ANTI-TICK PROPERTIES OF SELECTED EAST AFRICAN PLANTS AGAINST THE BROWN EAR TICK *RHIPICEPHALUS APPENDICULATUS* NEUMAN AND IDENTIFICATION OF SOME OF THEIR SECONDARY COMPOUNDS.

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THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DOCTOR OF PHILOSOPHY (Ph.D) DEGREE AT THE DEPARTMENT OF CHEMISTRY, MOI UNIVERSITY

1998

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DEDICATION

This work is dedicated to my father, the late Salim Kaoneka, my mother, Mboza and my children, Mboza, Mohamed and Salim.

Good reasons must, of force, give place to better.

Julius Caesar

ACKNOWLEDGEMENTS

My sincere thanks are due to Professor Ahmed Hassanali, my ICIPE first supervisor whose enthusiasm, encouragement and guidance during the course of this study gave me the motivation that finally led me to produce what is reported in this thesis. He has not only been very helpful in academics but also in my social life. It is not possible to enumerate all instances in which he had come forward to assist me whenever I was in problems.

Dr. B. Torto, my second ICIPE supervisor is greatfully acknowledged for his tireless efforts in ensuring that this work is a success. The unlimited assistance he extended to me has enriched this work a great deal.

I sincerely thank Professor M. Rajab, my university supervisor for his great interest in this work, very useful discussions and critical evaluation of progress made at various stages of this study. To achieve his goal, he has always been in touch with me. This gave me a lot of encouragement and energy to forge ahead.

I wish to acknowledge the assistance and encouragement extended to me by Dr. V. Musewe, Training Coordinator at the ICIPE through out the period of this study.

I am grateful to the Tropical Pesticides Research Institute fot granting me a study leave and financial support.

I am indebted to the Dutch Government through DSO for sponsoring my study.

My sincere thanks are due to Dr. Kwatelai Quartey of the Department of Chemistry, University of Trondheim, Norway, and Dr. Khambay of the Rothamstead Experimental Station, UK, for running NMR and MS of some of my isolates.

The assistance of Mr D. Munyinyi of Biomathematics Unit at ICIPE in the analysis of my research data is deeply appreciated.

I wish to thank Mr. Mathenge of the Department of Botany, Nairobi University, and S. Kibuwa, formerly with Tropical Pesticides Research Instutute, Arusha for collecting and identifying the plant materials used in this study.

Finally , I would like to acknowledge the kindness and cooperation I received from ARPPIS scholars and the entire staff of ICIPE and Behavioral Chemical Ecology Department, especially Mr Edward Nyandat and Wanyama B. O. Kaye.

Abstract

Hexane, ethyl acetate and methanol extracts of *Commiphora swynertonii* Burtt., *Turraea cormicopia* (L), *T. abyssinica*, *T. floribunda* and *Melia volkensii* Gurke were tested for anti-tick activity against the larvae and adult *Rhipicephalus appendiculatus* Neuman using the packet method bioassay. The hydrodistilled oil of the leaves of *C. swynertonii* was tested for acaricidal and repellency activities against adult *R. appendiculatus* using the packet and climbing bioassay method, respectively. Isolation and structural elucidation of pure components from stem bark of *C. swynertonii*, root bark of *T. floribunda* was attempted. Chemical composition of the hydrodistilled oil was carried out using GC and high resolution mass spectrometry. Residual effects of the extracts of *T. cormicopia*, *T. abyssinica*, *T. floribunda* and *M. volkensii* were investigated.

Hexane and ethyl acetate extracts of *C. swynertonii* exhibited a mean mortality of 71% and 54% respectively on two weeks old larvae of *R. appendiculatus*. These extracts were inactive against adults. The acaricidal activity of the hexane extract was comparable to the commercial acaricide, triatix at the concentration of 0.01% v/v. The hydrodistilled oil of the leaves of *C. swynertonii* showed acaricidal and repellency activities against the larvae and adult *R. appendiculatus*. The oil induced a mean mortality of 84% at the concentration of 1% v/v. The phytosterol constituents of *C. swynertonni*, 3β,4α,5-cholest-7-en-3-ol, β-sitosterol, cholest-7-en-3β-ol and γ-sitosterol exhibited low acaricidal activity against the larvae of *R. appendiculatus*.

Methanol and dichloromethane extracts of the root bark of *T. cormicopia* and *T. floribunda* delayed the number of adult female ticks attaching themselves to the ears of the rabbits. They also significantly delayed engorgement time and reduced hatchability of the eggs.

Seven pure compounds, six phytosterols and a diol were isolated from the stem bark of *C. swynertonii*. Three limonoids belonging to the class havanensin were isolated from the methanol extract of *T. floribunda* by preparative High Performance Liquid Chromatography. The limonoids were characterized using ^{1}H NMR, ^{13}C

NMR, 1-D and 2-D COSY, Heteronuclear Multiple Bond Correlation (HMBC) and Mass Spectrometry. These compounds were 28-nor-4α-carbomethoxy-1α,11β-di acetoxy-3,7-dihydroxy-12α-(2-hydroxy-3-methylbutanoloxy)-14,15epoxyhavanensin (39), 28-nor-3,7-dihydroxy-4α-carbomethoxy-1α,11β-diacetoxy 12α-(1-methyl propanoloxy)-$\Delta^{14,15}$ havanensin (40) and 28-nor-4α-carbomethoxy-1α,11β-diacetoxy 12α-(2-methylbutanoloxy)-14,15 epoxyhavanensin (41).

High resolution mass spectrometry of the oil obtained by hydrodistillation of the leaves of *C. swynertonii* revealed the presence of fourteen sesquiterpenoids. The major compounds were 9-(furanyl)-2,6-dimethyl-2,6-nonanone, 9-(furanyl-2)-2,6-dimethyl-2,6-nonadien-4-one and 9-(3-furanyl)-2,6-dimethyl)-2,5-nonadien-4-one.

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Discovery of ticks

Ticks have been recognized as ectoparasites of domestic animals since ancient times. Tick fever, for instance, was recorded in Egyptian papyrus scrolls dated 1550 BC. Early scholars were familiar with tick ectoparasites of a variety of domestic animals and described the damage caused by them.[1] Despite this early recognition, it was not until the second half of the nineteenth century, when the world cattle population increased rapidly to feed the human populations of the great industrial centres, that the diseases they transmit and their serious debilitating effect on cattle became a problem. There was a great deal of cattle movement during this period with susceptible, exotic breeds being imported into tick infested areas and exposed unwittingly to the hazards of tick borne diseases.[2] This was also the period that *Boophilus* ticks were discovered to be transmitting the Texas fever pathogen, *Babesia bigemina*. This was followed by a series of discoveries that ticks transmit various other pathogenic filaria, protozoa, bacteria, rickettsiae and viruses.[1]

1.2. Diseases caused by ticks

The major diseases transmitted by ticks to animals include East Coast Fever (ECF), heartwater, streptothricosis, babesiosis, benign tropical theileriosis and anaplasmosis.[1,2,3] These are highly pathogenic to African livestock and cause high mortality among them. Animals which survive are usually unthrifty for the rest of their life.[4]

The most important ticks in Africa are the Brown Ear Tick, *Rhipicephalus appendiculatus* Neuman and the Tropical bont tick, *Amblyomma variegatum*. *R. appendiculatus* is the most important species of ticks in Eastern, Central and Southern Africa.[5] It is a vector of the haemoprotozoan *Theileria parva* which is the causative agent of ECF. This disease is common in Kenya, Tanzania, Uganda, Rwanda, Burundi, Zaire, Malawi, Zambia, Mozambique and Southern Sudan. Although, there has been a belief that ECF is caused by *T. parva*, the biological complexity of this organism has led to the recent introduction of a trinomial classification which recognizes *T.parva parva*, *T. parva lawrencei* and *T. parva boris*. The first agent causes virulent ECF while the second and third agents cause corridor disease and Zimbabwean malignant theileriosis respectively.[6]

Ticks surpass all other arthropods in number and variety of diseases they transmit to animals, and rank second to mosquitoes as vectors of human diseases.[1] They constitute the most important livestock pest in Africa. Unlike tsetse, which infest only 40% of the African continent, ticks are to be found on livestock in the entire 30m sq km of the African continent.[7]

Ticks act both as vectors and reservoirs of these diseases since they are able to transmit the disease agents transovarially to their offspring.[8,9] For example, in the case of *Dermacentor andersoni* Stiles, the principal vector of Rock Mountain Spotted Fever rickettsiae (RMSF) , *Rickettsia rickettsi*, organisms ingested by the larvae may be passed to the nymph, then to the adult which in turn may transmit the pathogen transovarially to the larvae of the next generation.[9]

In Indiana, USA, several ticks have been known to transmit diseases to human beings. For example, Lyme disease is caused by the spirochete *Borrelia burgdorferi* and is transmitted to man via bites by nymphs of the bear (deer) tick, *Ixodes dammini* Speilman, Clifford, Piesman and Corwin. The lone star tick, *A. americanum* L., the American dog tick, *Dermacentor variabilis* Packard and the blacklegged tick, *Ixodes*

scapularis Say have also been implicated in Lyme disease transmission. Other tick borne diseases to man include tularemia which is caused by bacterium *Francisella tularensis* transmitted by *D. variabilis* and two rabbit ticks *Ixodes dentatus* Marx and *Haemaphysalis leporipalustris* Packard; and RMSF which is caused by *R. rickettsi*, transmitted by *A. americanum*, *I. scapularis* and *Rhipicephalus sanguineous* Latreille.[10]

1.3. Economic losses due to ticks

The cattle industry is devastated by ticks which affect about 800m cattle and similar number of goats and sheep around the world. Ticks cause most problems in the tropics.[11] In Africa alone, ticks infest about 90% of the estimated 200m cattle, about 70% of them from simultaneous infestation by several species. The infestation causes enormous production losses due to mortality and debility imposed on the affected animals. The debility arises from physical damages, such as loss of blood, irritation, damage of the hides and predisposition of the affected animals to bacteria, fungal and parasitic infection.[4]

Cattle tick occupies at least 1.3 m km^2 in northern Australia which supports close to 9m cattle or about 30% of Australian beef cattle herd. To control the ticks, the Australian government spent about US$40m in 1972.[12] Losses due to *Boophilus microplus* in Argentina and USA stood at US$ 180m and 90m respectively in 1980.[13] In Mexico, the Caribbean Basin and South America, it is estimated that *B. microplus* (Canestrini) infest about 70% of the 250m cattle.[14]

According to Sutherst[15] the breakdown of the economic losses caused by the cattle tick is as follows: increased labour cost = 36%; loss of beef = 20%; loss of dairy production = 16%; loss by death = 11% and increased draught loss = 5%. Springell in

his paper on the cattle tick in relation to animal production in Australia agrees with the above figures.[16]

There is, as yet, no reliable data on the economic effects of tick infestation on livestock in Africa. However, in view of the large number of animals infested by ticks in Africa and multiplicity of tick species available in the continent, it is generally believed to be high.[17]

The need to develop technically feasible, environmentally acceptable tick control strategies are, therefore, required so as to serve the cattle industry worldwide.

1.4. Significance of the study

Ticks have been and are still pests to animals (especially the domesticated ones) and to man. Many attempts to develop an efficient tick control method have been made but not a single one has been without problems. A review of literature shows that little work has been carried out on plants with anti-tick properties. This work was, therefore ,undertaken to investigate more about the potentialities of plants, particularly from the families Meliaceae and Burseraceae , known for their anti-insect properties, in the control of ticks. The knowledge obtained provides a basis for the development of plant based agents as components of an integrated tick management(ITM) package.

1.5. Objectives of the study

To date, no extensive work has been carried out to isolate active crude extracts/compounds with anti-tick properties from plants. To explore this goal, this study was undertaken with the following objectives:

(i) To screen n-hexane, dichloromethane and methanol extracts of *Commiphora swynertonii* Burtt. (Burseraceae) (stem bark); dichloromethane and methanol extracts of

the ripening fruits of *Melia volkensii Gurke (Meliaceae)*, hexane and methanol extracts of the root barks of *Turraea comicorpia*, *T. abbyssinica* and methanol extract of *T.floribunda* against the larvae of *R. appendiculatus*.

(ii) To screen the extracts of *M. volkensii*, *T.floribunda*, *T. cormicopia* and *T.abbyssinica* as in (i) against adults of *R. appendiculatus* and subseuently monitor their long term effects on these individuals.

(iii) To isolate pure compounds from the most active extracts of *C. swynertonii* and *T.floribunda* for chemical characterization.

(iv) To undertake dose-response studies using the active pure compounds isolated from *C. swynertonii*.

(v) To obtain the oil from the leaves of *C. swynertonii* by hydrodistillation and screen it against the larvae and adults of *R. appendiculatus* for anti-tick properties.

(vi) To identify the compounds present in the oil

CHAPTER 2

LITERATURE REVIEW

2.1 Acaricide application in tick control

Tick control is a continuing exercise worldwide because of the devastating effects of tick infestation on livestock production. Its control is presently dependent on the use of synthetic acaricides which are applied in dips, dusts, sprays or released from plastic tags and collars.[4,18] Since the turn of this century, cattle farmers in areas where ticks abound, arsenic compounds, rotenone, chlorinated hydrocarbons, organophosphates, carbamates and pyrethroids have been used. Application of synthetic acaricides to tick infested cattle is regarded as an easy and practical way of ridding animals of their ticks.[18,19]

Many synthetic acaricides have so far been used to control ticks. These include dioxathion,[13,20,21] toxaphene and malathion.[22] In the study of acaricides, Hadani and his co-workers, used benzyl benzoate, dimethyl phthalate (DMP) and N,N-diethyl-toluamide (DEET).[23] Some of the old acaricides such as DDT and BHC are no more available because of their high toxicity and persistence in the environment.[24]

The widespread use of acaricides especially in Africa has created a number of problems. First, acaricides like any other pesticides are toxic not only to the target pest but also to many other insects and animals as well. Secondly, target insects rapidly develop resistance to them.[25] Acaricides also cause environmental pollution and contaminate both meat and milk.[4] Exorbitant prices of acaricides make them a less appropriate method for tick control especially in Africa.[26,27] Socio-economic problems also contribute significantly to the failure of acaricides to effect a major impact on tick control in Africa. It is believed that the distribution of free and highly subsidised

acaricides in several African countries have discouraged farmers and herders from exploring sustainable alternatives. Furthermore, pastoralism in some parts of Africa, for example in the Sahelian countries, is a limiting factor in the use of communal dips which are normally built closer to various urban centres. In addition, in some countries, lack of water especially during dry seasons, vandalism, and the introduction of some form of tax for each cattle have made the use of acaricides impractical.[4]

2.2 Integrated tick management (ITM)

It has been proposed by some tick scientists that the most effective way of controlling ticks in Africa is through the application of low cost ITM. In addition to population dynamics seven potential components of ITM have so far been recognized. These include, immunology, behavioural manipulations with semiochemicals, natural resistance, ecological manipulation, farm management, biological and botanical control.[4]

2.2.1 Immunological approach

Research in Australia and at ICIPE has shown that, in principle, it is possible to protect Africa's livestock against ticks through vaccination. About 8 polypeptide antigens isolated from whole tick extracts or midgut have been found to be protective against the oviposition and feeding of adult ticks and moultability of immature stages after being screened on cattle. Furthermore, there are indications that the antigens could serve other purposes, such as, enhancing natural resistance of host to tick infestation, acting as a tick field diagnosis mediator and as a natural resistance selection mediator.[4]

2.2.2 Natural resistance approach

It has been established that most Zebu crossed breed cattle develop a high level of resistance to the cattle tick as compared to exotic breeds.[28]

It has been suggested that tick resistance qualities of Zebu cattle should be utilized for tick control. Similar qualities are shown by cross cross breeds of British cattle and Zebu which have been found to need less dipping than local British cattle on similar pastures.[18,20] However, a disadvantage of this approach is that it decreases productivity.[18,28] Although, this approach of tick control has been known for quite sometime, its use as a tool for tick control has not been exploited in Africa.[4]

In order for this approach to be a success in the long term management of tick, there is the need to direct research effort to the development of vaccines which enhance natural resistance.

2.2.3 Ecological approach

This approach is mainly concerned with manipulation of the environment and is directed to the non parasitic phases of ticks. The use of carbon dioxide traps briefly used in Zimbabwe is not yet widely applied in Africa. This approach also includes cultivation of plants inimical to the survival of non parasitic stages of ticks, such as , *Stylosanthes*[29] and *Gynandropsis gynandra*.[30]

2.2.4 Farm management approach

The basis of this component is the improvement of the farming management practices which decrease tick populations on pasture or on animals. It includes hand deticking. Deticking is undertaken almost daily by pastoralists but it is irregular in

agropastoral farming. This approach prevents successful mating and disrupts tick oviposition.

2.2.5 Biological control approach

This approach employs the use of predators, parasitoids and pathogens. The red billed oxpecker *Buphagus erythrorhynchus* and the yellow billed oxpecker *B. africanus* have been found to prey on the parasitic stages of ticks.[31,32] Apart from these birds , domestic chickens have been observed to feedon engorged stages of *R. appendiculatus*.[31,33] Other predators of ticks include the ants *Iridomyrmex detectors*, *Rheidole megacephala* and *Aphaenogaster longiceps* which feed on *B. decoloratus*. [31]

Although several tick parasitoids have been recorded, *Hunterellus hookei* has been recorded as a parasitoid of *A. variegatum*, is most sited.[34] Its biology and life cycle have been fully established. [35,36]

Pathogens like viruses, protozoa, bacteria and fungi have been found on dead ticks. [37,38] Viruses have been successfully used in several developing and third world countries for the control of insect pests. In addition, viral infections on ticks have been reported to have taken place both under laboratory and natural conditions.[31]

A more recent approach of using entomogenous fungi *Beauveria bassiana* and *Metarhizium anisopliae* has shown promising results in the control of *R. appendiculatus* and *A. variegatum*.[39] Toxins produced by *B. bassiana*, *M. anisopliae* and *Paecilomyces fumosoroseus* have been used against soft tick *Argas persicus*.[31]

2.2.6. Use of botanicals

Despite the fact that many pesticide products have been on the market for the last four decades, pests and vectors of human diseases are still a problem.[40] Alternative

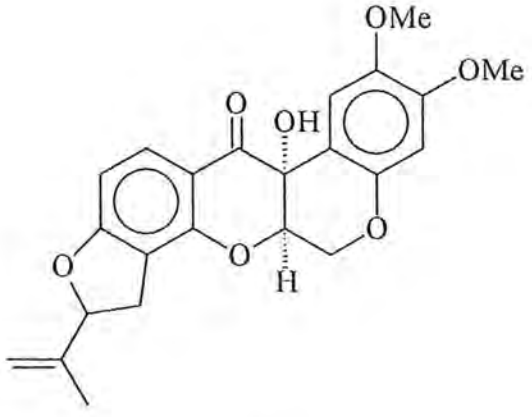
means such as the use of botanicals needs to be explored seriously. Meliaceae, Rutaceae, Asteraceae, Annonaceae, Labiatae and Canellaceae have been shown to contain potent chemicals for the control of arthropods.[41] Although there has been some advances in the use of botanicals in the control of insect pests, very little has been done to study the effects of these botanicals on ticks. However, there are a number of indications on the potential of botanicals as tick repellents and tickicides. [42]

Essential oils of *Gynandra gynandra* (fam. Capparaceae) and some Labiatae species have been found to exhibit repellent activity against adult *R. appeniculatus*, comparable to the commercial insect repellent, DEET. The highest repellency, 90.9% for *G. gynandra*, 100% for Labiatae plant oil and 96.2% for DEET were obtained at a dose of 0.1µl per 25cm long metal rod. [42] Mwangi and her co-workers [43] found that the hydrodistilled oil from the tropical shrub *Ocimum suave* had a repellency of 92.2% against *R. appendiculatus* at a concentration of 0.0001 (v/v). It was further found out that the oil caused a mortality of 100% against the the larvae of *R. appendiculatus* at a concentration of 0.2% (v/v). In another study , it was found out that all stages of *R. appendiculatus* and *A. variegatum* were repelled by *G. gynandra*. It was further found that some ticks which were continuously exposed to the leaves of this plant died, while surviving ones were found to be weak and inactive. The effectiveness of the plant leaves as a repellent and tickicide was relatively more pronounced on nymphs than on adults.[30]

It was found by Sutherst and his co-workers [29] that larvae of *B. microplus* ascending some tropical pasture legumes belonging to the genus *Stylosanthes* became trapped and immobilized in material secreted from the glandular trichomes of these plants. Trapped larvae were observed to die rapidly. In their assessment of the ability of 22 species of the genus *Stylosanthes* to trap larvae of *B. micrplus* or prevent them from ascending the plant stems, the same group found that *S. viscosa*, *S. scabra* and *S. guianensis* were the most active.[44]

Dipeolu et. al,[45] found that a mixture of ground tobacco leaves (fam. Solanaceae) and a mineral called magadi soda popularly known as "Kupetaba" was effective as a tickicide against all stages of *R. appendiculatus*. This substance which contains nicotine as the active ingredient prevented the completion of all feeding phases of tick, suppressed the oviposition capacity of the engorged ticks and drastically reduced the hatchability of eggs. Larvae and nymphs were killed within 24 hours of application with a large number of adult ticks being killed within 2-3 days in in vitro studies.

The plant *Neorautanenia mitis* (A. Rich) belonging to the family Fabaceae (Papilionaceae) is known as a fish poison, insecticide and remedy for syphilis in Central Africa. As a result of these properties, chemical investigation carried out by Puyvelde and his co-workers [46] led to the isolation of a tickicidal compound, 12a-hydroxyrotenone (1) from the hexane extract of this plant.



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Although only a few naturally occurring compounds have been purified as tickicides, there are, however many potent crude extracts and compounds which have exhibited biological activity against other arthropods. It is possible that such plant species could be potential sources of tickicidal principles. Tables 1 and 2 show active crude extracts and active principles respectively against different organisms.

TABLE 1. Active crude extracts against some arthropods

No.	Plant species	Family	Extraction solvent	Target arthropod	Concentration	Remarks	Ref.
1	*Melia azedarach* L.	Meliaceae	Acetone	*Culex pipens molestus* larvae	40ml/ml	insecticide	47
2	*M. volkensii* Gurke	Meliaceae	Acetone	*C. pipens molestus* larvae	30mg/ml	insecticide	47
3	*Azadirachta indica* A.Juss.	Meliaceae	Methanol	*Schistocerca gregaria* (Forsk.)	1%	repellent	48
4	*Phytolacca dedecandra*(L'Herit)	Phytolaceae	Water	*Biomphalaria glabrata* and *Bulinus truncatus*	20ppm	mulluscide	49
5	*Caryopteris divaricata* Maxim	Verbenaceae	Benzene	*Spodoptera litura* F.	1%	antifeedant	50
6	*C. erythraceae* Engler	Burseraceae	Hexane	*R. appendiculatus* Neuman	NM	repellent larvicide	51

Key: MN - Not mentioned

Table 1 (contd)

No.	Plant species	Family	Extraction solvent	Target arthropod	Concentration	Remarks	Ref.
7	*M. volkensii* Gurke	Meliaceae	hexane EtOAc(1:1)	*Aedes aegyptei*	2ml/ml in H2O instars	prolongs larval instars	52
8	*Lonchocarpus castilloi* Standley	Leguminosae	Methanol	*Cryptotermes brevis* Walker	2.5% v/v	antifeedant	53
9	*Tropaeolum majus*	Tropaeolaceae	Methanol	*Pieris rapae*	0.1gle	antifeedant	54
10	*Zea mays*	Gramineae	DCM	*Diabrotica vigifera vigifera*	NM	repellent	55

Key:

MN - Not mentioned
DCM - Dichloromethane
gle - gram leaf equivalent

Table 2. Natural active principles against some arthropods

No.	Compound	Plant Species	Family	Insect Pest	Conc.	Remarks	Ref.
1	Nicalbin A (2) (L.) Gaertin Var. albiflora	*Nicandra phsaloids*	Solanaceae *Varievestis* Mexican bean beetle	*Epilachna*	0.01%	Antifeedant	56
2	Azidraachtin (3)	*Azidarachta indica* A. Juss. (L.)	Meliacae	NM	NM	Antifeedant	57
				Schistocerca gregaria desert locust	40mg/l	Antifeedant	58
				Spodoptera frugipeda Fall army worm	0.35ppm	Antifeedant	59
				Heliothis virescens tobacco bud worm	0.8 ppm	growth inhibitor	60
3	Pedonin (4)	*Harrisonia abyssinica* Oliv.	Simarubaceae	*Eldama sacharia* and *Maruca testularis* pod borer	NM	Antifeedant	61
4	Harrisonin (5)	*Harrisonia abyssinica* Oliv.	Simarubaceae	*S. exempta* army worm	20ppm	Antifeedant	62
5	Trichlin A (6)	*Trichilia roka*	Meliaceae	*S. eridarina* Southern army worm & *Epilachna varivestis*	NM	Antifeedant	63

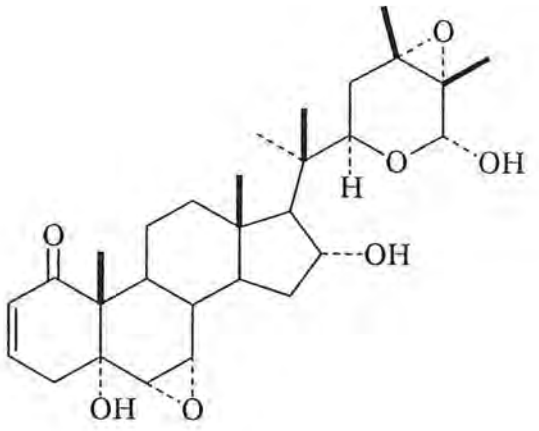
Table 2. (Contd).

No.	Compound	Plant Species	Family	Insect Pest	Conc.	Remarks	Ref.
6	Withanolide(7)	*Withania somnifera*	Solanaceae	*S. littolaris* Egyptian cotton leaf worm	NM	Antifeedant	64
7	Warbuganal(8)	*Warbugia stuhlmanii*	Canelaceae	*S. exempta*	0.1ppm	Antifeedant	65
		W. ugandeusis		*S. exempta*	0.1ppm	Antifeedant Antifungal Antiyeast	66
8	Plumbagin(9) *P. zeylanica*	*Plumbago capensis* Plumbagaceae	Plumbagaceae	Microbials larvae of *Aedes aegypti*	NM 0.01mg /ml	Antimicrobial Larvicidal	67
9	Obacunone(10)		Meliaceae	*Maruca testularis*	100mg/ disk	Antifeedant	68
10	Azedarachol(11)	*Melia azedarach Var. japonica*	Meliaceae	*Agrotis sejetum* Denis cutworm	500ppm	Antifeedant	69
11	Encecalin(12)	*Encelia farinosa* Adans	Asteraceae	*Paridroma saucia*	2.4mmol /gfr.wt.	Insecticidal	70
12	Xylomolin(13)	*Xylocarpus moluccensis* Roem	Meliaceae	*S. exempta*	100ppm	Antifeedant	71
13	Vismione B(14)	Vismia	Guttiferae	*Locusta migratoria*	10^{-3}M	Antifeedant	72
14	Shiromodiol diacetate(15)	*Parabolium trilobum* Nakai	Lauraceae Boisd	*S. littolaris*	0.125%	Antifeedant	73

Table 2. (Contd).

No.	Compound	Plant Species	Family	Insect Pest	Conc.	Remarks	Ref.
15	Trans 2-nonenal (16)	*Daucus carota*	Liliaceae	Carrot fly, *Psila rosae* (F) and *Periplaneta americana*	4000ppm 50ppm	Insecticidal repellent	74
16	Eugenol (17)	*Eugenia caryophytta*	Myrtaceae	maize weevil, *Sitophilus zeamais*	5ml/ disc	repellent	75
17	N-isobutyl-2E,4E,8E,102 Z-dodeca-2, 4,8,10 tetra-enamide (18)	*Spilathes maurit-iana*	Compositae	Third larvae of *Aedes aegypti*	10mg/ml	Larvicide	76
18	Imperatorin (19) and xanthoxyletin(20)	*Glausena assisata* (Willd.) Hook F. Ex Benth	Rutaceae	Army worm, *S. exempta*	NM	Antifeedant	77

Key: NM - Not mentioned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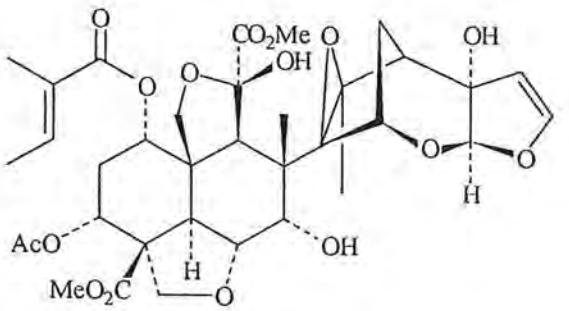
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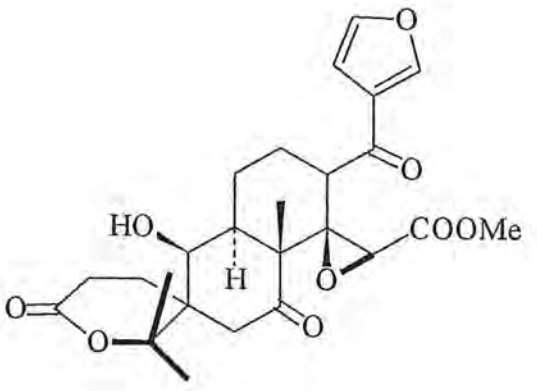
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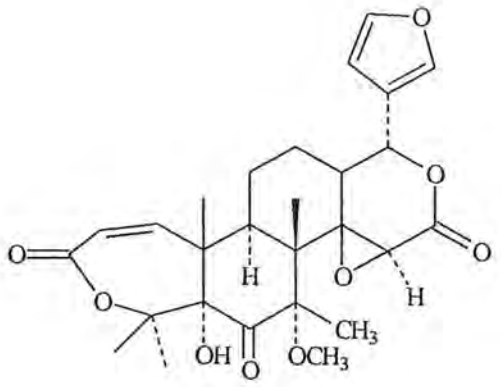

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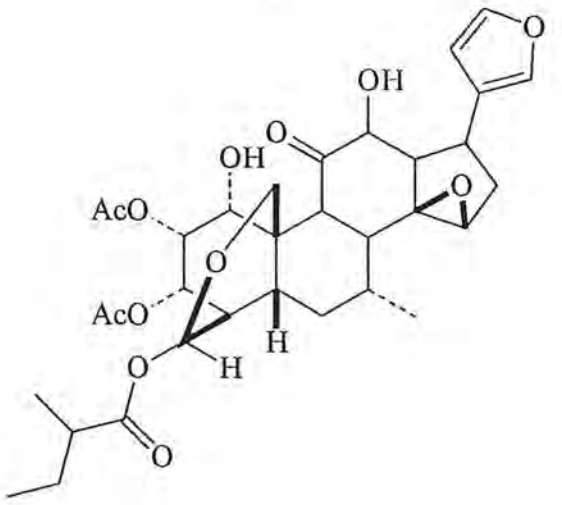

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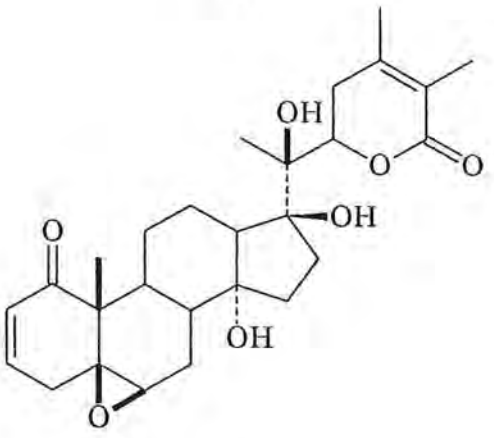

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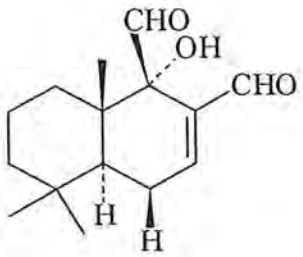

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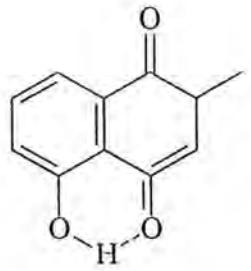

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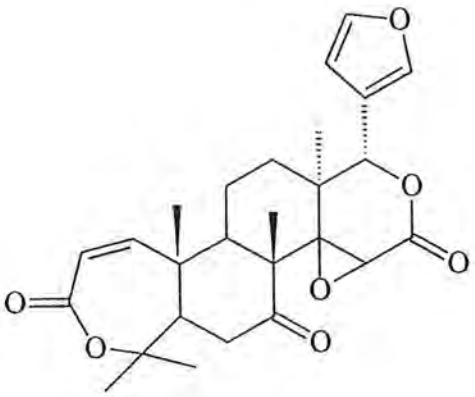

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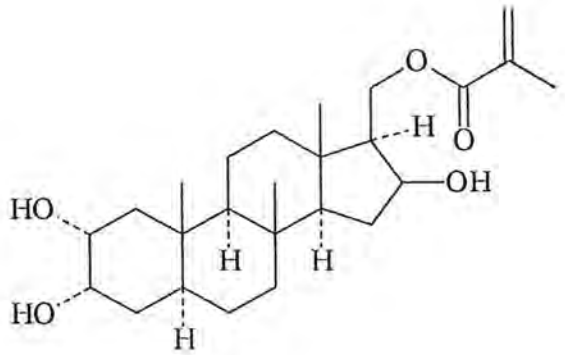

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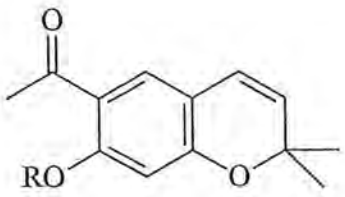

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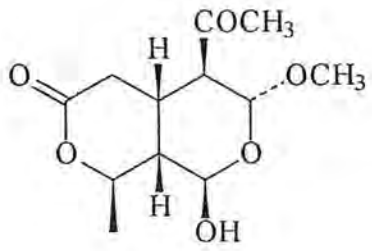

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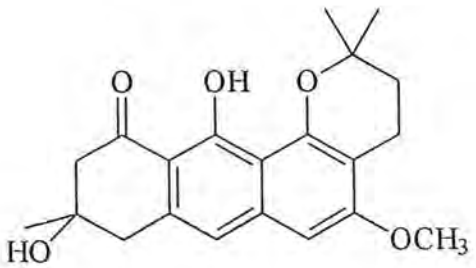

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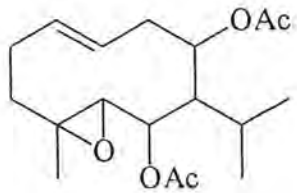

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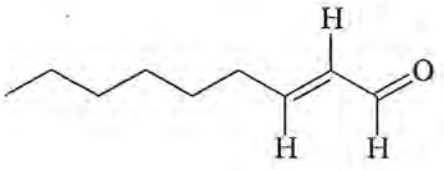

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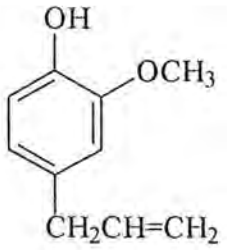

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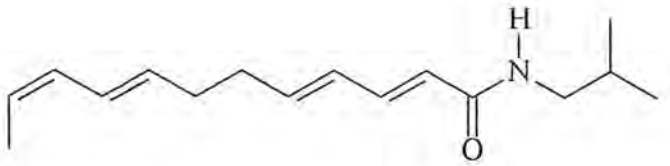

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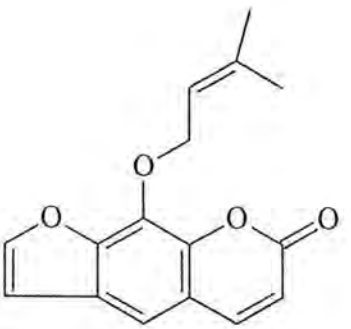

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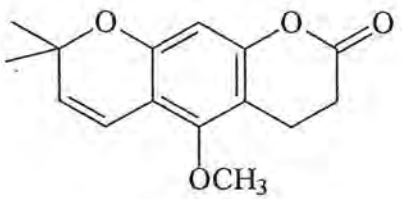

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2.2.7 Choice of plant families in the present study

In this study, two families, namely Meliaceae and Burserasceae were chosen. The choice was based on biological activities exhibited by some plants belonging to these families as outlined below.

2.2.7.1 Meliaceae

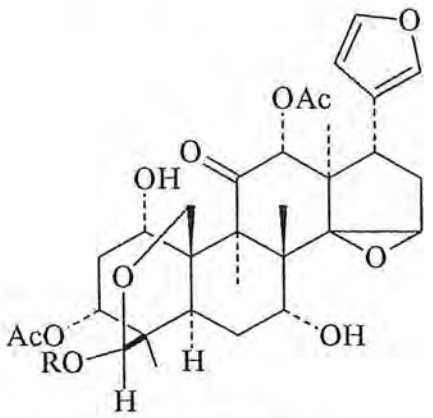
A number of Meliaceae species are known for their medicinal properties.[78] For instance, the roots of *Turraea mombassana* are used for the treatment of excess bile and as an emetic. A decoction of the roots is taken for the treatment of malaria and other fevers.[78] Some Meliaceae species have been used for the traditional treatment of cancer. These plants include, *Amoora rohituka*, *Khaya ivorensis*, *M. azedarach*, *Sandoricum indium* and *Trichilia birta*. In their evaluation of the Meliaceae as a source of potentially useful antineo-plantic agents, Pettit and his co-workers isolated a series of tetranotriterpenes that strongly inhibited growth of murine P-388 lymphocytic leukemia cells.[78]

Meliaceae species are also known to exhibit biological activities against many arthropods. Hexane and ethanol extracts of the seeds obtained from 10 species of this family were found to effect significant mortality, reduce larval development and increase the time for pupation of *S. frugiperda* (J.E. Smith) larvae at a concentration of 16ppm.[79] In another study by the same author, these extracts were found to inhibit feeding and effect mortality in the larvae of the stripped cucumber beetle *Acalymma vittatun*.[80]

The biological activities of the Meliaceae have been attributed mainly to the presence of tetranortriterpenoid derivatives known as limonoids.[81] This class of

compounds is also present in the Rutaceae family.[82] They are known to display marked biological activity against a variety of insects as illustrated below.

Sendalin (21) and trichirokanin (22) isolated from the rootbark of *Trichilia roka* inhibited growth of the pink bollworm, *Pectinophora gossypiella*, *S. frugiperda* and tobacco budworm, *Heliothis virescens*.[81]

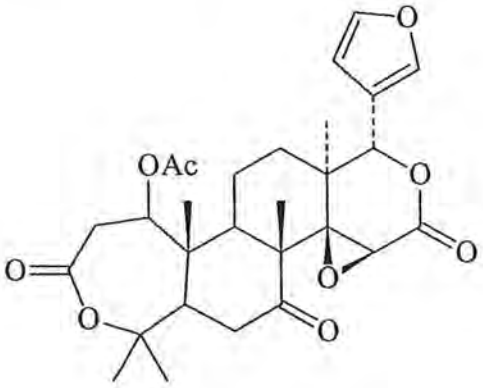


21 R=Ac

22 R=CO—CH(CH$_3$)CH$_2$CH$_3$

Serit and his co-workers [83] found that the lower precipitated layer of methanol extract of *Citrus natsudaidai* Hayata seeds applied to paper discs at a dosage ranging from 500 to 2000ml/disc, significantly deterred feeding by *Reticulitermes speratus* Kolbe nymphs. Three termite antifeedant limonoids namely obacunone (10), nomilin (23) and limonin (24) were identified as active principles. Obacunone had a minimum effective dosage (MED) of 150ml/disc, about two fold more active than nomilin.The MED ofr limonin was 1000ml/disk. The same compounds exhibited feeding deterrence to the Colorado potato beetle larvae, *Leptinotarsa decemlineata* (Say) at a concentration of 31.7mg/disc.[84,85] In another study, Serit and his co-workers [86] evaluated four limonoids by a no choice method as termite antifeedants using *Reticulitermes speratus* Kolbe. Obacunone showed highest degree of antifeeding

potency (PC_{95}=1133 ppm w/w), followed by nomilin (PC_{95}=4475 ppm), azadirachtin (PC_{95}=65,293 ppm) and limonin (with PC_{95} estimates beyond the bioassay limits).



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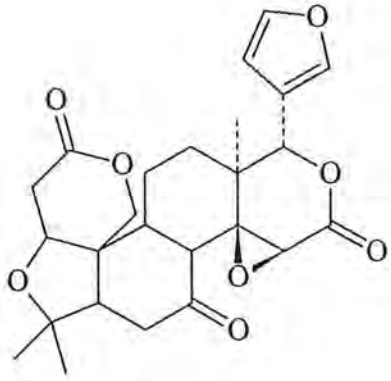

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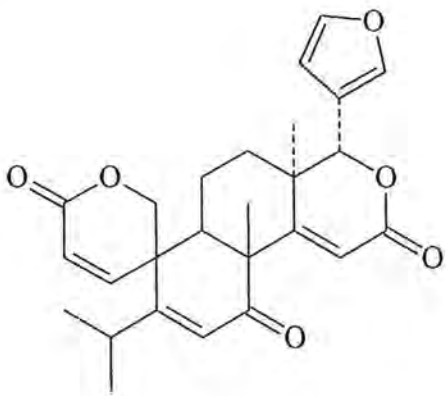
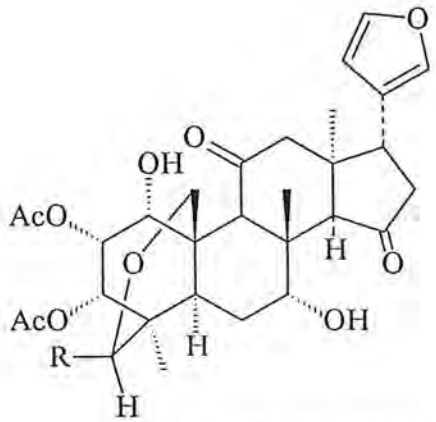
A review by Champagne and his co-workers[87] on the effects of 78 limonoids on insect feeding and growth shows that this class of compounds is very active against many insects especially lepidopterans. Out of 199 insects studied and found to be affected by limonoids, 147 were lepidopterans, 37 coleopterans, 12 heteropterans and 3 dipterans.

In long term feeding studies (fourth instar to pupa) Alford and Bentley [88] found that a high concentration of citrolin (25) (500ppm) extended larval development time of the spruce budworm, *Choristoneura fumiferana* (Clemens) by 40% over that of control.



25

The tetranortriterpine, meliatoxin A2 (26), induced a significant level of antifeedant activity in *S. litura* larvae at a concentration of 500ppm.[89]



R=CH_2CH_2CHCO

26

In recent years the use of limonoids in arthropod control has been recognized. Formulations of the broad spectrum anti-insect limonoid derivative azadirachtin are now available on the market.An azadirachtin rich formulation, Morgosan-O and is being used in the USA for non food crops and ornamentals. In India a granular formulation called Neemark with azadirachtin as the active ingredient is used for the control of pests of cotton, paddy, tobacco, groundnuts, sugarcane, chili, egg plant, vegetables and horticultural legumes.[90] More research on the activities of limonoids and other compounds from the Meliaceae and Rutaceae could offer a breakthrough in the control of arthropods.

2.2.8 Choice of species

Four plants, namely *T. floribunda, T. abyssinica, T. cormicopia* and *M. volkensii* were chosen for biological screening against *R. appendiculatus.*

2.2.8.1 *T. floribunda*

Though several compounds have been isolated and characterized, [91,92,93,94] there was still a need to isolate and characterise the rest of the compounds from this plant. In addition, a full assessment on the biological activities of compounds isolated from this plant could then be made.

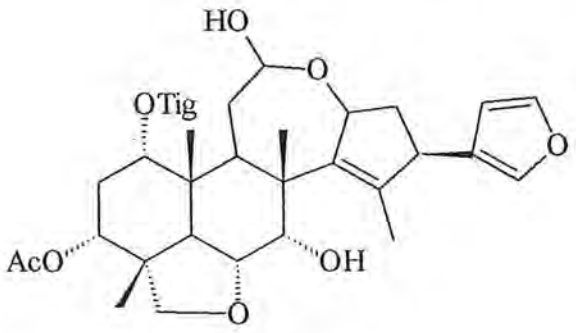
2.2.8.2 *M. volkensii* Gurke

M. volkensii (kirumbutu in Taita Kenya) is a tree found in the dry areas of East Africa. A small amount of water from which the bark has been boiled is said to cure pain and aches in the body. [95]

This plant has been shown to have some biological activities. An active fraction of extracts of dry *M. volkensii* fruits was reported to prolong larval instars of *Aedes aegypti* at a concentration of 2µl/ml in water. It was also reported that the LC_{50} larval mortality is 50µg/ml in 48 hours. [52] Feeding activity of nymphs and adult *Schistocerca gregaria* is reduced markedly after second instar nymphs receive topical treatment with a 2% aqueous solution of *M. volkensii* fruit kernel extract. It was also found that due to feeding inhibition, there was very poor growth, prolonged intermoult periods and high mortality especially during ecdysis. [96] Wilps, et. al [97] have also studied the effects of *M. volkensii* extracts on mortality and fitness of adult *S. gregaria*. They found that a formulation of the methanol extract of the ripening fruits of *M. volkensii* with palm oil exhibited a 40% mortality on S. gregaria which were in a flight mill.

Rajab and his co-workers [98] reported that volkensin, an isolate from the seed of *M. volkensii*, exhibited potent antifeedant activity against the nymphs and adults of *S. gregaria*.

The broad spectrum of biological activities (as earlier reported) has encouraged us to work on the dichloromethane extract of the fresh whole fruit of *M. volkensii*.



27

2.2.8.3 *T. cormicopia* and *T. abyssinica*

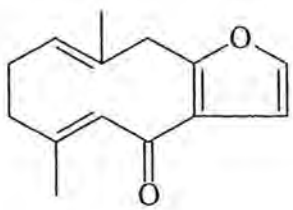
The choice of these plants were mainly due to two reasons. First, no chemical or biological investigation on either the root or stem bark has been recorded. Secondly, the plants belong to the family Meliaceae which is known to have limonoids with broad spectrum of biological activities.

2.2.9 Burseraceae

The genus *Commiphora* belongs to the family Burseraceae. Some of the species of this genus have been used for medicinal and perfumary purposes and have recorded some tickicidal properties.[99]

Preliminary bioassays on the hexane extract of the stem bark of *C. swynertonii* have also shown tickicidal activities [100] while the oil obtained from the leaves by hydrodistillation showed repellant activity. Similarly, plant species belonging to this genus that have already been investigated had shown either tickicidal or repellence activities against ticks.

Resinous gums of *C. erythraea* are used by the natives of North Eastern province of Kenya for the control of ticks. Three sesquiterpenoids, furanodienone (28), 2-o-acetyl-8,12-epoxygermacra-1(10)-4,7,11-tetraene (29) and 2-o-methyl-8,12-epoxygermacra-1(10)-4,7,11-tetraene (30) isolated from the hexane fractions of the gum showed moderate acaricidal activity against the larvae of *R. appendiculatus*.[101]



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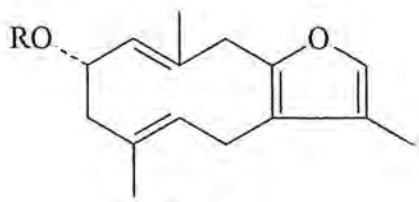
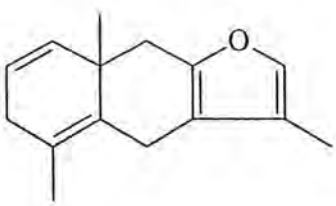


29 R=Ac

30 R=CH_3

Furanodienone has also been isolated from the myrrh oil of *C. myrrh* (Nees).[51] The hexane fraction of this oil exhibited repellent activity against adult ticks of *R. appendiculatus* and house flies, *Musca domestica*. The repellent activity has been attributed to the presence of the furanonsesquiterpenoids, furanogermacrene (31) linderstrene (32) and furanoeusdema-1,3-diene (33).



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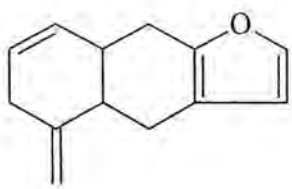

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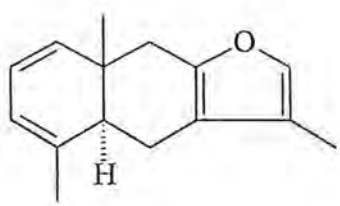

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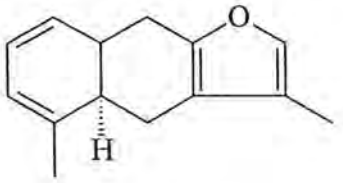
Other reported furanosesquiterpenoids are furanoeudesma-1,3-diene (34) and furanoeudesma-1,4-diene-6-one (35) have been isolated from essential oils of *C. molmol* Engler. [102]



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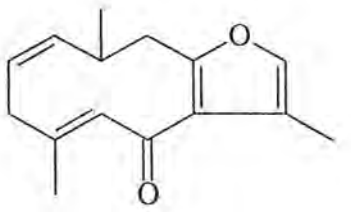
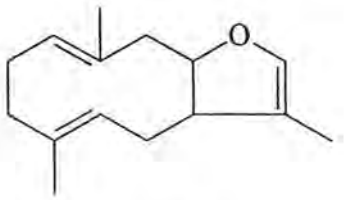


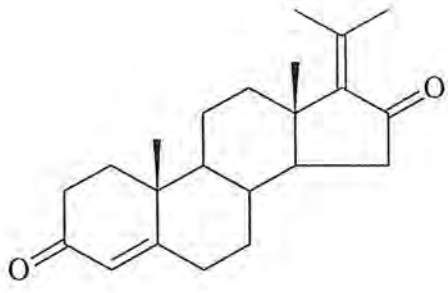
35

Furanodiene (36), isofuranogermancrene and linderstrene (32) have also been isolated from the essential oil of *C. abyssinica.* [103]



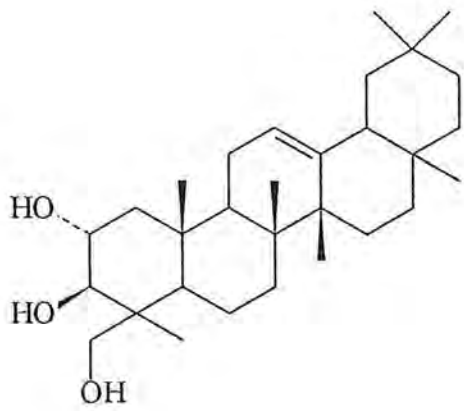
36

Biological and chemical investigations carried out on the gum resin of *C. mukul* led to the isolation of *Z*-guggulsterol (37) which showed significant hypolipaemic activity. [104]



37

2a,3b,23-Trihydroxyolean-12-ene (38) isolated from the root bark of *C. merkeri* exhibited a 28% anti-inflammatory activity on rats as compared to 68% of the reference material, phenylbutazone, both at a concentration of 100mg/kg.[105]



38

CHAPTER 3

RESULTS AND DISCUSSION

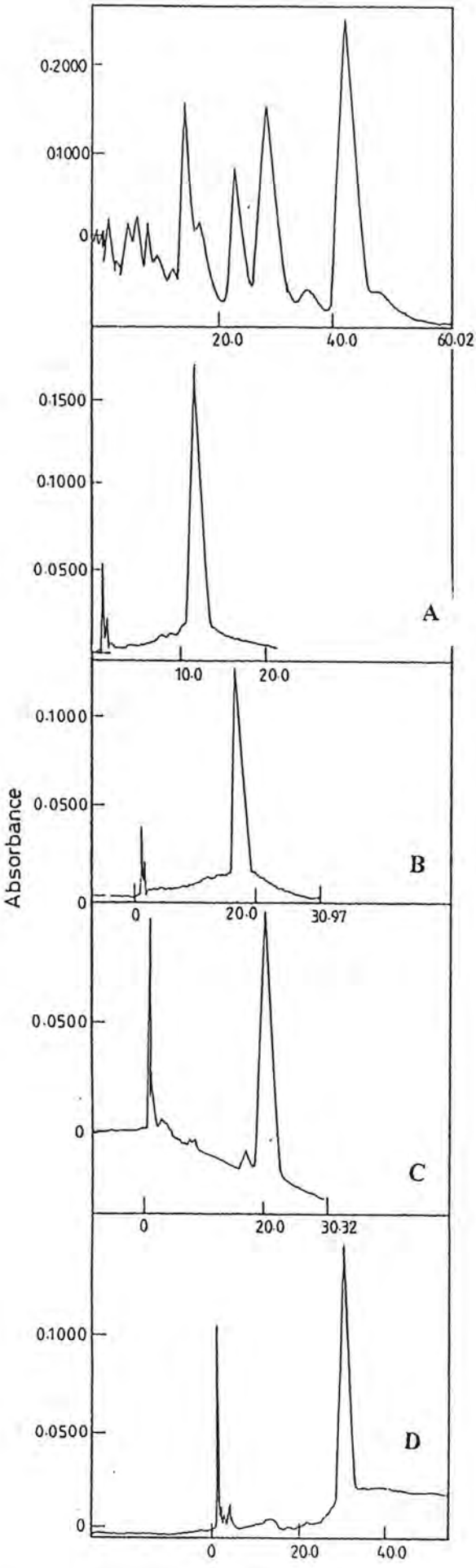
3.1 Isolates from the rootbark of *T.floribunda*

Liquid Chromatography-Mass Spectrometry (LC-MS) of the methanol extract of this plant revealed the presence of four major compounds (chromatogram 1). All the four limonoids were isolated. However, one decomposed on further purification.

The rootbark of *T.floribunda* is known to produce limonoids of the havanensin class with 11α, 12β substitution.[106,107,108] Both ^{1}H and ^{13}C spectra (Tables 3 and 4) confirm that the compounds isolated belong to the havanensin goup of limonoids.

3.1.1 Compound 39

Compound (39), molecular formula $C_{36}H_{50}O_{13}$ corresponding to molecular weight of 690 showed three multiplet absorptions (Figure 1a, 1b) at δ 6.26, 7.22 and 7.40 in the 300MHz 1H NMR spectrum (Table 3) indicating the presence of a furan ring. The presence of the furan ring was confirmed by ^{1}H NMR COSY where there were couplings between H-23 and H-21, H-23 and H-22 (Figure 2).



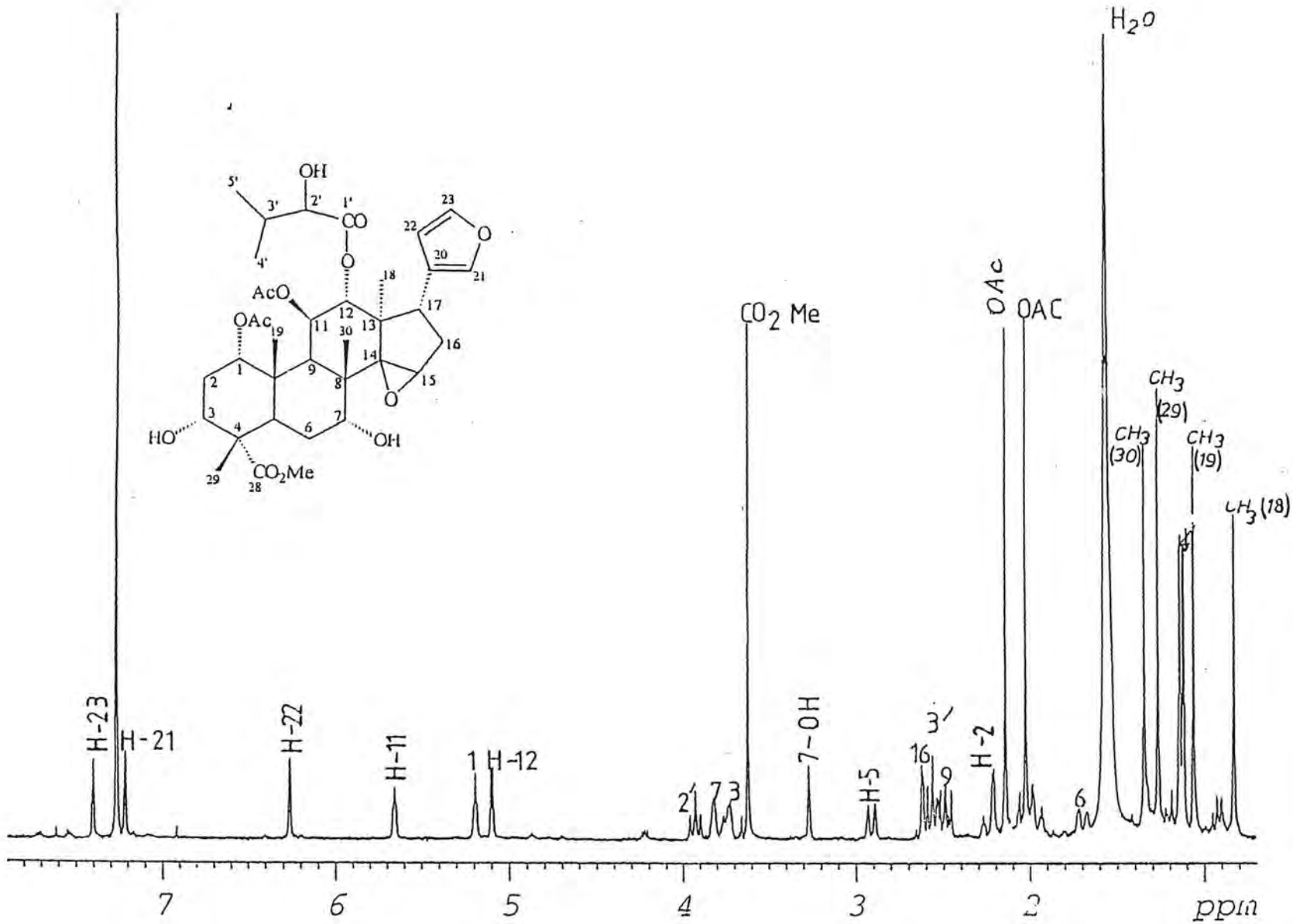
A = compound 39

B = compound 40

C = decomposed

D = compound 41

Chromatogram 1: LC-MS of the methanol extract of T. floribunda (acetonitrile/ water, 1:1)


H2O
CO2 Me
OAc
OAc
CH3 (29)
CH3 (30)
CH3 (19)
CH3 (18)
H-23
H-21
H-22
H-11
H-12
7-OH
H-5
H-2
ppm

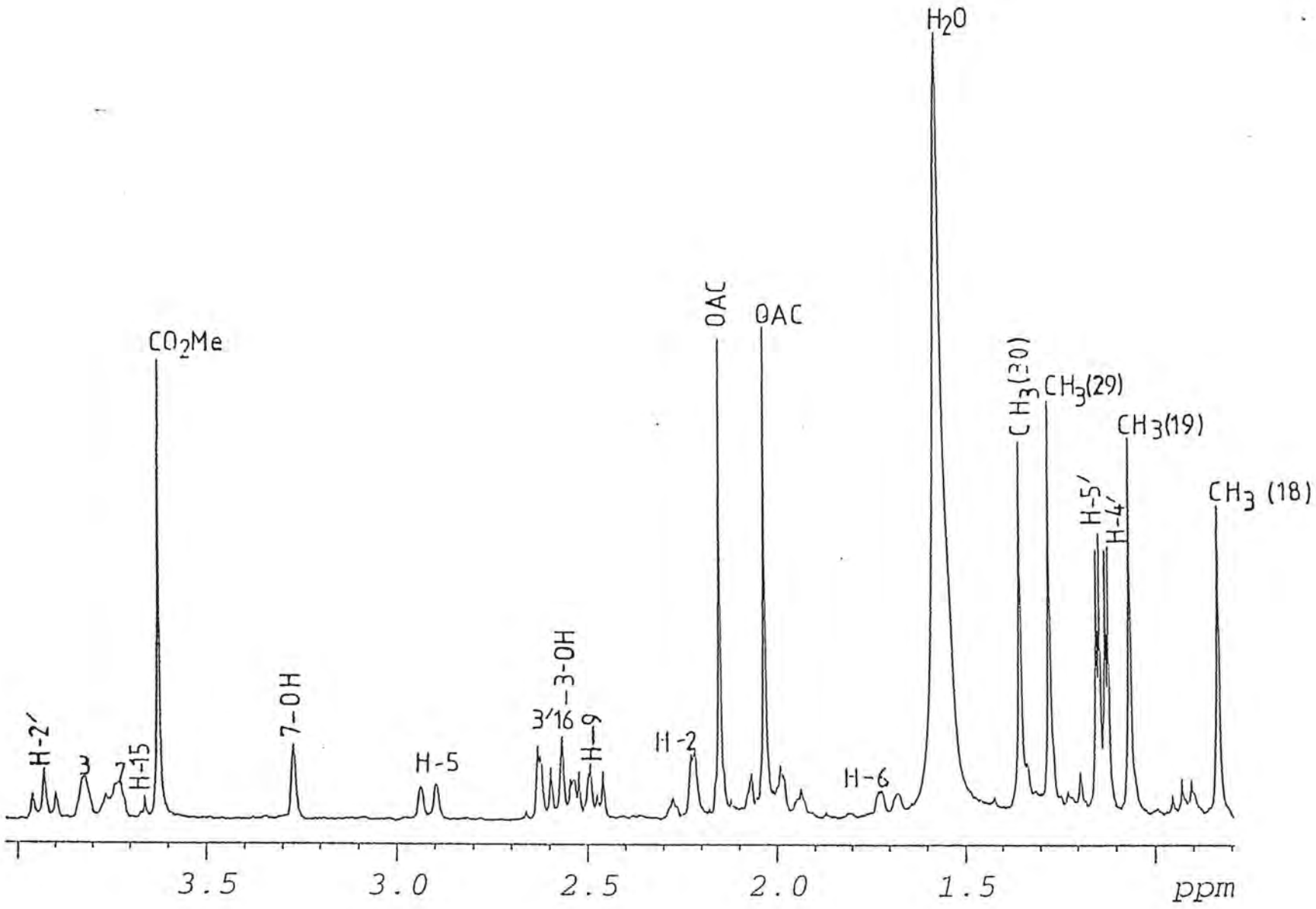


Figure 1b. Expanded ^{1}H NMR of compound 39 in $CDCl_3$

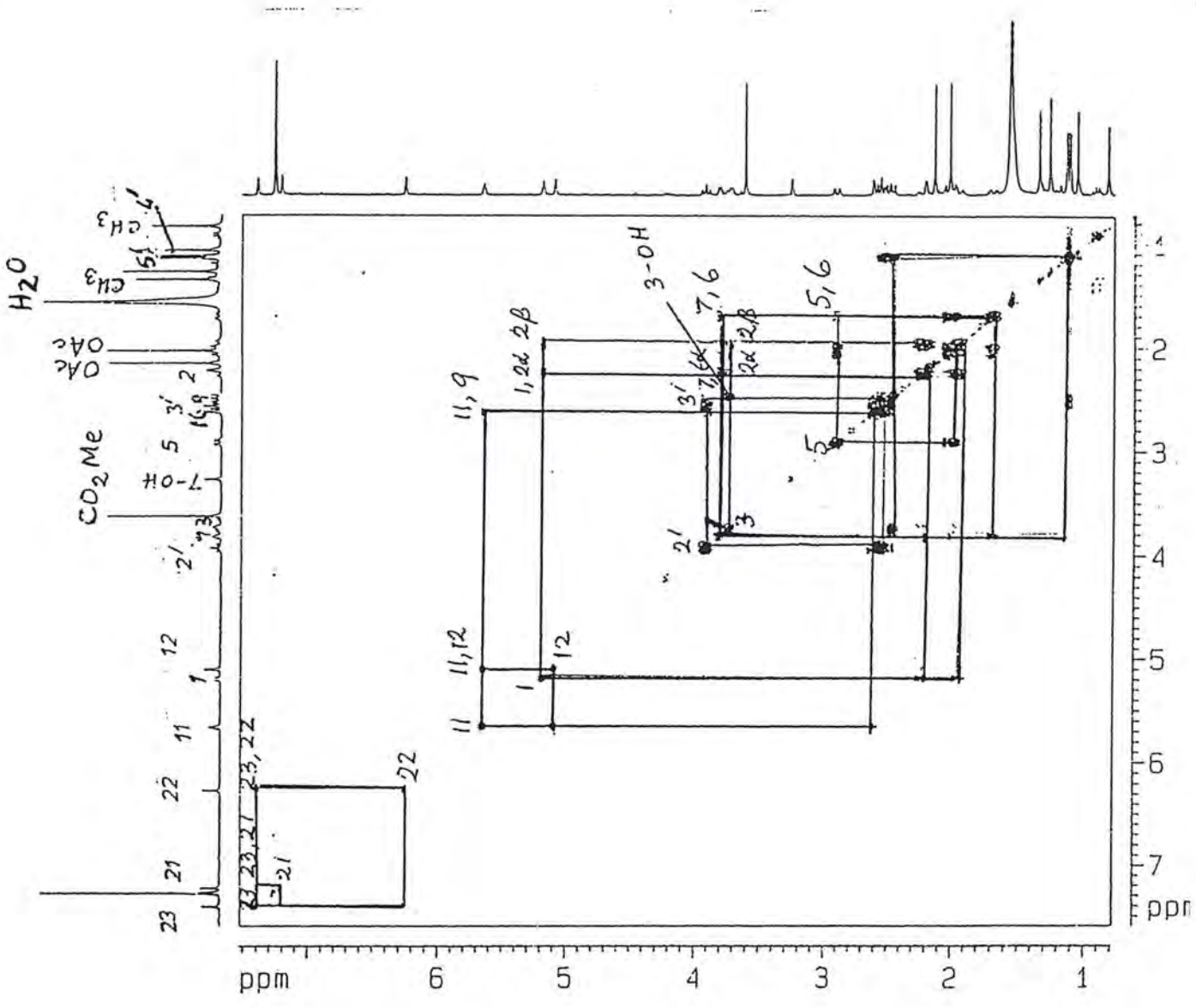


Figure 2. ^{1}H NMR COSY of compound 39 in $CDCl_3$

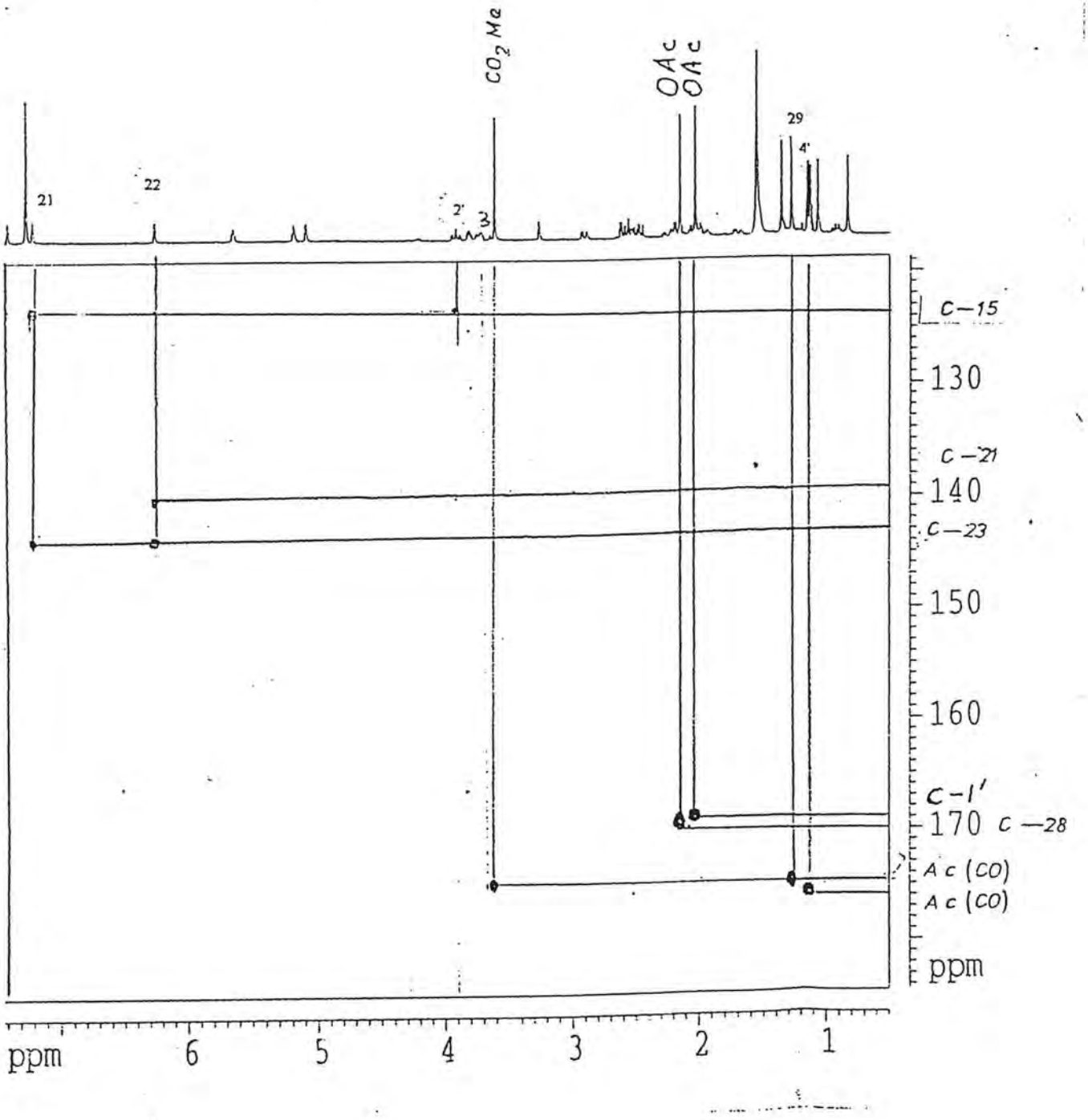


Figure 3a. HMBC correlation of compound 39

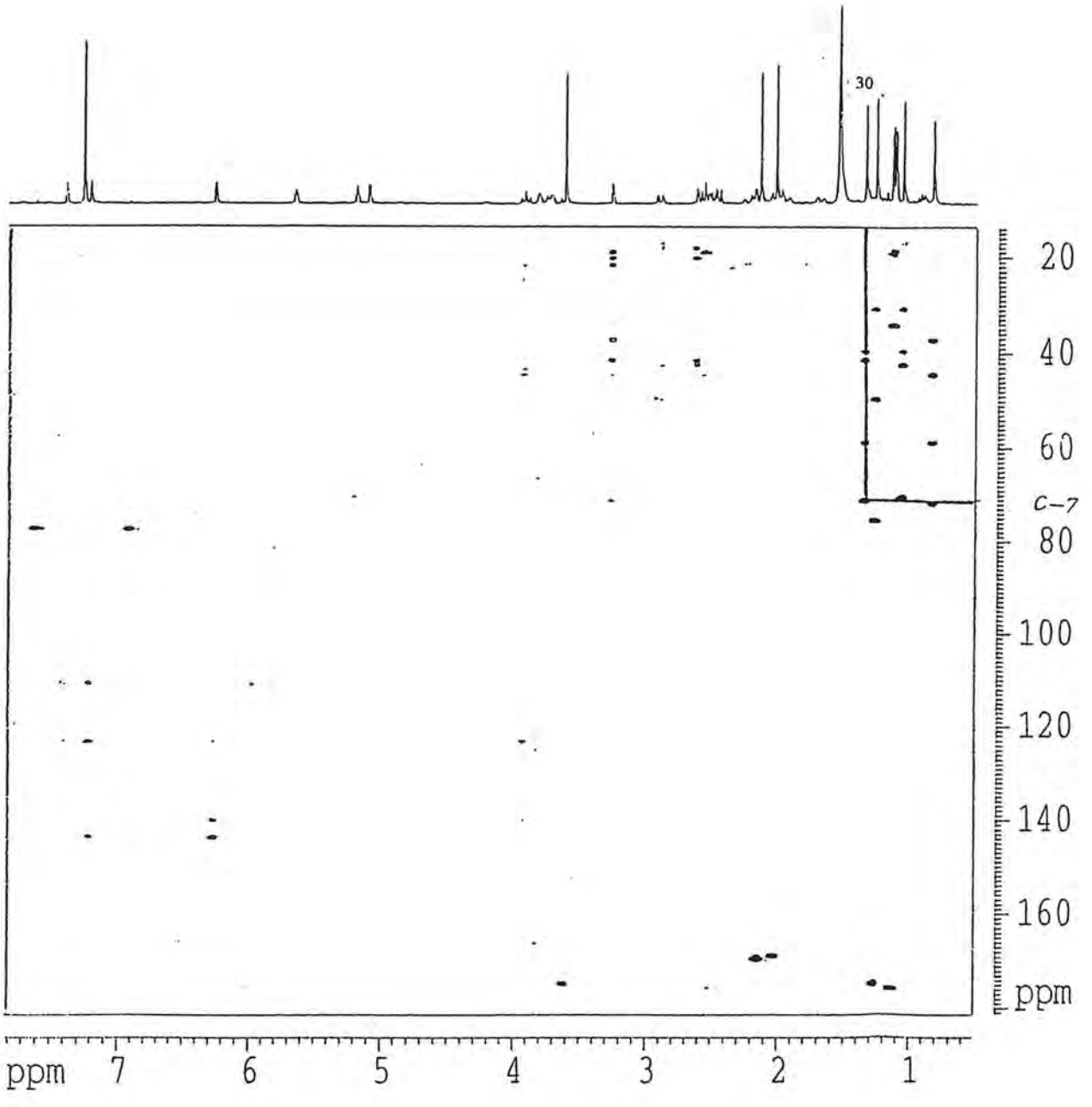


Figure 3b. Expanded HMBC correlation of compound 39

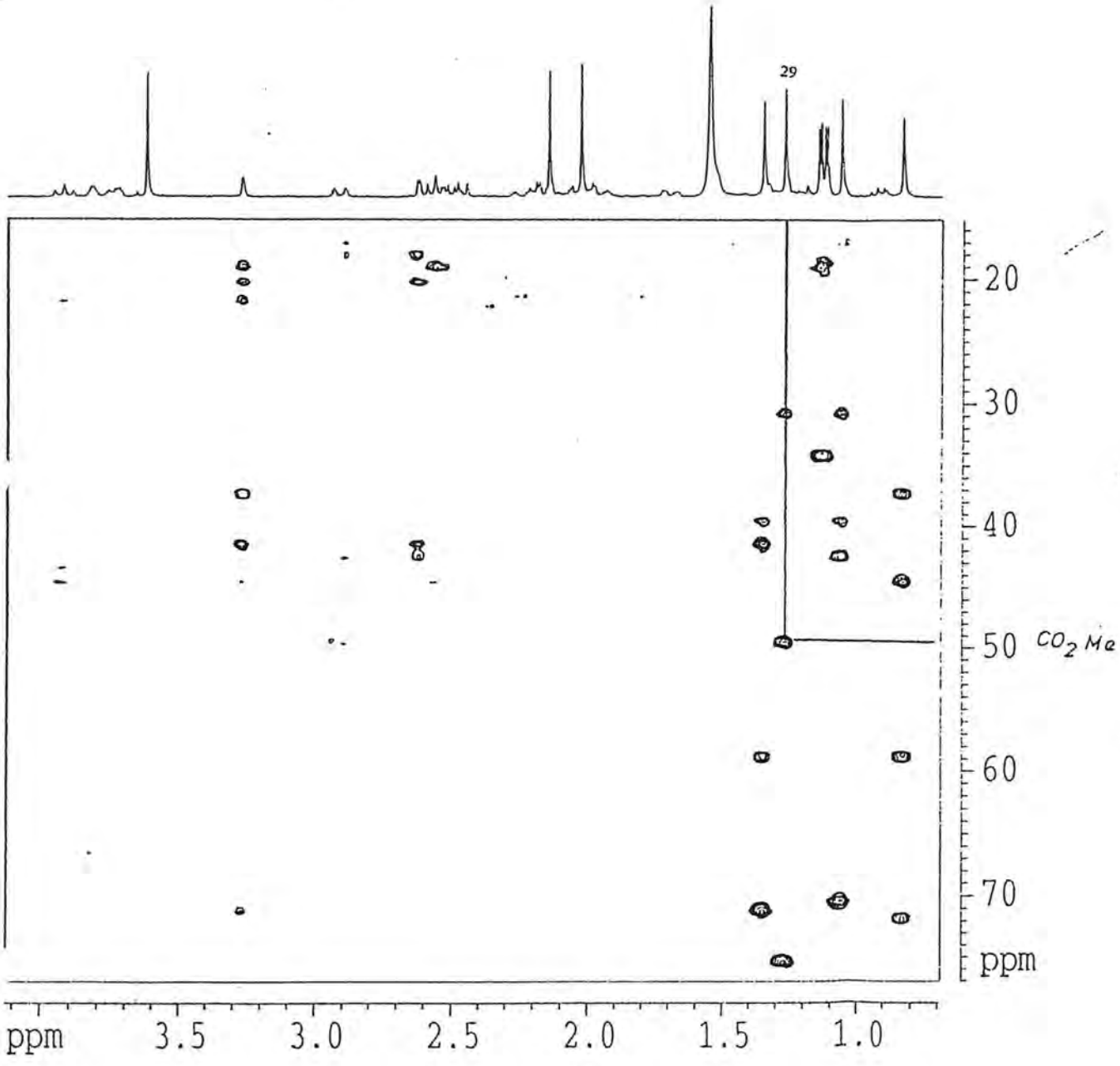


Figure 3.c. Expanded HMBC correlation of compound 39

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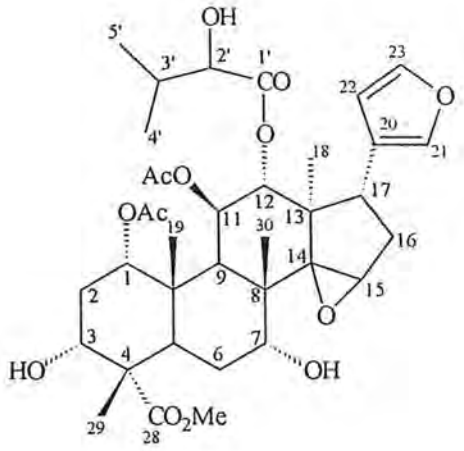


39

The Heteronuclear Multiple Bond Coupling (HMBC) NMR technique also confirmed the presence of the furan ring (Figure 2a, 2b, 2c). There is correlation for H-21 and C-23, H-22 and C-21, and H-22 and C-23 (Table 5). Singlets which had absorptions at δ 0.83, 1.07, 1.27 and 1.35 are typical for the four methyl goups normally present in the limonoids belonging to the havanensin class. [106,107,108] The absorption at δ 3.66 indicates the presence of the carbomethoxy group which is also typical for this class of limonoids. The position of 3-OH group was established by using ^{1}H NMR COSY and HMBC. ^{1}H NMR COSY correlations of H-1 with H-2 and H-2 with H-3 showed that ring A is substituted at C-1 and C-3. The absorptions at δ 2.03 and 2.15 indicate the presence of acetate groups which are typical of this class of compounds. HMBC correlation (Figure 4) of H-3 with Ac(CO) shows that the ester group is on C-1 and not C-3.

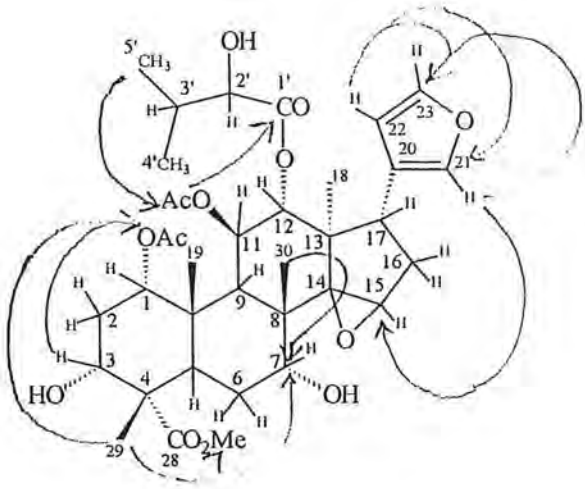


Figure 4. HMBC correlation of compound 39

The position of the second acetate group was confirmed by ^{1}H NMR COSY. COSY correlations of H-9 with H-11 and H-11 with H-12 showed that the esters were at C-11 and C-12. The multiplets at δ 3.81 and 3.82 indicated the presence of 2-OH groups. COSY correlation of H-7 with H-6 and H-6 with H-5 allowed the placement of one OH (δ 3.81).This was further confirmed by HMBC which showed that H-27 with C-7 and H-30 correlated with C-7. The other OH group was placed at C-3. The presence of two duplets at δ 1.12, J= 2.5 Hz and 1.13, J = 2.5 Hz means the presence of -CH- group splitting two - CH_3 groups placed in the same chemical environment. The triplet at δ 3.93, J = 10 Hz indicates that the proton which is being split is attached to an OH group. MS (Appendix 1) loss of a fragment with ion mass of 118 proved the presence of the side chain. The loss of the side chain (ion mass of 118) gave rise to ion mass 572. Loss of the side chain and ester group gave peak ion mass while ion mass 452 was the result of losing the side chain and two molecules of ester group. The ion mass 494 is a result of the loss of one moeity each of the side chain, ester goup and water.

Table 3. 1H NMR spectral data of limonoids 39-41

Proton	39	40	41
1	5.19m	4.80t (J=2.95Hz)	4.81m
2	2.23m	2.20m	2.22m
3	3.82m	3.79d (J=10.6Hz)	3.81m
5	2.92d (J=2.5Hz)	3.19dd(J=2.7,2.7Hz)	3.4m
6	2.46-2.51m	1.70-1.95m	1.87-1.96m
7	3.81m	3.67m	3.94m
9	2.53m	3.36d (J=4.5Hz)	2.97d(J=4.5Hz)
11	5.66t (J=3.0Hz)	5.21m	5.07m
12	5.11d (J=3.6Hz)	4.95dJ=4.0Hz)	5.15d(J=3.5Hz)
15	3.73m	3.71m	5.79m
16	2.59m	2.32-2.50m	2.19-2.51m
17	2.90m	2.95m	3.15m
18	0.83s	1.01s	1.14s
19	1.07s	1.20s	1.18s
21	7.22m	7.05m	7.32m
22	6.26m	6.34m	6.27m
23	7.40m	7.27m	7.17m
29	1.27s	1.22s	1.24s
30	1.35s	1.29s	1.43s
2'	3.93t (J=9.2Hz)	2.33m	2.17m
3'	2.63m	1.25s	1.27m
4'	1.12d (J=2.5Hz)	1.01s	0.81t(J=7.1Hz)
5'	1.13d (J=2.5Hz)	N/A	0.93d(J=7.1Hz)
Ac(1-)	2.03s	2.04s	2.02s
Ac(11-)	2.15s	2.09s	2.09s
COOMe	3.66s	3.71s	3.72s
OH(3-)	2.65br	2.62m	2.69d(J=10.7Hz)
OH(7-)	3.27br	3.12br	3.15m

Table 4 **^{13}CNMR spectral data for limonoids 40 and 41**

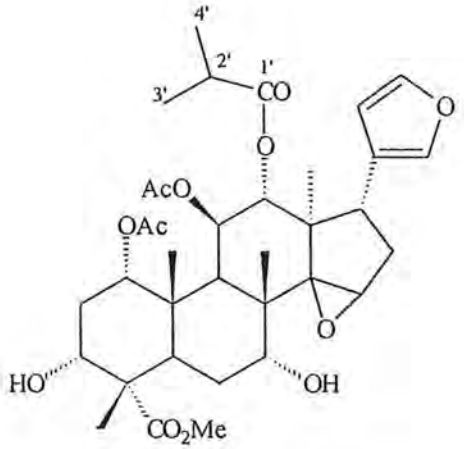
Carbon	40	41
1	74.13 (1)	73.89 (1)
2	27.01 (2)	27.18 (2)
3	72.05 (1)	73.31 (1)
4	41.93 (0)	41.01 (0)
5	39.32 (1)	39.16 (1)
6	27.25 (2)	26.95 (2)
7	73.18 (1)	74.42 (1)
8	49.05 (0)	50.62 (0)
9	40.87 (1)	41.26 (1)
10	40.64 (0)	43.91 (0)
11	74.13 (1)	74.56 (1)
12	79.20 (1)	83.78 (1)
13	51.35 (0)	51.43 (0)
14	74.13 (0)	156.39 (0)
15	63.41 (1)	124.21 (1)
16	34.29 (2)	37.33 (2)
17	34.01 (1)	50.76 (1)
18	16.69 (3)	16.79 (3)
19	17.71 (3)	17.19 (3)
20	127.93 (0)	123.86 (0)
21	140.34 (1)	140.29 (1)
22	112.10 (1)	111.39 (1)
23	142.45 (1)	142.13 (1)
28	169.32 (0)	169.13 (0)
29	18.70 (3)	17.38 (3)
30	28.30 (3)	28.29 (3)
1'	168.93 (1)	168.61 (1)
2'	32.49 (2)	31.21 (1)
3'	25.45 (3)	25.91 (2)
4'	16.45 (3)	11.82 (3)
5'	N/A	15.61 (3)
Ac(Me)	21.42 (3)	21.43 (3)
Ac(Me)	21.47 (3)	21.68 (3)
Ac(CO)	176.59 (0)	175.43 (0)
Ac(CO)	175.36 (0)	175.39 (0)
COOMe	52.12 (3)	52.13 (3)

Table 5 HMBC data of compound 39

Proton	Correlating carbon
7.21 (H-21)	123.1 (C-15)
7.21 (H-21)	144.0 (C-23)
6.26 (H-22)	140.0 (C-21)
6.26 (H-22)	144.0 (C-23)
2.0 (MeCOO)	168.9 (C- 1')
2.11 (MeCOO)	169.8 (C-28)
3.60 (H-3)	174.0 (AcCO)
1.30 (Me- 29)	175.0 (AcCO)
1.10 (H- 4')	175.5 (AcCO)
1.30 (Me -29)	49.9 (COOMe)
1.27 (Me -30)	72.0 (C- 7)

3.1.2 Compound 40

Compound (40) has a molecular formula of $C_{35}H_{48}O_{12}$ corresponding to molecular weight of 660.



40

HMBC (Table 6, Figure 5) correlations for H-3 with Ac(CO) and H-4 with Ac(CO) indicated the presence of a side chain. MS fragment ion of mass 88 showed

that it is a four carbon chain. The splitting of the protons of this side chain appeared at the same position as $-CH_3$ (19) and $-CH_3$ (29). ^{13}C NMR spectrum (Figure 7a, 7b) also indicated the presence of a side chain having four carbons. 1H NMR and 1H NMR COSY of compound 40 are shown on figures 6a, 6b and 8 respectively. HMBC spectrum is shown on figures 9a and 9b. The MS (Appendix 2) showed loss of water (ion mass 642), loss of acetate (ion mass 600), loss of acetate together with water (ion mass 582) and loss of side chain together with acetate (ion mass 512).

Table 6. HMBC data of compound 40

Proton (δ)	Corresponding carbon(δ)
7.05 (H-21)	143.1 (C-23)
6.34 (H-22)	143.1 (C-23)
2.18 (H-2)	170.0 (Ac(CO))
2.75 (H-17)	140.1 (C-21)
1.29 (H-30)	73.20 (C-7)
3.71 (H-15)	175.5 (Ac(CO))
3.65 (COOMe)	175.0 (Ac(CO))
1.20 (H-4')	175.0 (Ac(CO))
1.25 (H-3)	176.0 (Ac(CO))
4.69 (H-1)	72.05 (C-3)
4.92 (H-12)	74.50 (C-1)

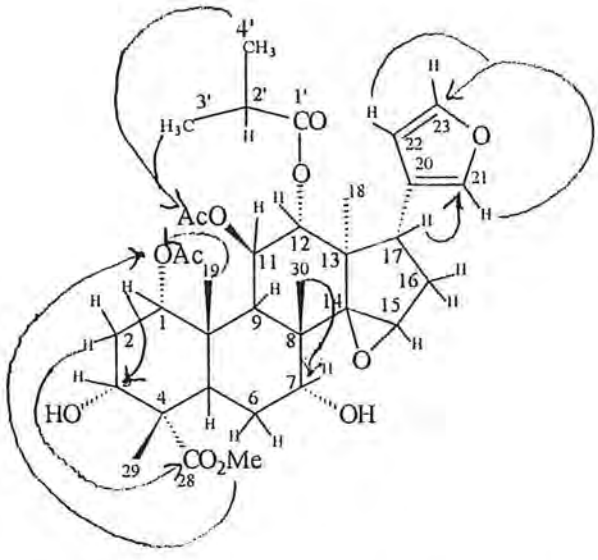


Figure 5. HMBC correlation of compound 40

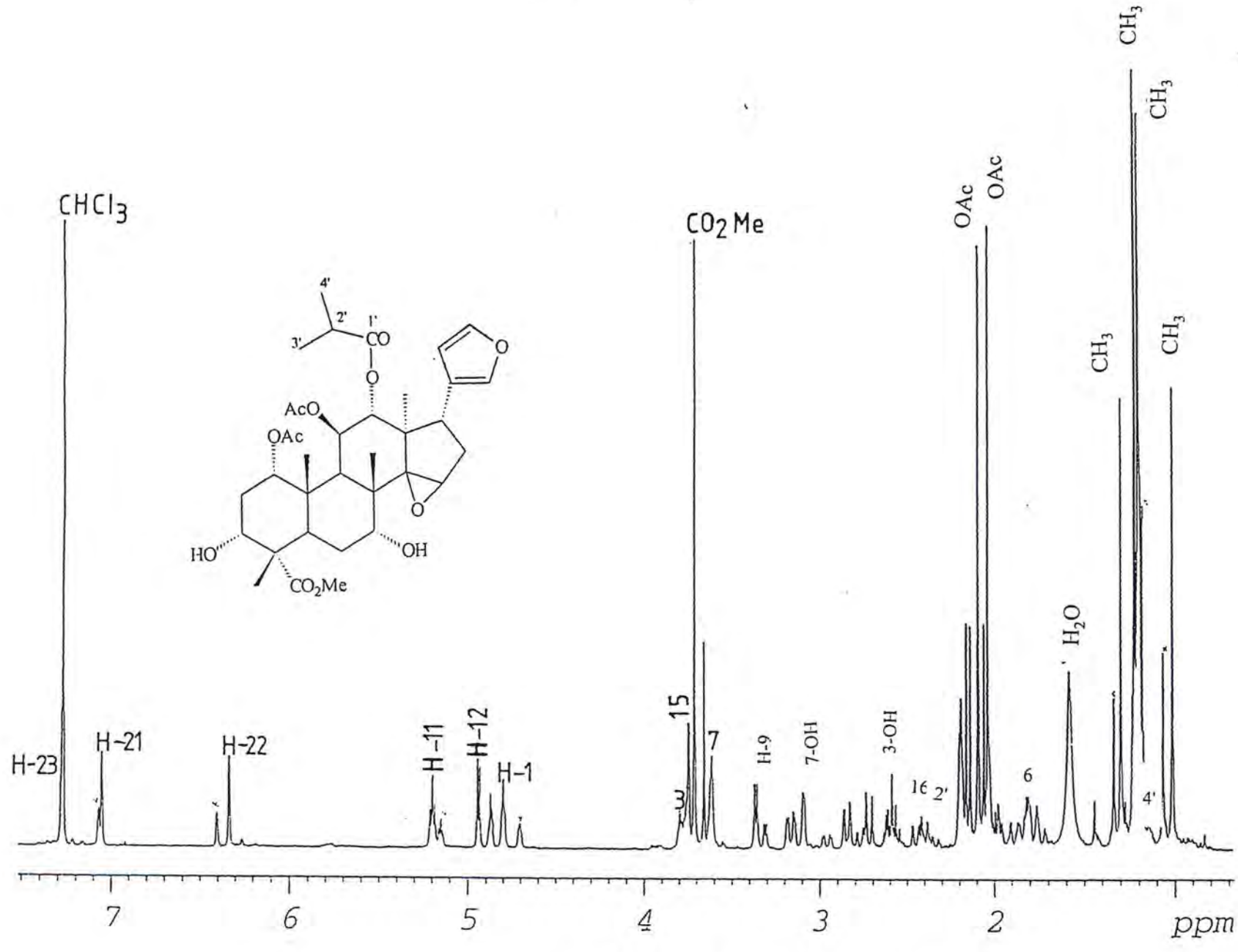


Figure 6a. ^1H NMR of compound 40 in $CDCl_3$

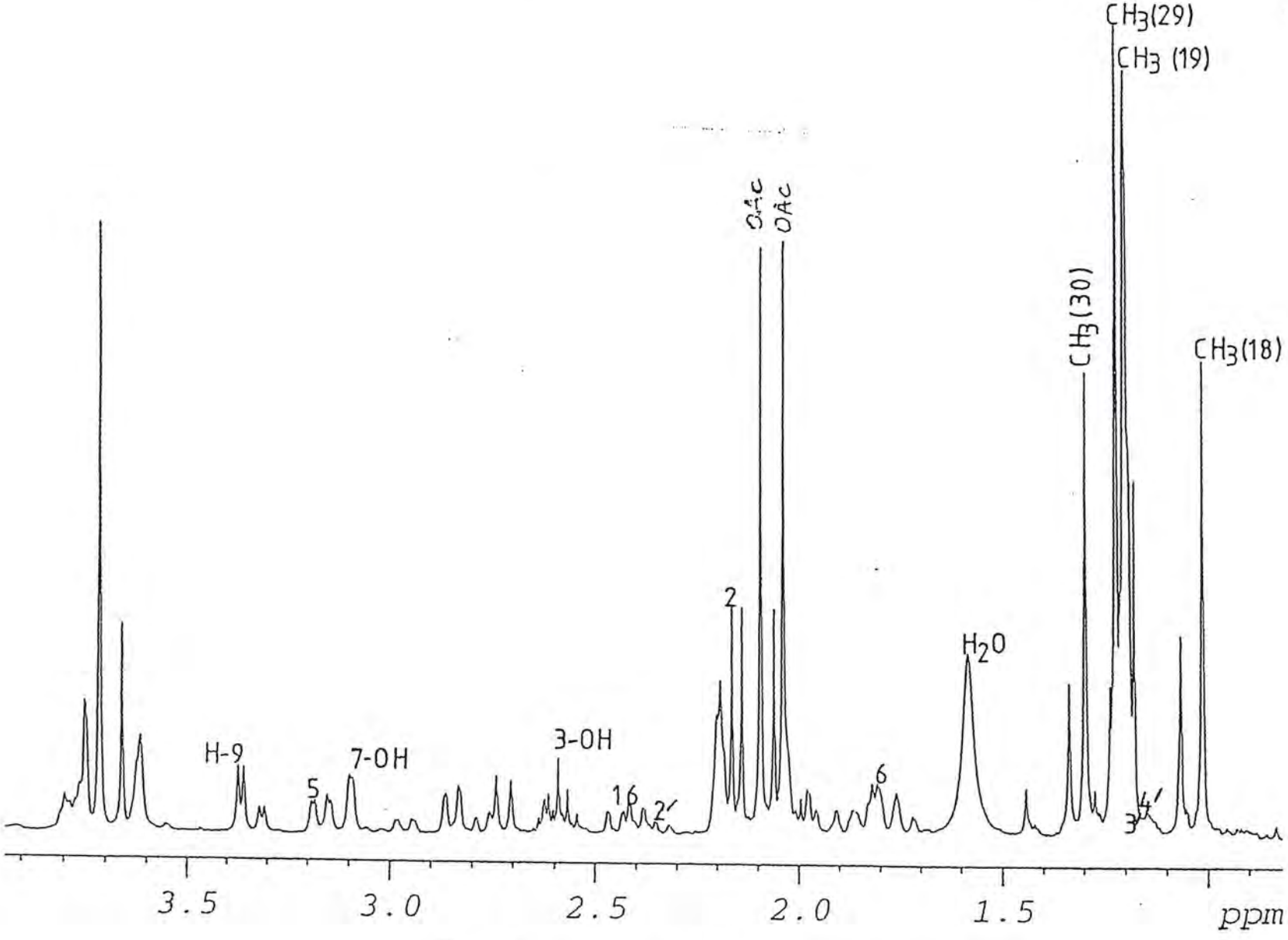


Figure 6b. Expanded ^1H NMR of compound 40 in $CDCl_3$

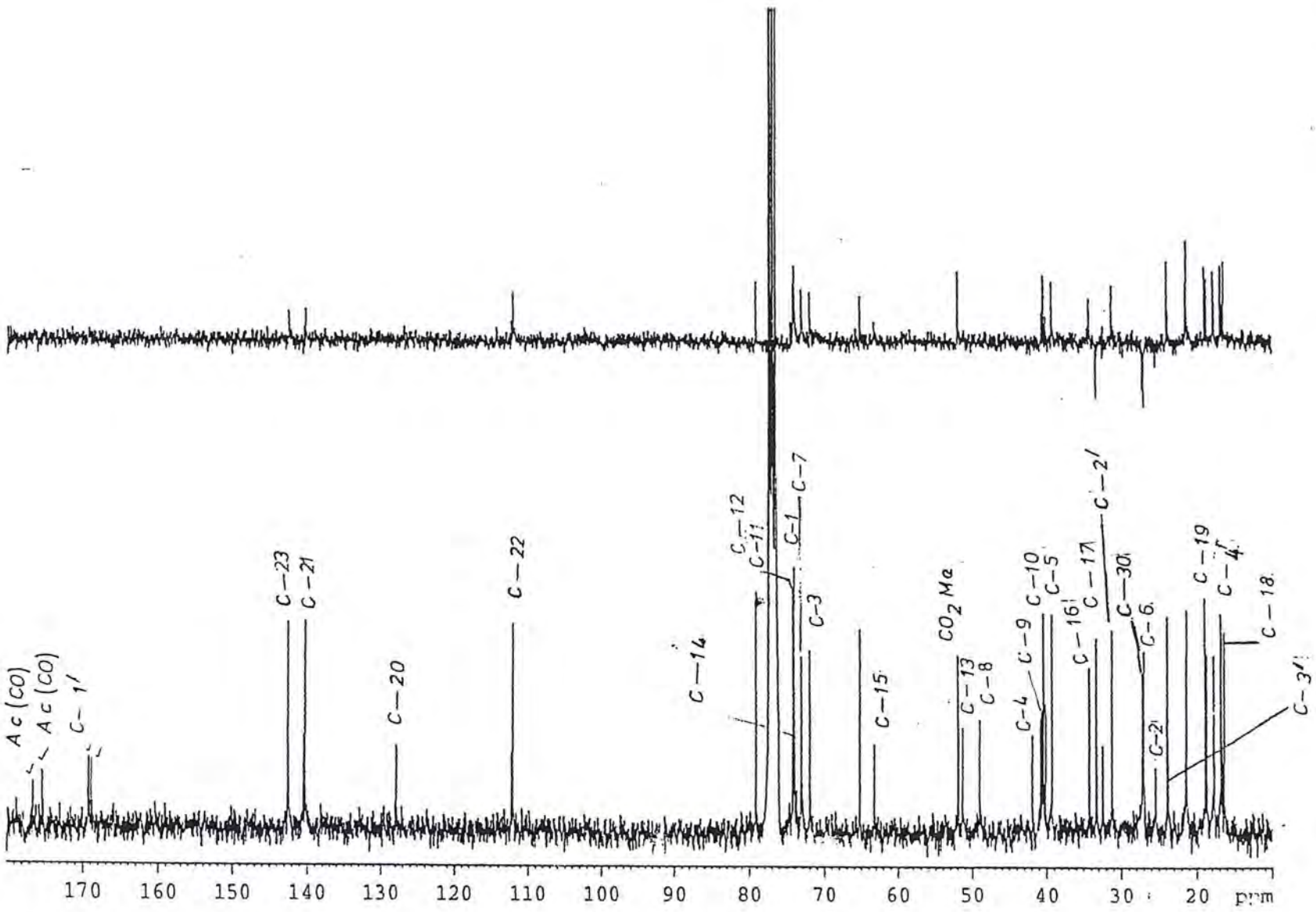


Figure 7a. ^{13}C NMR of compound 40 in $CDCl_3$

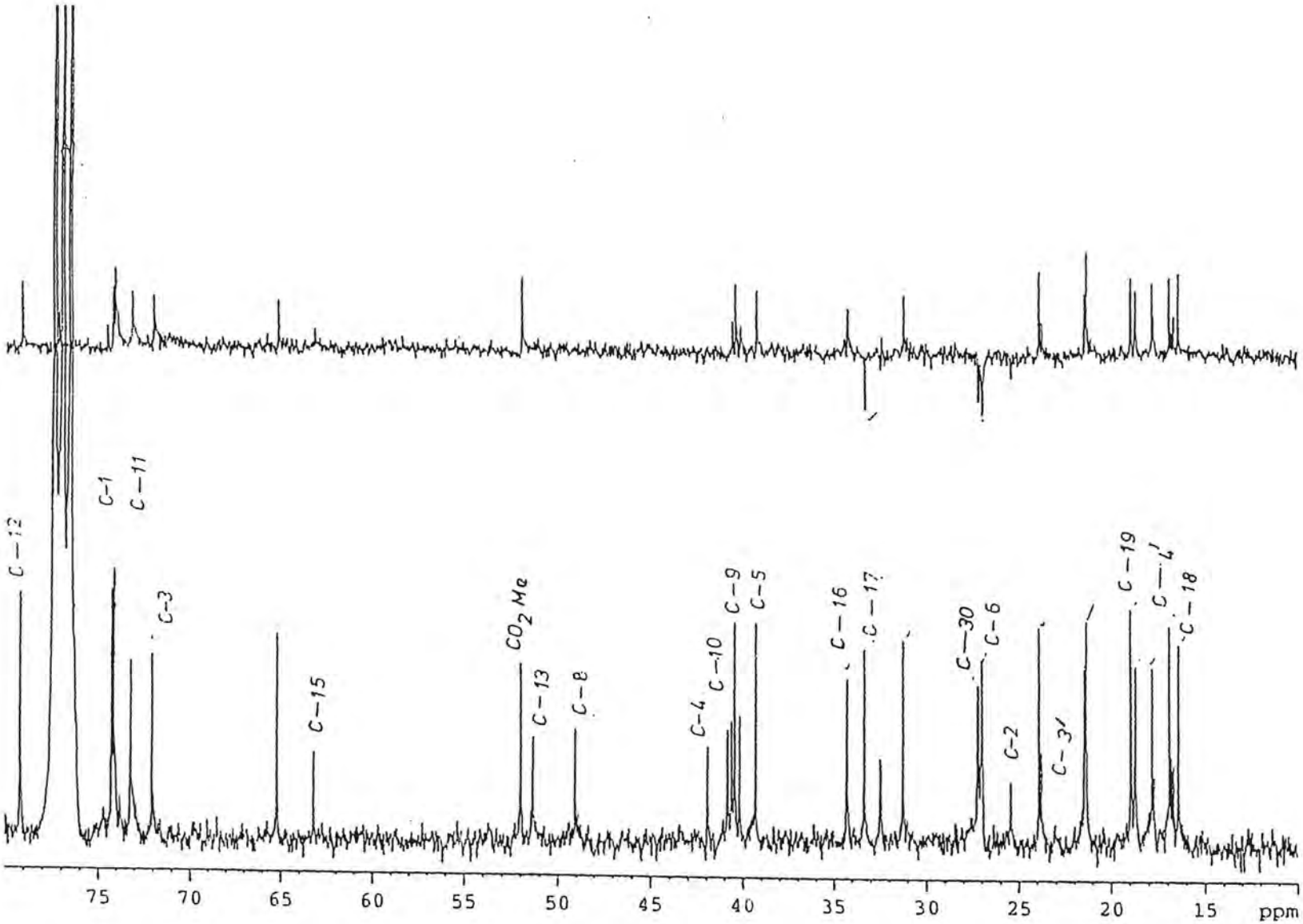


Figure 7b. Expanded ^{13}C NMR of compound 40 in $CDCl_3$

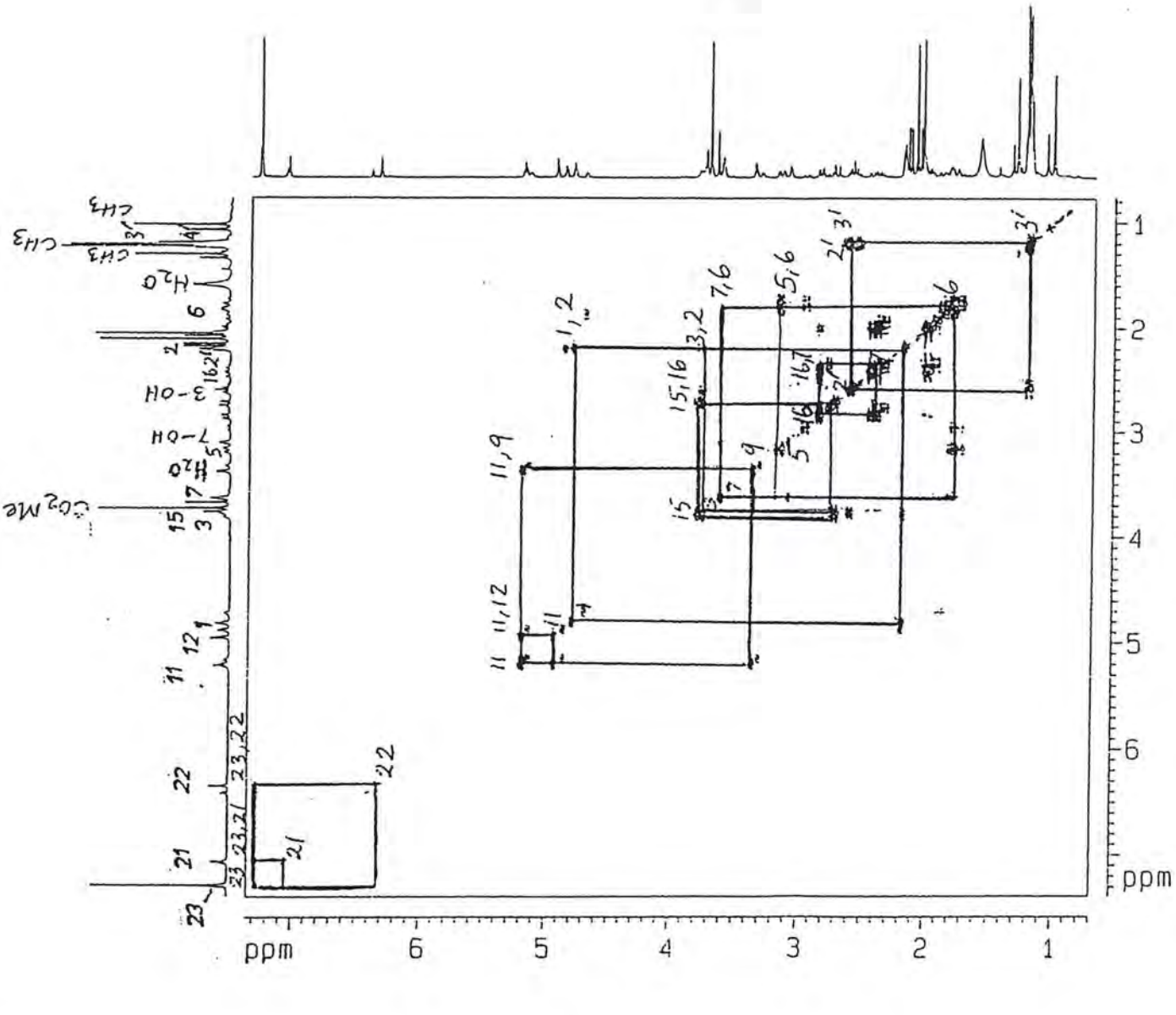


Figure 8. ^{1}H NMR COSY of compound 40 in $CDCl_3$

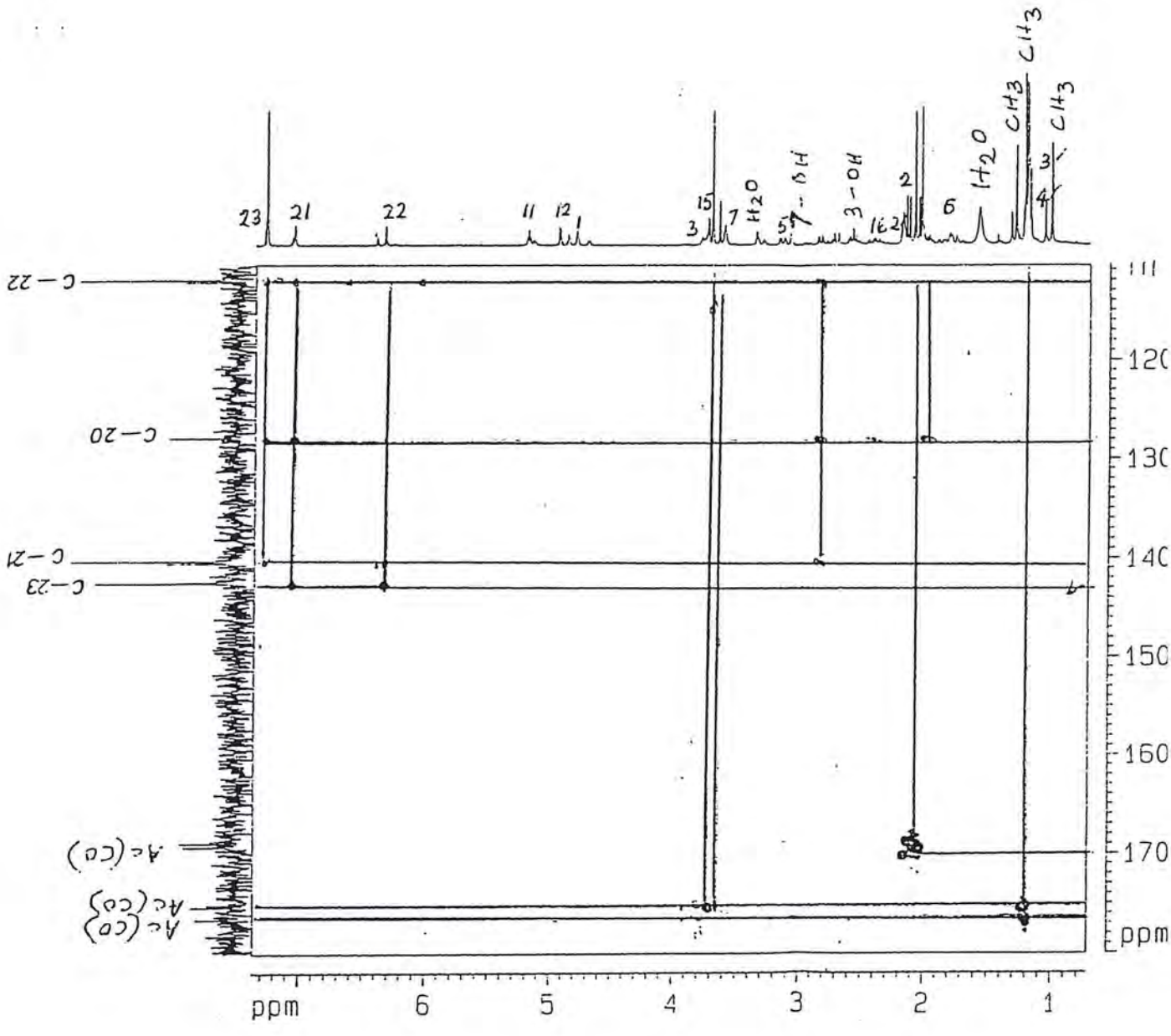


Figure 9a. HMBC correlation of compound 40

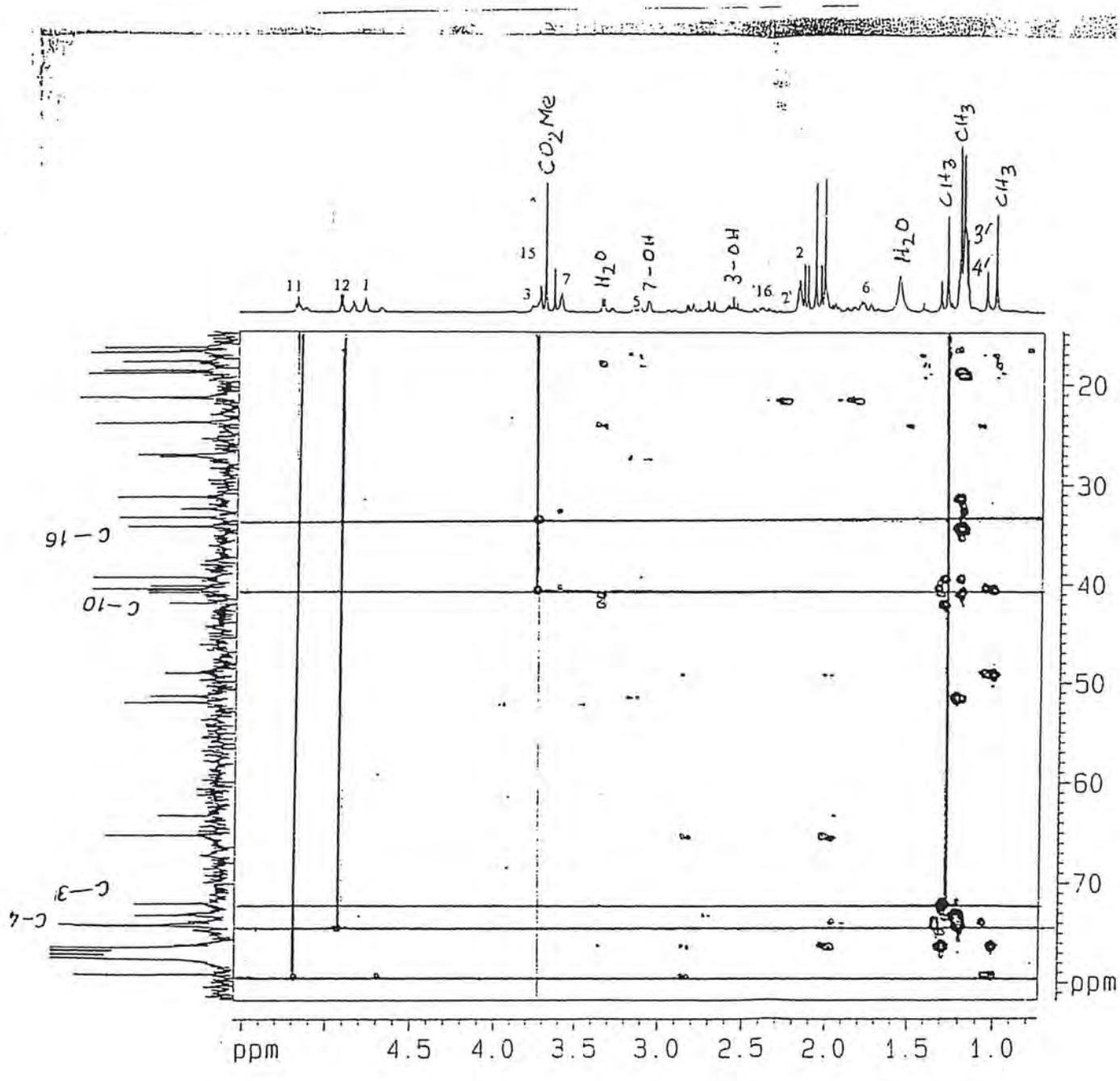
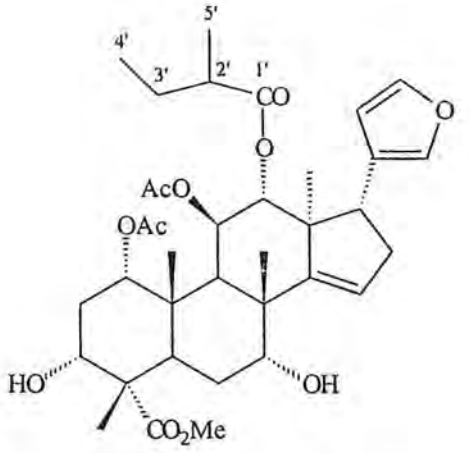


Figure 9b. Expanded HMBC correlation of compound 40

3.1.3 Compound 41

The spectral data (^{1}H NMR, Table 3, and ^{13}C NMR, Table 4) and MS (Appendix 3) of this compound agree well with a compound which was earlier isolated from the same plant.[106] ^{1}H NMR and ^{13}C NMR spectra of this compound are shown on figures 10a, b, c and 11a, b, c, d respectively. ^{1}H NMR COSY spectrum is shown on figure 12.



41

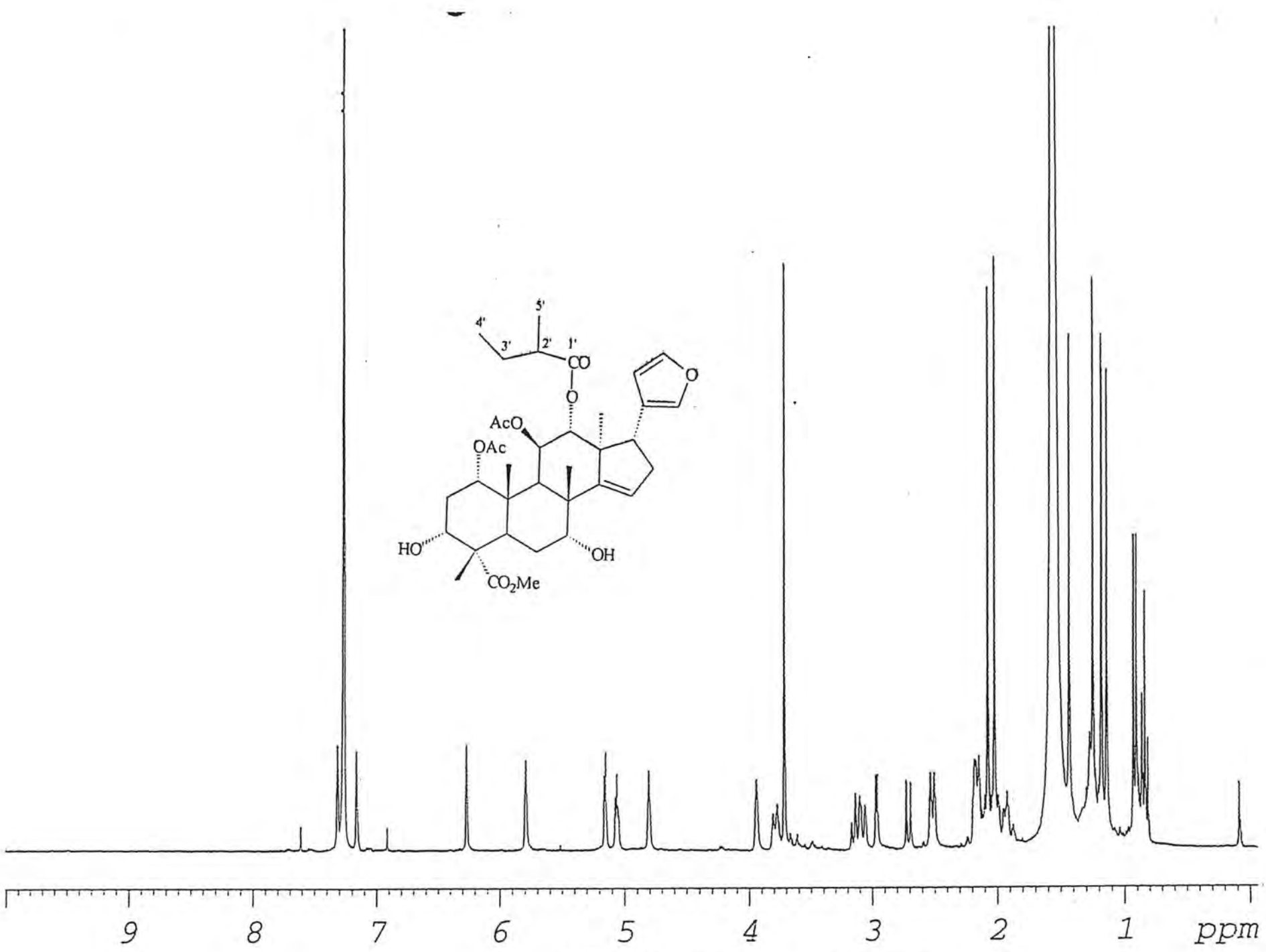


Figure 10a. ^{1}H NMR of compound 41 in $CDCl_3$

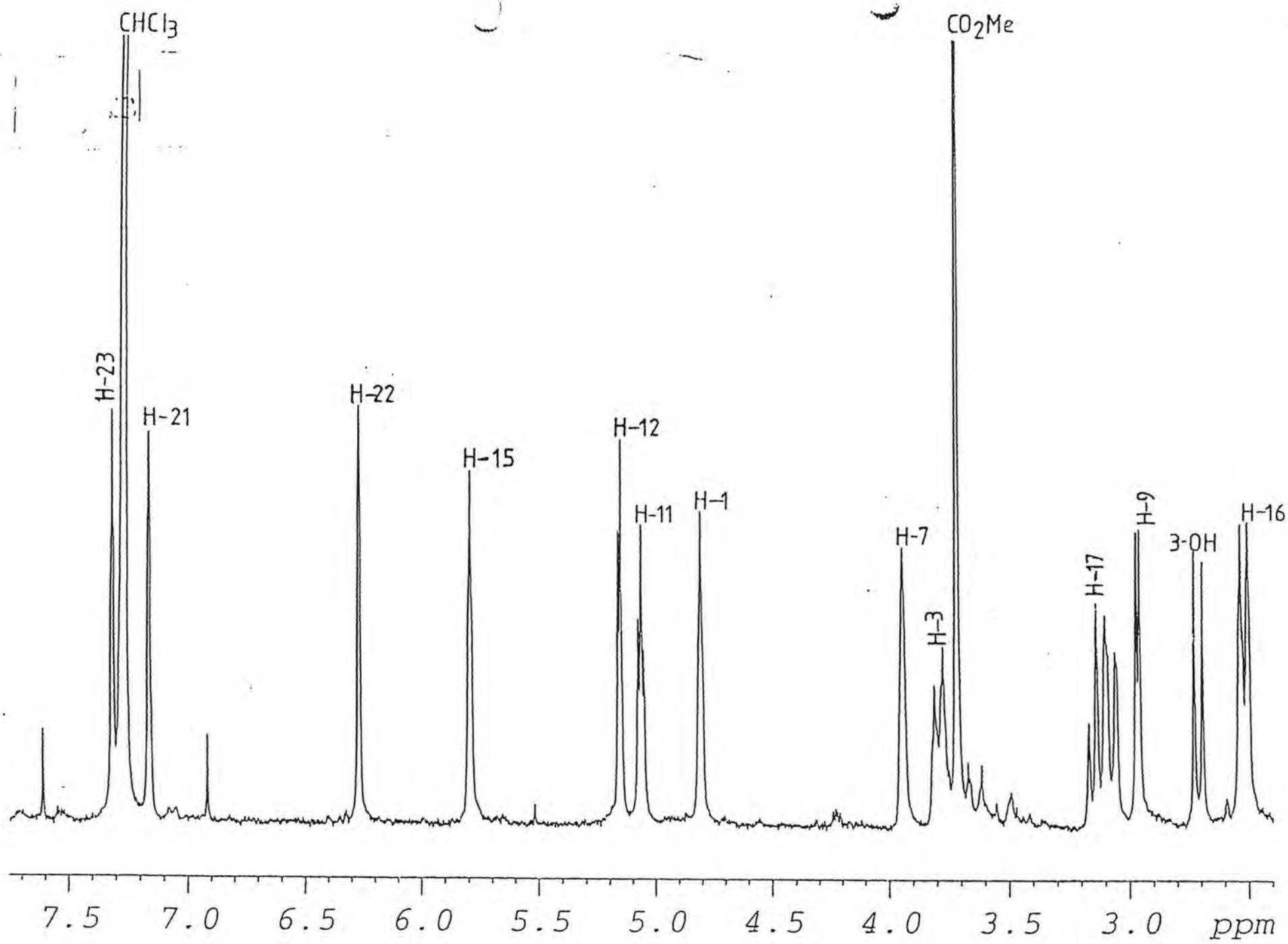


Figure 10b. Expanded ^{1}H NMR of compound 41 in $CDCl_3$

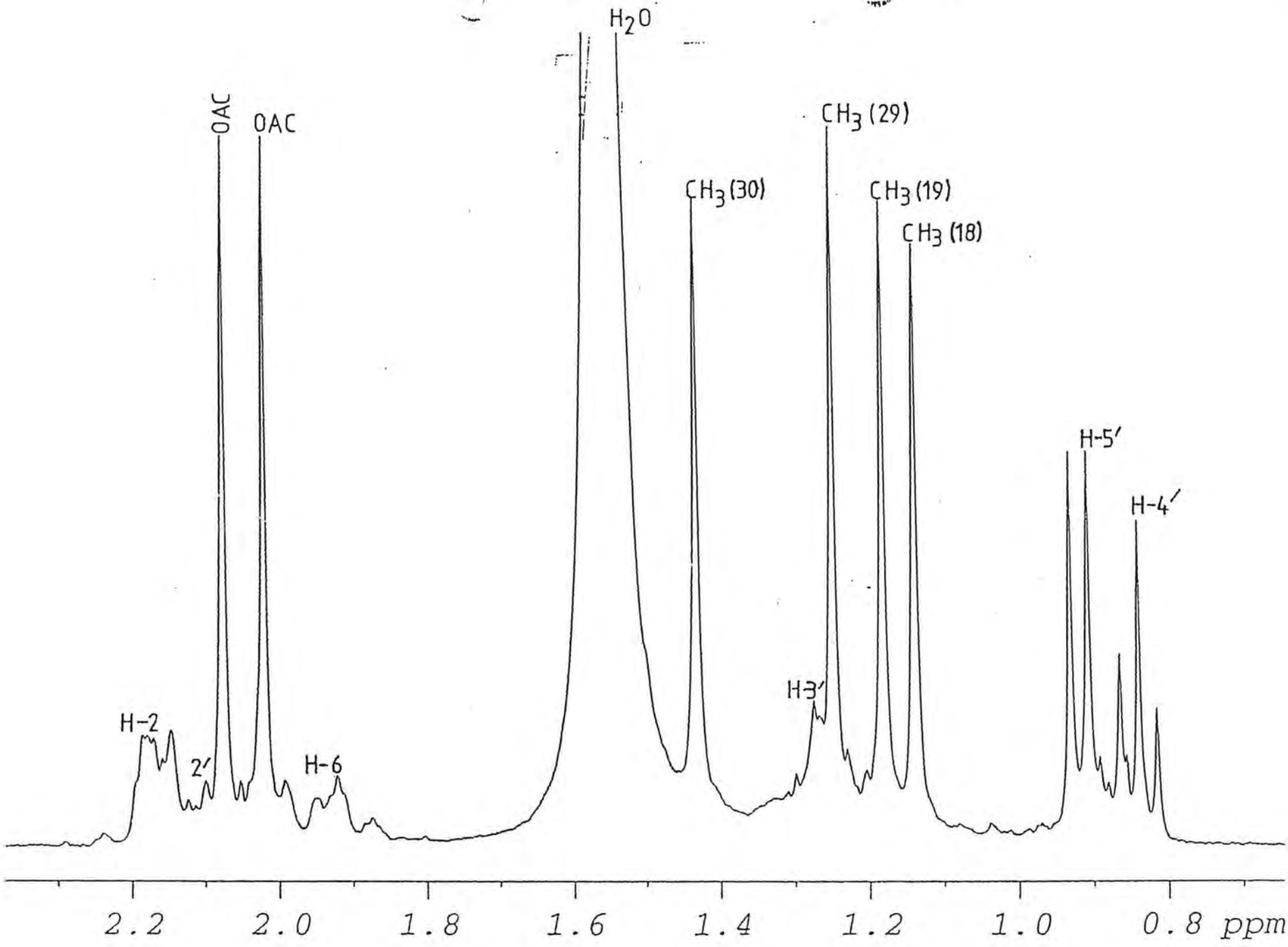


Figure 10a Expanded ^{1}H NMR of compound 41 in $CDCl_3$

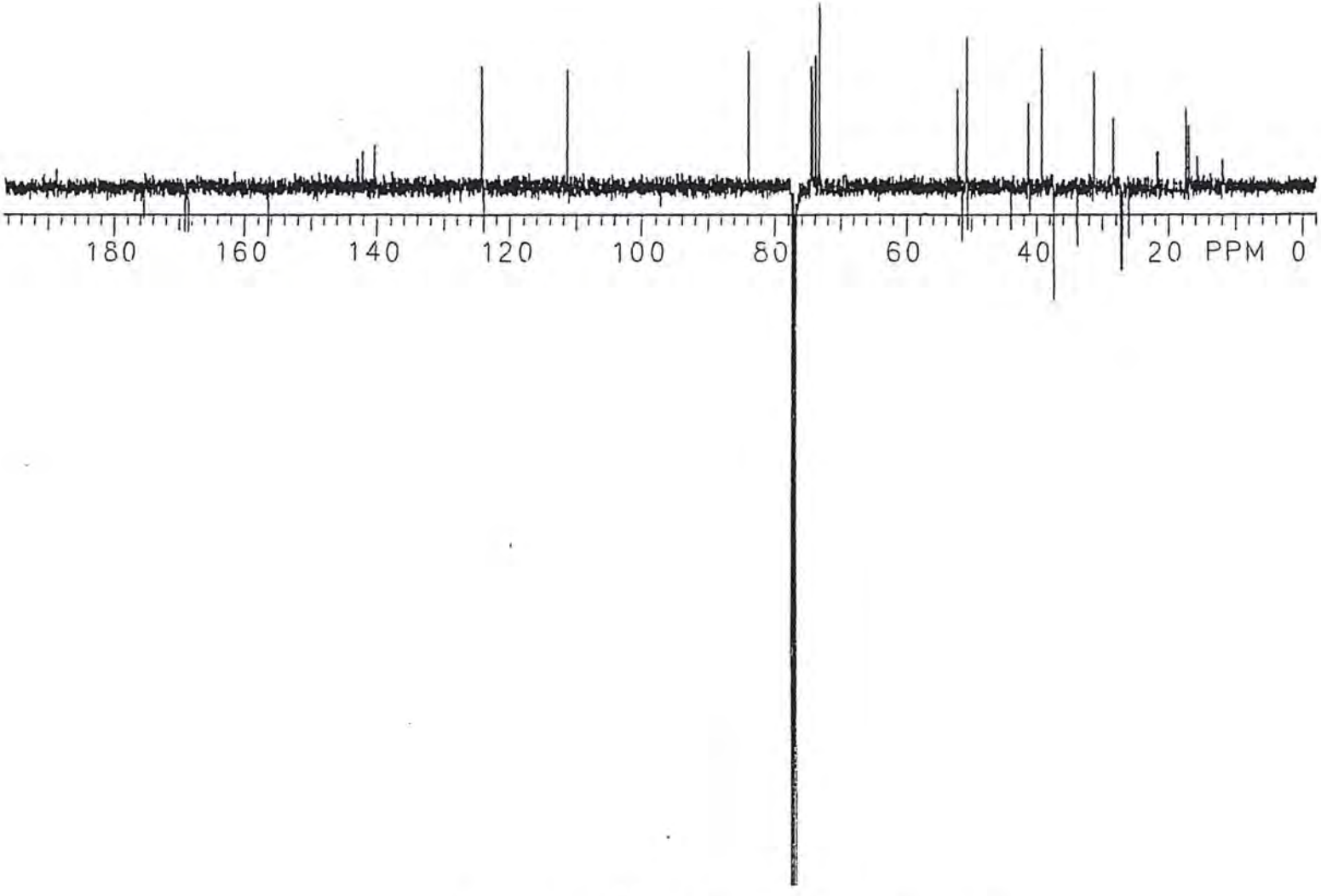


Figure 11a. ^{13}C NMR of compound 41 in $CDCl_3$

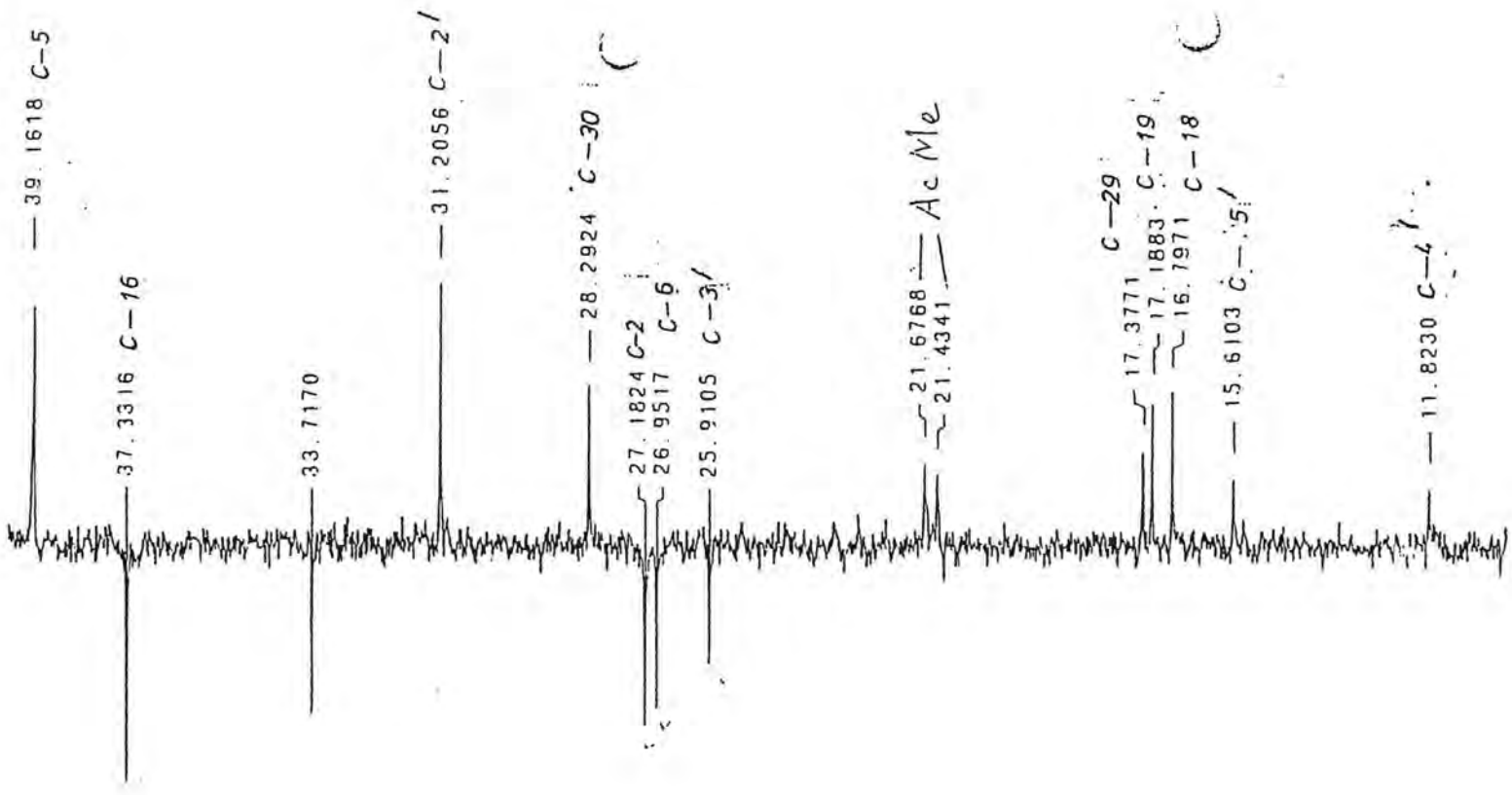


Figure 11b. Expanded ^{13}C NMR of compound 41 in $CDCl_3$

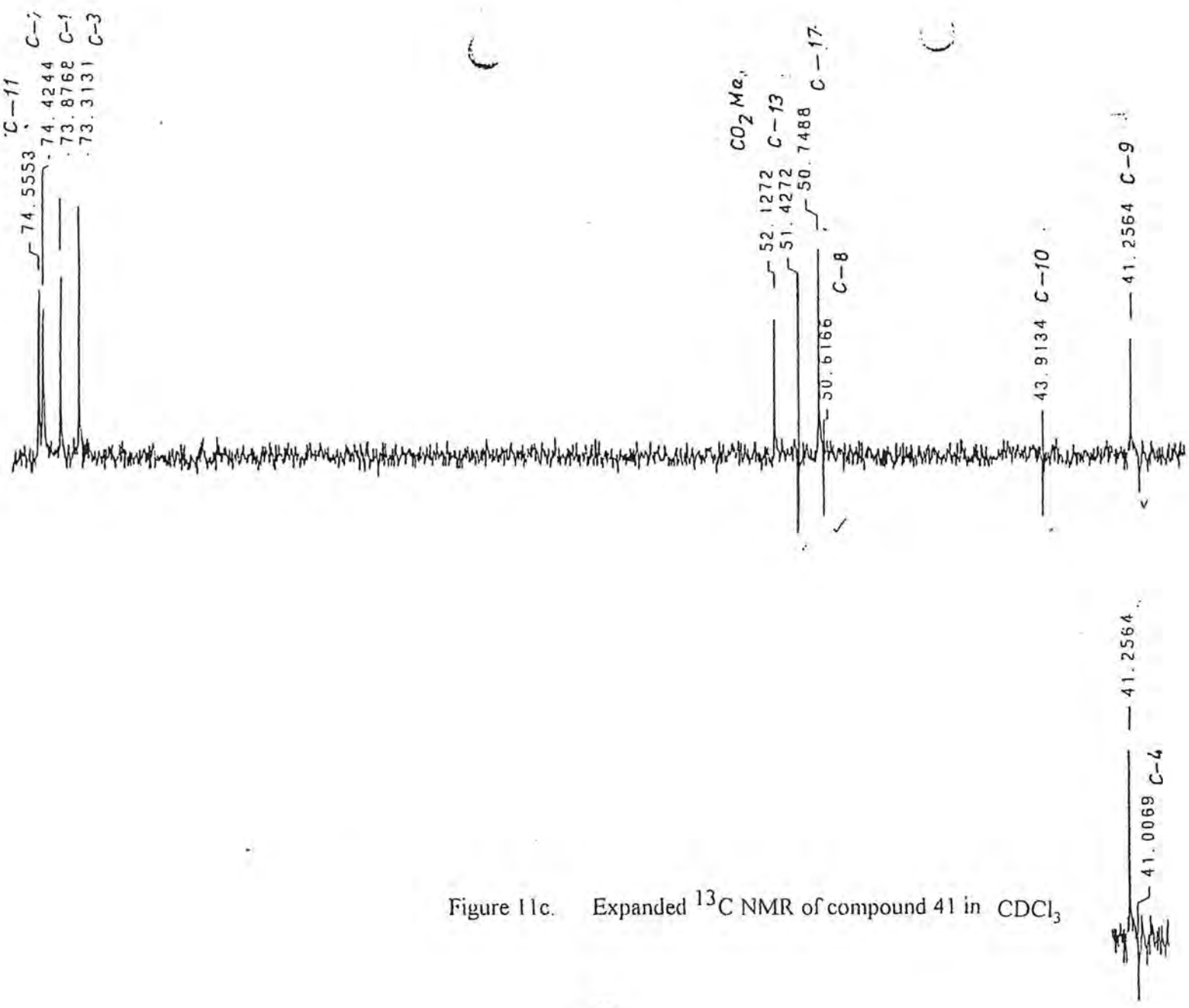


Figure 11c. Expanded ^{13}C NMR of compound 41 in $CDCl_3$

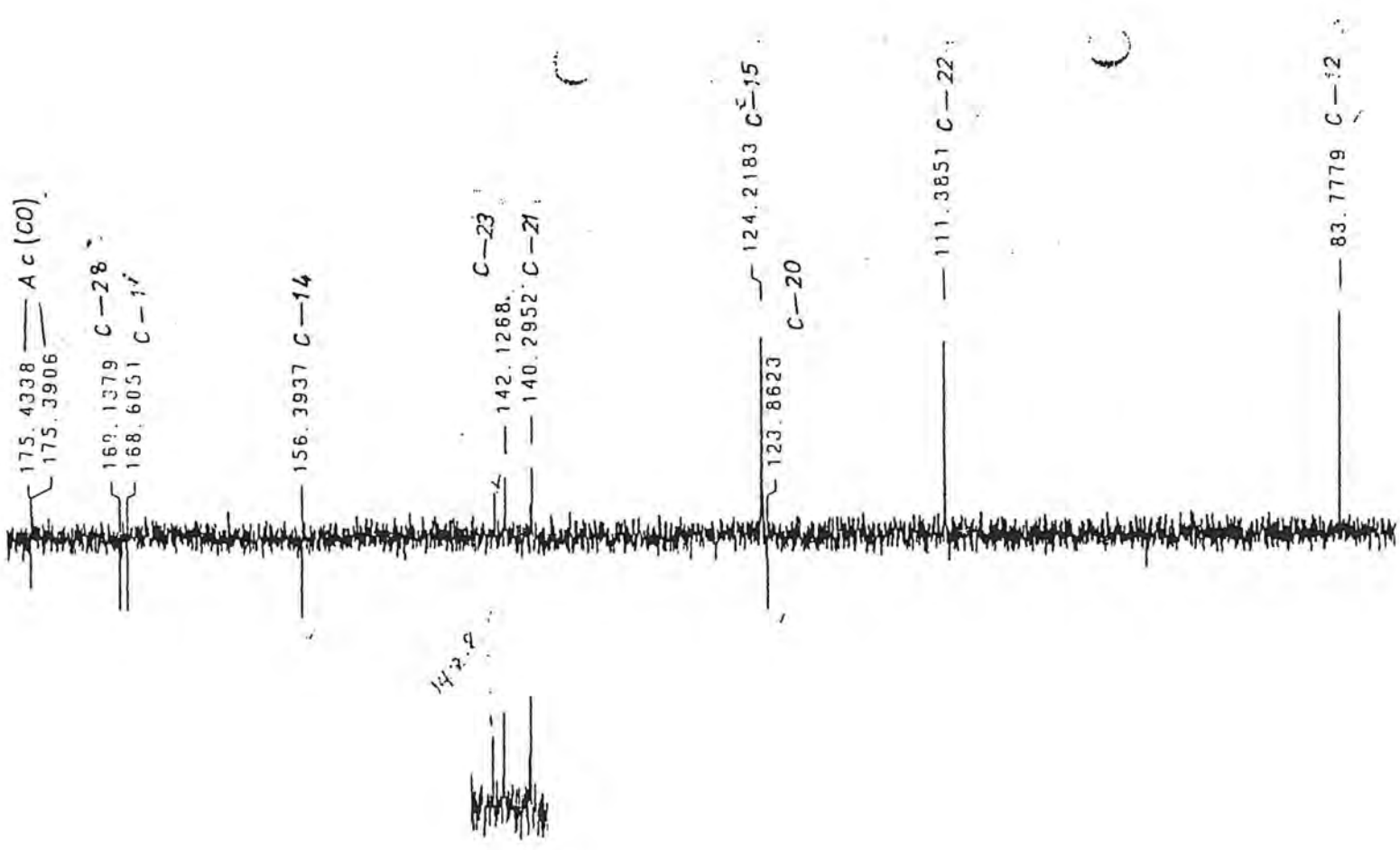


Figure 11d. Expanded ^{13}C NMR of compound 41 in $CDCl_3$

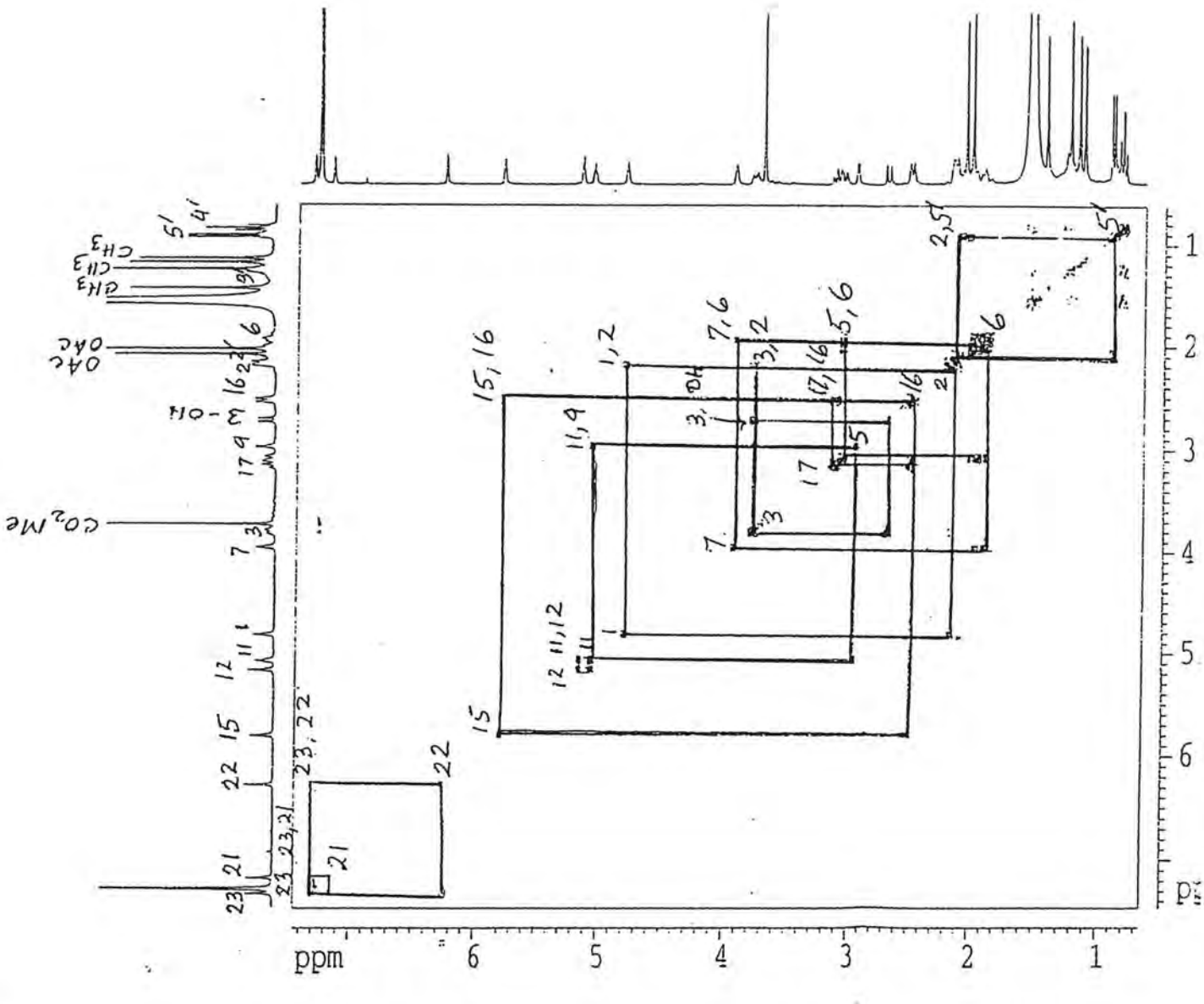


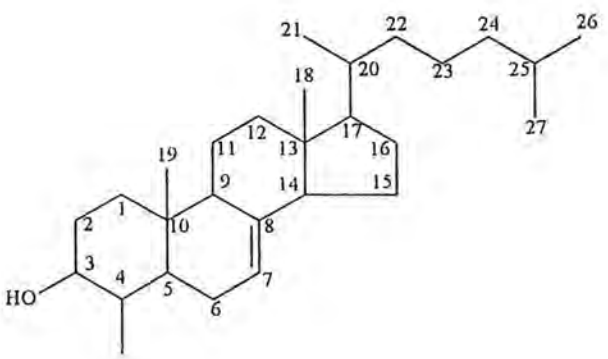
Figure 12. ^{1}H NMR COSY of compound 41 in $CDCl_3$

3.2. Compounds isolated from the stem bark of *C. swynertonii*

Gravitational column chromatography and preparative thin layer chromatography were employed in the fractionation of the hexane and ethyl acetate extracts of the stem bark of *C. swynertonii*. Seven compounds which comprised of 5 sterols and a diol (glycol) were isolated.

3.2.1 3β, 4α, 5-Cholest-7-en-3-ol (42)

The ^{1}H-NMR spectrum of (42) (Fig.13a, 13b) showed a doublet at δ 5.19 (J=1.8 Hz) and a multiplet at δ 3.51 (J=3.5 Hz), assignable to H-7 and H-3 respectively. These assignments are in agreement with previous assignments made for the sterols, condrillasterol and condrillasterone.[109] The protons of the methyl groups on C-10 and C-13 which appear as singlets at δ 0.70 and δ 1.0, respectively, are in agreement with assignments made for the sterols 24-ethyl-26-norcholesta-5,22E,25-trien-3β-ol [110]and methyl (E) 3β-acetoxy-$\Delta^{5,22}$-choladien-24-oate.[111] Assignments of the side chain were done by inspection of ^{13}C- NMR spectrum which were consistent with the assignments of corresponding acetates of stigmasterol and β-sitosterol.[112]



42

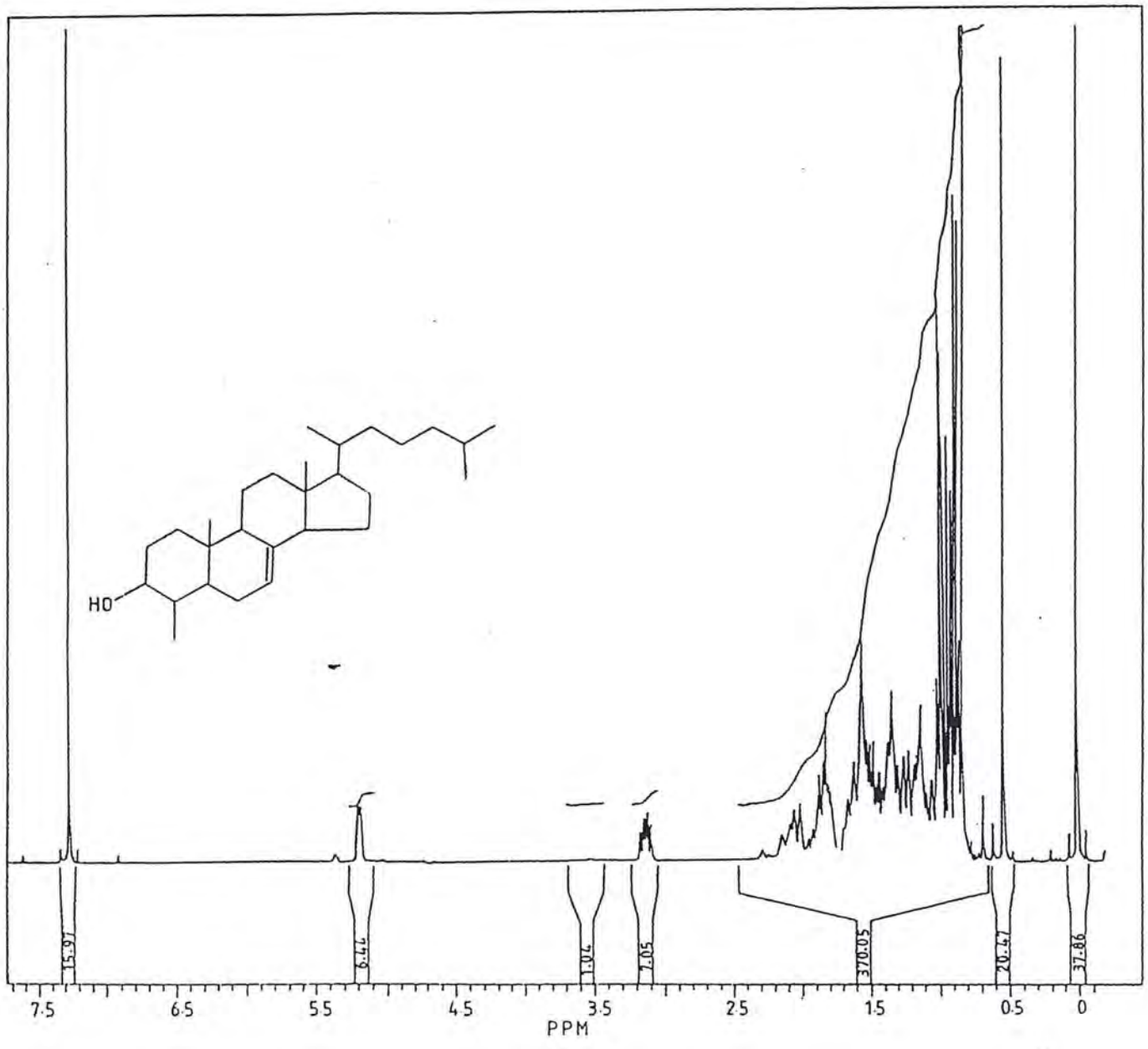


Figure 13a. ^{1}H NMR of compound 42 in $CDCl_3$

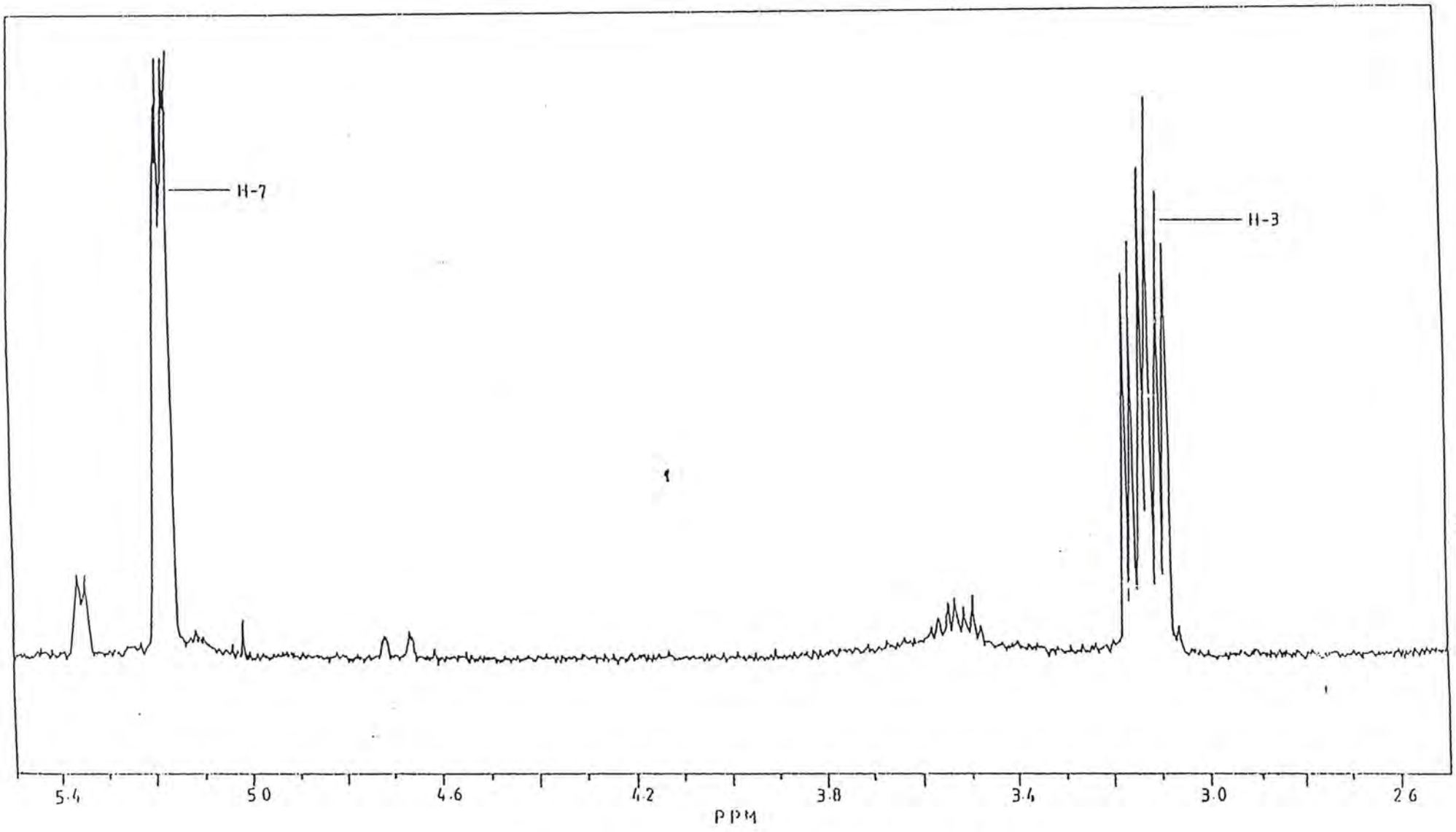


Figure 13b. Expanded ^{1}H NMR of compound 42 in $CDCl_3$

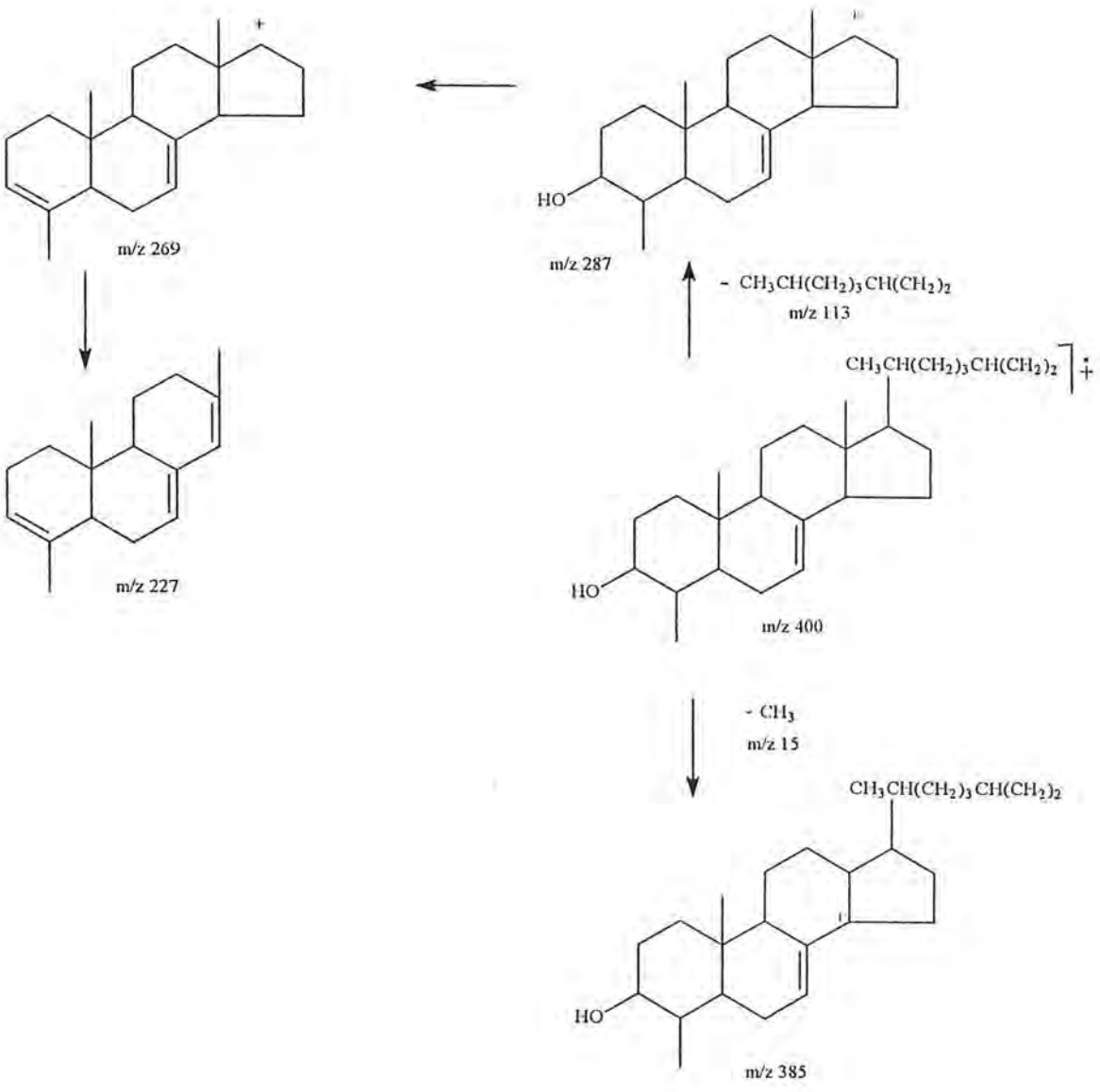
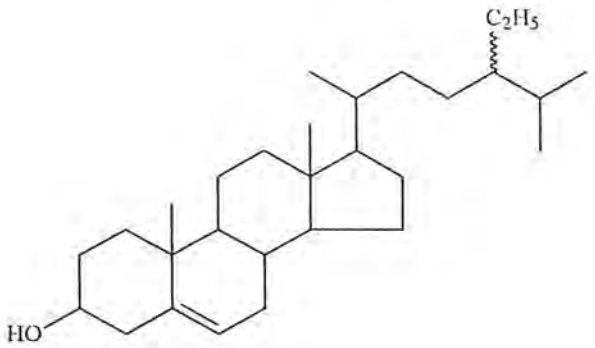


Figure 14. MS fragmentation pattern of 42

The characteristic mass spectralcfragment ions of compound 42 (Figure 14.) were at m/z 385, 287, 269 and 227 which are consistent with those of other Δ^5 and Δ^7-unsaturated steroids.[113]

3.2.2 β-Sitosterol (43)

^{1}H-NMR signals at δ 3.51 (J=3.6 Hz) and d 5.35 (J=1.9 Hz) were assigned to H-3 and H-5 respectively (Fig.2). The protons of the side chain were assigned as follows: δ 0.69 H-18, 1.02 H-19, 0.94 H-21, 0.86 H-26, 0.81 H-27 and 0.84 H-29 all which were in agreement with those assigned to β-sitosterol by Rubinstein and his co-workers.[114] ^{13}C-NMR inspection of this compound showed that the assignments are in consistent with those assigned to β-sitostrol by Rubinstein.[114]



43

The MS showed a molecular ion M^+at m/z 414 and characteristic fragment ions of Δ^5-unsaturated steroids shown on Figure 16.[113]

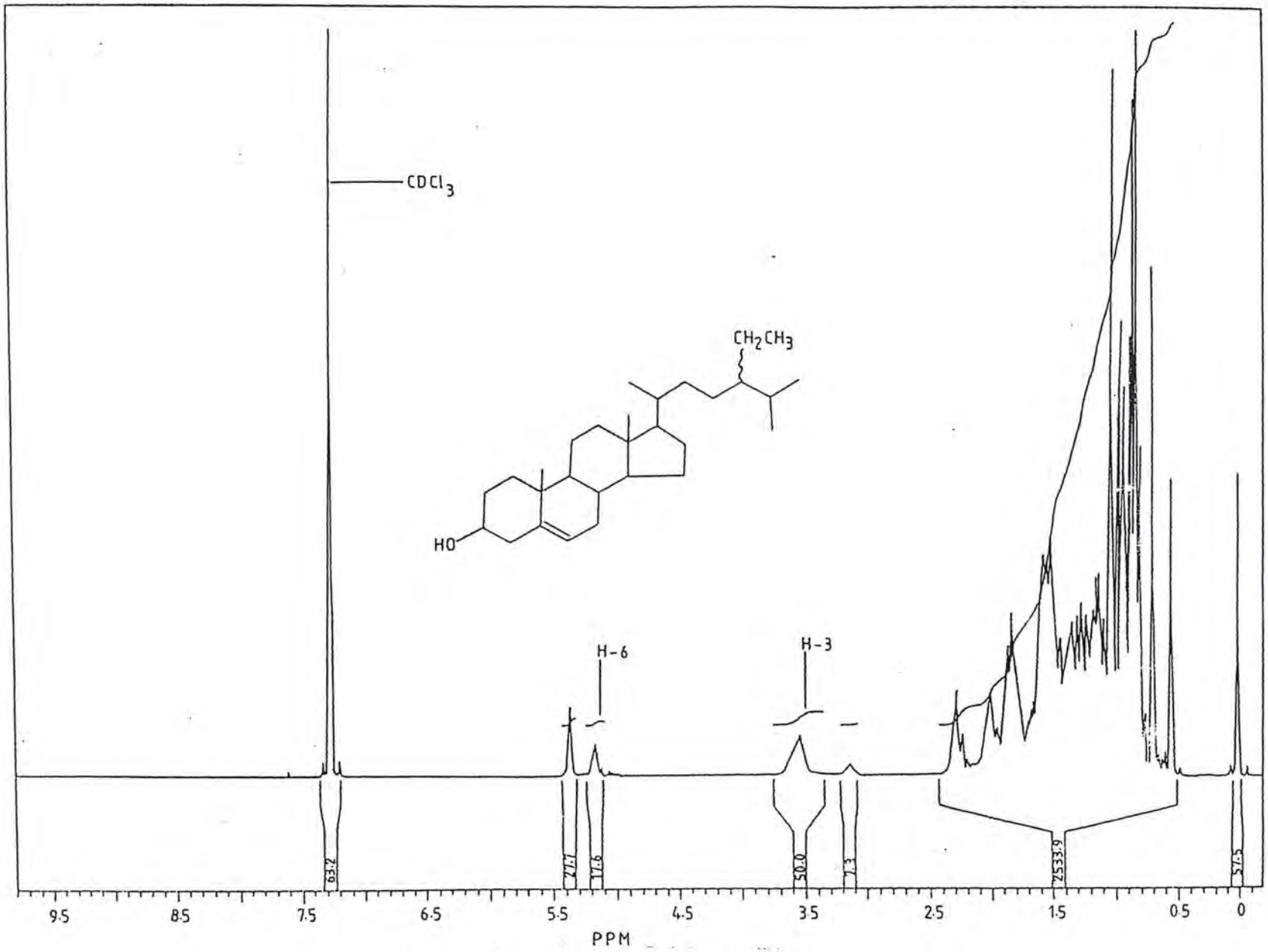


Figure 15. ^{1}H NMR of compound 43 in $CDCl_3$

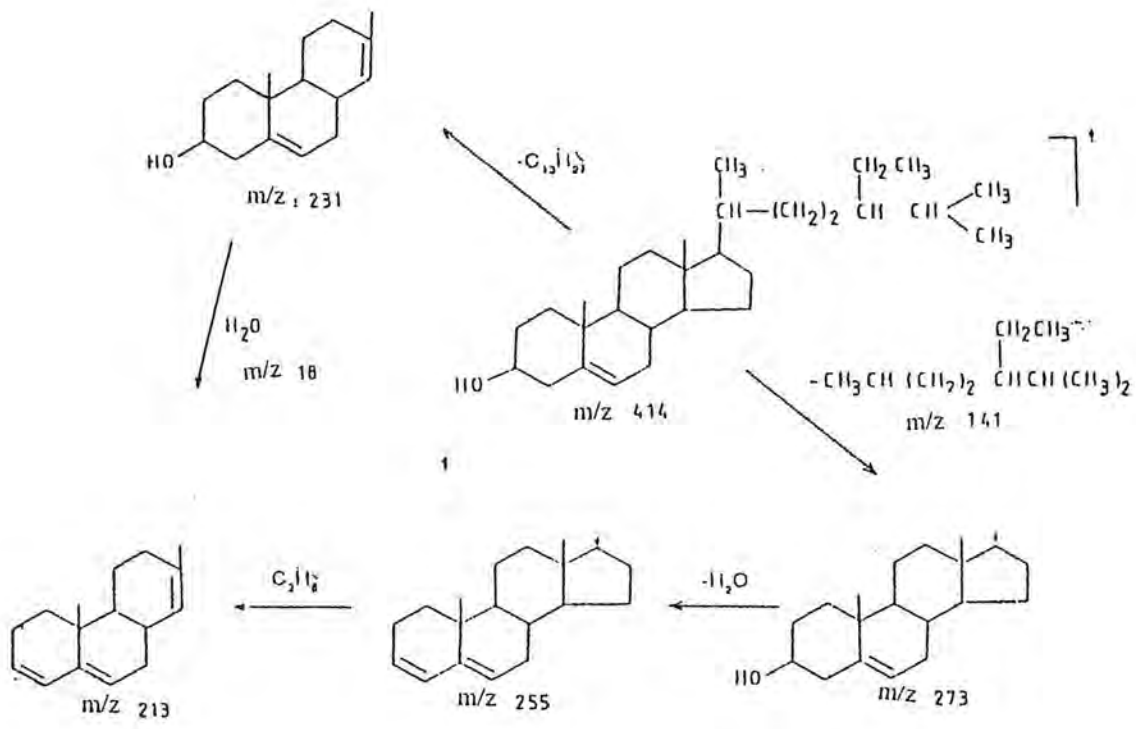
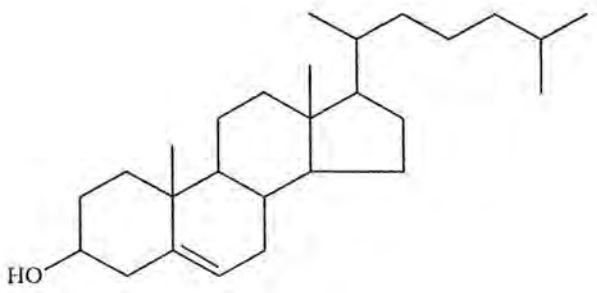


Figure 16. MS fragmentation pattern of compound 43

3.2.3 Cholest-7-en-3β-ol (44)

The ^{1}H-NMR spectrum (Fig.17) showed a multiplet at δ 3.61 (J=3.5 Hz) and a doublet at δ 5.18 (J=1.7 Hz) assigned to H-3 and H-5 respectively. The protons of the methyl groups atached to C-10 and C-13 which appeared as singlets at δ 0.83 and δ 1.01 respectively. Inspection of the ^{13}C-NMR spectrum showed that the signals were consistent with other closely related Δ^5-unsaturated compounds.[114]



44

The mass spectrum showed fragment ions at m/z 371, 353, 301, 273, 255 characteristic to Δ^5 and Δ^7-unsaturated steroids (Figure 18).[113]

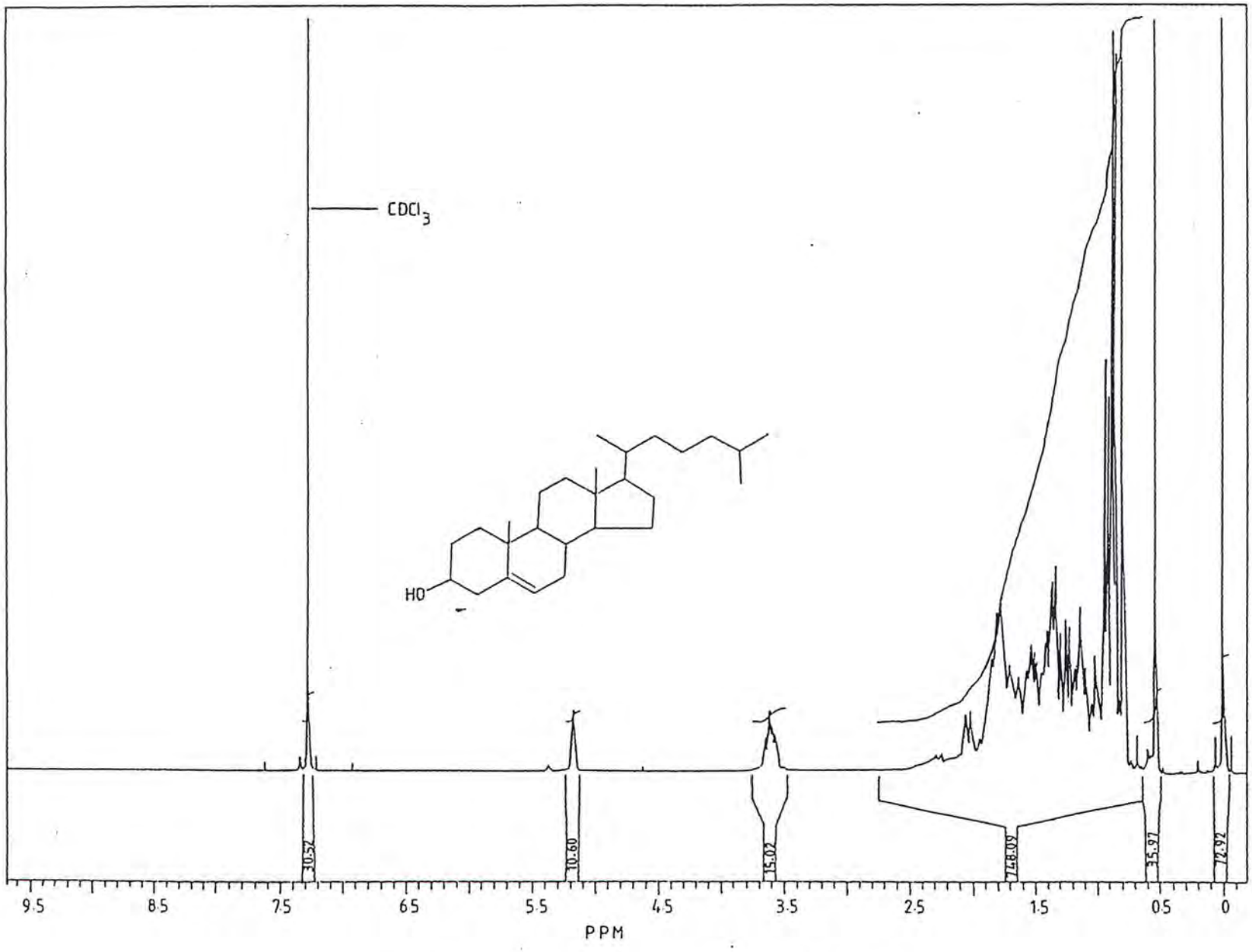


Figure 17a. ^{1}H NMR of compound 44 in $CDCl_3$

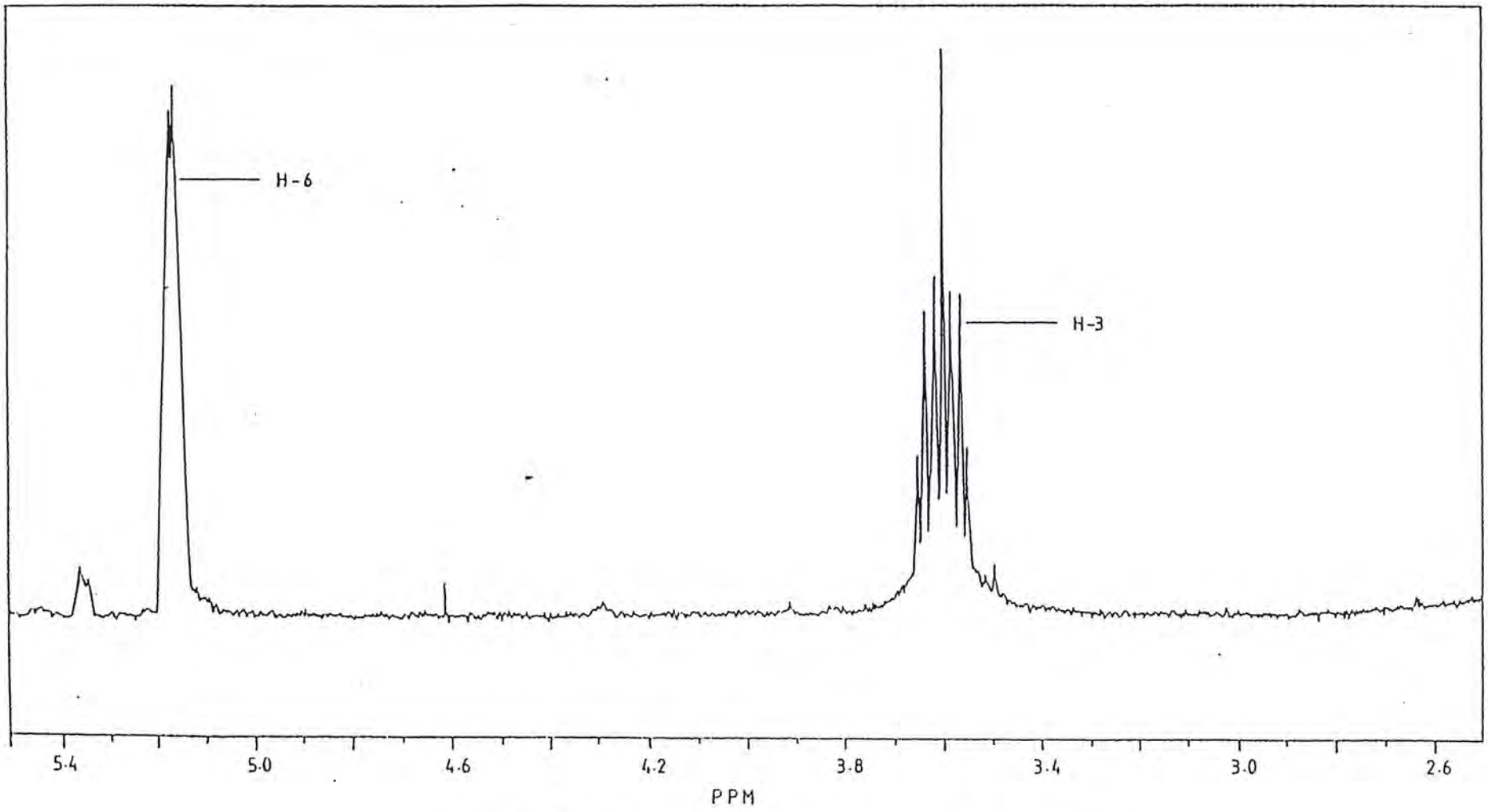


Figure 17b. Expanded ^{1}H NMR of compound 44 in $CDCl_3$

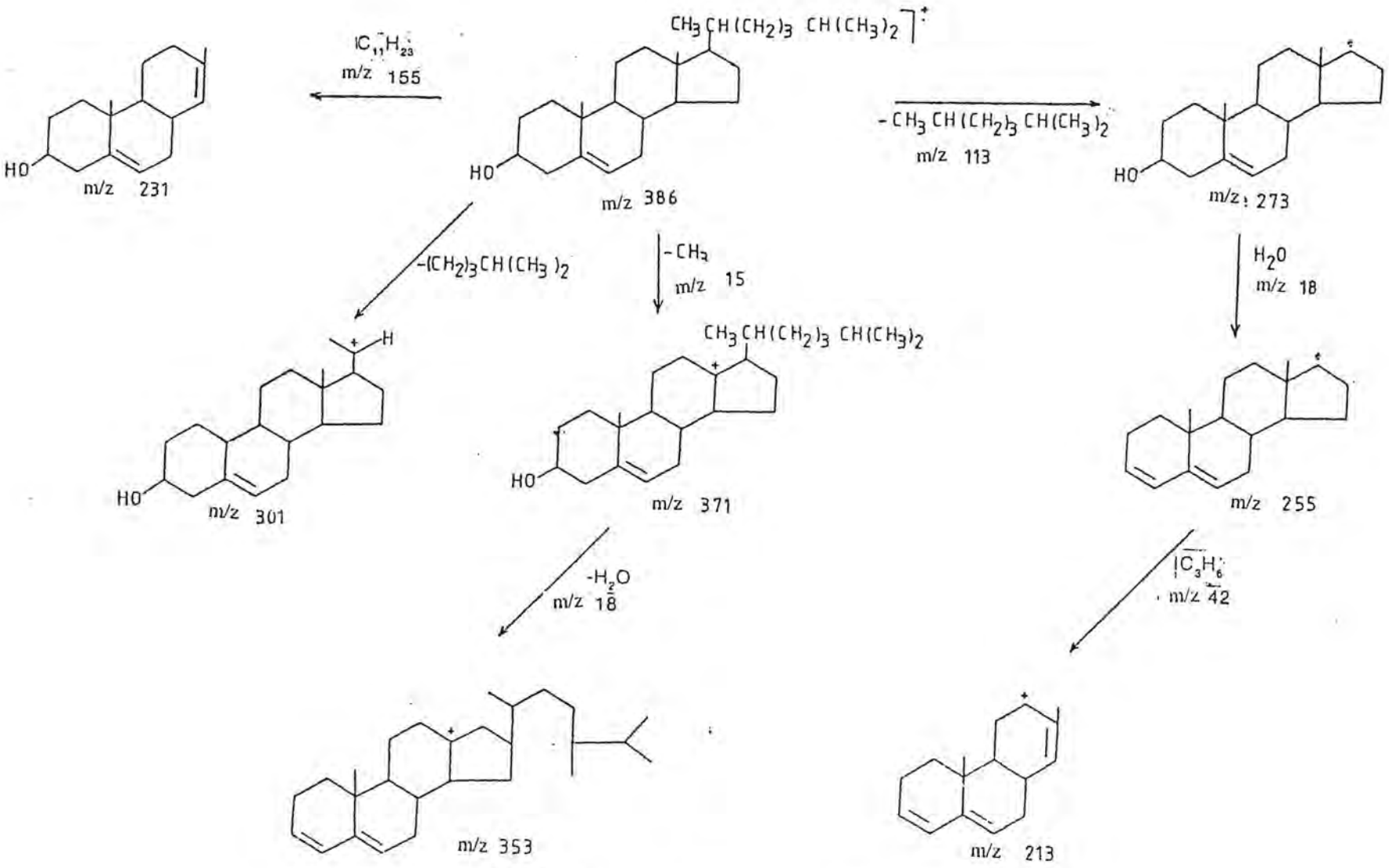
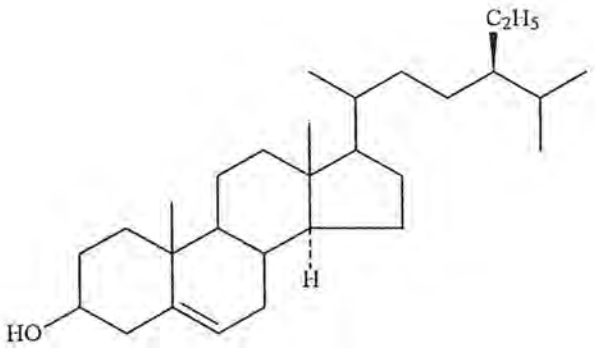


Figure 18. MS fragmentation pattern of compound 44

3.2.4 γ-Sitosterol (45)

The MS spectrum of this compound showed the same fragments as those of β-sitosterol (43).



45

^{1}H-NMR spectrum (Figure19) and MS fragmentation pattern are similar to those of β-sitosterol. The major difference between the spectra ^{1}H-NMR of β-sitosterol and γ-sitosterol is the appearance of the methyl C-26 and C-27 at δ 0.53 in β-sitosterol and δ 0.69 in γ-sitosterol attributed to the orientation of the CH_2CH_3 moeity at C-24. [115]

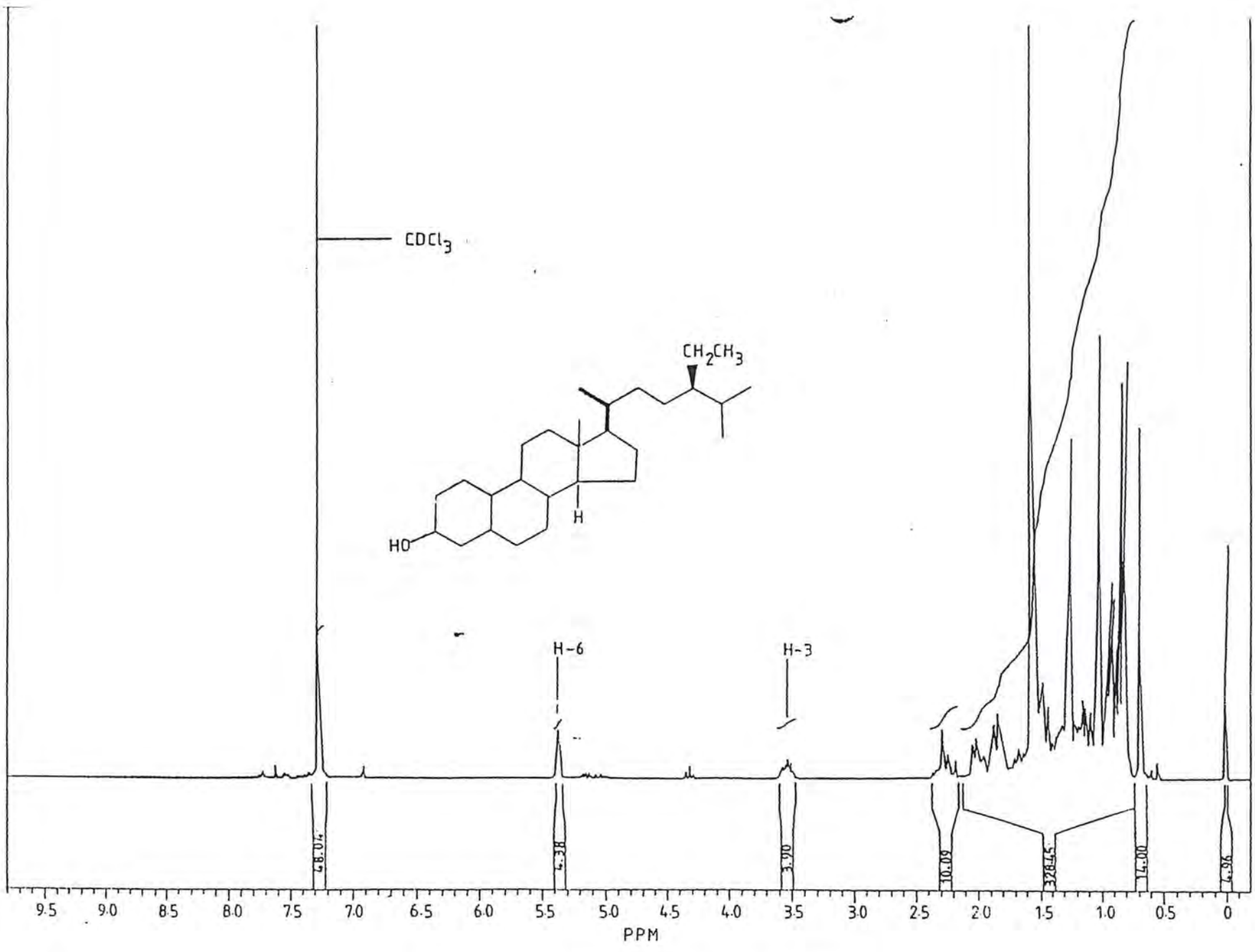
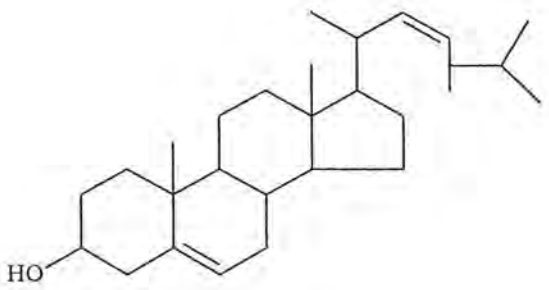


Figure 19. ^{1}H NMR of compound 45 in $CDCl_3$

3.2.5 Stigmasterol (46)

^{1}H-NMR spectrum (Figure 20) of this compound showed a doublet at δ 5.35 and a multiplet at 3.48 assignable to H-6 and H-3 respectively. The chemical shifts of the methyl protons attached at C-10 and C-13 are δ 0.82 and 1.01 respectively. The assignments of the carbons were made by direct comparison with the ^{13}C-NMR data of the same compound assigned by Holland and his co-workers.[112]



46

The characteristic ions typical of Δ^5 and Δ^7 -unsaturated steroids were at m/z 412 M^+, 273, 255, 231 and 213.

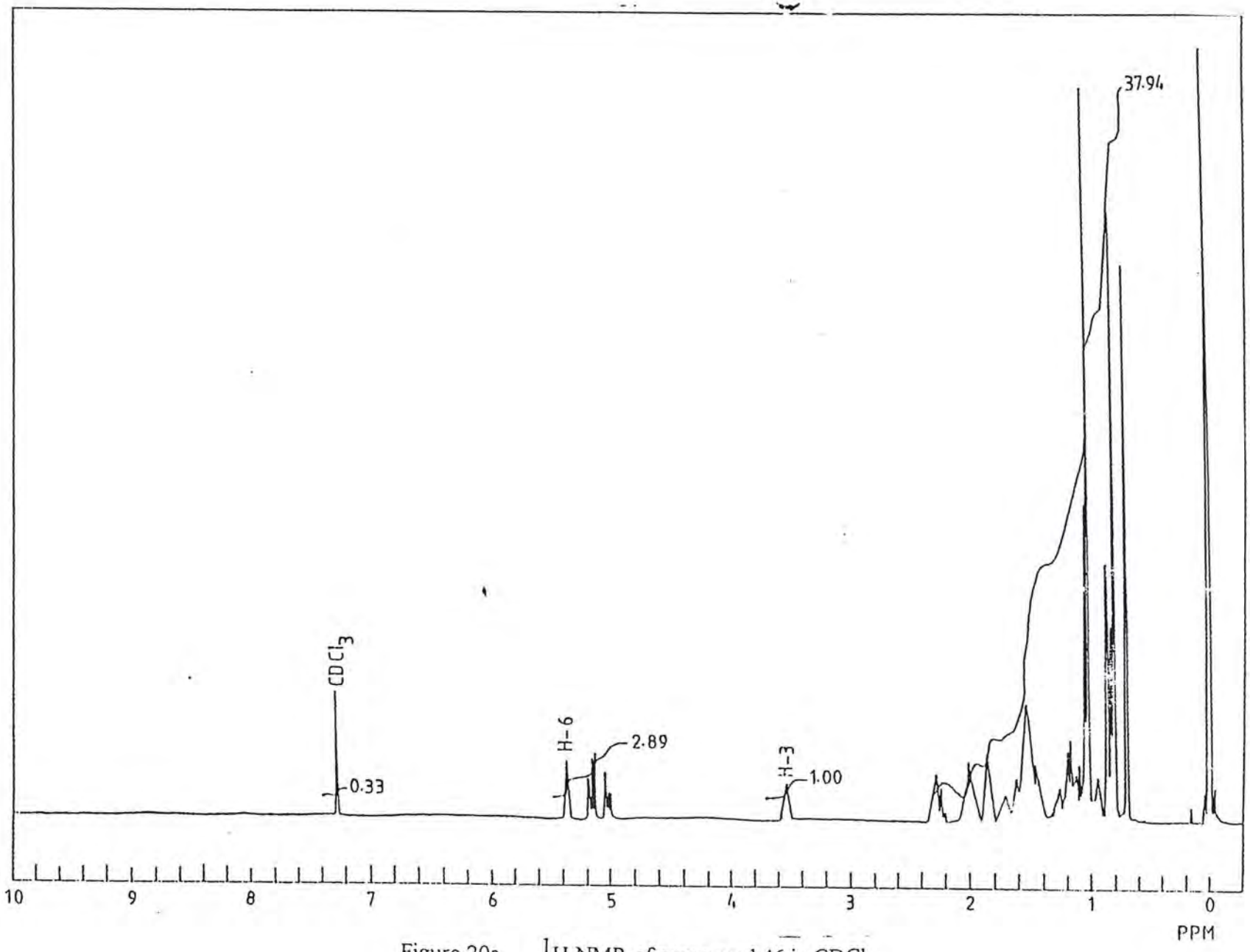


Figure 20a. ^1H NMR of compound 46 in $CDCl_3$

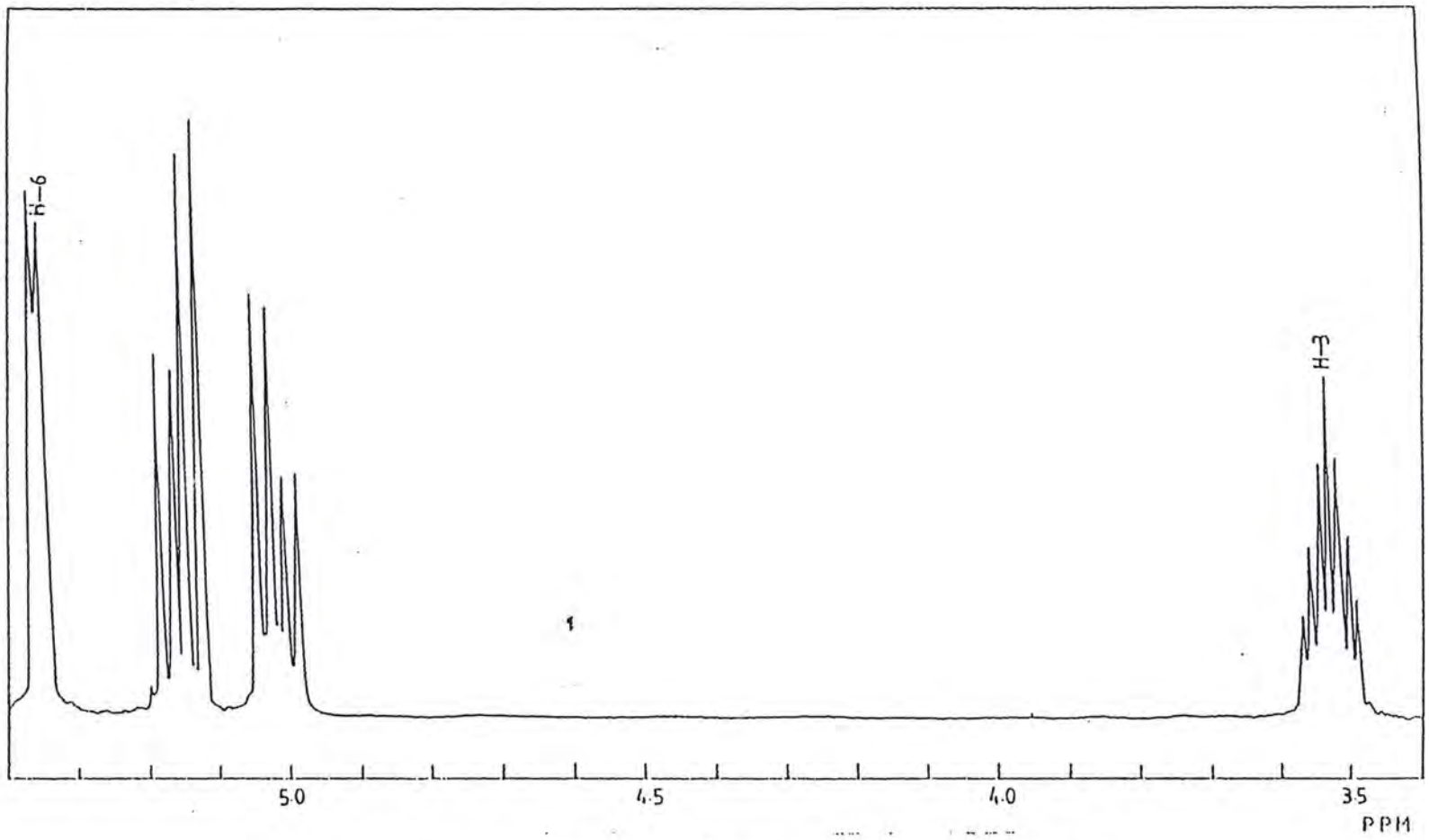


Figure 20b. Expanded ^1H NMR of compound 46 in $CDCl_3$

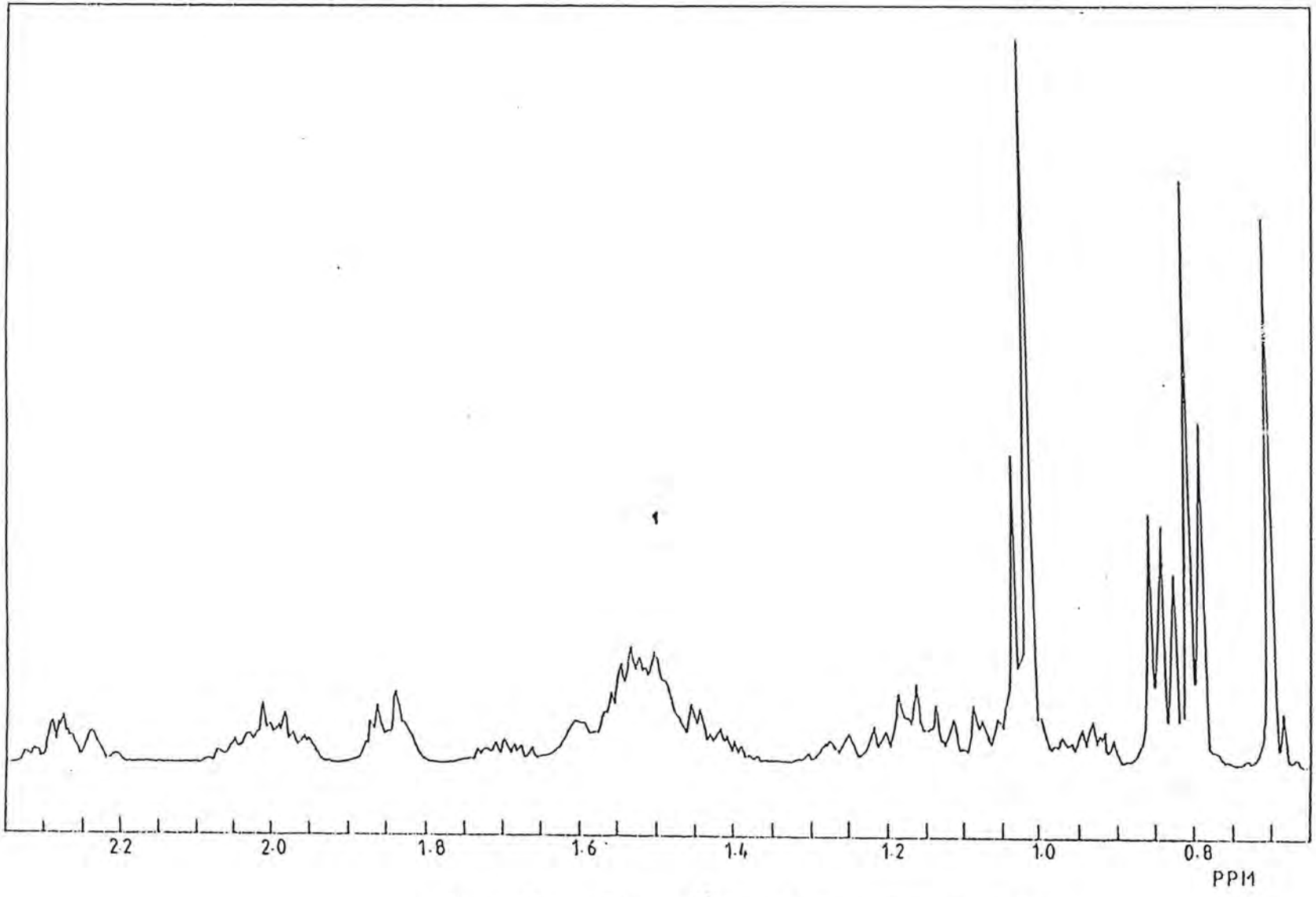


Figure 20c. Expanded ^{1}H NMR of compound 46 in $CDCl_3$

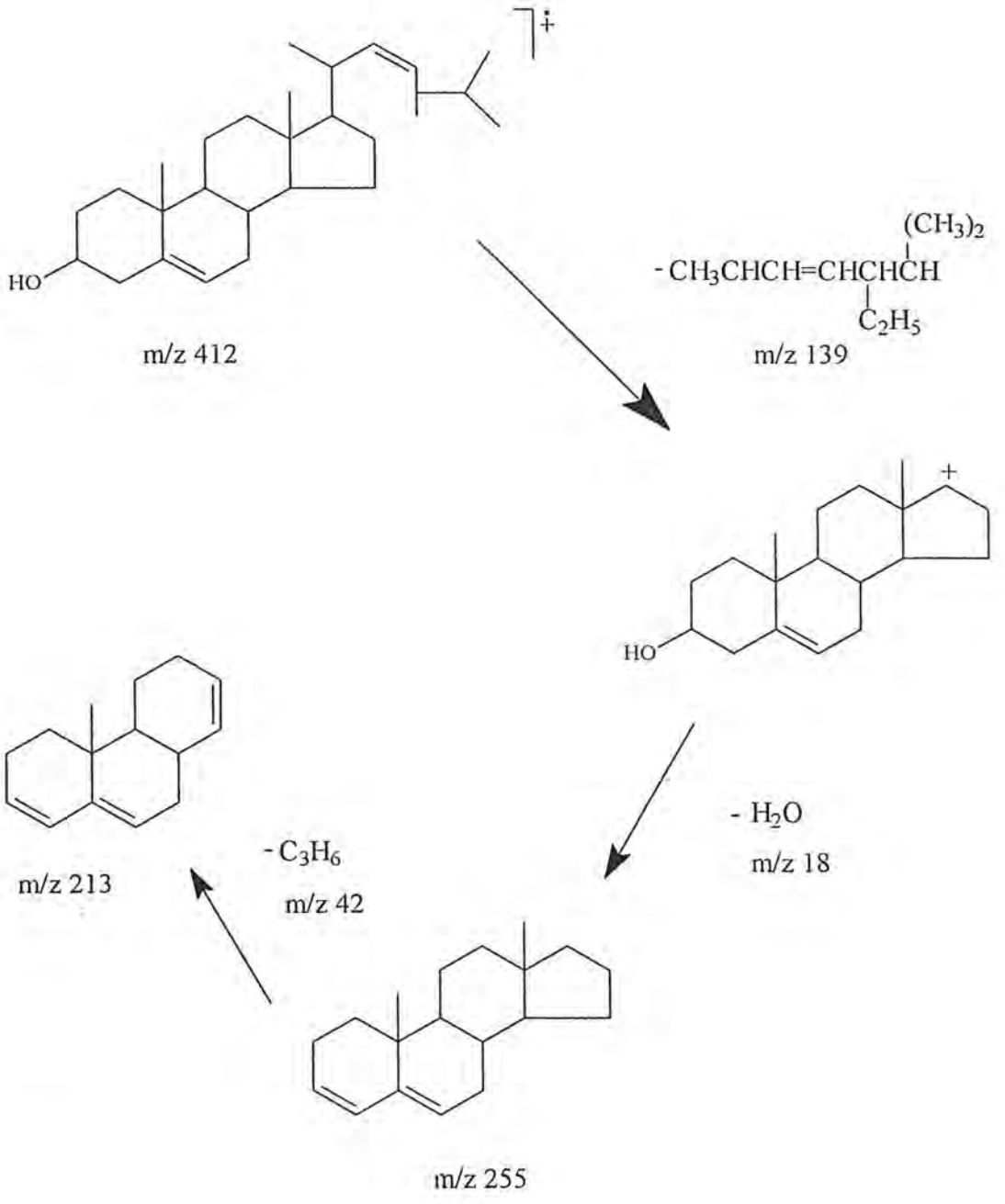
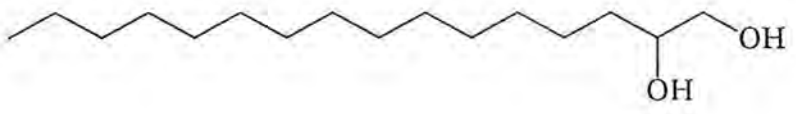


Figure 21. MS fragmentation pattern of 46

3.2.6 Hexadecane-1,2-diol (47)

The ^{1}H-NMR spectrum of compound (47) (Figure 22) showed peaks at δ 0.9 (t, CH_3), 1.3 (m, CH_2), 1.6 (m, -CH_2CH-OH and multiplets at δ 3.45(1H) and 3.7(2H) indicating the presence of two hydoxyl groups attached to two different carbons of an aliphatic chain.



47

The MS showed ions at m/z 256, 241, 166, 153, 125 and 111 (Figure 23) which are characteristic of aliphatic saturated hydrocarbons.

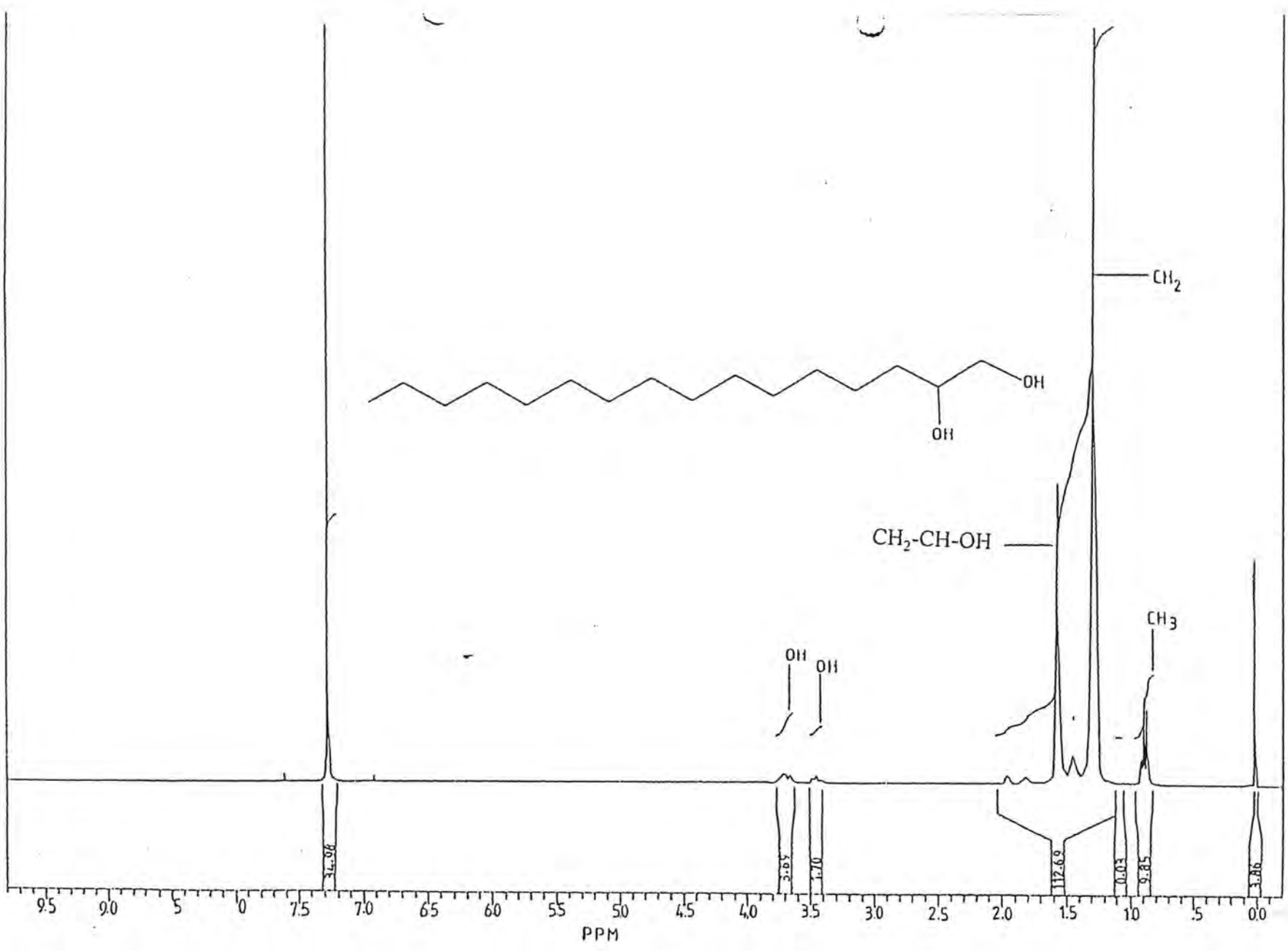


Figure 22. Expanded ^{1}H NMR of compound 47 in $CDCl_3$

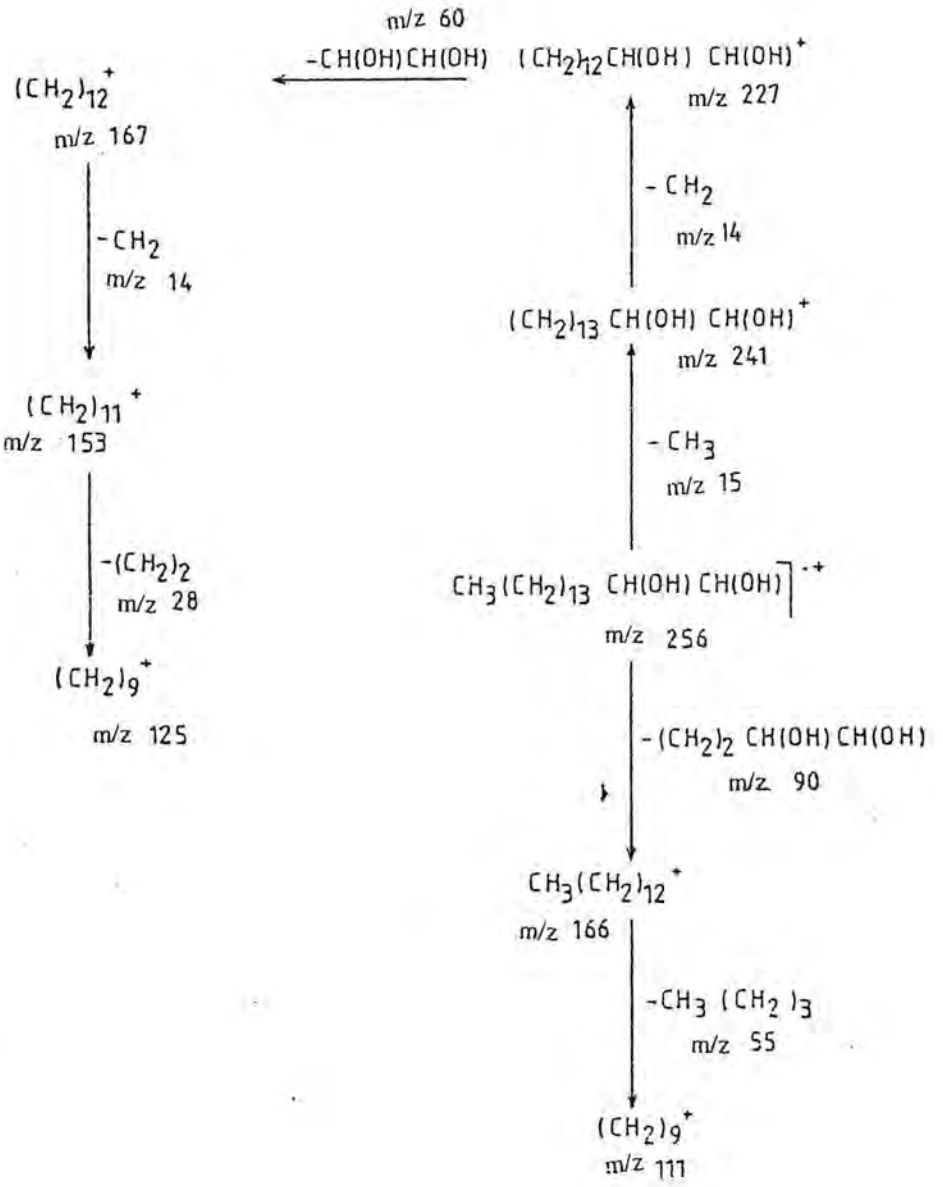


Figure 23. MS fragmentation pattern of compound 47

The effect of the position of double bond on the fragmentation pathway of the unsaturated steroids under electro impact has been studied in the Δ^4 and Δ^5 -isomers of cholestane, androstane and pregnane. Under certain experimental conditions it was found that fragmentation pathway of the Δ^4-unsaturated steroids differ from those of corresponding Δ^5-isomers. It was therefore possible to determine the position of the double bond in such compounds on the basis of their mass spectra.[113] The fragment ions of mass 303, 301, 289, 275, 219, 205 and 167 which contain rings C and D with the side chain characterize fragments of Δ^5 -unsaturated steoids. The mass of the ion will therefore depend on the substituent at C-17.[113]

In the mass spectra of Δ^7-unsaturated steroids the ion resulting from the elimination of the side chain becomes one of the characteristic ion. The fragment resulting from the elimination of the side chain together with ring D and leading to the formation of the ion consisting of rings A, B and C is considered the characteristic ion of Δ^7 -unsaturated steroids.

The doublet due to the splitting of the alkene protons present in ring B of Δ^5 and Δ^7 -unsaturated steroids appeared between δ 5.18 to 5.36 while the OH group appeared between δ 3.51 to 3.61. Due to the crowding of peaks resulting from the splitting of the side chain protons, assigments were mainly based on the ^{13}C-NMR data.

With the exception of the diol (47) the remaining isolates from *C. swynertonii* were steroids. Steroids include a wide range of naturally occurring compounds, among which are the sterols proper, the bile acids, the sex hormones, the adrenocortical hormones, the cardiac glycosides, the sapogenins, some alkaloids and other minor groups.[123] Many steroids contain an alkyl group at C-24.[113] In general, algae and fungi produce sterols with the 24b-configurations while in most higher plants the sterols have the 24a-configuration.[113]

3.3 GC-MS analysis of hydrodistilled oil of *C. swynertonii*

GC-MS (GC coupled to a Low Resolution Mass Spectrometer-LRMS) chromatogram of the oil obtained by hydrodistillation is shown (Figures 24a and 24b) (GC coupled to a High Resolution Mass Spectrometer-HRMS). The relative retention times and relative intensities of the compounds of the oil as analysed by LRMS are given in Tables 7a and 7b. Five sesquiterpenoids and four sesquiterpenoid derivatives were identified. With the exeption of α-copaene and isocaryophylene whose structure was confirmed by GC co-injection with authentic sample, the structures of the other compounds were deduced from two techniques namely, probability based matching system (PBM)[116] and the fragmentation pattern of each component.

The chromatographic retention times and relative intensities are shown in Table 7a and 7b.

Compounds represented by peaks 12-14 are isomers (Figure 24a and 24b). The PBM system technique involves comparison of unknown compounds against all possible reference spectra. This is supported by statistical evaluation of the matches actual reliability of the predictions based on the degree of match found between the unknowns and the reference spectra. however, confirmation of the structure using this technique needs a lot of care because of two reasons. First, there are difficulties in evaluating the reliability of matches due to errors which are often observed between the spectra of sterio- and geometric isomers and other very closely related compounds. Secondly, there are always some differences in experimental conditions between the unknowns and matching spectra.[116]

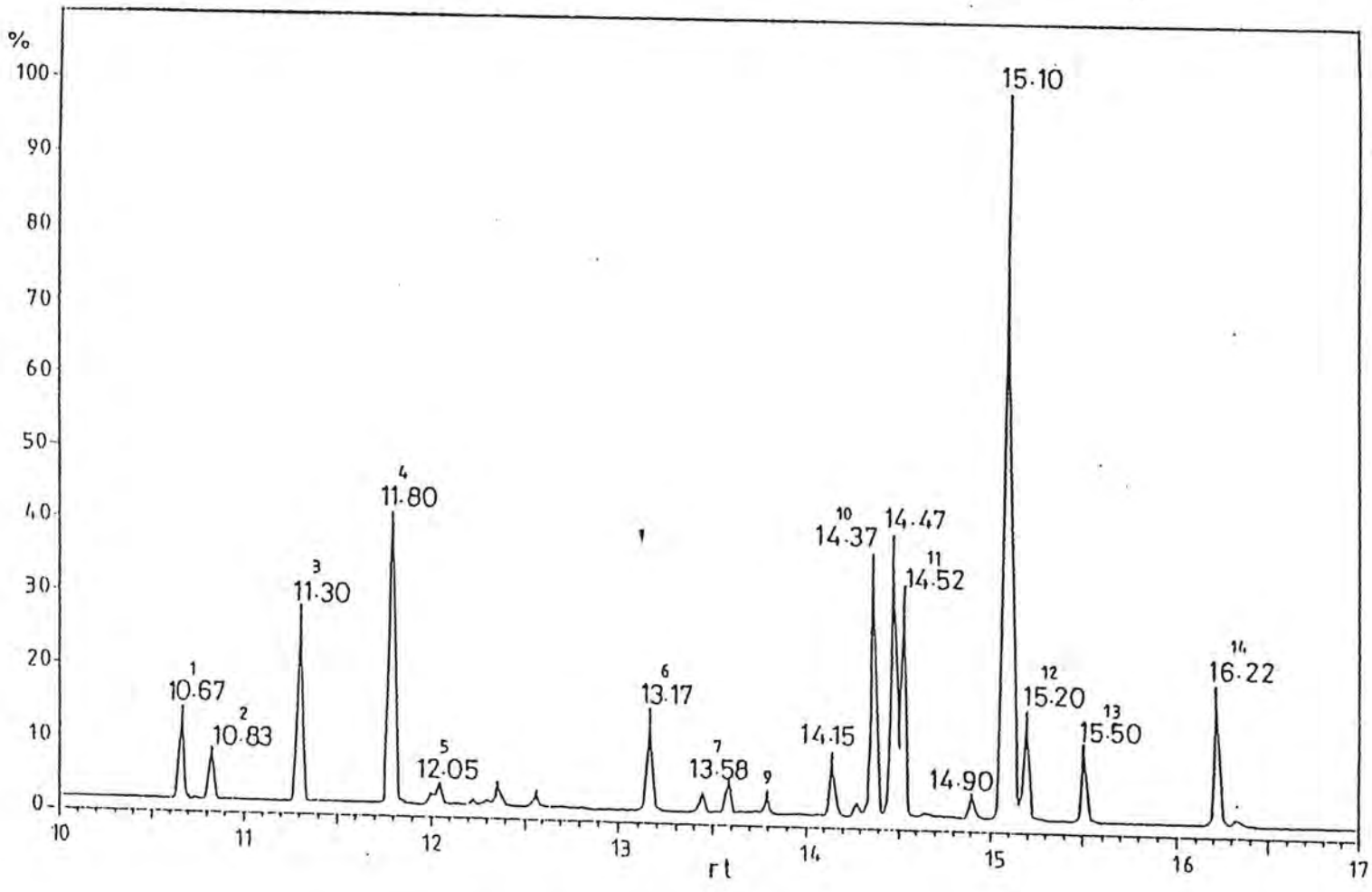


Figure 24a. GC profile of oil (low resolution)

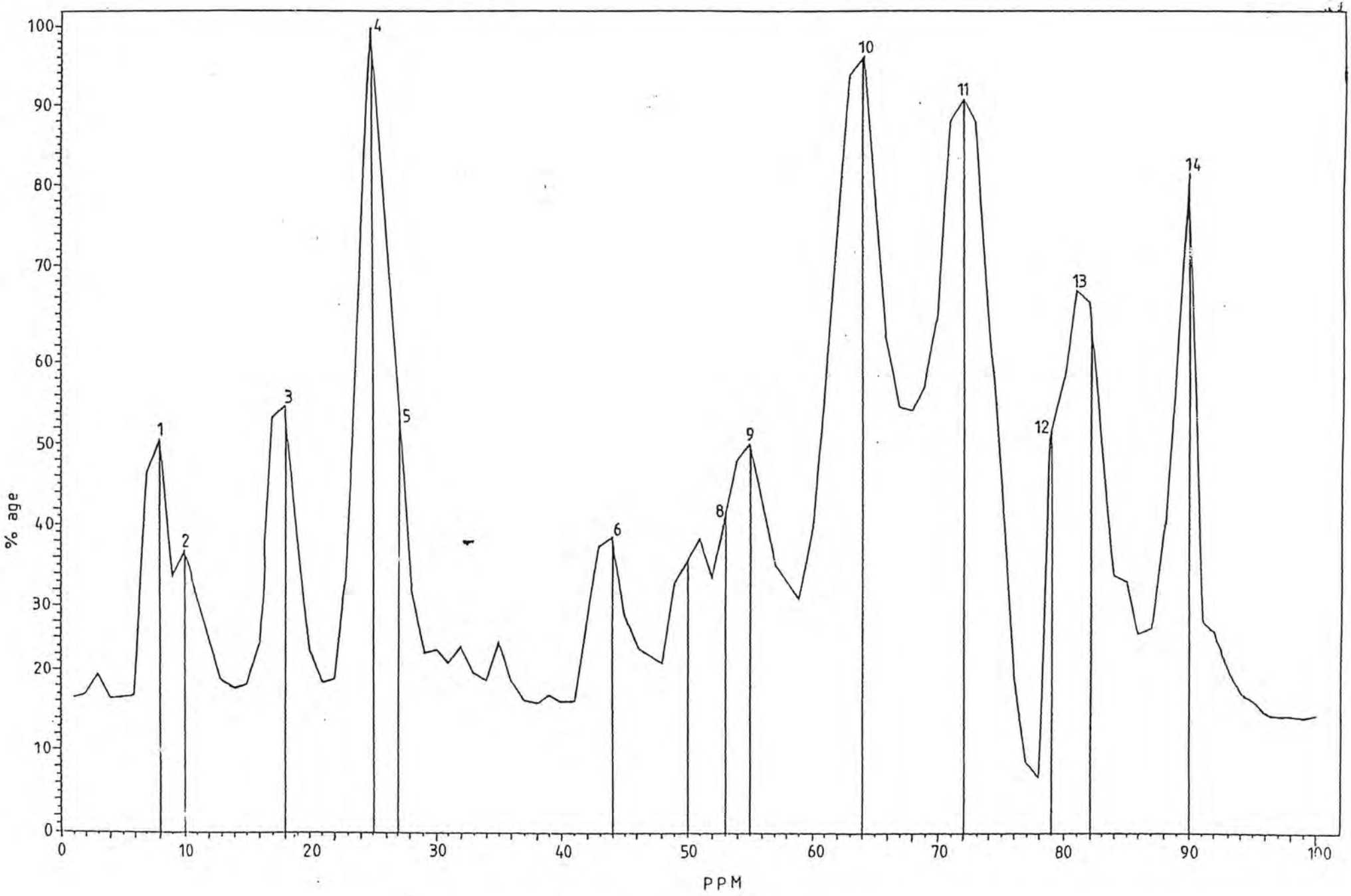


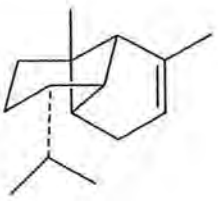
Figure 24b GC profile of oil (high resolution)

Table 7a. Gas chromatographic data of the oil

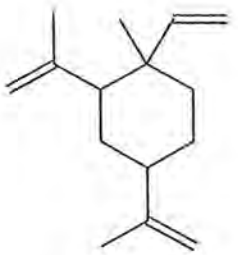
Peak	Component	Analysis type	RT	Structure
1	α-Copaene	GC-MS,RT	10.69	48
2	1-Ethenyl-1-methyl-diisopropyl 2,4-cyclohexane	HRMS	10.83	49
3	Isocaryophyllene	GC-MS,RT	11.30	50
4	1,1,4,8-Tetramethyl-4,7,10-cycloundecatriene	HRMS PBM	11.80	51
5	4-Methyl-1-1,5-dimethyl-4-hexenyl-benzene	HRMS PBM	12.05	52
6	Furan, 3-(4,8-dimethyl-3,7-nonadienyl)	HRMS PBM	13.17	53
7	Unidentified	HRMS PBM	13.58	-
8	Unidentified	HRMS PBM	13.60	-
9	Unidentified	HRMS PBM	13.80	-
10	Unidentified	HRMS PBM	14.37	-
11	Unidentified	HRMS PBM	14.52	-
12	9-(3-Furanyl)-2,6-dimethyl-2,6-nonadien-4-one	HRMS PBM	15.20	54
13	9-(3-Furanyl)-2,6-dimethyl-2,5-nonadien-4-one	HRMS PBM	15.50	55
14	9-(3-furanyl)-2,6-dimethyl-2,5-nonadien-4-one	HRMS PBM	16.22	55

Table 7b. Chemical composition of the hydrodistilled oil

Peak	Component	Relative Intensity	Structure
1	α-Copaene	117566	48
2	1-Ethenyl-1-methyl-diisopropyl 2,4-cyclohexane	39200	49
3	Isocaryophyllene	61046	50
4	1,1,4,8-Tetramethyl-4,7,10-cycloundecatriene	146165	51
5	4-Methyl-1-1,5-dimethyl-4-hexenyl-benzene	10749	52
6	Furan, 3-(4,8-dimethyl-3,7-nonadienyl)	135495	53
7	Unidentified	53816	-
8	Unidentified	71941	-
9	Unidentified	53812	-
10	Unidentified	388503	-
11	Unidentified	257059	-
12	9-(3-Furanyl)-2,6-dimethyl-2,6-nonadien-4-one	558591	54
13	9-(3-Furanyl)-2,6-dimethyl-2,5-nonadien-4-one	2108303	55
14	9-(3-Furanyl)-2,6-dimethyl-2,5-nonadien-4-one	34160	55



48



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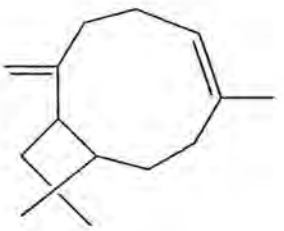

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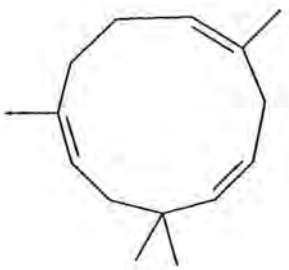

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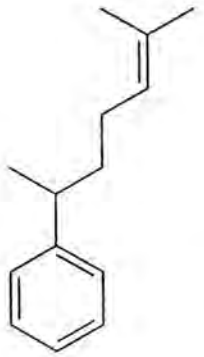

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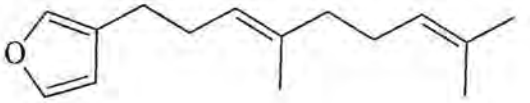

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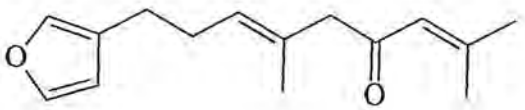

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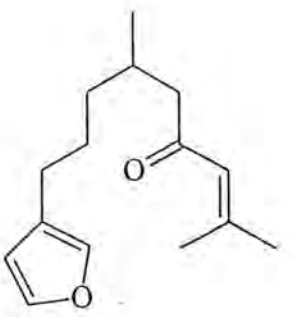

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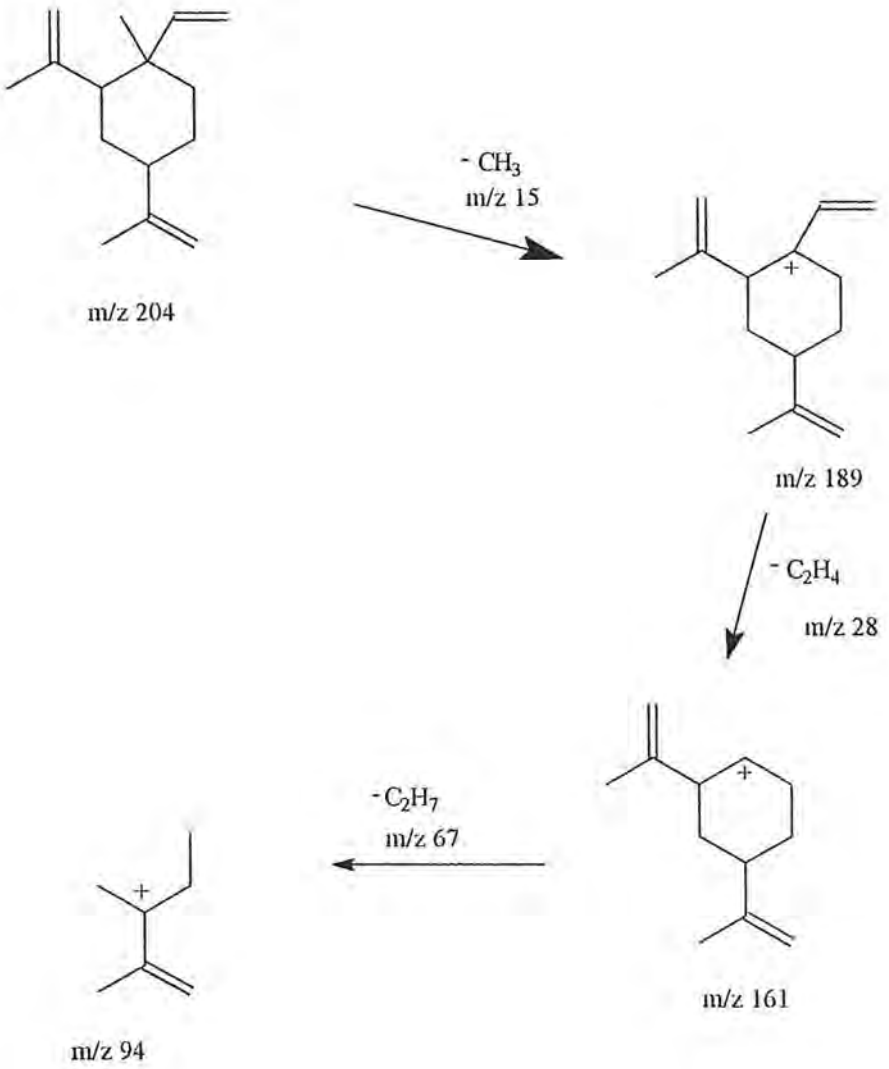

Figure 25. MS fragmentation pattern of 1-ethenyl-1-methyl-1,4-diisopropenyl cyclohexane

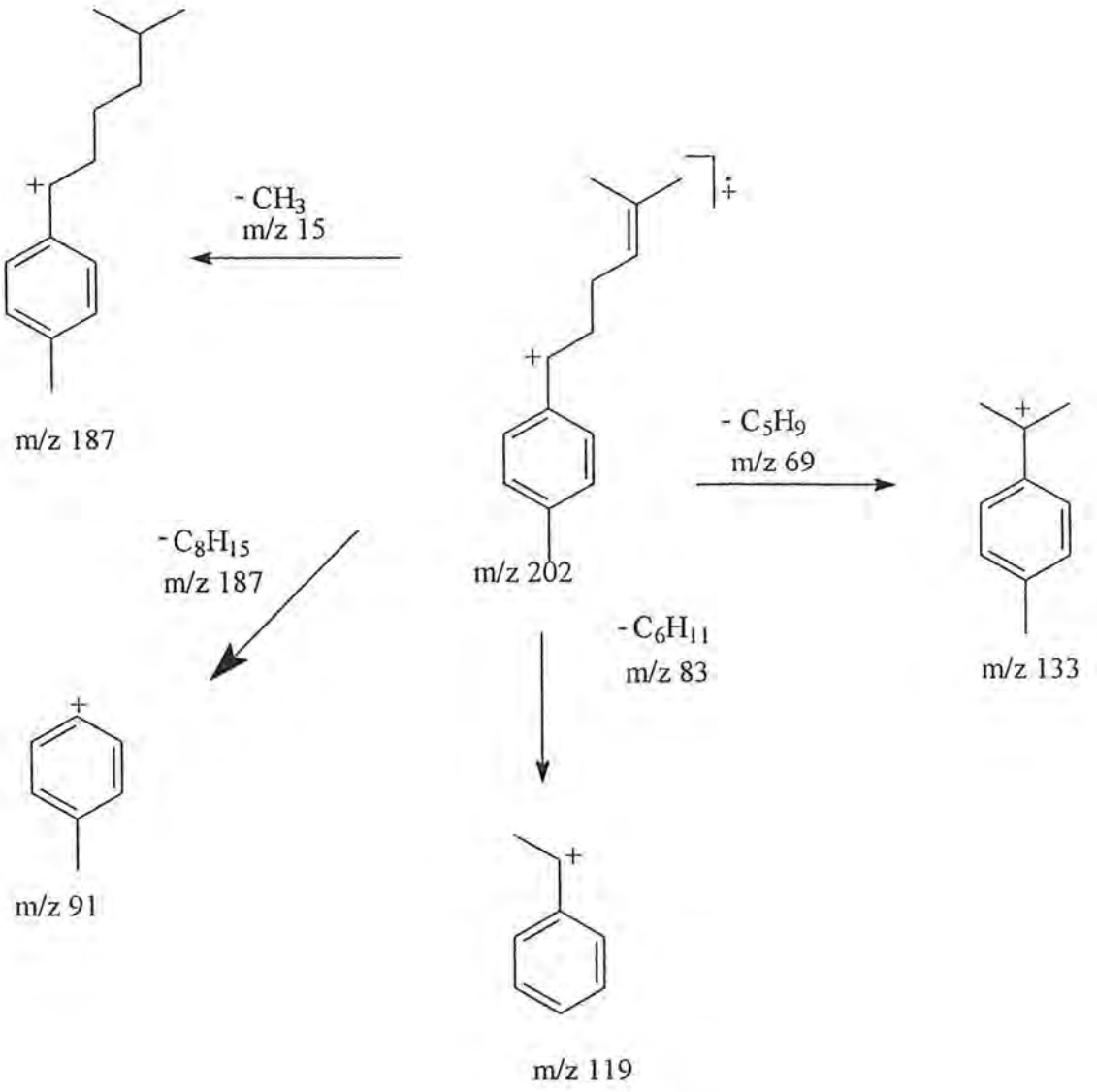

Figure 26. MS fragmentation pattern of 4-Methyl,1-(1,5-dimethyl-4-hexenyl) benzene

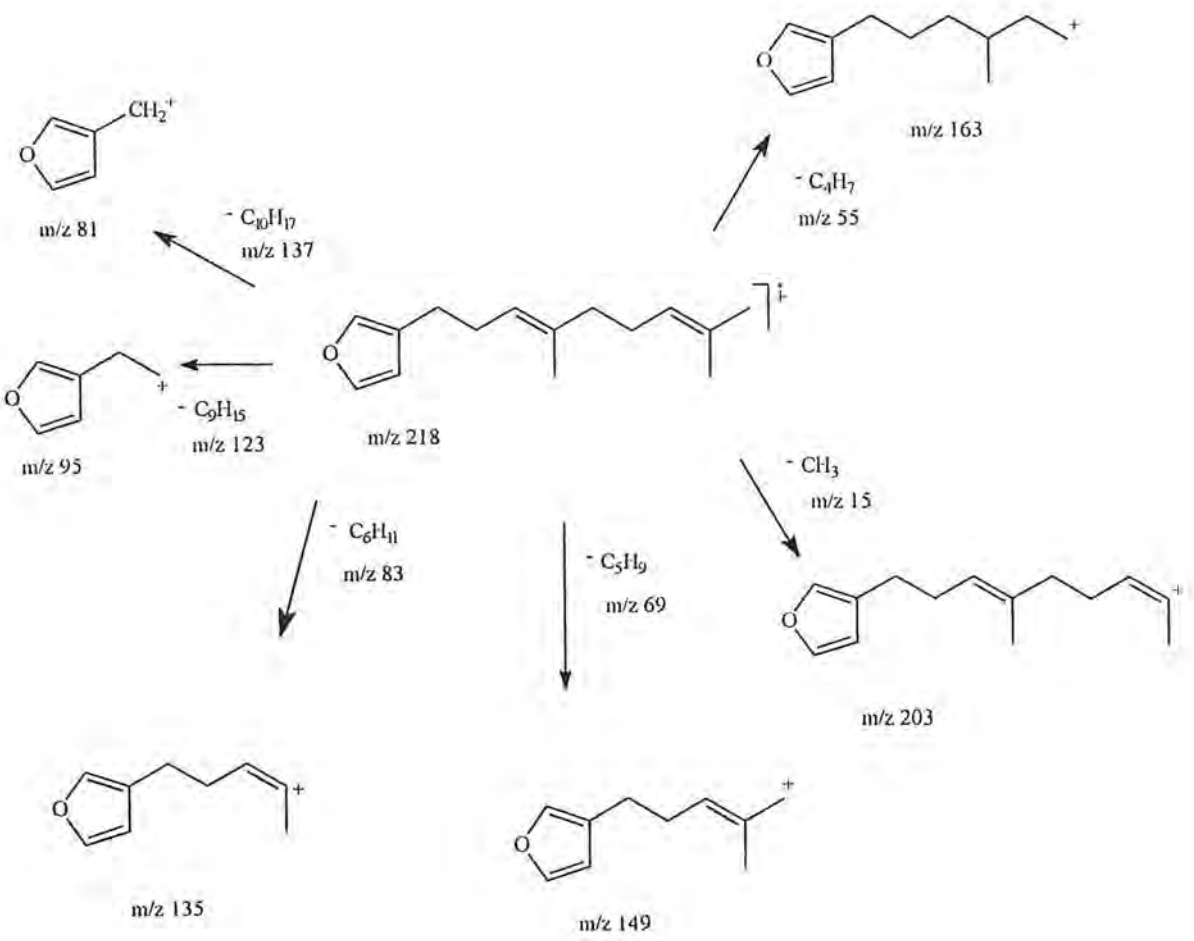

Figure 27. MS fragmentation pattern of 3-(4,8 dimethyl-3,7-nonadienyl) furan

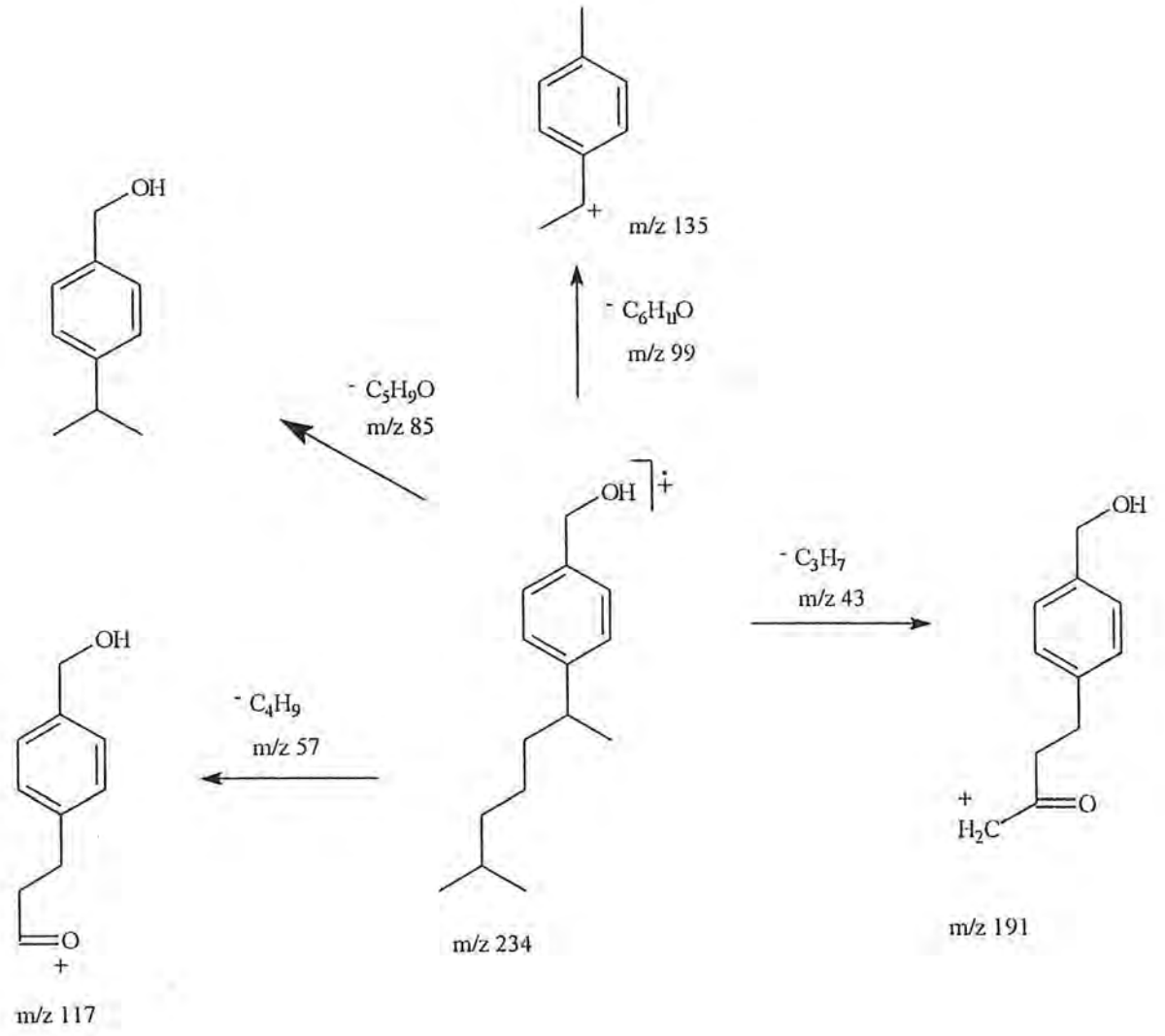

Figure 28. MS fragmentation pattern of an alcoholic benzene compound

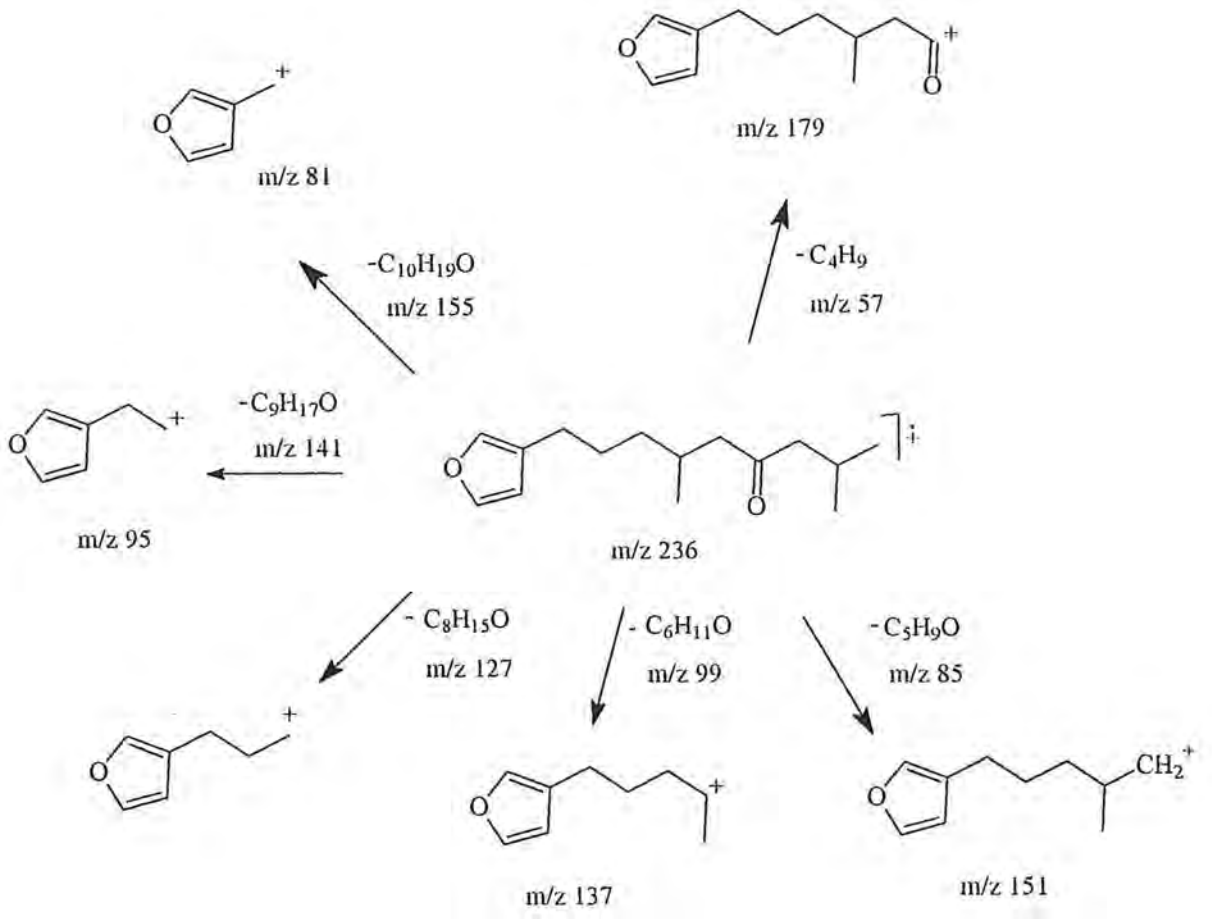

Figure 29. MS fragmentation pattern of a furanic compound without olefenic bonds

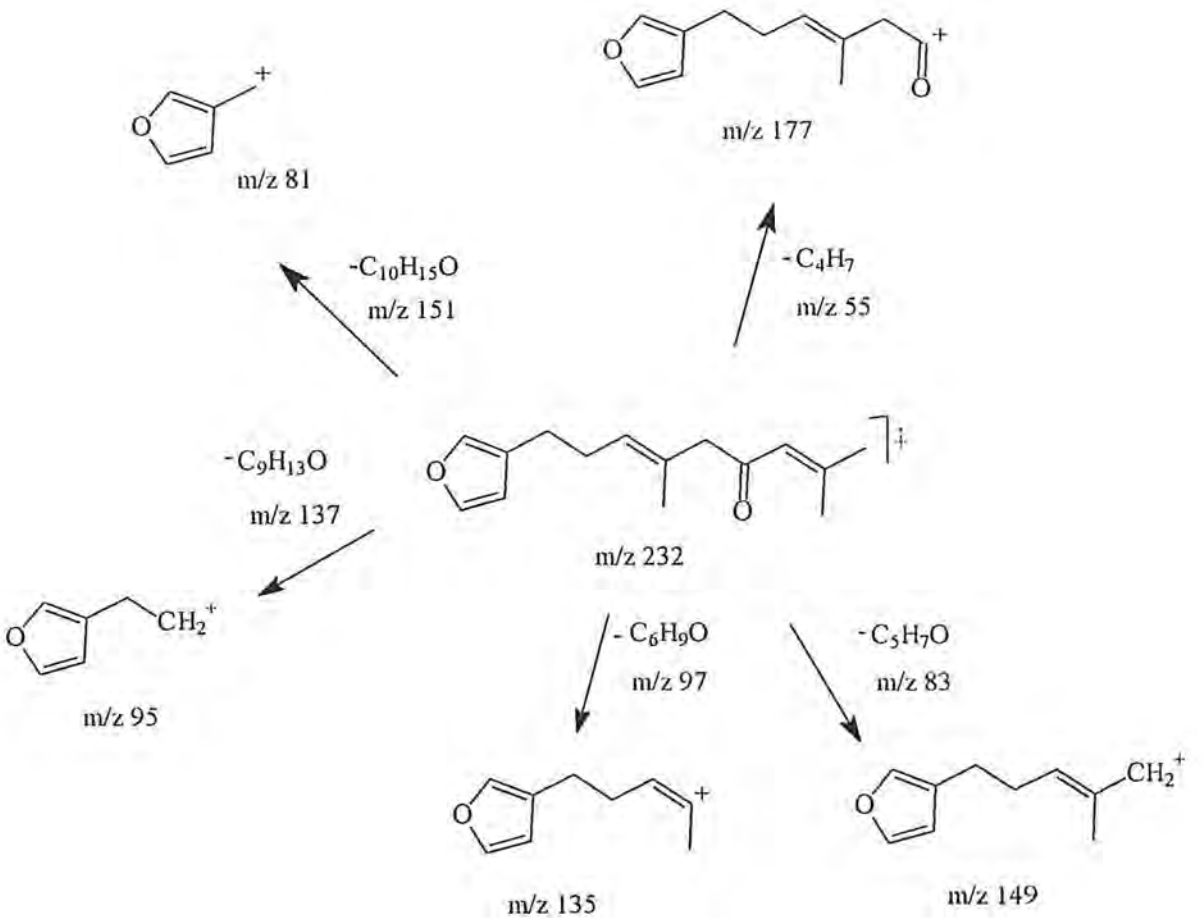

Figure 30. MS fragmentation pattern of 9-(3-furanyl)-2,6-dimethyl-2,6-nonadien-4-one

Copaene, a tricyclic sesquiterpenoid was amongst the several sesquiterpenoids and sesquiterpenoid derivatives identified .from the hydrodistilled oil of the leaves of *C. swynertonii*. The confirmation of the oil components was through high resolution mass spectrometry instead of the simpler and less cumbersome GC-MS and co-injection with standard samples technique. This was due to the fact that the components of this oil with the exception of copaene and isocarryophyllene were not readily available on the market.

Copaene was isolated for the first time in 1914 from the African copaiba balsam oil.[117] Since then, it has been identified in essential oils of different plants.[118]

3.4 Anti-tick activities of *C. swynertonii*, *T. cormicopia*, *T. floribunda*, *T. abyssinica* and *M. volkensii*

In this study we report acaricidal activity of hexane and ethyl acetate extracts and pure components of the stem bark of *C. swynertonii*. The acaricidal and repellency activities of the oil obtained by hydrodistillation of the leaves of this plant are also reported. Structural elucidation of the pure components and identification of the components of the oil is also discussed. In this study, reduced effects of methanol extracts of *T. cormicopia*, *T. abyssinica*, *T. floribunda*, *M. volkensii* and dichloromethane extract are discussed. The study also included isolations of pure principles from the methanol extract of *T. floribunda*.

3.4.1 Repellency activities

Tables 8a and 8b summarise repellency activities of the oil obtained by hydrodistilation from the leaves of *C. swynertonii* and the two identified components of the oil, α-copaene and isocaryophyllene. α-Copaene and isocaryophyllene exhibited repellency activity against *R. appendiculatus* at the concentrations of 0.001% v/v and 10% v/v, respectively. The repellency activity of α-copaene was comparable to the commercial insect repellant, DEET.

Table 8a. Mean percentage repellencies (± S.E) of the oil obtained from the leaves of *C. swynertonii*, two of its identified components and DEET to *R. appendiculatus*

	Test material			
Dose v/v (µl)	Oil	Isocaryophyllene	α-Copaene	DEET
10	87.3 ± 2.7^{b}	55.7 ± 0.7^{c}	100.0 ± 0^{a}	100.0 ± 0^{a}
1	71.0 ± 3.5^{b}	43.8 ± 1.8^{c}	100.0 ± 0^{a}	100.0 ± 0^{a}
0.1	48.3 ± 3.8^{c}	33.2 ± 0.8^{d}	83.8 ± 3.1^{b}	100.0 ± 0^{a}

Mean values with the same letters within the same dose are not significantly different at 5% level.

Table 8b. Mean percentage repellencies (± S.E) of a-copaene and DEET

	Test material	
Dose v/v(ml)	α-Copaene	DEET
10	100.0 ± 0^{a}	100.0 ± 0^{a}
1	100.0 ± 0^{a}	100.0 ± 0^{a}
0.1	83.8 ± 3.09^{b}	100.0 ± 0^{a}
0.01	66.8 ± 1.11^{b}	94.9 ± 0.86^{a}
0.001	50.8 ± 1.77^{b}	86.0 ± 0.95^{a}

Mean values with the same number within the same dose are not significantly different at 5% level.

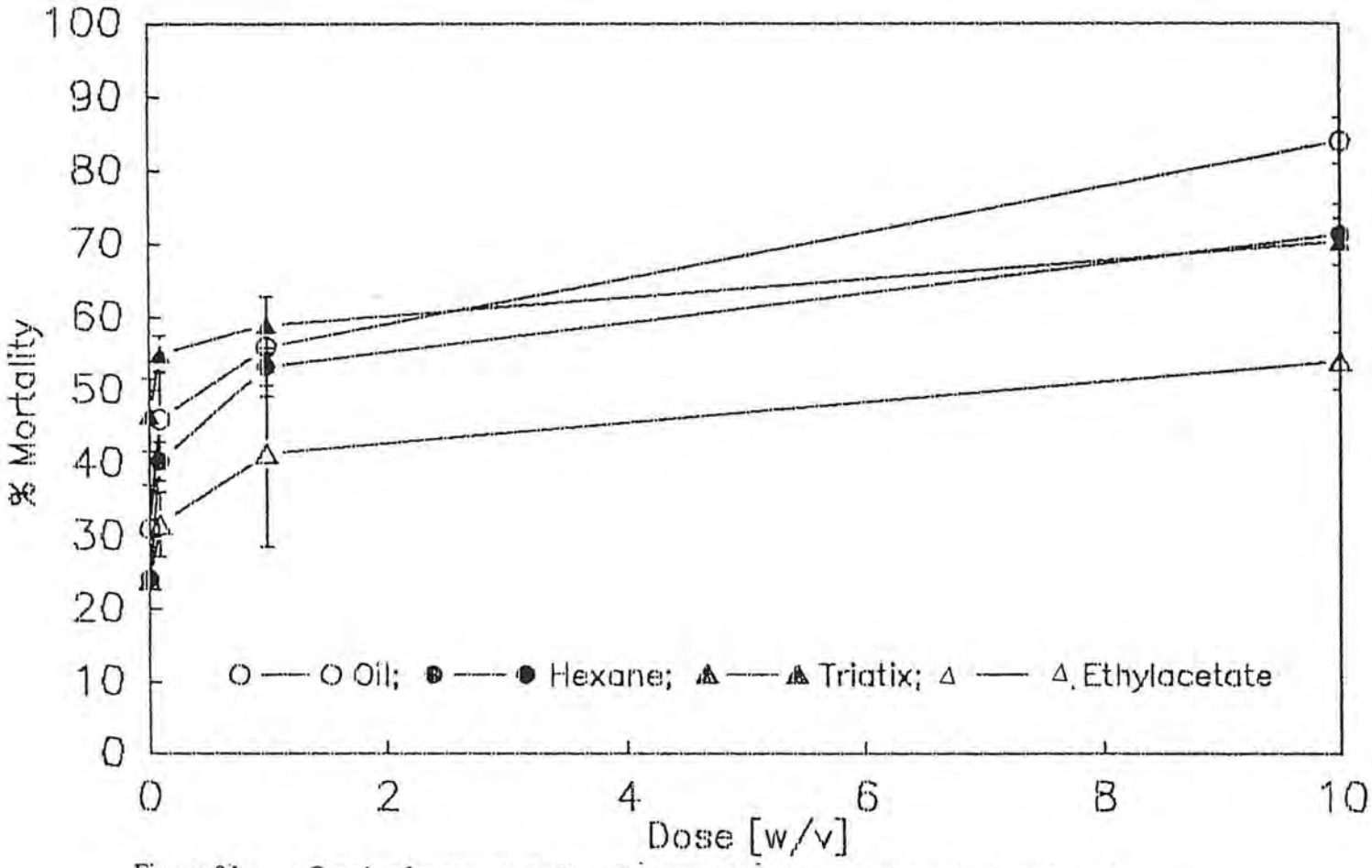

Figure 31. Graph of mean mortality of larvae of *R. appendiculatus* (adjusted data)

A study by Lwande and his co-workers [119] found out that *Cleome monophylla* (Capparidaceae) essential oil and its constituents were tick (*R. appendicullatus*) and maize weevil (*Sitophilus zeamais*) repellants. They also found out that 1-α-terpenol, 2-dodecanone and carvacrol had repellencies comparable to DEET at concentrations of 0.1% v/v and 0.01% v/v.

Some sesquiterpenoids isolated from the genus *Commiphora* showed repellency activity against *R. appendicullatus*. For instance, sesquiterpenoids, furanogermacrene , linderstrene and furanoeudesma-1,3-diene isolated from the hexane fraction of the myrrh oil of *C. myrrh* showed repellency activity towards *R. appendicullatus* and house flies, *Musca domestica*.[51]

3.4.2 Acaricidal activities of *C. swynertonii*

The hexane and ethyl acetate extracts (Table 9) of the stem bark *C. swynertonii* and the oil obtained by hydrodistillation of the leaves of *C. swynertonii* killed larvae of *R. appendiculatus* but were inactive against the adults.

Table 9 **LD_{50} of extracts of *C. swynertonii*, triatix and the oil from leaves of *C. swynertonii***

	LD_{50}	Lower limit	Upper limit
Hexane extract of *C. swynertonii*	0.02519	0.01511	0.03830
Ethyl acetate extract of *C. swynertonii*	0.08933	0.05713	0.1343
Methanol extract of *C. swynertonii*	12.29153	-	-
OIL	0.03573	0.02681	0.046656
TRIATIX	3.5*10-5	$1.5*10^{-5}$	$6.9*10^{-5}$

Dipeolu et. al,[45] reported that "Kupetaba", a ground mixture of dried tobacco leaves and a mineral called "Magadi" soda prevented completion of feeding, suppressed oviposition and hatchability of eggs in *R. appendiculatus* and also caused high mortality in this species of ticks. At a concentration of 6.25% the oil of hexane extract of the dry wood of *Margaritaria discoidea* (Euphorbiaceae) killed 100% nympal *R. appendiculatus*, 100% adult *R. appendiculatus* and 100% adult *Amblyomma variegatum*.[120] It was also found that application of a 50% concentrated oil extract on rabbit ears caused a complete inhibition of attachment by adult *R. appendiculatus* and *A. variegatum* for at least 4 days, and when applied on adult ticks engorging on rabbits, it induced mortalities of 70% and 97% in *A. variegatum* and *R. appendiculatus*, respectively. Malonza and his co-workers[30] reported that *Gynandropsis gynandra*, a shrub plant, is capable of killing and repelling all instars of *R. appendiculatus* and *A. variegatum*.

In this study, we found that the hexane extract exhibited a stronger acaricidal activity than the ethyl acetate or methanol extracts suggesting that the active compounds are non polar. The activity of the hexane extract was comparable to that of Triatix, a commercial acaricide, at 0.01% (v/v). Carol and co-workers[121] reported that the hexane extract of the gum of *C. erythreae* Engler has both larvicidal and repellant activities against *A. americanum*, *Dermacentor variabilis* and a repellence activity against adults of *Ixodes dammini*, *D. variabilis* and *A. americanum*.

The LD_{50} of the test materials are tabulated on Table 9. The LD_{50} of triatix (0.00035µl/ml) was the highest while methanol extract of the stem bark of *C. swynertonii* was the least active with an LD_{50} of 12.29153µl/ml.

The acaricidal activities of the sterols, 3β, 4α, 5-cholest-7-en-3-ol, β-sitosterol, cholest-7-en-eb-ol and γ-sitosterol were relatively weak against *R. appendicullatus*. Stigmasterol and hexadecane-1,2-diol were not tested because they were in minute

quantities. β-Sitosterol showed a slightly higher activity as compared to the others (Table 10).

Four out of the six Δ^5 and Δ^7 -unsaturated phytosterols isolated from the stem bark of *C. swynertonii* showed weak acaricidal activity against *R. appendicullatus*. However, the activity of β-sitosterol was slightly higher as compared to the other phytosterols isolated from this plant. The mortality of of *R. appendicullatus* larvae due to the effect of the screened phytosterols is shown on Table 10. The level of mortality displayed by these products is weak compared to percentage mortality of the crude extracts. This was probably due to synergistic effect of constituents. In addition, the minor components isolated in insufficient amounts could not screened due to insufficient amounts.

Table 10 Acaricidal activities of some pure compounds isolated from *C. swynertonii*

Compound	Mean mortality
Control	0
41	23.89 ± 1.4^{c}
42	39.82 ± 2.05^{a}
43	30.56 ± 3.21^{b}
44	14.29 ± 2.03^{d}

3.4.3 Residual effects of the extracts of the meliaceae species

Extracts of *T. cormicopia*, *T. floribunda*, *T. abyssinica* and *M. volkensii* were inactive against the larvae and adult *R. appendiculatus*. Due to this reason, long-term observations on the effects of these extracts were carried out.

3.4.3.1 Effect of extracts on tick attachment to the ears of rabbits

It was found that during the first 12 to 36 hours after the female ticks were placed on the ears of rabbits, the methanol extracts of *T. cormicopia* and *T. floribunda* delayed the ticks from attaching themselves to the ears of rabbits in day three (Table 11) as compared to the other extracts. However, all the extracts were not significantly different from one another in days one and two.

Table 11. Mean number (± S.E) of ticks attached to the ears of ticks (Transformed data)

Extract	Day 1	Day 2	Day 3
CONTROL	2.91 ± 0.09	1.77 ± 0.09	0.71 ± 0
M. volkensii (dichloromethane)	1.95 ± 0.08	2.41 ± 0.06	1.34 ± 0.12
M. volkensii (methanol extract)	2.79 ± 0.15	1.73 ± 0.26	0.71 ± 0
T. abyssinica (methanol extract)	2.52 ± 0.49	1.71 ± 0.53	0.99 ± 0.29
T. cormicorpia (methanol extract)	2.24 ± 0.25	1.93 ± 0.22	1.55 ± 0.19
T. floribunda (methanol Extract)	2.54 ± 0.11	1.77 ± 0.09	1.46 ± 0.12

Mean values with the same letter are not significantly different at 5%

3.4.3.2 Effect of the extracts on engorgement

Methanol extracts of *T. cormicopia* and *T. floribunda* slowed down the engorgement of the ticks during the first two days but lost activity thereafter.

Table 12. Mean number (± S.E) of engorged ticks on a daily basis (Transformed data)

	Time (days)						
Extract	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
CONTROL	2.91 ± 0^{a}	1.68 ± 0.96^{a}	1.65 ± 0.21^{a}	0.71 ± 0^{c}	0.71 ± 0^{b}	0.71 ± 0^{c}	0.71 ± 0^{b}
M. volkensii (methanol extract)	1.94 ± 0.18^{b}	$0.88 \pm .17^{bc}$	1.68 ± 0.09^{ab}	1.72 ± 0.33^{a}	1.05 ± 0.17^{b}	0.88 ± 0.17^{bc}	0.88 ± 0.17^{b}
T abyssinica (methanol extract)	1.77 ± 0.09^{b}	1.86 ± 0.16^{a}	1.56 ± 0.19^{ab}	1.34 ± 0.12^{ab}	1.05 ± 0.17^{b}	0.71 ± 0^{c}	0.88 ± 0.17^{b}
M. volkensii (methanol extract)	1.22 ± 0^{c}	$1.05 \pm .17b^{c}$	1.68 ± 0.09^{ab}	0.88 ± 0.17^{bc}	1.05 ± 0.17^{b}	1.77 ± 0.09^{a}	1.65 ± 0.21^{a}
T. cormicopia (methanol extract)	1.05 ± 0.17^{c}	0.71 ± 0^{c}	1.34 ± 0.2^{b}	1.68 ± 0.09^{a}	1.95 ± 0.08^{a}	1.05 ± 0.17^{bc}	0.71 ± 0^{b}
T. floribunda (methanol extract)	0.87 ± 0^{c}	0.71 ± 0.14^{c}	1.34 ± 0.14^{b}	1.78 ± 0.11^{a}	2.19 ± 0.21^{a}	2.20 ± 0.17^{a}	2.25 ± 0^{a}

Mean values with the same letters within the same day are not significantly different at 5% level.

3.4.3.3 The effect of extracts on the weight of ticks

The effect of the extracts were not significantly different from the control. (Table 13)

Table 13. Mean weight (± S.E) of ticks

Extract	Mean weight of ticks
CONTROL	1.0 ± 0.1^{a}
T.cormicopia (methanol extract)	0.98 ± 1.8^{a}
M.volkensii (methanol extract)	0.98 ± 0^{a}
M. volkensii (methanol extract)	0.97 ± 0^{a}
T.abyssinica (methanol extract)	0.97 ± 0.02^{a}
T.floribunda (methanol extract)	0.97 ± 0.01^{a}

Mean values with the same letters are not significantly different at 5% level.

3.4.3.4 Effect of extracts on oviposition

On the first day, the extracts were signifficantly different from the control. The dichlomethane extract of *M. volkensii*, and the methanol extracts of *T. cormicopia* and *T. floribunda* (Table 14) were significantly different from the other extracts.

Table 14. Mean number (± S.E) of ticks which have oviposited (Transformed data)

	Time (days)				
Extract	Day 1	Day 2	Day 3	Day 4	Day 5
Control	2.92 ± 0^{a}	0.86 ± 033^{b}	1.46 ± 0.33^{a}	0.71 ± 0^{a}	0.71 ± 0^{b}
M. volkensii (methanol extract)	1.86 ± 0.58^{b}	1.17 ± 0.58^{ab}	2.0 ± 1.20^{a}	1.57 ± 1.20^{ab}	0.71 ± 0^{b}
T. abyssinica (methanol extract)	1.77 ± 0.33^{c}	1.34 ± 0.33^{ab}	1.27 ± 0.88^{a}	1.05 ± 0.33^{b}	0.71 ± 0^{b}
M. volkensii (methanol extract)	1.22 ± 0^{c}	1.86 ± 0.57^{a}	1.64 ± 0.88^{a}	1.17 ± 0.57^{b}	1.77 ± 0.33^{a}
T. cormicopia (methanol extract)	1.17 ± 0.58^{c}	2.08 ± 1.15^{a}	1.47 ± 1.16^{a}	1.34 ± 0.33^{b}	1.71 ± 0^{b}
T. floribunda (methanol extract)	1.05 ± 0.33^{c}	1.56 ± 0.33^{ab}	1.85 ± 0.57^{a}	2.19 ± 0.33^{b}	0.71 ± 0^{b}

Mean values with the same letters within the same day are not significantly different at 5% level.

3.4.3.5 Effects of extracts on the number of eggs laid

The number of eggs laid by ticks exposed to methanol extracts of *T. abyssinica* (Table 15) was significantly different from the other extracts.

Table 15. Approximate (mean S.E ±) of eggs layed

Extract	Mean (S.E ±) number of eggs
CONTROL	6692.6 ± 243.77[a]
M.volkensii (dichloromenthane extract)	6169.4 ± 673.06[a]
T.cormicopia (methanol extract)	6018.2 ± 162.38[a]
M. volkensii (methanol extract)	5933.4 ± 478.94[a]
T. floribunda (methanol extract)	5221.4 ± 88.82[a]
T. abyssinica (methanol extract)	3459.4 ± 764.35[b]

Mean values with the same letter are not significantly different at 5% level

3.4.3.6 Effect of extracts on hatchability

The percentage hatchability of eggs laid by ticks which had been exposed to methanol extract of *T. floribunda* and *T. cormicopia* were significantly different from the other extracts.

Table 16. Mean (S.E ±) percentage hatchability

Extract	Mean (%) hatchability
CONTROL	73.0 ± 4.64^{a}
M.volkensii (methanol extract)	69.0 ± 4.0^{ab}
T.abyssinica (methanol extract)	59.0 ± 10.04^{abc}
M. volkensii (methanol extract)	45.0 ± 11.62^{bcd}
T.cormicopia (methanol extract)	34.0 ± 4.85^{cd}
T.floribunda (methanol extract)	29.0 ± 3.32^{d}

The results show that the methanol extracts of *T. floribunda* and *T. cormicopia* exhibited higher activities in delaying attachment of ticks on the ears of rabbits, engorgement, oviposition and hatchability. The extracts did not have any effect on weight and number of eggs layed. Methanol extract of *T. abyssinica* reduced the weight of ticks and number of eggs layed. On the other hand, methanol and dichloromethane extracts of *M. volkensii* had least residual effects.

The results of this study indicate that there is no direct relationship between attachment of ticks on the ears of ticks, oviposition, and hatchability on one hand and the weight of engorged ticks blood meal intake and number of eggs layed on the other hand. However, the results show the presence of constituents with long term residual effects on the ticks studied.

CHAPTER 4

4.0 GENERAL DISCUSSION

In the present study, anti-tick activities of the oil obtained by hydrodistillation of the leaves of *C. swynertonii* were carried out on the larvae and adult *R. appendiculatus*. Hexane, ethyl acetate and methanol extracts of the stem bark of *C. swynertonii* and their pure isolates were screned for anti-tick properties against *R. appendiculatus*.

Residual effects of the methanolextracts of *T. floribunda*, *T. cormicopia*, *T. abyssinica* and the dichloromethane extract of *M. volkensii* were carried out on *R. appendiculatus*.

The chemistry of these plant species included analysis of the chemical composition of the essential oil, isolations and characterisation of compounds from the hexane extract of *C. swynertonii* and the methanol extract of the root bark of *T. floribunda*.

4.1 Biological activities of the Meliaceae species

The methanol extracts of *T. floribunda*, *T. cormicopia*, *T. abyssinica* and dichloromethane extract of *M. volkensii* showed no activity when they were screened against the larvae and adult *R. appendiculatus*. The female ticks which were previously treated with these extracts were observed for long term residual effects.

It was found out that the methanol extracts of *T. floribunda* and *T. cormicopia* delayed the previously exposed ticks to the extracts from attaching themselves to the ears of rabbits, slowed down engorgement of adult ticks and reduced oviposition and hatchability of eggs. These delays distrupted the life cycle of *R. appendiculatus*.

Plants belonging to Meliaceae family have been shown to have broad biological spectrum. These activities are attributed to the presence of a class of compounds known as limonoids which are commonly found in this family. These compounds are also common in Rutaceae family.

Four limonoids belonging to the havanensin class were isolated from the the methanol extract of the root bark of *T. floribunda* using HPLC. One of the compounds decomposed on being purified.

The compounds isolated had high molecular weights of more than 650 and they all had 11a and 12b substitution which is typical of this class of compounds. The isolates differed mainly on the nature of the side chain present at C-12 and the presence of either a double bond or an epoxide between C-14 and C-15.

Due to material and time constraints these compounds were not assessed for long term residual effects.

Recommanations

From the results of this study, the following recommendation could be made:
A well formulated crude methanol extracts of *T. floribunda* and *T. cormicopia* may be assessed in field conditions.

Future line for study

The following lines for future research could be suggested based on results of the present study:

1. To assess the residual effects of the pure isolates from the methanol extract of *T. floribunda*,
2. To creen more meliaceae species for long term effects on adult ticks,

3. To assess these materials on othert notorious spicies of ticks like *A. variegatum*.

4.2 Biological activities of C. swynertonii

The present study has showed that the oil obtained by hydrodistillation of the leaves of *C. swynertonii* exhibited both acaricidal and repellant activities against *R. appendiculatus*.

At a concentration of 10v/v (µl) the oil showed a high repellency activity of 87.3%. Its activity diminished to 48.3% at the concentration of 0.1v/v (µl). a-Copaene , identified as one of the components of the oil showed an activity comparable to that of DEET at the concentration of 1v/v (µl). Its activity diminished to 50.8% at the concentration of 0.001 v/v (µl).

The hexane extract of the stem bark of *C. swynertonii* exhibited a higher acaricidal activity than ethyl acetate and methanol extracts of the same plant at the concentration of 10w/v (µl).

Isolates from the stem bark of *C. swynertonii* exhibited weak acaricidal activity against *R. appendiculatus*. This was probably due to active components present being in so small quantities that they could not be isolated or due to synergestic effects.

Five of the six compounds isolated from the stem bark of *C. swynertonii*, were found to have an OH group attached to C-3, two methyl groups attached to C-10 and C-13 and a long aliphatic chain to C-17. The only differences arise from the position of the double bond and the side chain. These compounds are common in several plant species.

A total of nine componds were identified from the hydrodisilled oil obtained from the leaves of *C. swynertonii*. The presence of α-copaene and isocaryophyllene were confirmed by GC co-injection with authentic samples. The remaining componds were identified using PBM and high resolution fragmentation pattern of each components because the corresponding standards were not available.

Bioassay data was based on α-copaene and isocaryophyllene because they were readly available.

Recommandations

From the present laboratory investigations, the following recommandations could be made:

1. The essential oil from the leaves of *C. swynertonii* may be produced for use by farmers after its validation in the field.
2. Being volatile, α-copaene may be formulated and used as a general arthropod repellant.

Suggestions for future study

The following lines for future research could be made based on results of this study:

1. To study the activities of all the components of the essential oil,
2. To screen the exudate of the stem bark of *C. swynertonii* against *R. appendiculatus*

4.3 Conclusion

1. The essential oil from the leaves of *C. swynertonii* has both acaricidal and repellant activities against adult *R. appendiculatus*.
2. The methanol extracts of *T. floribunda* and *T. cormicopia* have long term residual effects on adult *R. appendiculatus* and as such they could be incorporated in the existing intergrated tick management strategies.

3. Plant materials could provide cheap, readly available, safe and easy to handle anti-arthropod products.

CHAPTER 5

EXPERIMENTAL

5.0 EXPERIMENTAL

Thin layer chromatography (TLC) was performed on MN precoated silica gel 60 F254 plates (0.25mm thickness), flush chromatography on silica gel 60 (Merck) (0.063-0.200mm, 70-230 mesh, ASTM), silica gel 60 (0.040-0.063mm, 230-400 mesh, ASTM) and preparative chromatography using MN preparative TLC (0.25mm x 20cm x 20cm). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on NMR spectrometer AC-P 300 with Aspect 3000 (Bruker, Karlsruhe) equipped with automated sample changer and QNP-head for nuclei ^{1}H, ^{13}C, ^{19}F and ^{31}P. Mass spectrometer spectra were recorded on a mass spectrometer VG QUATTRO (FISONS, Manchester) coupled with gas chromatography 5890 11 (Hewlett-Packard, Boblingen) with autosampler CTC AS200.

5.1 BIOLOGICAL ACTIVITY TESTS

5.1.1 Repellency assays

Repellency assays were carried out on the oil against adult *R. appendiculatus* in the climbing bioassay.[119] The climbing system (Figure.31) consisted of two 26cm long metal rods covered with glass tubes, 7cm apart, attached to a metal base placed in a tray of water. A 1cm wide filter paper ring placed 10cm up each rod served as carrier for the test material and control.

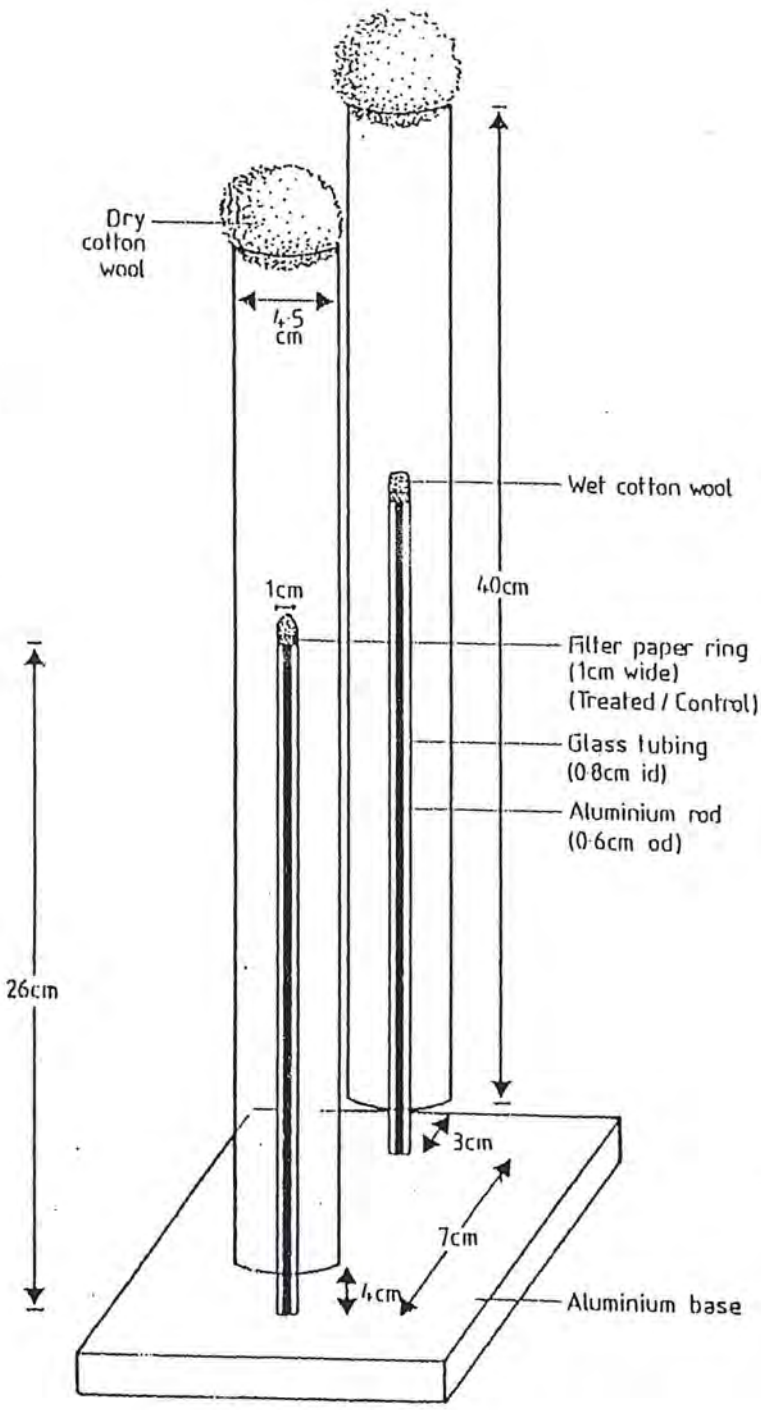

Figure 32. Aggregation bioassay assembly

Stock solutions were prepared by dissolving 100µl of each test material in 1000µl of dichrolomethane. Serial dilutions were carried out on each stock solution to give 7 doses by drawing 100µl aliquots from each subsequent prepared dose and dissolving it in 1000µl of dichloromethane. 100µl of each dose applied on the filter paper ring was tested against control.

Tests were performed on isocaryophyllene, (-)-α-copaene and DEET which was prepared by standard procedures described previously by Hassanali and his co-workers.[75] About 80 ticks were released into the base of the climbing system assembly. They were left there for 1 hour. Repellency (%) was calculated from the formula [(C-T)/(C+T)]x100 where C represents the number of ticks that arrived on top of the control rod and T represents the number that arrived on top of the rod containing paper strip treated with test material.

5.1.2 Acaricidal activity

Bioassays were carried out on n-hexane, EtOAc and MeOH extracts of the stem bark of *C. swynertonii* for acaricidal properties using the standard method described by Stone.[122]

Pieces of filter papers of desired sizes (9cm x 6cm and 6cm x 4cm) were cut from filter papers (Whatman 1). These papers are folded in such a way that a packet is formed when the open ends are closed using three dog bull clips.

Three of such papers were treated with n-hexane, EtOAc and MeOH extracts at a time. A stock solution of each extract was prepared by dissolving 1mg of sample in 1:2 solution of olive oil and trichloroethylene (TCE). Serial dilutions were made on each stock solution to give the different doses by drawing 100µl aliquots from each subsequent prepared dose and dissolving it in 1000µl of olive oil : TCE (1:2) solution.

The control comprised of olive : TCE (1:2) 500µl of each dose of the test material was applied on the filter papers. TCE was allowed to evaporate leaving behind the test material.

Tests were performed on the extracts by introducing 25 adult ticks through the open end of a treated piece of filter paper and closing it using a third clip. Two week old larvae were introduced into the test area by using a fine brush. The packets were closed as previously described for adults. The packets were laid down flat on a clean bench for 24hours and 48hours for larvae and adults respectively. The bioassays were carried out at room temperature (24-26^{O}C).

Each packet was opened after the exposure period and inspected under a 2x lens. Mobile larvae were picked off by a fine brush, transferred to a wet cotton wool pad and counted. Adult ticks were counted using the naked eye.

5.1.3 Long term effects on adult ticks

10% (w/v) solutions of the test materials (extracts) were prepared as described in 4.1.2. The extracts were:

(i) dichloromethane and methanol extracts of *M. volkensii*

(ii) methanol extracts of the root bark of *T. cormicopia*

(iii) methanol extract of *T. abyssinica*

(iv) methanol extract of *T. floribunda*

Tests were performed on the extracts by introducing 20 adult females of *R. appendiculatus* into the filter papers (described in 4.1.2) which had previously been treated with 500µl of 10% w/v solutions of extracts.

Each packet was opened after 48 hours and the treated ticks were used to infest the ears of rabbits. Three rabbits were used for each extract.

The time used by ticks to attach themselves to the ears of rabbits was recorded. Other data recorded included time taken by each tick to engorge, time taken to oviposit and the total number of eggs laid by each tick.

5.2 ISOLATIONS

5.2.1 Isolates from *T. floribunda*

Preparative High Performance Liquid Chromatography was performed on the Beckman System Gold with a programmable solvent system 126 and a diode array detector module 168, Beckman Instruments, Inc., Fulleton. USA. A CN 10, 4mm x 0.25 in OD x 25cm length column was fitted on the intrument.

A 20 µl of the solution of the methanol extract of *T. floribunda* was used in each injection at a flow rate of 1ml/minute. Four limonoids were isolated, three of them in their pure form. The fourth limonoid (Appendix 4) decomposed on further purification.

Compound (39): Molecular formular $C_{36}H_{50}O_{13}$
EIMS, m/z (relative intensity): 690 (5%) $(M)^+$, 572 (7%) $(M\text{-}C_5H_{10}O_3)^+$, 512 (38%) $(M\text{-}C_5H_{10}O_3 - AcOH)^+$, 452 (14%) $(C_5H_{10}O_3 - 2AcOH)^+$.

Compound (40): Molecular formular $C_{35}H_{48}O_{12}$
EIMS, m/z (relative intensity): 660 (7%) M^+, 614 (25%) $(M\text{-}AcOH)^+$, 572 (11%) $(M\text{-}C_5H_{10}O_2)^+$, 554 (12%) $(M\text{-}H_2O\text{-}C_5H_{10}O_2)^+$, 512 (34%) $(M\text{-}C_5H_{10}O_2\text{-}AcOH)^+$, 452 (10%) $(M\text{-}C_5H_{10}O_2\text{-}2AcOH)^+$.

Compound (41): Molecular formular $C_{36}H_{50}O_{11}$

18-Nor-4α-carbomethoxy-11β-acetoxy12α-(2-methylbutanoloxy)-14,15-eoxyhavenesin -1-acetate. EIMS, m/z (relative intensity): 658 (24%) $(M)^+$, 598 (35%) $(M\text{-}AcOH)^+$, 580 (22%) $(M\text{-}2H_2O\text{-}AcOH)^+$, 556 (20%) $(M\text{-}C_5H_{10}O_2)^+$,538 (29%) $(M\text{-}H_2O\text{-}C_5H_{10}O_2)^+$, 496 (49%) $(M\text{-}C_5H_{10}O_2\text{-}AcOH)^+$, 436 (19%) $(M\text{-}C_5H_{10}O_2\text{-}2AcOH)^+$.

5.2.2 Isolates from *C. swynertonii*

The stem bark and leaves of *C. swynertonii* were collected from Kiteto in December, 1992. The stem bark was air dried and ground to a fine powder at Kenyatta University.

Extractions

About 1.2kg of the powder was extracted sequentially in n-hexane, ethyl acetate and methanol. Each extract was concentrated to dryness by rotary evaporation to afford hexane (8g), ethyl acetate (240g) and methanol (300g) extracts.

Isolations

Both flash and gravitational chromatography were employed in the isolations of pure compounds. About 6.8g of the hexane extract was flash chromatographed using silica gel 60 mesh (Merck) and n-hexane : ethyl acetate gradient solvent system with increasing amounts of ethyl acetate. Six fractions were obtained. Chromatography of the ethyl acetate extract (6g) on silica gel using hexane : ethyl acetate (3:7) at increasing amounts of ethyl acetate yielded 7 fractions.

Purification

Fraction 1 of the hexane extract could not be purified using either gravitational chromatography or preparative TLC. Crystallization of fractions 2-5 of the hexane extract in MeOH (Merck) gave white crystals of compounds (44) - (49). The weights of these compounds were as follows: Compound (44) (25mg), (45) (117mg), (46) (25mg) , (47) (16mg) and (48) (6mg).

Compound (42): Molecular formular $C_{28}H_{48}O$

3β,4α-cholest-7-en-3-ol (42) Mp.134-136°C. MS: m/z 400 (M^+) 386, 367, 287, 269, 245, 227, 213, 173, 161, 147. ^{1}H-NMR (300 MHz, CDC_{13}); δ 0.53 (s, 3H), 0.85 (s, 3H), 0.97 (s, CH-CH_3,), 1.01 (s, CH-CH_3,), 3.13 (m, -CH-OH, J=3.5Hz), 5.21 (dd, -C-CH, J=1.8Hz), 7.3 (s) CDC_{13} impurity.

Compound (43): Molecular formular $C_{29}H_{50}O$

β-Sitosterol (43). Mp 139-141°C. MS: m/z 414.0(M^+), 400, 386, 381, 329, 303, 273, 255, 245, 231, 213, 199, 173, 163. ^{1}H-NMR(300MHz, CDC_{13}); δ 3.15 (m, impurity), 3.51 (br, CH-OH), 5.20 (d, C=C-CH_2), 5.35 (d, C=CH).

Compound (44): Molecular formular $C_{27}H_{47}O$

Cholest-7-en-3b-ol (44). Mp 143-145°C. MS: m/z 387 (M^+), 371, 353, 329, 301, 274, 273, 255, 232, 231, 213, 187, 173, 161. ^{1}H-NMR (300MHz, $CDCl_3$), δ 3.3 (q, CH-OH),5.18 (d, C=C-CH_2), 5.35 (d, C=CH).

Compound (45): Molecular formular $C_{29}H_{50}O$

γ-Sitosterol (45). Mp 144-146°C. MS: m/z 414 (M^+), 400, 396, 381, 329, 303, 275, 273, 255, 231, 213, 173. ^1H NMR (300MHz, $CDCl_3$);δ 3.5 (br. CH-OH), 5.35 (C=C-CH_2)

Compound (46): Molecular formular $C_{29}H_{48}O$

Stigmasterol (46). Mp. 170-172°C. MS: m/z 412 (M^+), 400, 395, 383, 381, 329, 303, 275, 273, 255, 231, 213, 173.

Compound (47): Molecular formular $C_{16}H_{33}O_2$

Hexadecane-1,2-diol (47). MS: m/z 256 (M^+), 255, 241, 227, 167, 153, 127, 125, 111.3. ^1H NMR (300MHz, $CDCl_3$); δ 0.891(d, J=6.4Hz), 1.3 (s, CH_2), 1.59(s, -CH_2-OH), 3.45 (m, - OH), 3.7 (m,- OH).

5.3 Steam distillation of the leaves of *C. swynertonii*

Fresh leaves (600g) of C. swynertonii were collected from Kiteto in December, 1992. The leaves were steam distilled and the distillate was collected over hexane (Merck)(Figure 32). The oil was dried over anhydrous sodium sulphate (Na2SO4) and filtered. The filtrate was further purified by distillation to yied 2.1g of a yellowish green oil.

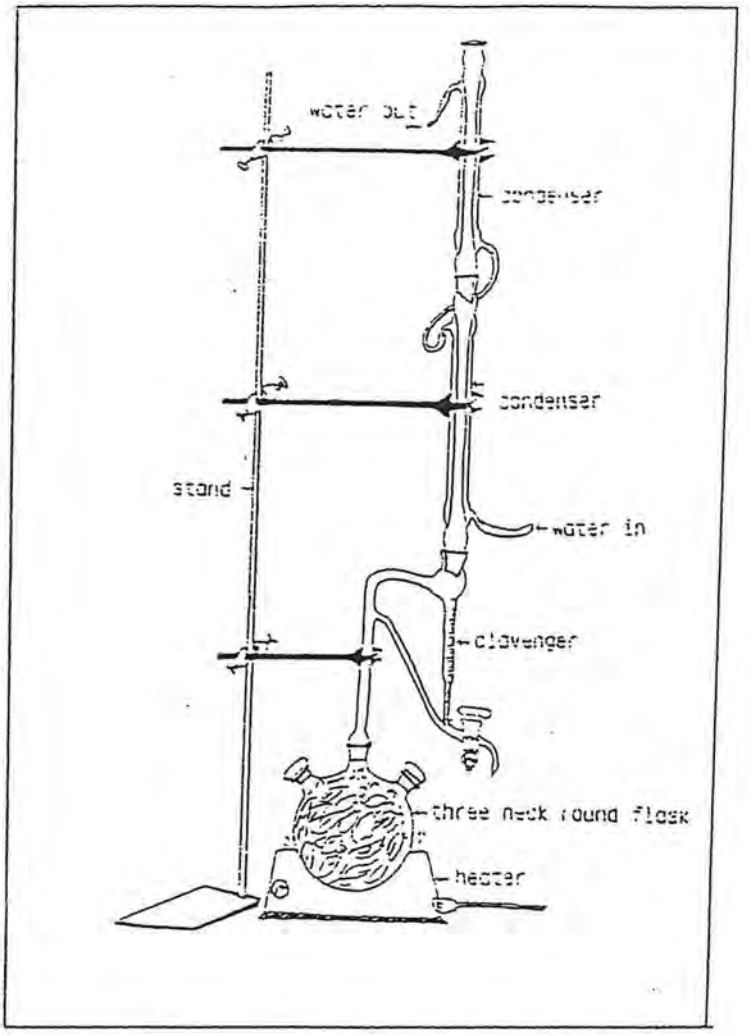

Figure 33. Apparatus for collecting steam distillate from *Commiphora*.

5.3.1 Analysis of the hydrodistilled oil

The oil was analysed by GC-HRMS to give 14 major components. The structures of the sesquiterpenoids and their derivatives were deduced by the probability based matching system (PBM) 100 and examination of the fragments of the respective components. The fragments of these components are shown below.

Compound (48): Molecular formular $C_{15}H_{24}$

α-Copaene (48). Mass 204.18189 tabulated (tab), 204.18781 calculated (cal)

m/z 204 (M^+), 189, 162, 161, 147, 141, 136, 133, 131, 120, 119, 105, 93.

Compound (49): Molecular formular $C_{15}H_{24}$

1-Ethenyl-1-methyl-2,4 diisopropenyl-cyclohexane (49). Mass 204.18353 tab,

204.18781 cal.m/z 204 (M^+), 190, 175, 161, 147, 135, 122, 93, 67, 42.

Compound (50): Molecular formular $C_{15}H_{24}$

Isocaryophyllene (50). Mass 204.19173 tab, 204.18781 cal. m/z 204 (M^+), 189, 175, 147, 133, 121, 105, 94, 79, 69, 54.

Compound (51): Molecular formular $C_{15}H_{24}$

1,1,4,8-Tetramethyl-4,7,10-cycloundecatriene (51)

Mass 204.18651 tab, 204.18781 cal, m/z 204 (M^+), 175, 147, 136, 121, 108, 106, 93, 79, 68, 51.

Compound (52): Molecular formular $C_{15}H_{24}$

4-Methyl-1-1,5-dimethyl-4-hexenyl-benzene (52). Mass 204.18553 tab,

204.18781 cal.m/z 204(M^+), 202, 190, 175, 159, 145, 132, 103, 83, 68, 42.

Compound (53): Molecular formular $C_{15}H_{22}O$

Furan, 3-(4,8 dimethyl, 3,7-nonadienyl)-,E (53) . Mass 218.16618 tab, 218.16707 cal.

m/z 218(M^+), 203, 175, 149, 136, 123, 94, 51, 41.

Compound (Unidentified)

(Peak 7) Rt 13.58

Mass 234.15810 tab, 234.16199 cal - $C_{15}H_{22}O_2$

235.19594 tab, 235.19093 cal - $C_{12}H_{27}O_4$

m/z 234(M^+), 213, 192, 177, 149, 135, 105, 91, 78, 42, 41.

Compound (Unidentified)

Unidentified (Peak 8)

($C_{15}H_{24}O$)

Mass 234.15971 tab

234.16199 cal

m/z 234 (M^+), 220, 204, 177, 147, 138, 133, 123, 107, 95, 66, 54, 42.

Compound (Unidentified)

Unidentified (Rt 14.37)

($C_{15}H_{25}O_2$)

Mass 237.18517 tab

237.18546 cal

m/z 236(M^+), 230, 204, 179, 148, 134, 121, 85, 82, 56, 41.

Compound (Unidentified)

Unidentified (Rt 14.52)

($C_{15}H_{23}O_2$)

Mass 235.17681 tab

235.16980 cal

m/z 234 (M^+), 218, 191, 177, 148, 125, 121, 95, 83, 81, 56, 41.

Compound (54): Molecular formular $C_{15}H_{20}O_2$

2,6-Nonadien-4-one, 9-(3-furanyl)-2,6-dimet (54). Mass 232.14372 tab, 232.14633 cal

m/z 232 (M^+), 217, 188, 177, 149, 134, 103, 83, 66, 53.

Compound (55): Molecular formular $C_{15}H_{20}O_2$

2,5-Nonadien-4-one, 9-(3 furanyl)-2,6-dimet (55). Mass 232.14968 tab, 232.14633 cal

m/z 232 (M^+), 217, 189, 177, 149, 138, 123, 83, 66, 54, 41.

Compound (55):

Mass 233.14669 tab,233.15416 cal, m/z 232(M^+), 217, 189, 177, 148, 138, 123, 83, 82, 54, 41.

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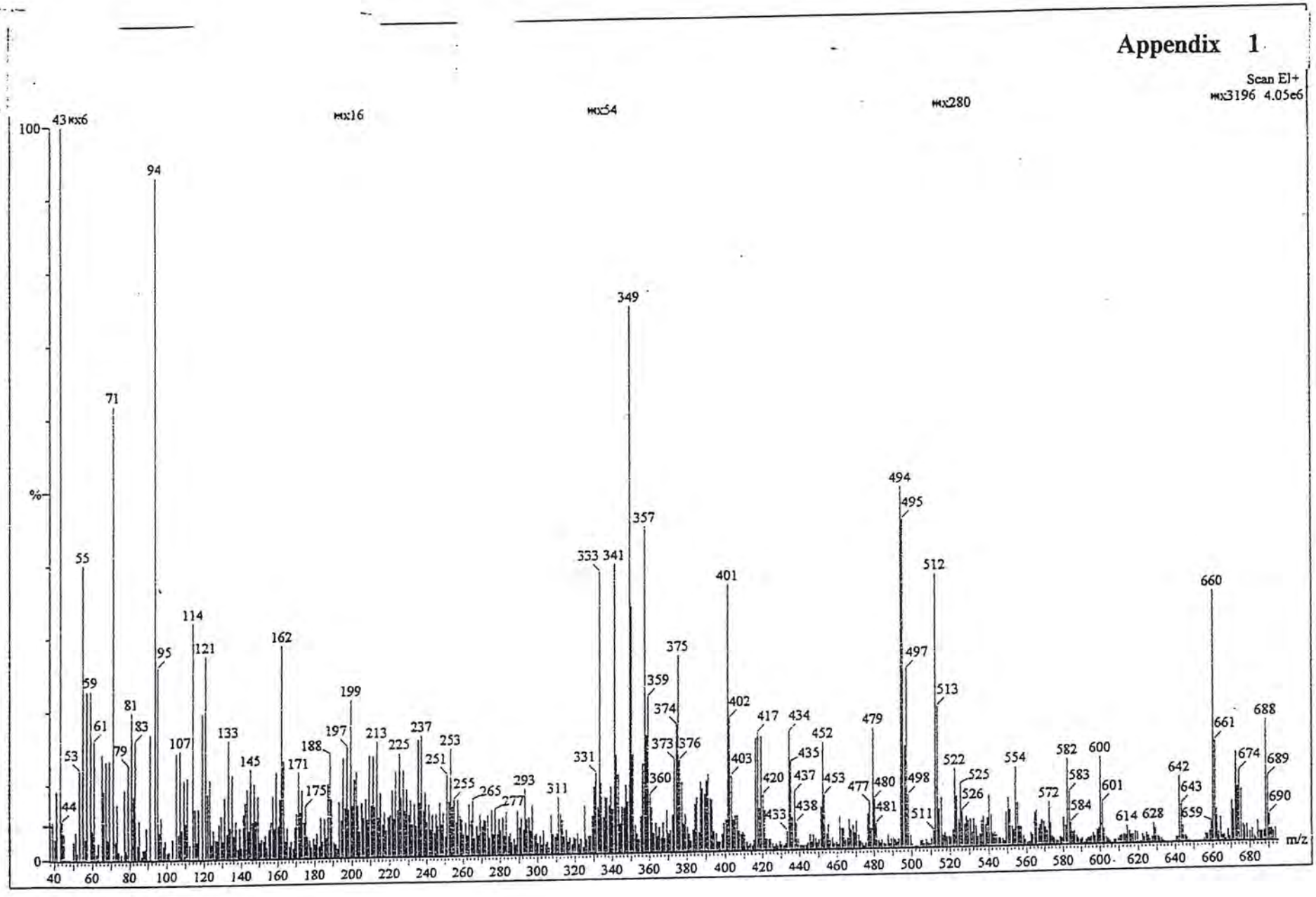
Appendix 1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mx280
43 mx6
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%
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m/z
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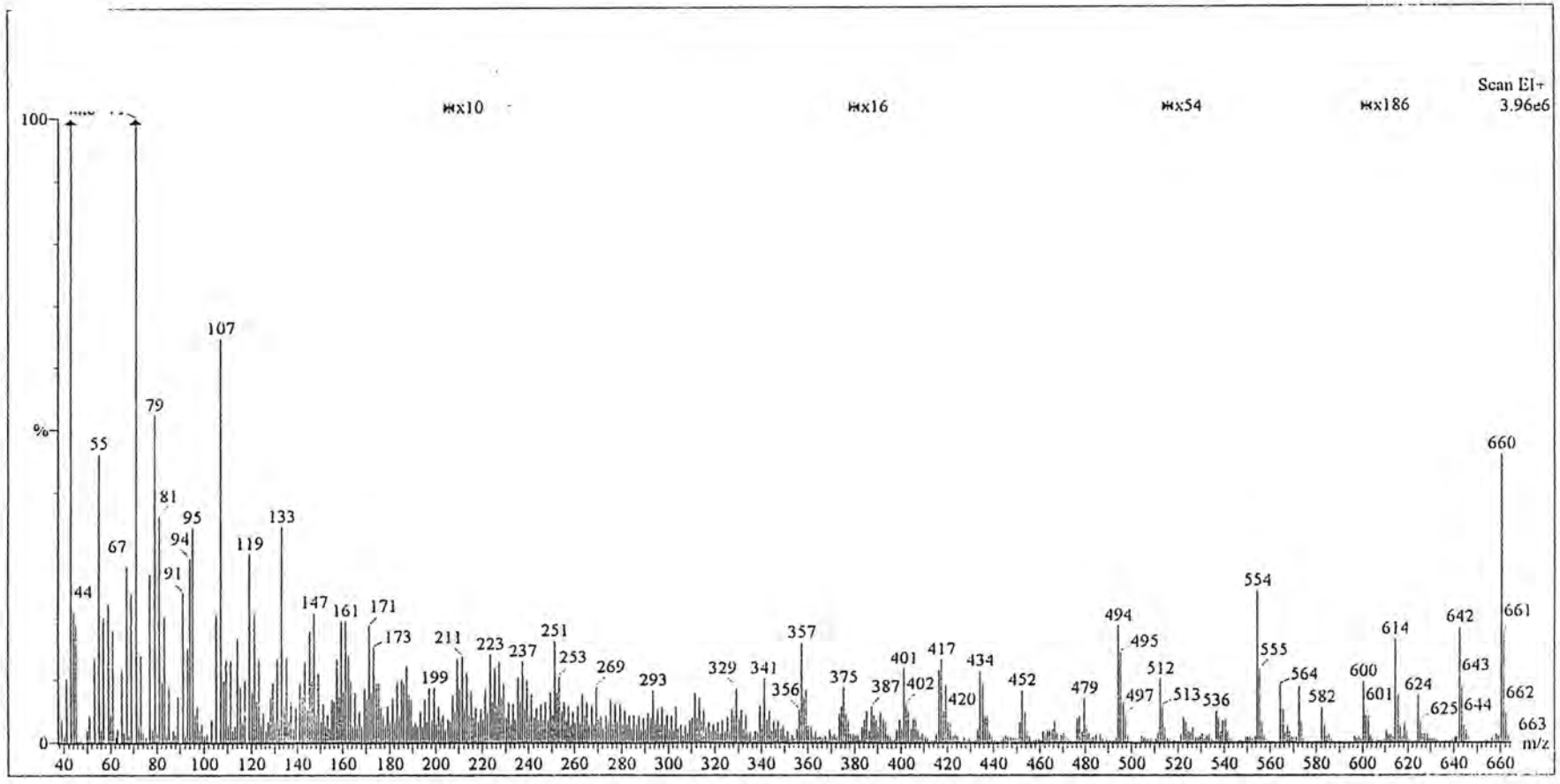
44 53 55 59 61 71 79 81 83 94 95 107 114 121 133 145 162 171 175 188 197 199 213 225 237 251 253 255 265 277 293 311 331 333 341 349 357 359 360 373 374 375 376 401 402 403 417 420 433 434 435 437 438 452 453 477 479 480 481 494 495 497 498 511 512 513 522 525 526 554 572 582 583 584 600 601 614 628 642 643 659 660 661 674 688 689 690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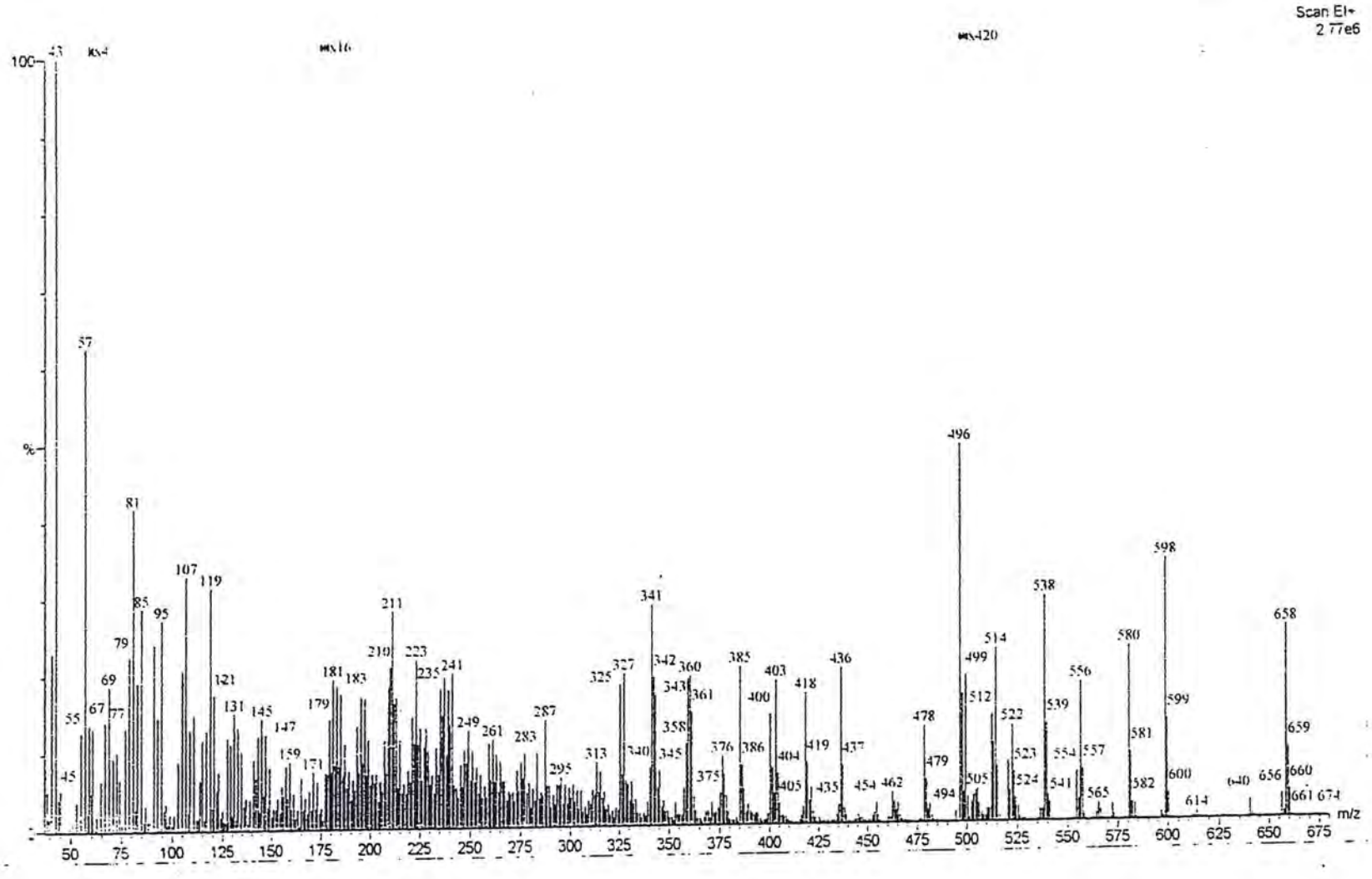
Appendix 3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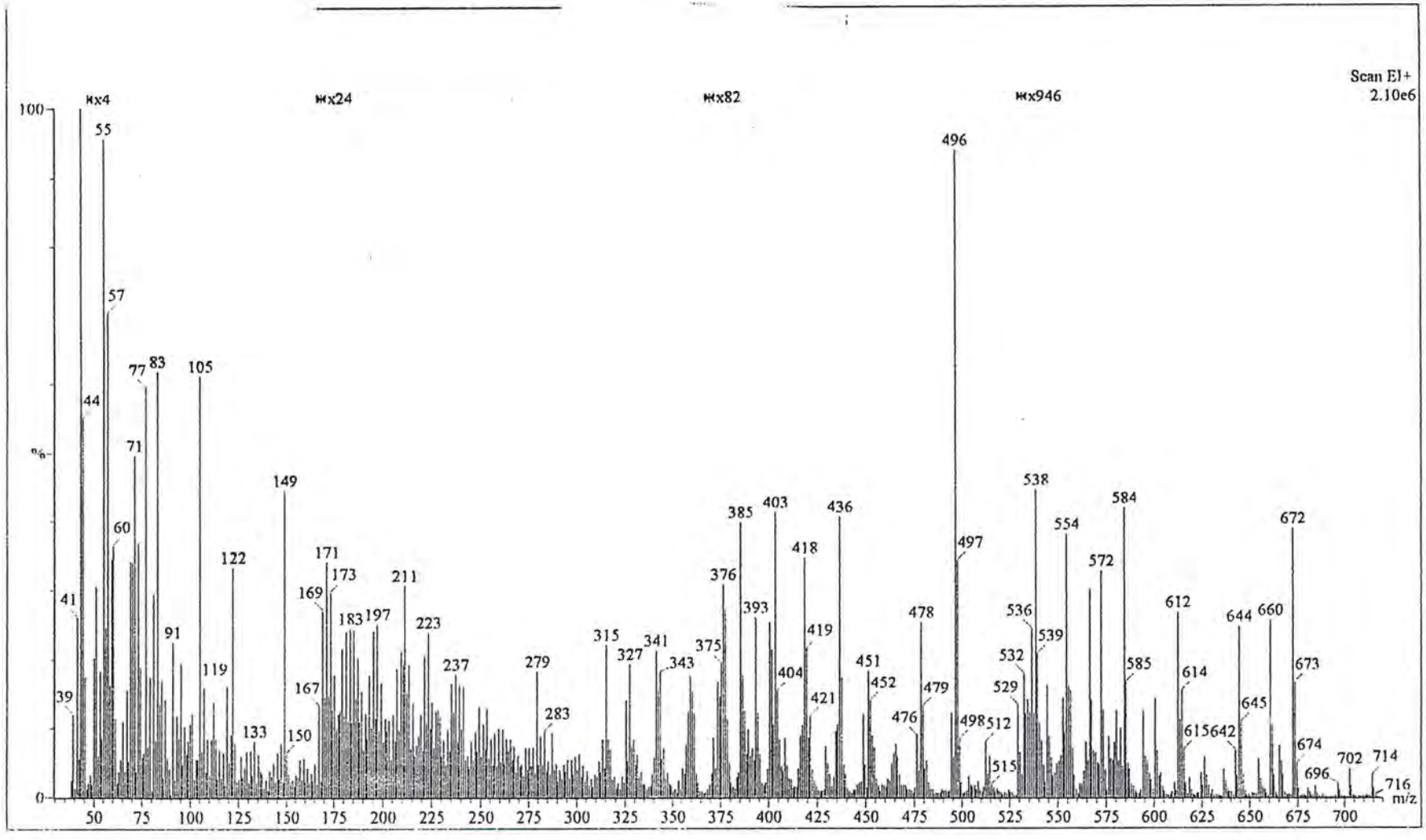
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m/z
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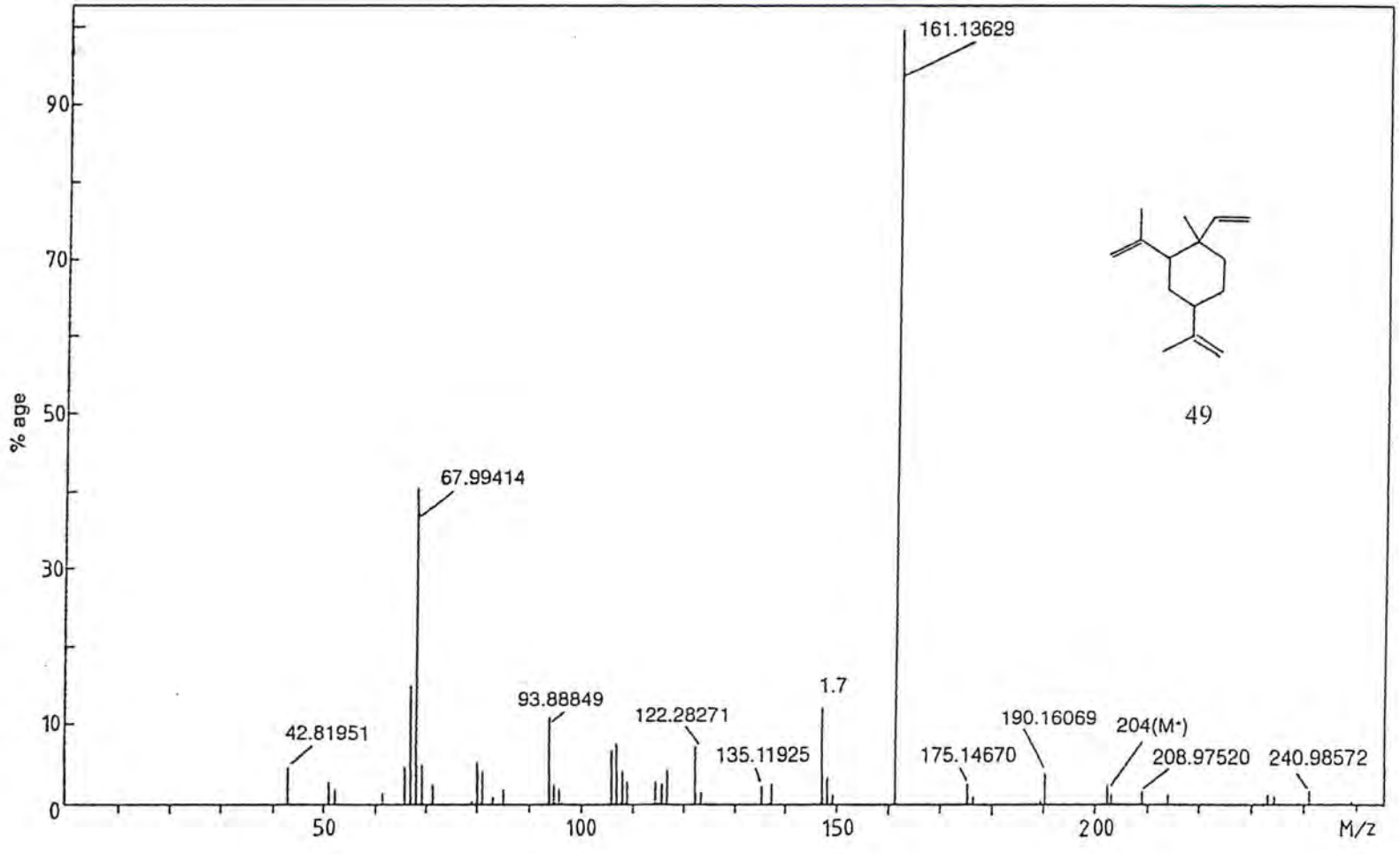
Appendix 5



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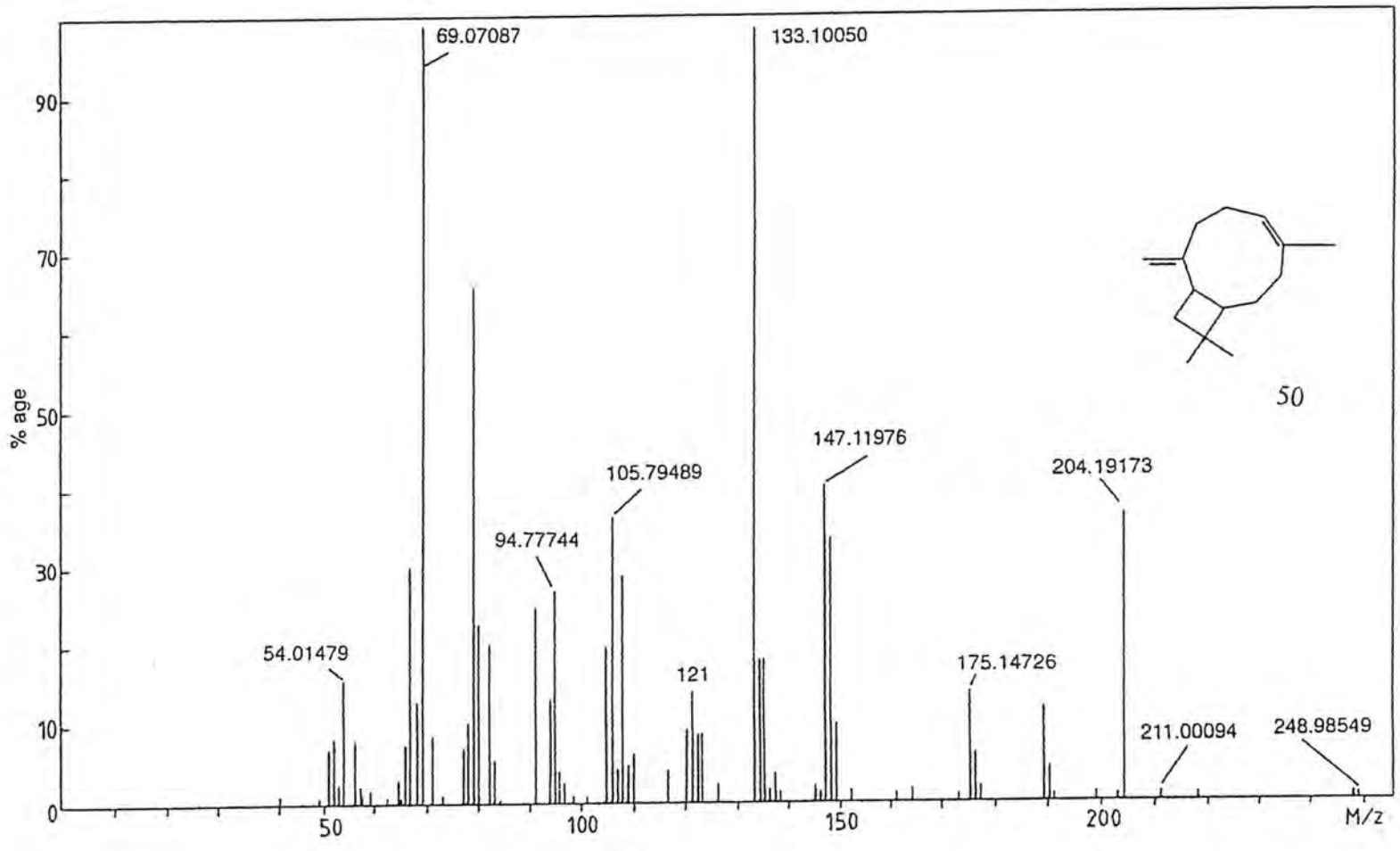
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121
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211.00094
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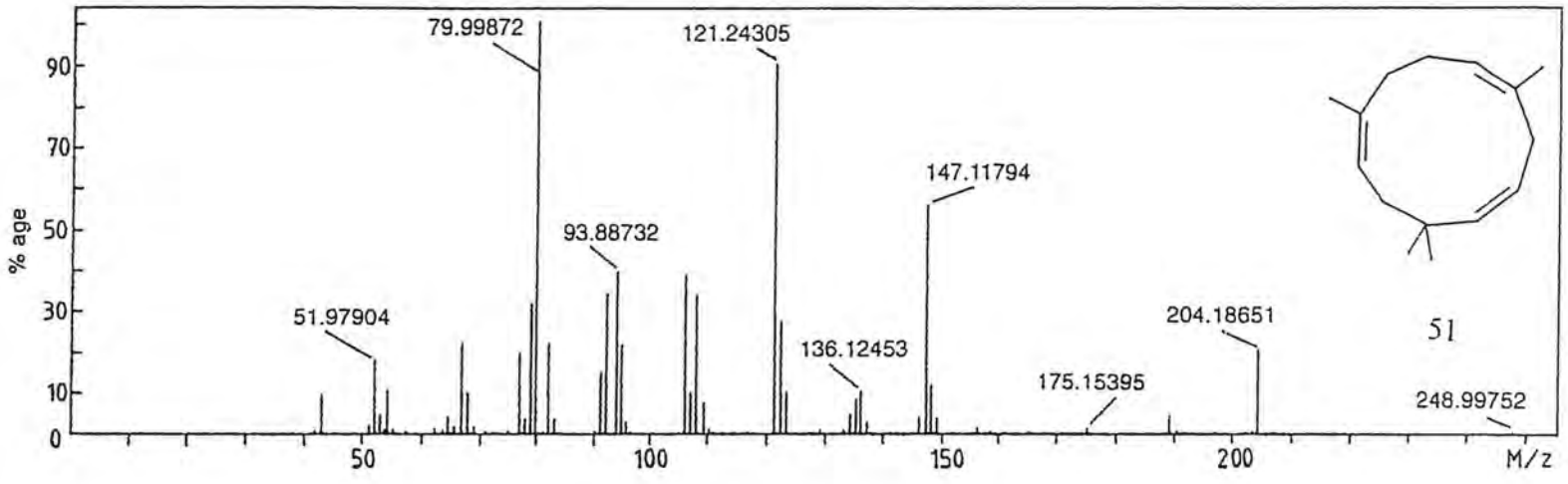
50
100
150
200
M/z

50

Appendix 7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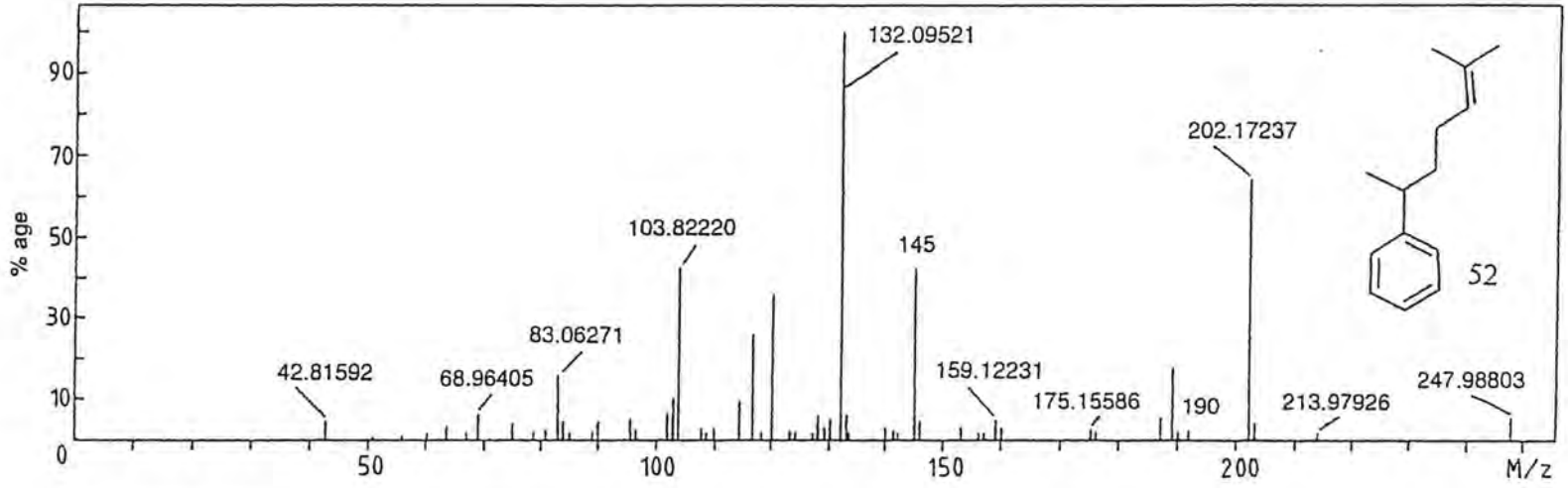
% age
M/z
51

Appendix 8



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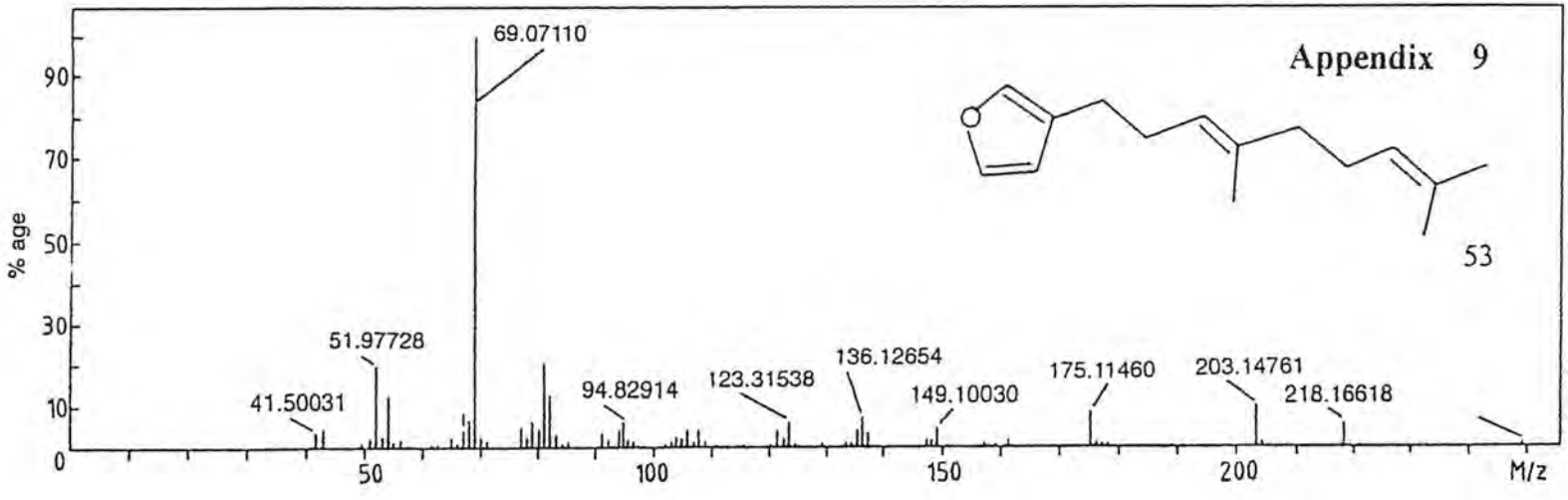

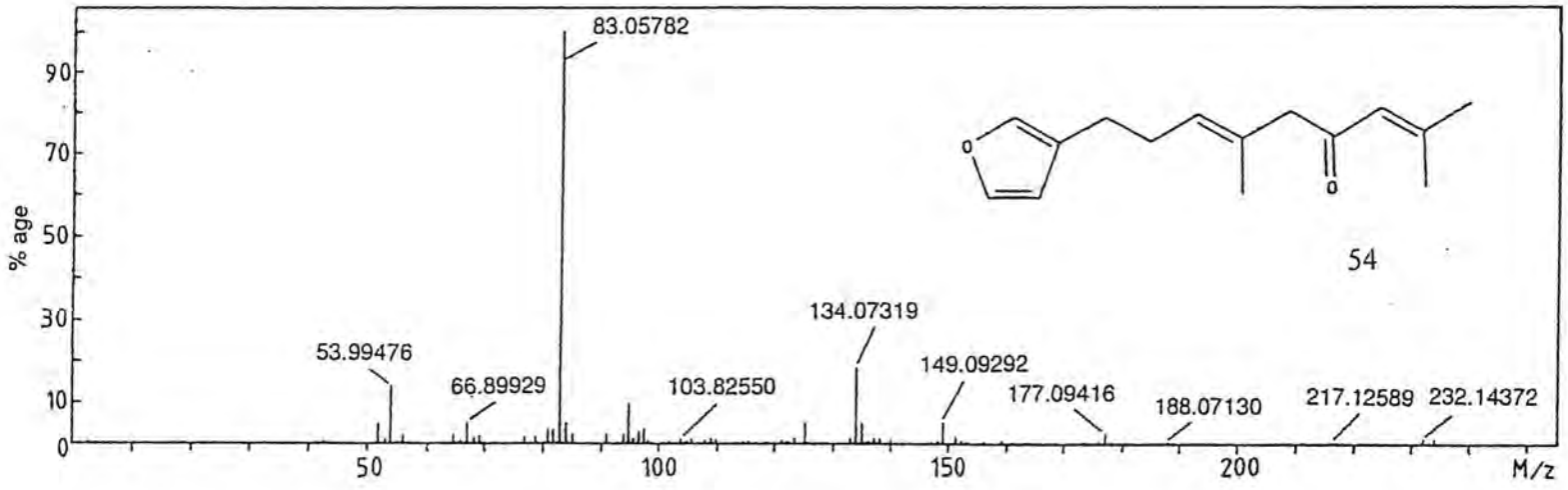
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232.14372
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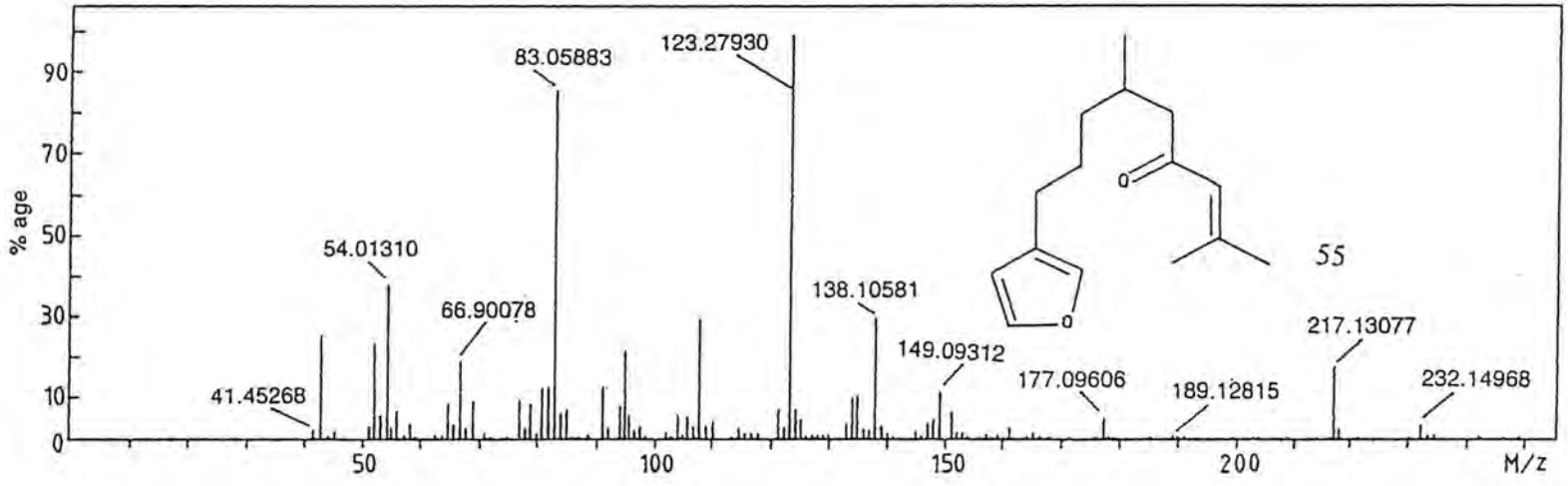
100
150
200
M/z
O
O

54

Appendix 11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150
200
M/z
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41.45268
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232.14968
O
O
55