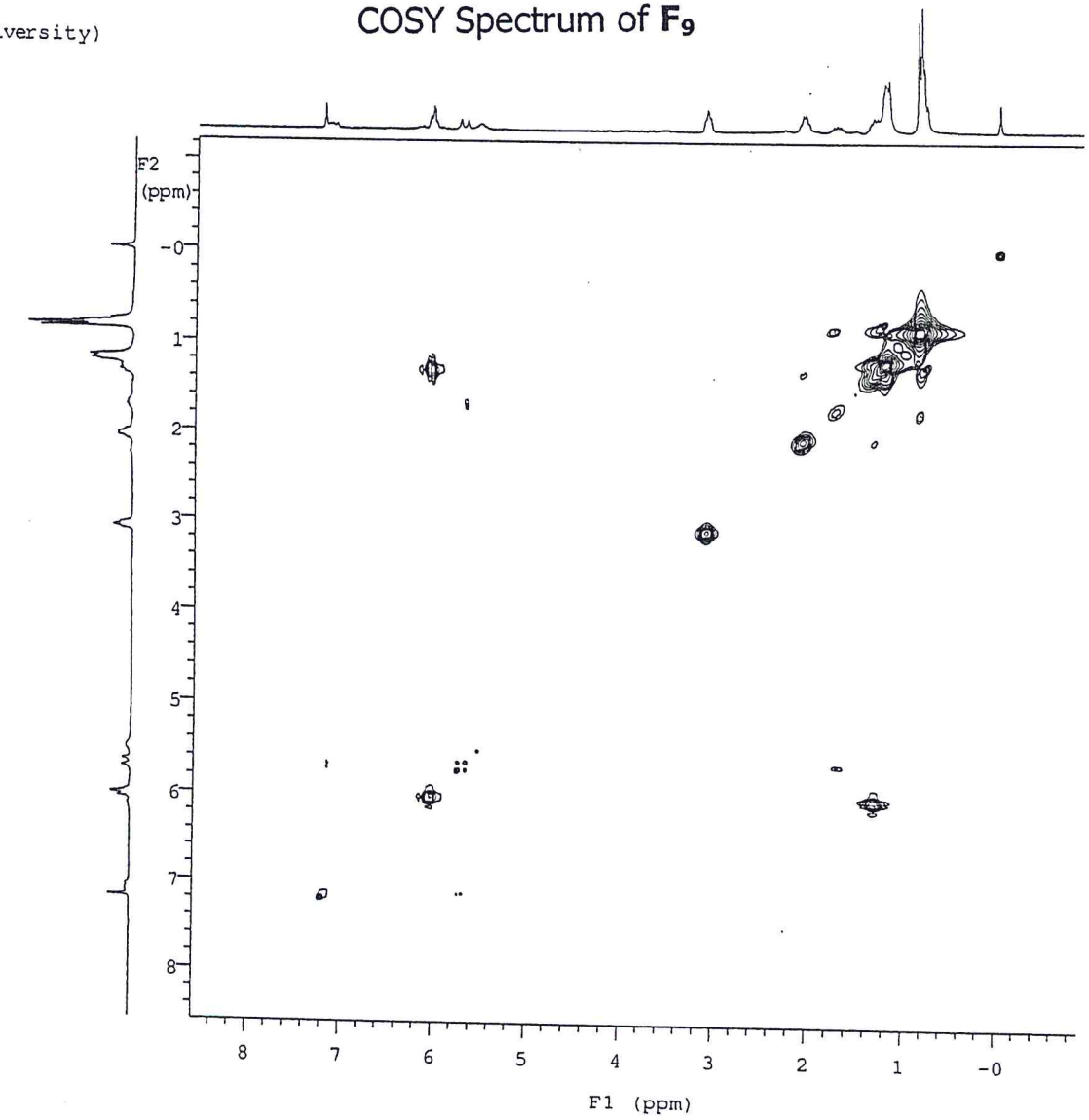
Name: Dennis Okinyo (KenyattaUniversity)
Sample code: 160/168
Solvent: CdCl3

Pulse Sequence: relayh

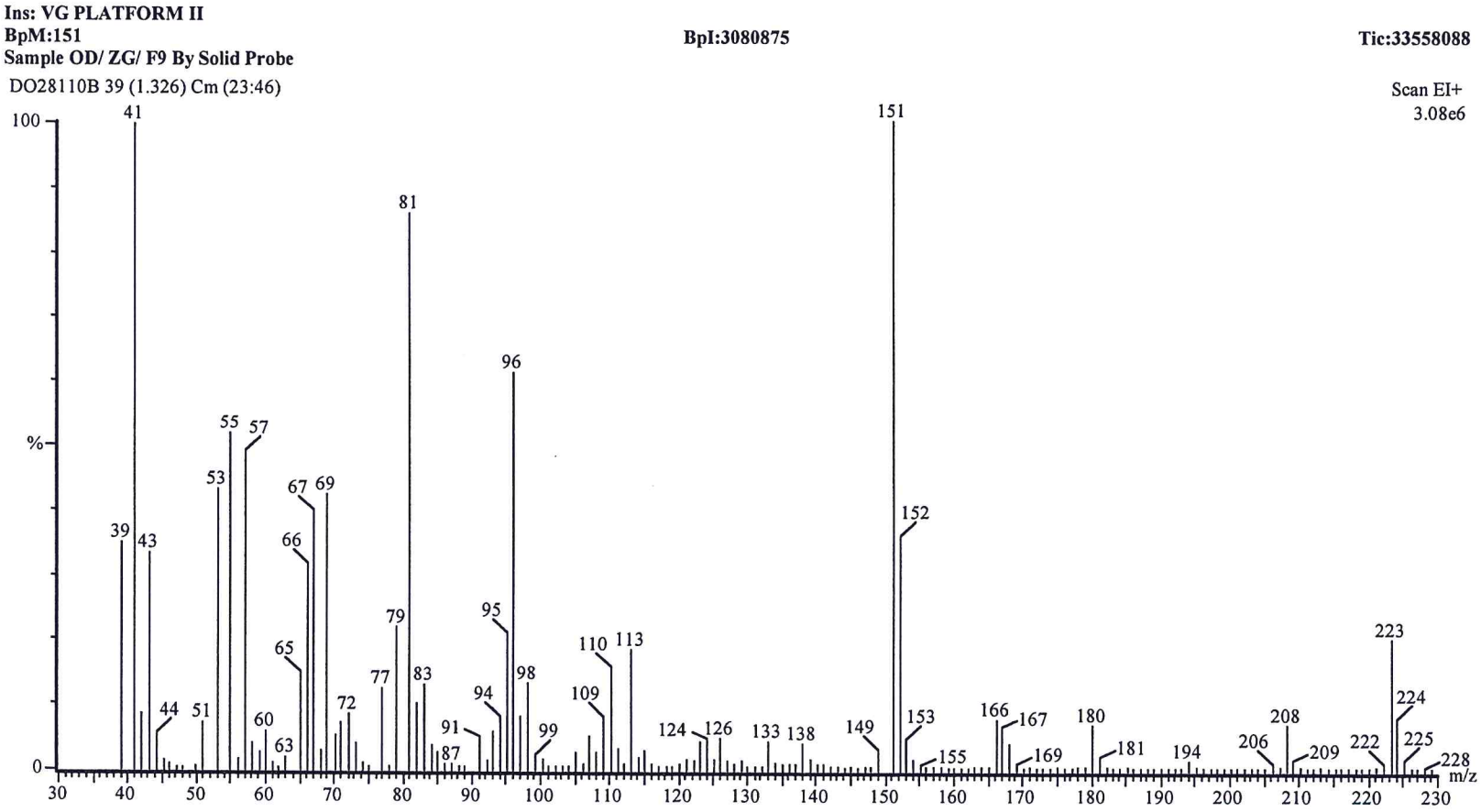
Solvent: CDCl3
Ambient temperature
Mercury-200 "uonnmr200"

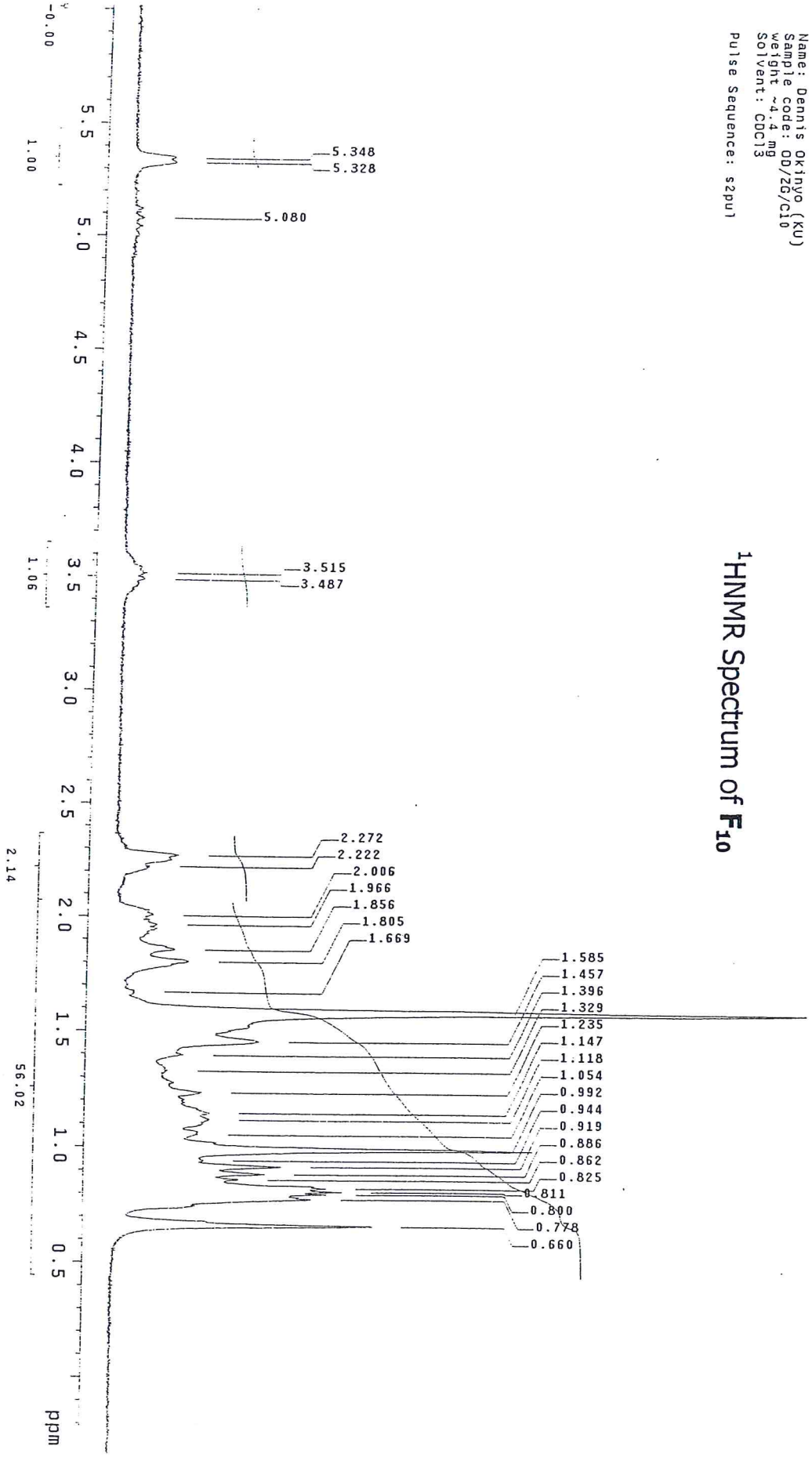
PULSE SEQUENCE: relayh
Relax. delay 1.500 sec
COSY 90-90
Acq. time 0.506 sec
Width 1961.5 Hz
2D Width 1961.5 Hz
4 repetitions
128 increments
OBSERVE H1, 200.0557810 MHz
DATA PROCESSING
Sq. sine bell 0.257 sec
Shifted by -0.267 sec
F1 DATA PROCESSING
Line broadening 0.3 Hz
Sq. sine bell 0.075 sec
Shifted by -0.079 sec
FT size 1024 x 1024
Total time 18 min, 30 sec

COSY Spectrum of F_9

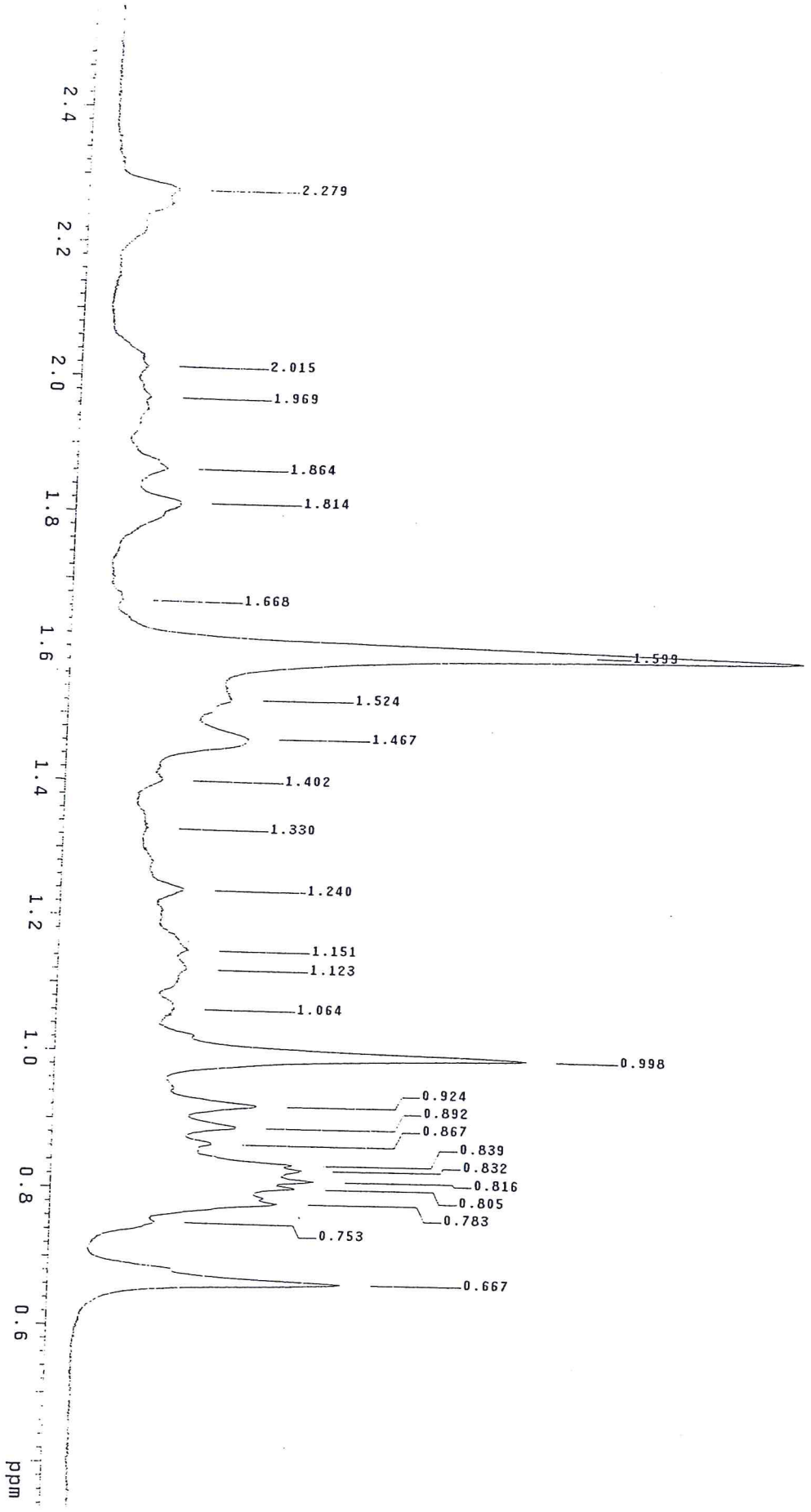


Mass spectrum of F_9





^{1}HNMR Spectrum of **F$_{10}$**

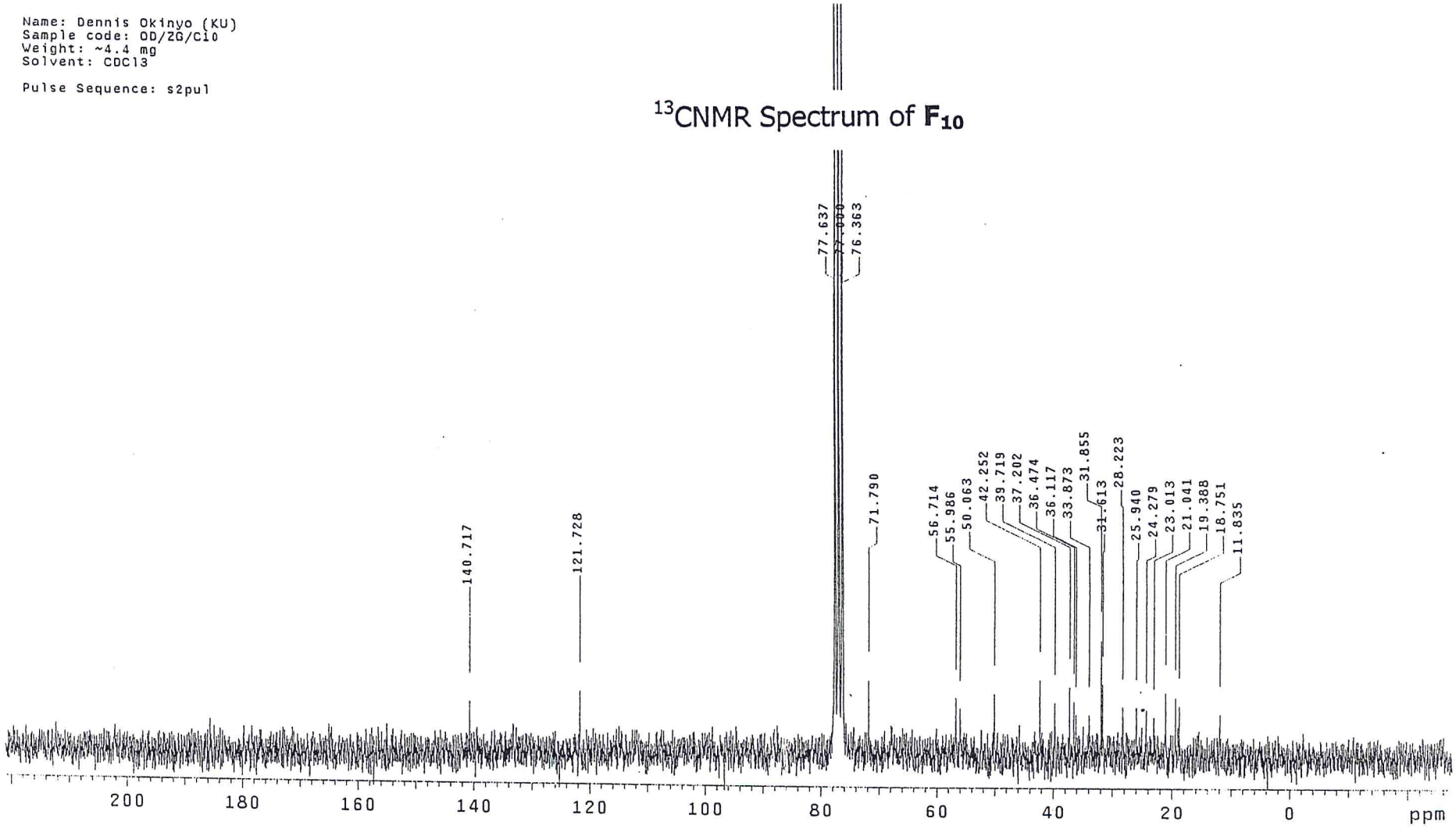


^{1}HNMR Spectrum of **F_{10}**

Name: Dennis Okinyo (KU)
Sample code: OD/ZG/C10
Weight: ~4.4 mg
Solvent: CDCl3

Pulse Sequence: s2pul

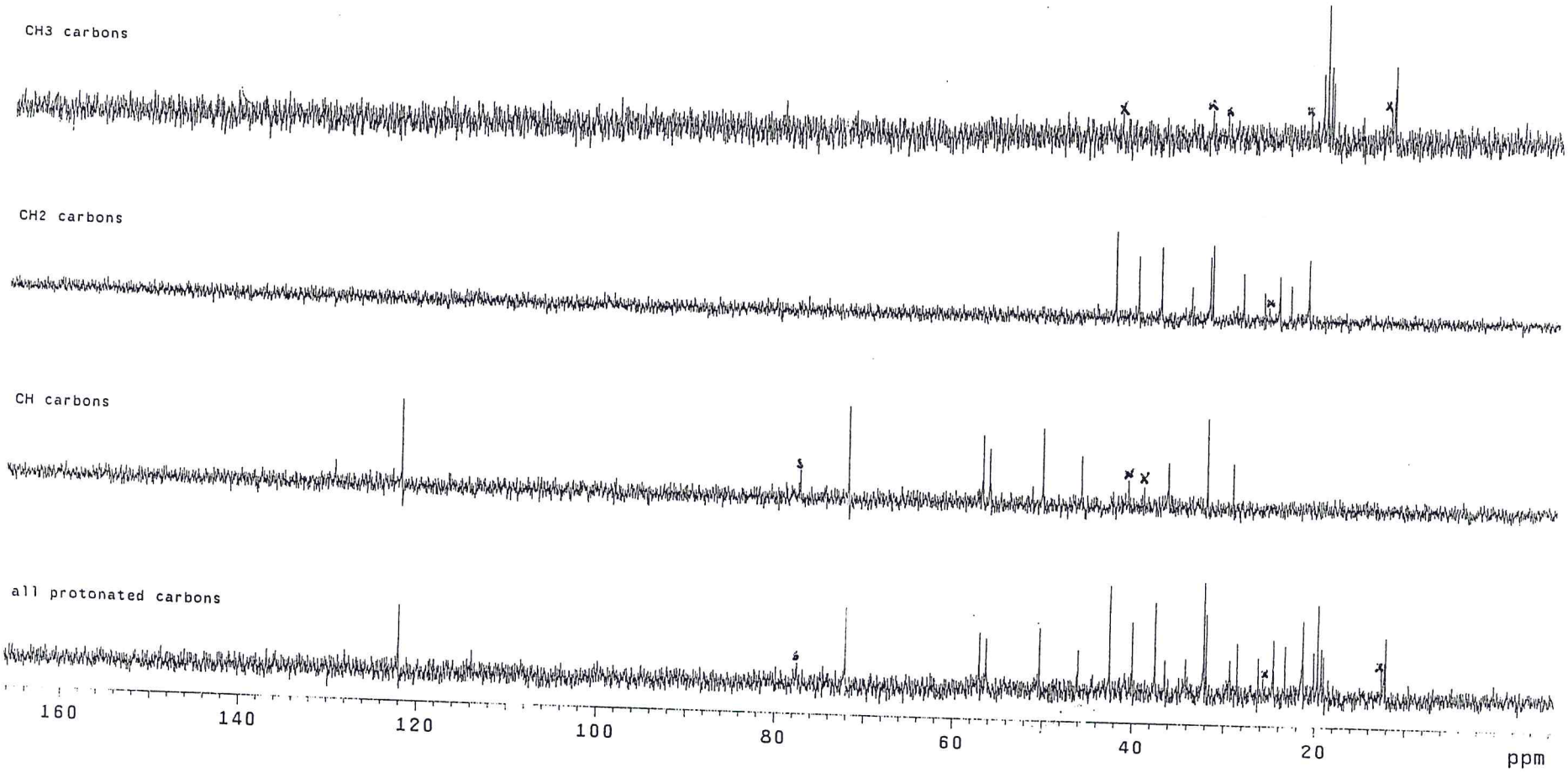
^{13}CNMR Spectrum of **F_{10}**



Name: Dennis Okinyo (KU)
Sample code: OD/ZG/C10
Weight: ~4.4 mg
Solvent: CDCl3

Pulse Sequence: dept

DEPT Spectrum of F_{10}



ADEPT spectrum analysis of F_{10}

ADEPT SPECTRUM ANALYSIS

index		frequency	ppm	intensity
1	D	6136.5	121.979	43.540
2	T	5726.8	113.834	5.785
3	Q			9.619
4	D	3624.1	72.038	46.411
5	D	2865.7	56.963	34.638
6	D	2828.5	56.224	27.563
7	D	2530.8	50.306	38.697
8	D	2314.3	46.002	24.757
9	T	2138.1	42.500	46.655
10	T	2010.7	39.968	33.583
11	T	1884.4	37.457	38.831
12	D	1830.3	36.381	22.272
13	T	1716.7	34.123	17.661
14	D	1616.4	32.130	20.472
15	T			33.748
16	T	1603.1	31.866	40.545
17	D	1473.6	29.292	22.590
18	T	1433.3	28.490	25.577
19	T	1317.6	26.190	15.375
20	Q			5.547
21	T	1234.3	24.534	24.191
22	T	1170.0	23.258	19.356
23	Q			6.162
24	T	1071.3	21.295	33.540
25	Q	1010.3	20.082	36.551
26	Q	988.5	19.650	70.572
27	Q	967.8	19.238	37.853
28	Q	956.2	19.006	29.396
29	Q	630.3	12.529	16.790
30	Q	614.9	12.223	26.209
31	Q	608.5	12.096	38.252

Number of protonated carbons: 31

CH : 9
CH2: 12
CH3: 10

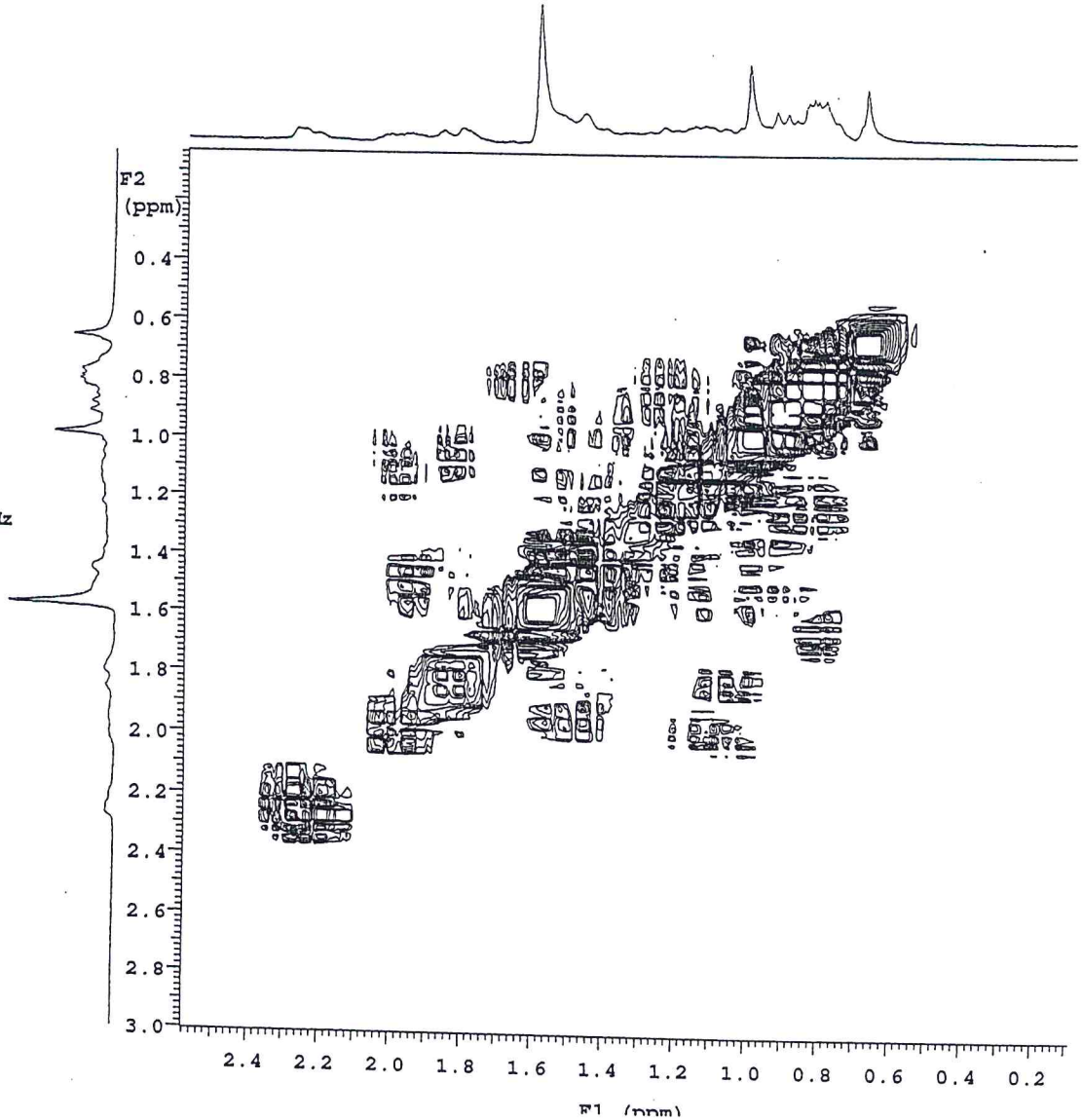
COSY Spectrum of F_{10}

Name: Dennis Okinyo (KU)
Sample code: OD/ZG/C10
weight ~4.4 mg
Solvent: CDCl3

Pulse Sequence: relayh

Solvent: CDCl3
Ambient temperature
Mercury-200 "uonnmr200"

PULSE SEQUENCE: relayh
Relax. delay 2.000 sec
COSY 90-90
Acq. time 0.493 sec
Width 2010.8 Hz
2D Width 2010.8 Hz
4 repetitions
83 increments
OBSERVE H1, 200.0557702MHz
DATA PROCESSING
Sine bell 0.247 sec
F1 DATA PROCESSING
Sine bell 0.021 sec
FT size 2048 x 2048
Total time 0 min, 0 sec



Mass spectra of F_{10}