CHEMICAL INVESTIGATION OF *ISIHLAMBEZO* OR TRADITIONAL PREGNANCY-RELATED MEDICINES

A thesis submitted in partial fulfillment of the requirements for the degree of

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by

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DECLARATION

The experimental work described in this thesis was carried out in the Chemistry Department at Mangosuthu Technikon and the Physiology Department at the University of Kwa-Zulu Natal under the supervision of Professor M. F. Dutton.

These studies represent original work by the author and have not been submitted in any other form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.

Signed: ----------

K. B. Brookes

I hereby certify that the above statement is correct.

Signed: ----------

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ABBREVIATIONS

Ac	acetate
ACh	acetylcholine
CI-MS	chemical ionization mass spectrometry
COSY	correlated spectroscopy
d	doublet
dd	double doublet
DABCO	diazabicyclo-octane
DEPT	Distortionless Enhancement by Polarisation Transfer
DMF	dimethylformamide
DMSO	dimethylsulphoxide
EI-MS	electron impact mass spectrometry
FAB-MS	fast atom bombardment mass spectrometry
HMBC	heteronuclear multiple bond coherence
IR	infrared
m	multiplet
MeOH	methanol
NC-MS	negative ion chemical mass spectrometry
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
q	quartet
t	triplet
TLC	thin layer chromatography
TMS	tetramethylsilane
UV	ultraviolet

ABSTRACT

This study was undertaken to redress the scant knowledge regarding the chemistry and mode of action of pregnancy-related traditional medicines, or *isihlambezo* (Zulu), which are used by 60 to 80% of women in South Africa. The three selected plants are among the six most frequently cited species from the approximately 90 used by traditional healers. The purpose of the study was to identify components which could cause uterine contractions, those with nutritional value for the foetus and mother, and those with any toxic effects.

Plant root extracts were purified via silica gel column chromatography and bioassays were carried out on the fractions, using isolated rat uterine tissue. Purified compounds were identified via spectral techniques, and some were characterised by comparison to authentic standards using HPLC, and others by matching their GC-MS spectra to library standards. Thirty-eight compounds were identified in total, the majority of these being novel to the species concerned. Those isolated from *Combretum kraussii* were **1** sitosterol, **2** combretastatin, **3** 3',4-tri-O-methylellagic acid, **4** combretastatin B-1, **5** combretastatin A-1, **6** 3,3'-di-O-ellagic acid lactone, **7a** ellagic acid lactone, **7b** ellagic acid, **8** and **9** a mixture of combretastatin B-1 and A-1 glucosides, **10** and **11** partly characterised glucosides of ellagic acid. Those isolated from *Gunnera perpensa* were **12** 3',4-tri-O-methylellagic acid, **13** ellagic acid lactone, **14** 1,1'-biphenyl-4,4'-diacetic acid, **15** p-hydroxybenzaldehyde, **16** Z-methyl lespedezate, **17** and **18** partly characterized higher glucosides of Z-methyl lespedezate. Those isolated from *Rhoicissus tridentata* were **19** (-)-epigallocatechin, **20** (+)-gallocatechin, **21** procyanidin B3, **22** procyanidin B4, **23** (+)-catechin hydrate, **24** (+)-mollisacacidin, **25** (+)-epicatechin, **26** fisetinidol-(4α-8) catechin, **27** (-)-fisetinidol, **28** fisetinidol-(4β-8)catechin, **29** gallic acid, **30** epicatechin-3-O-gallate, **31** partly characterized hydrogel of glucose, **32** sitosterol, **33** sitosterolin, **34** γ-sitosterol, **35** oleanolic acid, **36** lupen-3-one, **37** 20-*epi*-ψ-taraxastananol and **38** triacontanol.

The compounds with the greatest *in vitro* uteroactivity were predominantly proanthocyanidins or phenolic glucosides. It is proposed that effects of phenolic glucosides could be due to the interaction of the sugar moiety as well as the phenolic moiety with the receptor site in muscle tissue. The corresponding phenolic aglycones

isolated were only moderately uterotonic, or unreactive by comparison. Non-polar compounds such as sitosterol and sitosterolin showed minimal enhancement of the uterine response at low concentrations, and inhibition at higher concentrations.

Aqueous root extracts of the plants were all found to be non-toxic according to cell-viability tests using monkey vero cells and human fibroblasts. Extracts are therefore considered safe for human consumption, although it is recommended that *Rhoicissus tridentata* be used with caution because it showed the lowest cell viability of the three species, and uterine hyperstimulation has been attributed to this species, as well as CNS depression and respiratory arrest.

Ions which could be nutritionally beneficial in pregnancy, calcium, iron, and phospate, were present in low in aqueous extracts. Levels of calcium and potassium ions were considered to be too low to directly stimulate uterine muscle. Proanthocyanidins, combretastatins, ellagic acid derivatives and phytosterols, with health-promoting properties, were also identified.

PUBLICATIONS AND PRESENTATIONS

The following research outputs have arisen thus far from the current study. Journal publications and some of the oral and poster publications are included. A fourth publication on *Rhoicissus tridentata* is almost complete and will be submitted for publication in the near future.

Journal Publications

1 Veale D J H, Havlik I, Katsoulis L C, Kaido T, Arangies N S, Olivier D W, Dekker T, Brookes K B, and Doudoukina O V. 1998. "The Pharmacological Assessment of Herbal Oxytocics used in South African Traditional Medicine", *Biomarkers and Environment*, 2.3 p.42, from the Symposium on Alternative Medicine: Fact or Fallacy at Charles University, Pilsen, Czechoslovakia, July 1998.

2 Brookes K B, Doudoukina O V, Katsoulis L C and Veale D J H. 1999. "Uteroactive Constituents from Combretum kraussii", *South African Journal of Chemistry,* 52 (4), 127-132.

3 Brookes K B and Smith A N. 2003. "Cytotoxicity of pregnancy-related traditional medicines" *South African Medical Journal*, 93, 25-33.

Presentations

1 Indigenous Plant Use Programme, 2nd Annual Mini-Symposium at Natal University, Nov. 1995. Oral presentation by Brookes K B entitled: "Some Aspects of the Chemistry of *Isihlambezo*". Co-authors: Doudoukina O V, Katsoulis L C and Veale D J H.

2. SACI 33rd Convention Jan.29-Feb.2 1996. Poster presentation entitled: "Biologically Active Components from Plants used in Traditional Pregnancy-related Herbal Medicines or *Isihlambezo*". Brookes K B, Doudoukina O V, Katsoulis L C and Veale D J H.

3 Joint Kwa-Zulu Natal Biochemistry and Microbiology Symposium,15-17October 1997. Oral presentation by Brookes K B: "Isolation of Compounds from Combretum kraussii,

a Uteroactive Traditional Medicine" at the University of Natal, Durban. Co-authors: Doudoukina O V, Katsoulis L C and Veale D J H.

4. 7th International Chemistry Conference in Africa, University of Natal, July 1998. Poster: "Uteroactive Traditional Medicines: Combretum kraussii roots. Co-authors: Brookes K B, Doudoukina O V, Katsoulis L C and Veale D J H.

5. "The Year 2000: A time to reflect on the past and future of Natural Products Chemistry in South Africa", February 2000. Oral presentation by Brookes K B: "Uteroactive components in traditional medicine: "*isihlambezo*". Co-authors:. Doudoukina O V, Katsoulis L C, and Veale D J H.

OUTLINE OF CONTENTS

CHAPTER 1 INTRODUCTION

1.1 BACKGROUND AND REVIEW OF TRADITIONAL MEDICINES

Plants have been mankind's principal source of medicine for many thousands of years, in fact for all of human history, except for the last century. There are many Biblical references to the natural medicinal power of plants, as in Ezekiel 47:12 which refers to trees of all kinds whose "fruit will serve for wood and their leaves for healing". Herbal preparations and traditional medicines are used world-wide for example in China, Japan, Hawaii, India, Africa and North America, and many remedies are specific for pregnancy and childbirth.

A prolific number of modern clinical medicines have their origins in the folk medicines of traditional healers. These include digoxin and related cardiac glycosides isolated from the foxglove; quinine from the bark of the Peruvian cinchona tree; salicylic acid, the active ingredient in aspirin, from the bark of the willow tree and opium and morphine from the opium poppy, *papaver somniferum* (McMurry and Castellion 1995). The opium poppy has been used as an analgesic since at least the 17th century. However morphine was not isolated from this plant until 1803, being one of the first instances of the purification of the active principle of a drug. The complex structure of morphine was only deduced well over a hundred years later, and finally confirmed via an independent synthesis in 1952 (Solomons 1992). Several combretastatins, isolated from various species in the Combretaceae family, have notable antineoplastic activity, so play an important role in cancer chemotherapy, as discussed in Section 1.3.1.

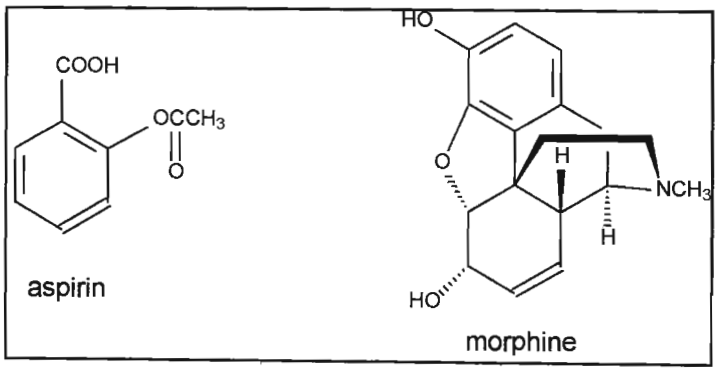


Figure 1 Modern medicines with traditional origins

Natural product research is an active field in South Africa, and has led to the characterisation of many biologically active compounds isolated from medicinal plants, an example being

rooperol, which has anti-cancer properties, obtained from the bulb of *Hypoxis hemero-callida* (Duminy 1996, Mulholland and Drewes 2004). However South Africa generally lags behind other countries in the formal recognition of traditional medicines, although the recent National Research Foundation's Innovation Fund grant to facilitate drug discovery will certainly assist in this respect. In China there are at least eight medicinal plant research institutes, and about 40% of all common diseases are treated with natural medicines. This is cost-saving because it avoids using expensive patented drugs. Tanzania likewise funds research in traditional medicines (Gericke 1995). In America a branch of the National Institutes of Health oversees this type of research (Matthews *et al.* 1999). The Kenyan government plans to table a bill in Parliament which, if it becomes law, will allow herbal medicines to be available in government hospitals for the first time (Kamau 2002). This proposal is backed by the World Health Organisation (WHO), that recognises traditional medicine as a solid amalgamation of dynamic medical know-how and ancestral experience.

Traditional medicines are widely used in South Africa by approximately 80% of the population, particularly by Zulu and Xhosa women. In Kwa-Zulu Natal alone the roots of more than 200 indigenous species as well as the bulbs of 55 indigenous species are sold commercially as traditional medicines (Cunningham 1988).

Traditional healers prepare aqueous root extracts using a selection from approximately 90 plant species and dispense them to women in the last trimester of pregnancy in order to promote maternal and foetal good health and a quick uncomplicated labour. Although most herbal species appear to be safe and seldom if ever result in serious toxicity, some are known to be toxic. There is a common misconception that because the remedy is natural it is safe. In an investigation of approximately 4000 poisoning cases admitted to Ga Rankuwa Hospital from 1987 to 1992 the mortality rate was highest (58%) in the traditional medicines category (Brandt and Muller 1995). The vast amount of research by pharmaceutical companies regarding the safety of drugs for pregnant women is in stark contrast to the paucity of knowledge about herbal remedies. An international workshop on the use and safety of medicinal herbs recommended that these therapeutic herbs should be tested, like other pharmaceuticals, to characterise their acute and chronic toxicities (Matthews *et al.* 1999). Herbal medicines are used by millions of people throughout the world and some countries such as China and the U.S.A. test and monitor the use of such medicines, informing health care providers of the benefits and risks involved.

This structured approach needs to be more fully developed in South Africa to ensure both efficacy and safety in the use of natural medicines. An exciting recent development in this direction was the opening of Mwelele Kweliphesheya (crossing over of two medical cultures)

at Cato Ridge, Kwa-Zulu Natal in 2002. Medicinal plants are cultivated there, which will assist in their protection. A traditional medicine hospital, as well as a college for training of healers is also planned, and the project has provincial government support. Traditional healers are also being trained at Silver Glen Nature Reserve, Kwa-Zulu Natal, in the recognition and propagation of medicinal plant species. This aids the preservation of certain species which are threatened because of over-harvesting. Medbase, a database of the 260 most important medicinal plant species in South Africa, has also been developed by the National Botanical Institute and the Department of Environmental Affairs and Tourism. Ultimately this rich heritage of traditional knowledge, the source of so many modern drugs, should achieve formal recognition by central government, and these developments would strengthen health-care in general in this country.

1.2 RATIONALE AND OBJECTIVES FOR RESEARCH

Isihlambezo is a general Zulu term for all pregnancy-related traditional medicines, which are usually water extracts of a mixture of selected plant roots, and are prescribed by *inyangas* or traditional healers. The primary function of *isihlambezo* is to ensure adequate foetal growth and well-being and promote general maternal health and a quick uncomplicated labour. Chopped plant roots of about four different selected species are boiled in water in approximately 10% solution, and then taken several times daily as a tonic in the last trimester of pregnancy. The concentration of the decoction may be increased at term to speed up labour. Other terms relating to remedies which are generally considered to be more potent than average are *imbelekisane*, used to treat prolonged labour, and *enembe*, the latter even being reputed to cause abortions. These two remedies are regarded as dangerous by traditional healers in Kwa-Zulu Natal (Veale *et al.* 1992).

The prime motivational force for undertaking this research was the scant knowledge of the chemistry, mode of action, or even the safety of the most popular species used in *isihlambezo* herbal medicines. The initial stimulus to begin a study on selected *isihlambezo* species arose from the curiosity of Christine Varga, an American Fulbright student who was carrying out research towards a PhD in Biological Anthropology. Her surveys and questionnaires in clinics in Durban and outlying districts showed that the overwhelming majority of pregnant black women used *isihlambezo* decoctions during pregnancy and childbirth, whether or not they attended "Western" clinics. Morris and Mdlalose noted regarding *isihlambezo* that "little is documented about this herbal decoction and its use" (Morris and Mdlalose 1991). Earlier preliminary investigations by Larsen and co-workers showed that *isihlambezo* solutions had an oxytocin-like effect on isolated rat uterine tissue, causing electrically measured contractions

(Larsen *et al.* 1983). Veale, Kaidu and co-workers subsequently demonstrated that aqueous extracts of *Clivia miniata, Agapanthus africanus, Pentanisia prunelloides* and *Gunnera perpensa*, all commonly used in *isihlambezo* decoctions, stimulated smooth muscle contraction in isolated rat uterine tissue (Veale *et al.* 1989, Veale *et al.* 1992 and Kaidu *et al.* 1997). These investigations lend support to the healer's claims that the use of *isihlambezo* leads to a faster and easier delivery. In all cases the uterotonic activity was associated with the water soluble extracts.

Pacheco noted that the existing chemistry of *Gunnera* species was scarce when she undertook a study of three species in the Juan Fernandes Islands (Pacheca 1989). Furthermore it was claimed in a recent book of South African medicinal plants that "nothing appears to be known about the chemical compounds in the wild grape" or *Rhoicissus tridentata* (van Wyk *et al.* 1997). A literature survey confirmed that there was little available chemical information for the three species finally selected for this research.

Initially it was intended that five plant roots be studied. These were selected as being popular among the herbalists according to Christine Varga's surveys as well as from other documented evidence (Larsen *et al.* 1983, Watt and Breyer-Brandwyk 1962, Cunningham 1988, Hutchings 1996, Veale *et al.* 1998). However the research was narrowed down to three plant species after preliminary investigations had been carried out, the main reasons being lack of availability of two of the plants, and the fact that the remaining three were among the top six most frequently cited species in traditional medicines used as herbal oxytocics by approximately 80% of South African Zulu and Xhosa populations (Cunningham 1988, Veale et al 1998). Preliminary uterine test results for the original five plants selected are shown in Section 2.5.2.

The two plants which were eliminated and Zulu names thereof were:

Asclepias fruticosa	ishinga
Typha latifolia	ibhuma

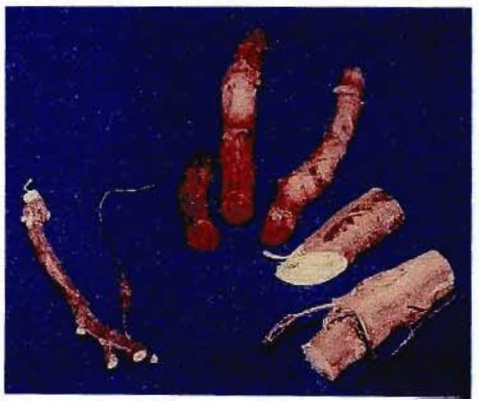
The final selection of plant species was:

Gunnera perpensa	ugobo
Rhoicissus tridentata	isinwazi
Combretum kraussii	umdubu

A survey of 57 plants, out of a total of up to approximately 90 used by healers in *isihlambezo* mixtures indicated that sixteen of these are toxic and might therefore have adverse effects on the mother and unborn foetus (Veale *et al.* 1992). *Callilepis laureola* or *impila* for example has been responsible for many fatalities due to hepato-renal failure. Other toxic effects linked to *isihlambezo* are low neonatal birth-weights, foetal meconium staining, which is a sign of possible foetal distress and hypoxia, and fatal uterine rupture (Veale *et al.* 1998). Uterine hyperstimulation is a possible explanation for some of these toxic effects, and it could be attributed to the use of herbal remedies. *Combretum erythrophyllum* and *Rhoicissus cuneifolia* remedies have been associated with recorded cases of poisoning/toxicity (Watt and Breyer-Brandwyk 1962) so it was evident that further investigation was needed regarding the toxic principles within these plants. Furthermore it was decided to measure levels of components such as calcium ions, which could promote good health in pregnant woman.

For the three selected plants the objectives of this study were:

- To characterise compounds which had an oxytocin-like effect on uterine tissue
- To establish whether plant extracts had any toxic effects
- To establish whether plant extracts had nutritional value.



Roots of *Rhoicissus tridentata* (young roots), *Gunnera perpensa* and *Combretum kraussii.*

1.3 REVIEW OF THE FAMILIES OF SPECIES STUDIED

1.3.1 Combretum kraussii (Combretaceae)

The belief held by healers in Africa and India that species of the Combretaceae family had

anti-cancer properties was substantiated in the 1980's by a comprehensive survey of stilbenes and bibenzyls isolated from the heartwood of the South African tree, *Combretum caffrum*, utilising the National Cancer Institute's biological evaluation system (Pettit *et al.* 1987a and 1987b). The need to find effective cancer therapeutic drugs for rapidly spreading forms of cancer, such as malignant brain tumours, was the driving force behind this survey. Although several species were used in traditional medicines, Combretaceae had received very little prior chemical investigation at that stage (Pettit *et al.* 1982). From a methylene chloride-methanol extract of *C. caffrum* rootbark, Pettit *et al.* isolated a series of compounds with antineoplastic and/or cell growth inhibitory properties.

At that stage only about 25 of the 250 *Combretum* species had been the subject of scientific study, with the most noteworthy effort concerning the isolation of a series of bibenzyl and related phenanthrene and 9,10-dihydrophenanthrene derivatives, sixteen of these being novel, from *C. psidioides, C. hereroense and C. molle* (Letcher *et al.* 1972a, 1972b and 1973). Examples of the novel compounds identified were the phenanthrene **A**, bibenzyl **B**, and 9,10-dihydrophenanthrenes **C** (Fig.1.3.1) which were isolated from the heartwood of *C.molle*, shown below (Letcher and Nhamo 1972a). Leaves of C.molle are used to dress wounds, and the leaf and root together are believed to be an antidote for snakebite by traditional healers.

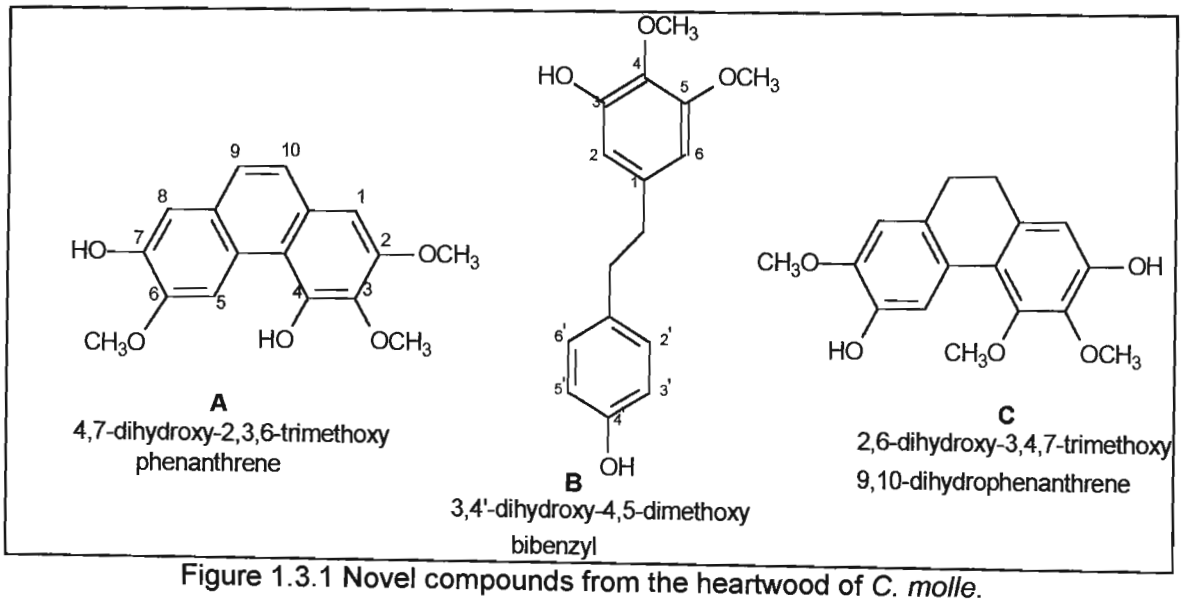


Figure 1.3.1 Novel compounds from the heartwood of *C. molle*.

Combretastatin was suggested by Pettit and co-workers as the possible biosynthetic precursor for bibenzyl derivatives and related 9,10-dihydrophenanthrenes from the genus *Combretum* (Pettit *et al.* 1982). Bibenzyls were also proposed as immediate precursors of the phenanthrenes and 9,10-dihydrophenanthrenes. Letcher and Nhamo used unambiguous syntheses to confirm the structures of several naturally occurring phenathrenes, and

interestingly also highlighted the relatively easy interconversion between the three classes of compounds, stilbenes, phenanthrenes and bibenzyls, through their work (Letcher and Nhamo 1972a).

Synthesis of a naturally occurring stilbene (Fig.1.3.2), involved the coupling of two aromatic compounds, 3,4-dihydroxybenzaldehyde and 3,4,5-trihydroxyphenylacetic acid, via a Perkin condensation carried out by refluxing with acetic anhydride and trimethylamine to form the substituted cinnamic acid, α-(3,4,5-trimethoxyphenyl)-3,4-dihydroxycinnamic acid. This was first acetylated and then refluxed under nitrogen with quinoline and copper chromite to yield the stilbene diacetate after decarboxylation. A solution of this stilbene was then irradiated with light and treated with iodine, resulting in photochemical oxidation to form the phenanthrene diacetate.

It was also shown during the synthesis of another similar stilbene, 2,3,4,7-tetramethyoxy-stilbene, that it could be converted to the corresponding 2,3,4,7-tetramethyoxy-bibenzyl by catalytic reduction of the stilbene double bond with hydrogen and platinum oxide. These chemical interconversions add support to the proposed biochemical links between these classes of compounds.

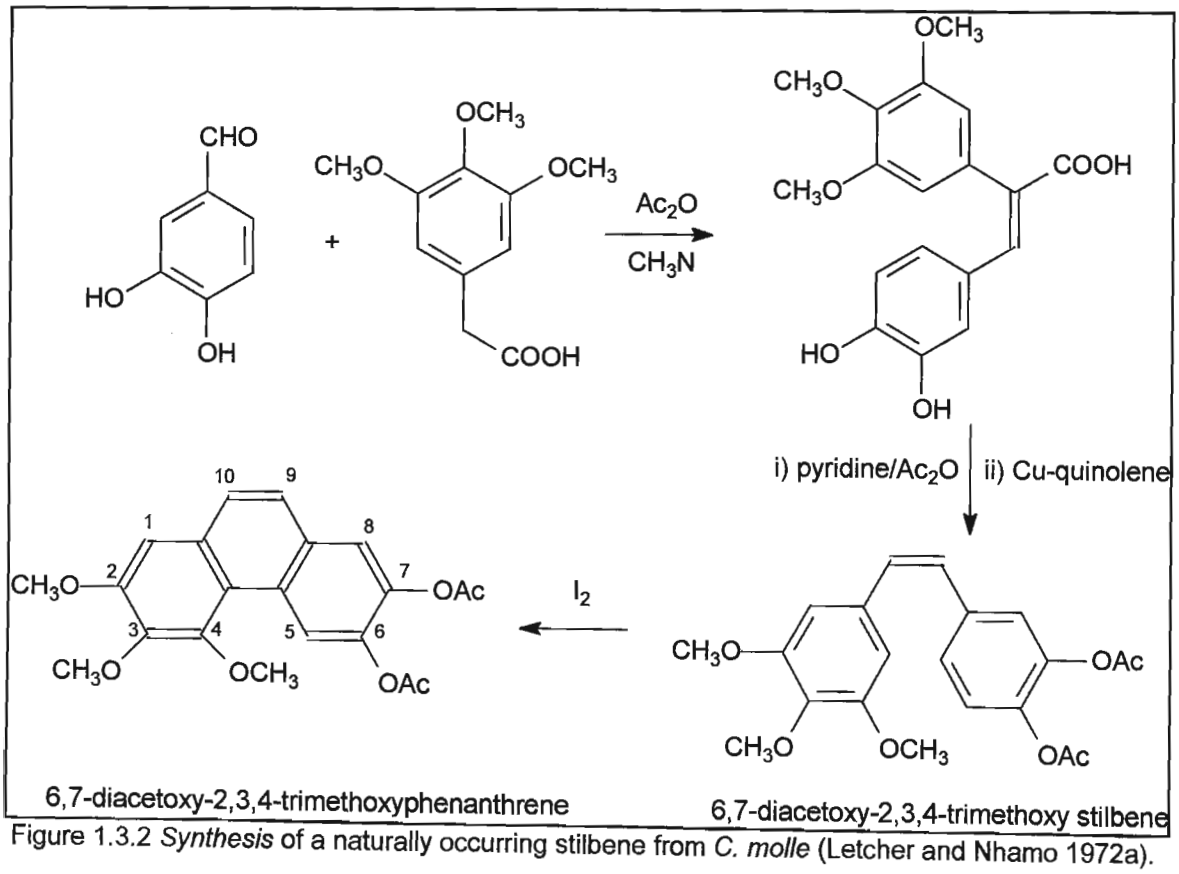
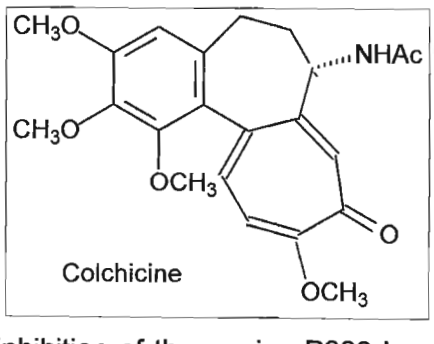


Figure 1.3.2 *Synthesis* of a naturally occurring stilbene from *C. molle* (Letcher and Nhamo 1972a).

The synthesis of combretastatin A and B series stilbenes and dihydrostilbenes can also be achieved via Wittig condensations between an aromatic aldehyde and an aromatic phosphonium halide (Verotta and Rogers 1997).

The genus *Combretum* is the source of many bioactive compounds. Many *Combretum* metabolites show molluscicidal, anti-inflammatory, cytotoxic and other physiological activities, which are now discussed. Leaf and fruit extracts of *Combretum* species are rich sources of pharmacologically active compounds, leading to the anticipation that the roots would prove to be an equally rich source (Verotta and Rogers 1997). A number of compounds with antineoplastic and anti-inflammatory properties have been isolated from various species, notably *Combretum caffrum* and *Combretum kraussii* (Guatteo 1996).

The microtubule system of eucaryotic cells is a potential target for antineoplastic agents. Drugs which interfere with microtubule function, virtually all interfere with the dimeric protein, tubulin, the major microtubule component. A large number of these agents are plant-derived, for example the *Colchicum* alkaloid colchicine (Verotta and Rogers 1997). There are common structural features that underlie this biological activity. Some of the important contributors to activity are two or more planar rings which are tilted with respect to each other, and a trimethoxy system on the A ring as shown in colchicine below. Combretastatins, podophyllotoxins and steganacin also have these structural features. The ethene bond must be in the cis configuration for binding, as in combretastatin A-1.



Colchicine

Combretastatin and several related compounds (Fig.1.3.3) are most interesting from the viewpoint of central nervous system antineoplastic activity, with combretastatin A-1, shown below, being particularly active. Combretastatin was the first active plant constituent identified from *C. caffrum* stemwood (Pettit *et al.* 1982). It caused substantial astrocyte reversal, and inhibition of the murine P388 lymphocytic leukaemia cell line, and also exhibited minimal cytotoxicity (0-5%). This *cis*-stilbene had *in vivo* activity against the murine P388 lymphocytic leukaemia (PS cell line) and was the most potent inhibitor of tubulin poymerisation known at that stage, leading to complete inhibition at 1.5μM concentrations. Both combretastatin A-1 and B-1 were potent inhibitors of microtubule assembly *in vitro* and among the most potent inhibitors of the binding of colchicine to tubulin, the major protein of the mitotic spindle, at the time (Pettit *et al* 1987a). Combretastatin A-1 was more active than combretastatin B-1 in its interactions with the tubulin assembly, in agreement with its greater antineoplastic activity.

Both compounds were significantly more potent than the previously described combretastatin.

Combretastatin, combretastatin A-1, A-2, A-3, B-3 and B-4 all exhibited anti-mitotic properties (Pettit *et al.* 1987a,1988). Several phenanthrene and dihydrophenanthrene constituents also exhibited cell growth inhibitory properties (Pettit *et al.* 1986).

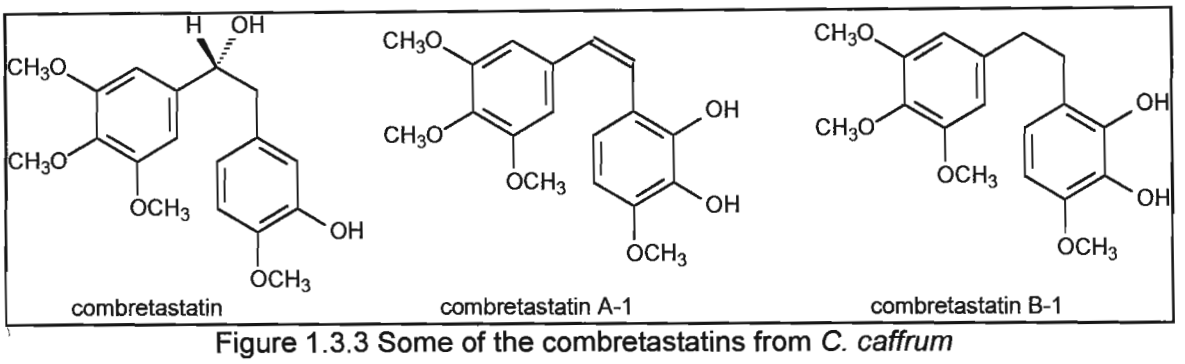


Figure 1.3.3 Some of the combretastatins from *C. caffrum*

In addition to the previously described anti-mitotic activity of combretastatins regarding cancer control, a new approach to cancer treatment is the role combretastatins play as angiogenesis inhibitors (Cirla and Mann 2003). Angiogenesis is a complex process by which new blood vessels are formed. Cancer is an angiogenesis-dependent disease, and therefore there is a great deal of interest currently in developing angiosuppressive agents, such as angiostatin and endostatin, for cancer therapy. Many other novel agents are also undergoing clinical trials worldwide at institutions such as Harvard and the National Cancer Institute. Combretastatins such as combretastatin A-1, B-1 and A-4 play an important role in this area of research as angiogenesis inhibitors, and therefore also tumour growth inhibitors.

Another prominent *C. caffrum* constituent, 3,3',4'-tri-O-methylellagic acid, was found to be PS-inactive. Ellagic acid occurs naturally in some foods such as strawberries, grapes and walnuts, and has antimutagenic and anticarcinogenic properties. Its effectiveness against the potent mutagen aflotoxin B_1 was demonstrated via a Salmonella microsuspension assay. The results supported the hypothesis that the inhibition of aflotoxin B_1 could involve the formation of a chemical complex between ellagic acid and aflotoxin B_1 (Loarca-Pina *et al.* 1996).

Subsequently, stilbenes, bibenzyls and their glucosides having the ability to inhibit cell growth were isolated from *C. kraussii* seeds, including the new combretastatin B-5 (Pellizoni *et al.* 1993). In a study in which mouse leukaemia L1210 cells were exposed to combretastatin B-1 (Fig.1.3.3), and combretastatin B-1-2'-O-β-D-glucopyranose, both compounds were found to exhibit *in vitro* cytotoxicity, with combretastatin B-1 being twice as active as the glucoside. Although combretastatin B-1 was also shown to inhibit polymerisation of microtubule protein,

whereas no effect on microtubule assembly was detected for combretastatin B-1 glucoside at the concentrations tested (Pellizoni *et al* 1993). More recently it was widely reported in the press in 2002 that combretastatin A-4 is the most potent inhibitor of colchicine binding to tubulin yet discovered, and is undergoing clinical trials in the UK.

The pharmacological action of combretastatin B-1 was also demonstrated on dorsal root ganglion neurones, human myotubes and TE 671 muscle tumour cells. Combretastatin B-1 produced remarkable effects on the action potentials of these excitable muscle cells (Guatteo *et al* 1996). It acted as a K^+ channel blocker for both inward and outward currents for the muscle tissues tested, and the action potential of sensory neurones was lengthened by this inhibition of the repolarising currents. Combretastatin B-1 reversibly increased the duration of the action potential in rat sensory neurones by approximately 300% suggesting abnormal excitability of the peripheral nervous system. It had negligible effects on Ca^{++} and Na^+ channels. This type of K^+ channel inhibition has been described in relation to certain polyunsaturated fatty acids such as arachidonic acid, cardiac anti-arrhythmic drugs such as ambasilide and tedisamil, and anti-tumour drugs such as tamoxifen. The pharmacological implications of these results are interesting when considering the potential effects on uterine muscle tissue.

Bibenzyls and 9,10-dihydrophenanthrenes are well-known phytoalexins, or fungitoxic agents (Sticher 1995). Seven 9,10-dihydrophenanthrenes and one bibenzyl derivative were isolated from *Sobralia violacea* L. (Orchidaceae), used by the Kallawayan Indians to treat heart diseases. All compounds in these two groups, with the exception of the glucosides, showed strong antibacterial and antifungal activity (Hostettman *et al* 1995).

In addition to the bibenzyls and related compounds already described, a variety of triterpenoid acids and their glucosides, mainly of the cycloartane (*C. molle*) and oleanane type (*C. imberbe*) and also aminoacids (*C. zeyheri*) have been isolated from *Combretum* species (Pelizzoni *et al* 1993). Alkaloids, tannins and flavonoids have been identified in *C. micranthum*. The genus is exceptionally rich in triterpenoid acids, of which a relatively small number have been identified (Rogers and Steyn 2002). Many of the triterpenoid acids isolated from *Combretum* leaves have physiological activity so could be of commercial value. Several of the triterpenoid acid mixtures have strong inhibitory effects on germination and seedling growth (Pelizzoni *et al* 1993, Verotta and Rogers 1997). Mollic acid-3-O-β-D-glucoside from *C. molle* has anti-inflammatory and molluscicidal properties (Panzini *et al.* 1993). The oleanane, imberbic acid (1α, 3β-dihydroxyolean-12-en-29-oic acid), and the cycloartane, mollic acid (1α-hydroxycycloart-24-en-30-oic acid), are shown in Figure 1.3.4.

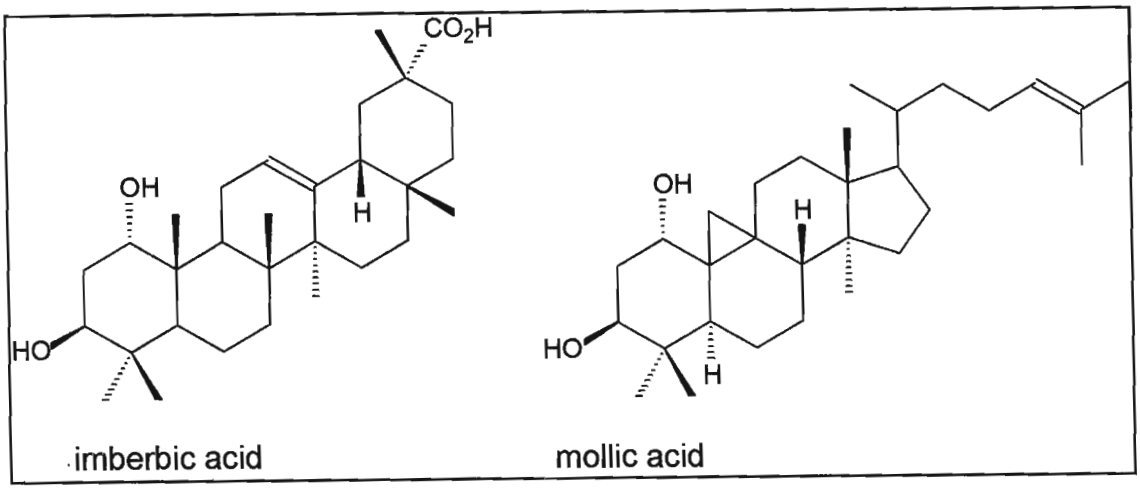
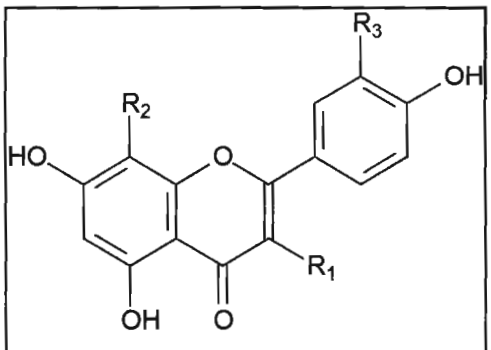


Figure 1.3.4 Triterpenoid acids from *C. imberbe* and *C. molle*.

1.3.2 Gunnera perpensa (Gunneraceae)

Gunnera perpensa is reported to yield the bitter principle celastrin (Hutchings 1996). It is not clear exactly what this is however. A white crystalline principle obtained from the medicinal plant *Celastrus scandens* (Celastraceae) or "false bittersweet" was originally named celastrin by Wayne in 1872 (Felter and Lloyd 1898). Other researchers found the bark to contain starch, gum, sugar, colouring material, volatile oil, two resins, one acid and the other neutral, and a body resembling caoutchouc. The colouring matter of *Celastrus scandens* was due to the vegetable colouring matters, xanthin and carotin.

An investigation of the evolution of the flavonoid chemistry in the genus *Gunnera* in the Juan Fernandez Islands, Chile, led to the identification of 15 flavonoids from the three subgenera, *Misandra, Panke and Perpensum*, studied (Pacheco 1989). Flavonoid chemistry assists the understanding of systematic relationships and evolutionary trends among taxa, and existing information on the chemistry of *Gunnera* was scarce. The flavonoid pattern of isolated compounds was characterised by the presence of glycosides of quercetin, kaemferol and isorhamnetin, two partially identified flavone glucosides, the aglycones of kaemferol and isorhamnetin and two unknown phenolic compounds. Compounds appearing as discrete spots on paper chromatography were eluted with methanol and further purified by polyamide TLC when necessary. Ultraviolet spectra were run on all compounds isolated and their structures are shown in Fig.1.3.5.



Compounds from 12 *Gunnera* species in the Juan Fernandes islands

	R_1	R_2	R_3
quercetin-3-O-arabinoside	ara	H	OH
quercetin-3-O-glucogalactoside	glu-gal	H	OH
quercetin-3-O-digalactoside	gal-gal	H	OH
quercetin-3-O-diglucoside	glu-glu	H	OH
quercetin-3-O-diglycoside	gly-gly	H	OH
kaempferol-3-O-glycoside	gly	H	H
kaempferol-3-O-diglycoside	gly-gly	H	H
kaempferol	OH	H	H
isorhamnetin-3-O-glucoside	glu	H	OCH_3
isorhamnetin	OH	H	OCH_3
flavone A	H	OH	OH
flavone B	H	OH	OH

Figure 1.3.5 Compounds from 12 *Gunnera* species

The flavonoid data had taxonomic significance, with the distribution of flavonoids among the three subgenera, *Misandra, Panke* and *Perpensum* showing delimitation by morphological character. Certain species had a much reduced flavonoid profile, which is a common trend among island species where there are smaller predator or pathogen populations. Phenolic constituents have at least some defensive role, and in an environment where predators and pathogens are reduced, the continuing selection for synthesis of complex arrays of phenolic constituents might be absent (Pacheco 1989).

Gunnera mandicata is used in the folk medicine of the Kallawayan Indians to treat infections. An investigation of the methanol extract of *Gunnera mandicata* resulted in the isolation of 15 phenolic compounds (Fig.1.3.6), predominantly glycosides, with all except the last two listed below being reported for the first time from the genus *Gunnera* (Hostettmann *et al.* 1995).

The compounds isolated from *Gunnera mandicata* were:

Shikimic acid

1-O-galloyl-β-D-glucopyranoside

1-O-caffeoyl-β-D-glucopyranoside

3-O-[6"-O-(caffeoyl)-β-D-galactosyl]-quercitin

3-O-[6"-O-(caffeoyl)-β-D-glucosyl]-quercitin

1,6-di-O-caffeoyl-β-D-glucopyranoside

3-O-[6"-O-(galloyl)-β-D-glucosyl]-kaempferol

3-O-[6"-O-(galloyl)-β-D-galactosyl]-kaempferol

6-O-caffeoyl-1-O-(6'-O-caffeoyl-α-D-glucopyranosyl)-β-D-glucopyranoside

rutin

kaempferol-3-O-rutinoside

3-O-(β-D-galactosyl)kaempferol

3-O-(β-D-glucosyl)kaempferol

3-O-(β-D-galactosyl)quercitin

3-O-(β-D-glucosyl)quercitin

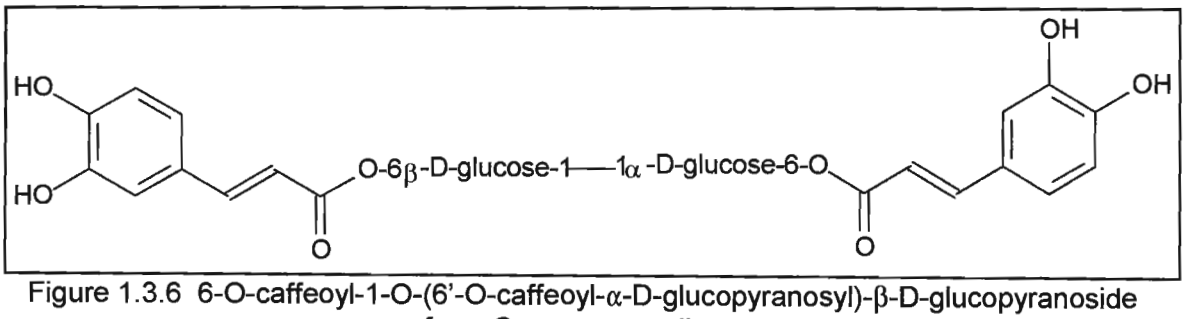


Figure 1.3.6 6-O-caffeoyl-1-O-(6'-O-caffeoyl-α-D-glucopyranosyl)-β-D-glucopyranoside from *Gunnera mandicata*

Although the predominant compounds in Gunneraceae are polar phenolics, other less polar compounds have also been isolated. *Gunnera peltata* Phil., a Chilean species which grows on the Juan Fernandes Islands, yielded five known pentacyclic triterpenoids from the methanol-ethyl acetate-hexane 1:1:1 extract of the aerial parts of the plant (Bittner *et al.* 1993). The compounds isolated were oleanolic acid, ursolic acid, lupeol, erythrodiol and 2α, 3α-dihydroxy-urs-12-ene, as well as sitosterol and sitosterol glucoside (Fig.1.3.7).

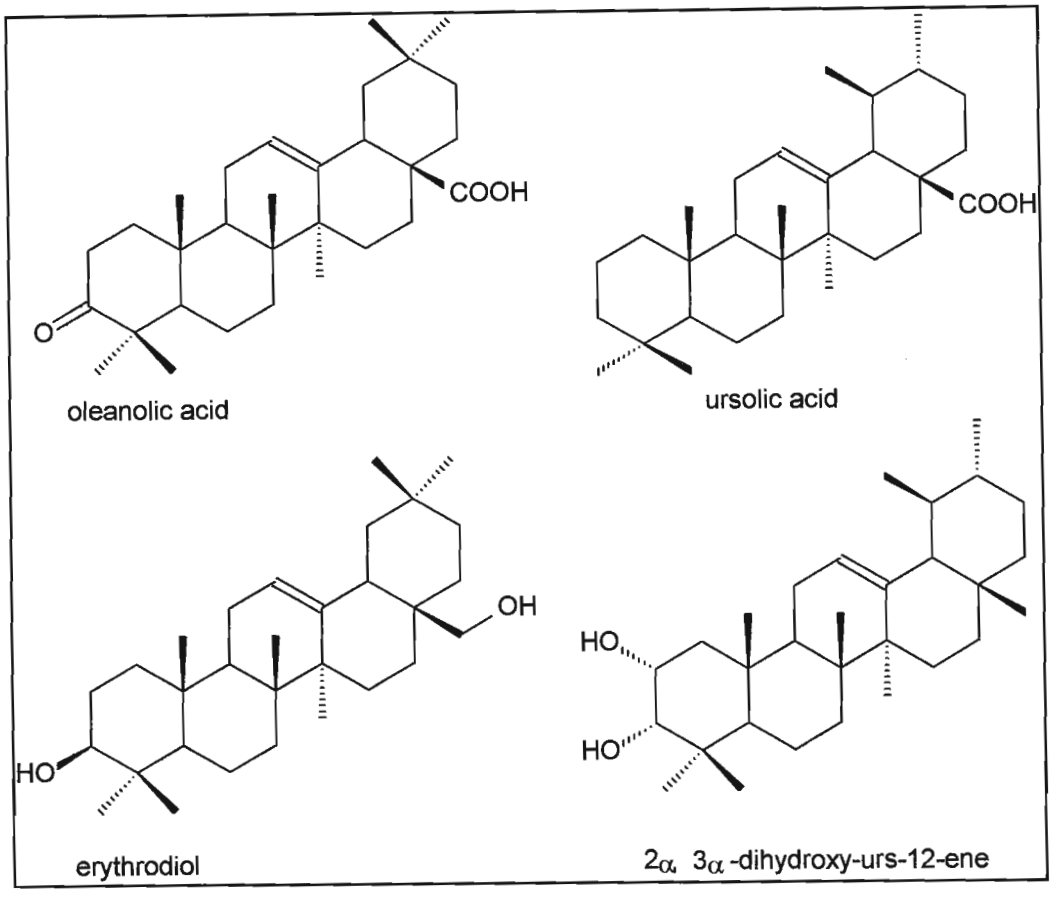


Figure 1.3.7 Triterpenoids from *Gunnera peltata*

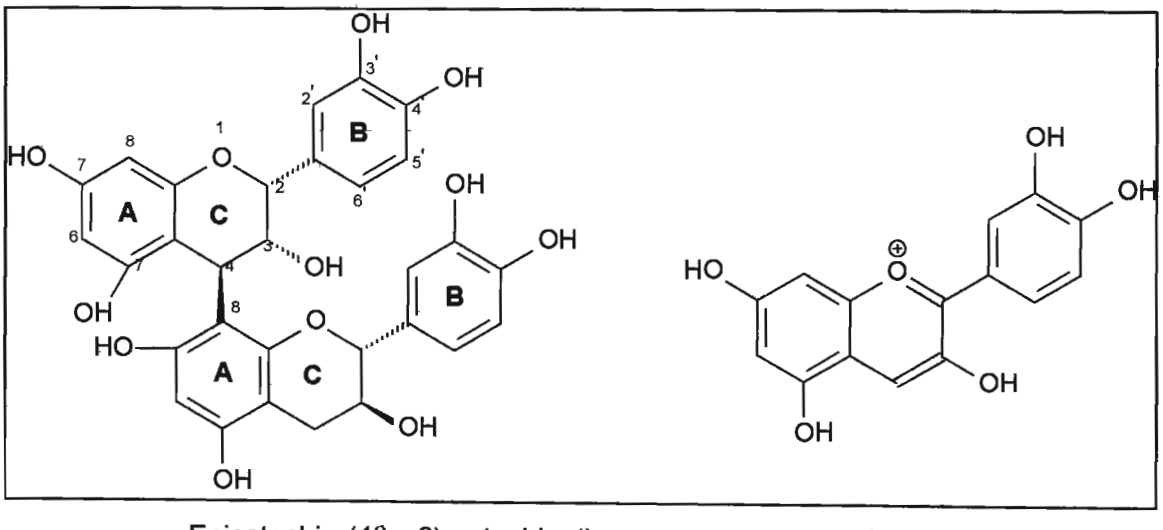
1.3.3 ***Rhoicissus tridentata* (Vitaceae)**

Proanthocyanidins are well-established components of the Vitaceae family which generally lacks ellagic acid derivatives. Although hydrocyanic acid is known in the genus *Cissus* L., the Vitaceae family is seldom cyanogenic and rarely has saponins. Vitaceae also lack anthraquinones, alkaloids, and iridoid compounds (Hutchings 1996). Fruit juice of the genus *Cissus* L. can cause irritant dermatitis and throat inflammation, and various species are reported as poisonous. *Cissus quadrangularis*, an African medicinal plant, contains a water-soluble glycoside as well as ascorbic acid, carotene, calcium, a potent anabolic steroid and triterpenoids Δ-amyrin, Δ-amyron, onocer-7-ene-3-α-21-α-diol and onocer-7-ene-3-α-21-β-diol. This species showed acetylcholine-like activity on isolated rabbit uterus and intestine. The water-soluble glycoside was non-toxic when administered orally to mice, rats and guinea pigs, but caused convulsions and death within five minutes of intravenous administration (Hutchings 1996). *Cissus rheifolia* has known anti-tumour activity and *Cissus trifoliata* anti-inflammatory activity.

Proanthocyanidins and flavans constitute the fastest developing area in flavonoid chemistry in terms of the number of new natural products reported. (Harborne 1994). Flavonoids are a

large group of natural products which are widespread in higher plants and have diverse biological functions. The ubiquitous proanthocyanidins, within this group, are thought to be the largest sub-group of plant phenolics (Buckingham 1994). Proanthocyanidins are found in the bark and heartwood of a variety of tree species, and extracts of these find commercial application in the leather tanning industry. Their well-documented anti-oxidant properties have led to a variety of pharmaceutical products, for example grape-seed extracts, which are rich in health-promoting proanthocyanidin monomers and oligomers.

Proanthocyanidins are traditionally known as condensed tannins, and consist of flavan-3-ol units. The most commonly occurring proanthocyanidins are based on catechin and/or epicatechin units. Chirality exists at C-2 and C-3 of the saturated C-ring of the flavan-3-ol unit, which usually has the 2R, 3S configuration. Compounds with a 2R, 3R configuration are prefixed *epi*, and those with a 2S, 3R configuration are prefixed *ent* (Harborne 1994). Interflavanoid linkages are indicated by the IUPAC αβ nomenclature as in sugar chemistry, with the direction of the bond in parentheses. Generally the two benzene rings are at right-angles to each other in the three-ringed system. There is some confusion in the literature when flavanol units are not oriented in the conventional (A, C, B ring) way when considering linkage to the next unit. A (4β→8) linked proanthocyanidin dimer is shown in Fig.1.3.8.



Epicatechin-(4β→8)-catechin dimer Cyanidin

Figure 1.3.8 Proanthocyanidin dimmer and flavylium chromophore

Anthocyanidins are intensely coloured plant pigments responsible for flower colour in most angiosperms. The presence of *in vivo* organic acids generates the three-ringed flavylium cationic chromophore, for example cyanidin (3,3',4,5,7-pentahydroxyflavylium ion), shown above. The flavylium cation has eight conjugated double bonds and hence exhibits visible colour (Fig1.3.8).

Flavonoids frequently occur naturally associated with sugars, and there are over 1500 glycosides of the flavones and flavanols (Buckingham 1994). Flavan-3-ol units vary according to the number and position of hydroxyl and methoxyl substitents. There are relatively few sugar-free anthocyanidin aglycones. The sugar-containing anthocyanins vary from simple monoglycosides such as chrysanthemin, cyanidin-3-glucoside, to the very large ternatin A-1 which is substituted with seven glucose, four p-coumaric acid and one malonic acid moiety. About one third of anthocyanins contain malonic acid, or other dicarboxylic acids, linked through sugar molecules, and therefore exhibit zwitterionic properties.

Darne and Glories used HPLC analysis to identify glucosides in three cultivars of *Vitis vinifera* L., family *Vitaceae*, used in wine-making *i.e.* Merlot Noir, Cabernet Sauvignon and Gamay Freaux (Darne and Glories 1988). Anthocyanins were extracted with 1% ethanolic HCl. Both the leaves and the grape berries were analysed over three seasons, and it was found that the concentrations of the five different anthocyanins in the leaves and grapes were different in the mature plants. The total anthocyanin content of leaves increased as the grape berries matured, then decreased until the leaves fell in autumn. This is illustrated (Table 1.3.1) for the Cabernet Sauvignon cultivar.

Table 1.3.1 Total Anthocyanin content of leaves of Cabernet Sauvignon cultivar.

Season	Total Anthocyanin Content µg/g dry leaves
Spring	3723
Maturity	14414
Fall	9998

The total anthocyanin content of leaves is over three times greater when comparing spring to the summer season when the fruit reaches maturity.

For HPLC analysis of the individual anthocyanins a linear solvent gradient was used which ranged from formic acid–water 5:95 to formic acid-methanol-acetonitrile-water 3:55:10:30. Results showed that the five dominant compounds in the leaves of all three cultivars were monoglucosides. Results are shown (Table 1.3.2) for the mature Cabernet Sauvignon cultivar in the grape-harvesting season. The other two cultivars showed a similar pattern, although leaves of the two red-juice cultivars were richer in anthocyanins than leaves of the non-colouring Gamay Freaux cultivar.

Table 1.3.2 Anthocyanin composition in leaves and berries of Cabernet Sauvignon at maturity.

Anthocyanin	Leaves %	Berries %
delphinidin-3-glucoside	5.2	16.4
cyanidin-3-glucoside	22.1	4.2
petunidin-3-glucoside	2.7	7.9
peonidin-3-glucoside	43.6	8.6
malvidin-3-glucoside	14.8	29.6
Total %	88.5	66.7

Peonidin glucoside was present in the highest amounts in the mature Cabernet Sauvignon leaves whereas malvidin glucoside was highest in the berries.

Although many flavanoids are monomers, dimers, trimers and polymers have been described. The highest molecular weight flavonoids are the oligomeric and polymeric proanthocyanidins, and oligomers up to hexamers and heptamers have been found in plants. Polymers consist of a series of flavan-3-ols usually joined by a C-C bond between C-4 of one flavan unit and C-8 of the next (Buckingham 1994). Proanthocyanidins release anthocyanidins when treated with acid and may be astringent to the taste (Foo and Porter 1978). Astringency and tanning properties are associated with the higher molecular weight polymers (Czochanska *et al.* 1980). The great majority of proanthocyanidins consist of procyanidins and prodelphinidins, the latter group having an extra hydroxyl in the B-ring at the C-5 position compared to catechin. Many procyanidins are esterified and the most common esters are the O-gallates. The simplest procyanidins are dimers which commonly have 4→8 linkages, and all are found in grapes.

Proanthocyanidins can be fully hydrolysed to the constituent anthocyanidin monomer units in 5% butanolic HCl at 100 °C (Foo and Porter 1978). They can also be cleaved under milder conditions by thiolysis using phenylmethanethiol or phloroglucinol and acetic acid in ethanol at 100 °C (Czochanska *et al.* 1980). It was later discovered that thiolysis could be achieved more easily at 50 °C if methanol instead of ethanol was used as the solvent (Porter *et al.* 1991). Phenylmethanethiol or phloroglucinol act as "unit catching agents" by forming adducts with the hydrolysis carbocation fragments which can be readily identified by spectroscopic methods. Both the position and stereochemistry of the interflavanoid bonds can be deduced from thiolysis. This technique is particularly useful for large molecules for which ^{1}H NMR spectra

are very complex and difficult to interpret because of overlapping signals and broadening effects due to rotational isomerism (da Silva *et al.*1991).

Porter and co-workers used Sephadex LH-20 to separate catechin and epicatechin based polymers from fresh unfermented cocoa beans (*Theobroma cacao* seeds) using methanol-water 1:9 to 100:0 step gradient (Porter *et al.* 1991). Generally lower molecular weight flanonoids are soluble in ethyl acetate whereas the larger polymers are soluble in aqueous acetone, so a partial separation can be achieved on this basis. The progress of the column separation was followed by HPLC with methanol-1% aqueous acetic acid 1:4 as the mobile phase. During the cocoa fermentation and roasting process dimers and higher oligomers are hydrolysed to yield catechin and epicatechin monomers, and the red-brown product is the characteristic colour of chocolate.

The highest mass ion that could be detected from the various fractions of the cocoa bean extract by negative ion FAB was m/z 2017 ($[M-H]^-$ for a heptamer). From analysis of the data from the column it was estimated that approximately 52% of the cocoa metabolites were monomers to hexamers and the remainder heptamers and above. Three new procyanidins were isolated and characterised by one and two-dimensional spectroscopy as well as analysis of material after hydrolysis. One of the novel procyanidins was 3-O-β-D-galactopyranosyl-*ent*-epicatechin-(2α→7,4α→8)-epicatechin,(Fig.1.3.9).

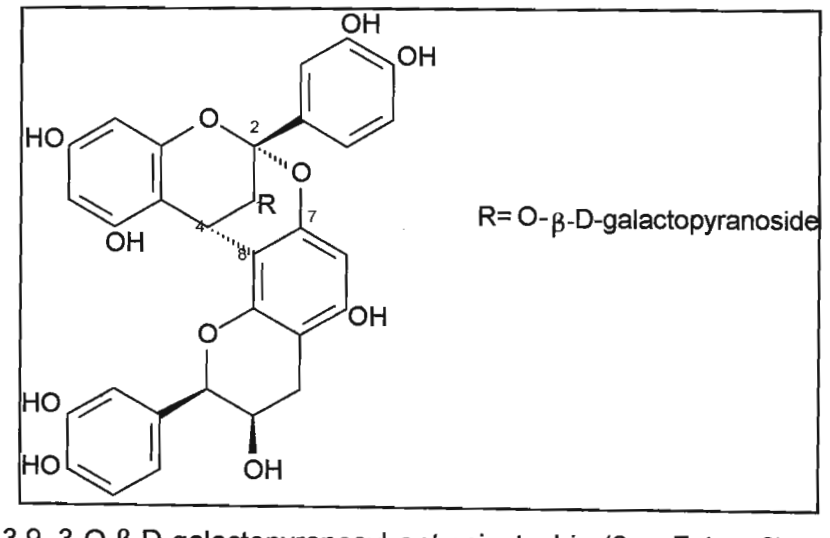


Figure 1.3.9 3-O-β-D-galactopyranosyl-*ent*-epicatechin-(2α→7,4α→8)-epicatechin, a novel procyanidin from cocoa beans

There are four criteria required to define the gross structure of a proanthocyanidin polymer (Czochanska *et al.* 1980):

1. the ratio of procyanidin to prodelphinidin units
2. the stereochemistry of the heterocyclic ring of the monomer units
3. the structure of the chain-terminating flanan-3-ol unit
4. the number average molecular weight

All the above criteria can be deduced from the ^{13}C NMR spectra of the polymer except for the third one. However the structure of the chain-terminating unit may be obtained from the degradation products. Thiolysis followed by GLC (gas liquid chromatography) enables the structure of the terminal unit to be established.

The structure of [epicatechin-(4β→8)]$_3$-epicatechin from cocoa beans was elucidated using these principles (Porter *et al.* 1991). The four C-3 signals were clearly discerned at 66.2, 71.9, 72.3 and 73.0 ppm, and the resonances at 66.3 and 73.0 were consistent with 4β→8 linkages between units. Thiolysis gave epicatechin-(4β→8)-epicatechin-4β-thiobenzyl ether and epicatechin-(4β→8)-epicatechin as the only dimeric products (Fig.1.3.10). From this it could be unequivocally concluded that all linkages were 4β-8, and so the structure below was confirmed as [epicatechin-(4β→8)]$_3$-epicatechin.

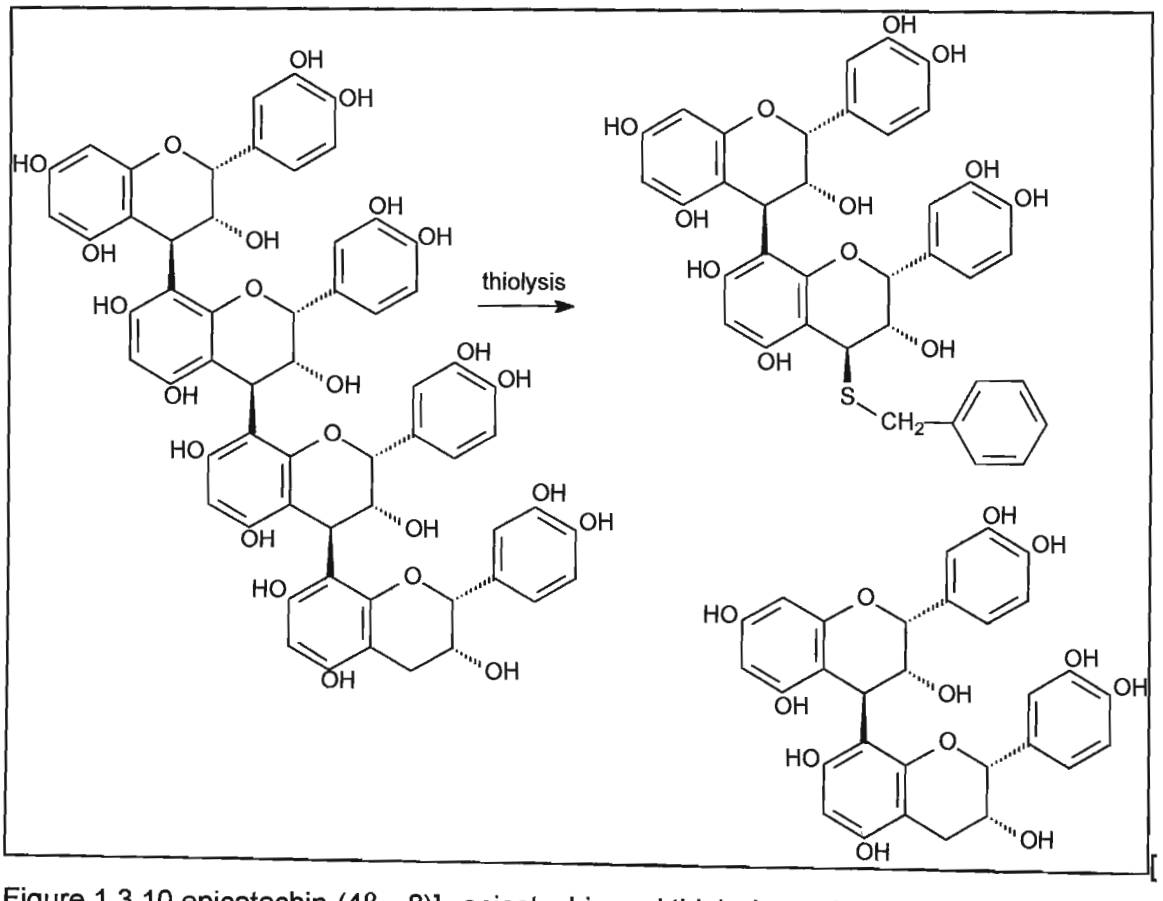


Figure 1.3.10 epicatechin-(4β→8)]$_3$-epicatechin and thiolysis products, epicatechin-(4β→8)-epicatechin-4β-thiobenzyl ether and epicatechin-(4β→8)-epicatechin.

A series of procyanidin dimers and trimers were identified for the first time in grape (*Vitis vinifera*, family Vitaceae) seed extracts (da Silva *et al.* 1991). After column chromatography on Fractogel column fractions were further purified by semi-preparative HPLC. For all isolated compounds cyanidin was obtained by complete acid hydrolysis, showing that they all contained only (+)-catechin and/or (-)-epicatechin units, i.e. they were all procyanidins. The structures were elucidated by complete acid hydrolysis, enzymatic hydrolysis, partial acid catalysed degradation with phloroglucinol and phenylmethanethiol, FABMS and ^{1}H NMR. Separation of the procyanidins and their phloroglucinol and phenylmethanethiol adducts was achieved by TLC and HPLC. The following novel procyanidin dimers mad trimers were identified:

Dimers: B5 [epicatechin-(4β→6)-epicatechin]
B6 [epicatechin-(4α→6)-catechin]
B8 [catechin-(4α→6)-catechin]

Trimers: [epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin]
[epicatechin-(4β→8)-epicatechin-(4β→6)-catechin]
[epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin]
[epicatechin-(4β→6)-epicatechin-(4β→8)-catechin]

In addition five galloyl proanthocyanidins were isolated: B1-3-O-gallate, B2-3-O-gallate, B2-3,3'-di-O-gallate, B4-3'-O-gallate and [epicatechin-(4β→8)-epicatechin-3-O-gallate-(4β-8)-catechin]. Also the presence of (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-O-gallate, procyanidin dimers B1, B2, B3, B4, B7, B2-3'-O-gallate, procyanidin trimer C1 and [epicatechin-(4β→8)-epicatechin-(4β→8)-catechin] was confirmed.

Red wines of the Vitaceae family have biological properties favouring protection against major causes of adult mortality such as coronary heart disease (CHD) and cancer. Goldberg and co-workers developed a quantitative gradient elution reversed phase HPLC technique with diode array detection for biologically active phenolic constituents in commercial wine (Goldberg *et al.* 1996). They achieved good separations for cis and trans resveratrol and their diglucosides, catechin, epicatechin, quercitin and rutin. The antioxidant properties of wine are well known and it is also well established that moderate consumption of wine reduces mortality from CHD. Furthermore there is some evidence that those who drink wine may have a lower CHD mortality than those who drink other alcoholic beverages. The constituents unique to wine have therefore attracted intense interest. Catechin, epicatechin, quercitin and resveratrol have

been shown to protect low density lipoproteins against oxidation more effectively than α-tocopherol. Some of the eight compounds detected, as well as an extract of red wine phenolics, promote vascular relaxation. Resveratrol and quercitin inhibit platelet aggregation *in vitro* and are likely to be protective against CHD. Red wine phenolics have been shown to protect rats against unfavourable lipid profiles that develop as a result of diets high in fat and cholesterol, with resveratrol, quercitin and morin being significant in this regard. Quercitin and its glucoside rutin also have potential anti-cancer effects.

1.4 BIOSYNTHESIS OF AROMATIC NATURAL PRODUCTS

Natural products comprise a vast number of diverse compounds, but in spite of this a remarkable link exists between all of them in that the same major classes of compounds recur in all forms of life. The shared biosynthetic pathways in all living organisms suggest a common origin of life. The four major classes of natural products, with examples shown in the table below, are the polyketides, shikimates, isoprenoids and alkaloids (Clayden *et al.* 2000, Bu'Lock 1965)

Table 1.4.1 Major classes of natural products:

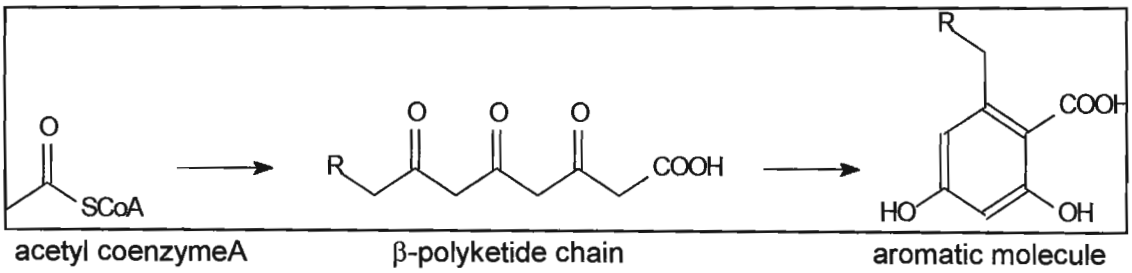
	Polyketides	Shikimates	Isoprenoids	Alkaloids
Precursor	Acetate	Shikimic acid	Mevalonate	Aliphatic amino acids
Examples	phenols, aromatics, fatty acids, prostaglandins, leukotrienes.	aromatics, tannins, lignans, cinnamic acid derivatives, aromatic amino acids.	terpenoids, steroids.	peptides, proteins, alkaloids.

Primary metabolites, biosynthesised via common routes in all living organisms, are found within these major classes. Universal examples, essential for all life forms, are nucleic acids, proteins, carbohydrates and lipids. Secondary metabolites on the other hand are quite distinct from primary metabolites. Whereas primary metabolism is associated with normal cellular function, secondary metabolism only occurs during senescence, and gives rise to unusual or unique metabolites not found in somatic organisms. These metabolites have restricted taxonomic distribution and no obvious metabolic role (Bu'Lock 1965), but they frequently play a defensive role in the species where they occur. Secondary metabolites are synthesized from a relatively small number of key intermediates in primary metabolic pathways, and frequently display notable physiological effects. Many drugs therefore originate from secondary metabolites, with classic examples being the alkaloids morphine, found in only two

species of poppy, and quinine from the Cinchona tree (Solomons 1992). The uteroactive compounds isolated in this study are also regarded as secondary metabolites.

The families of the three species studied in this research all contain aromatic compounds, and the biosynthesis of two major classes of these, lignins and tannins, is now considered.

Approximately 100 years ago it was first established that β-polyketoacids and β-polyketones could be converted into aromatic compounds under mild conditions that were believed to imitate nature remarkably (Fig.1.4.1). The generic name "polyketide" was given to all substances derived from a linear polyketide chain of repeating $-(CH_2CO)_n-$ units arising from acetic acid (Bu'Lock 1965).



acetyl coenzymeA β-polyketide chain aromatic molecule

Figure 1.4.1 Conversion of acetyl units to aromatic compounds

The knowledge of a common polyketide precursor established relationships between apparently quite different structural types such as flavones and stilbenes (Fig.1.4.2).

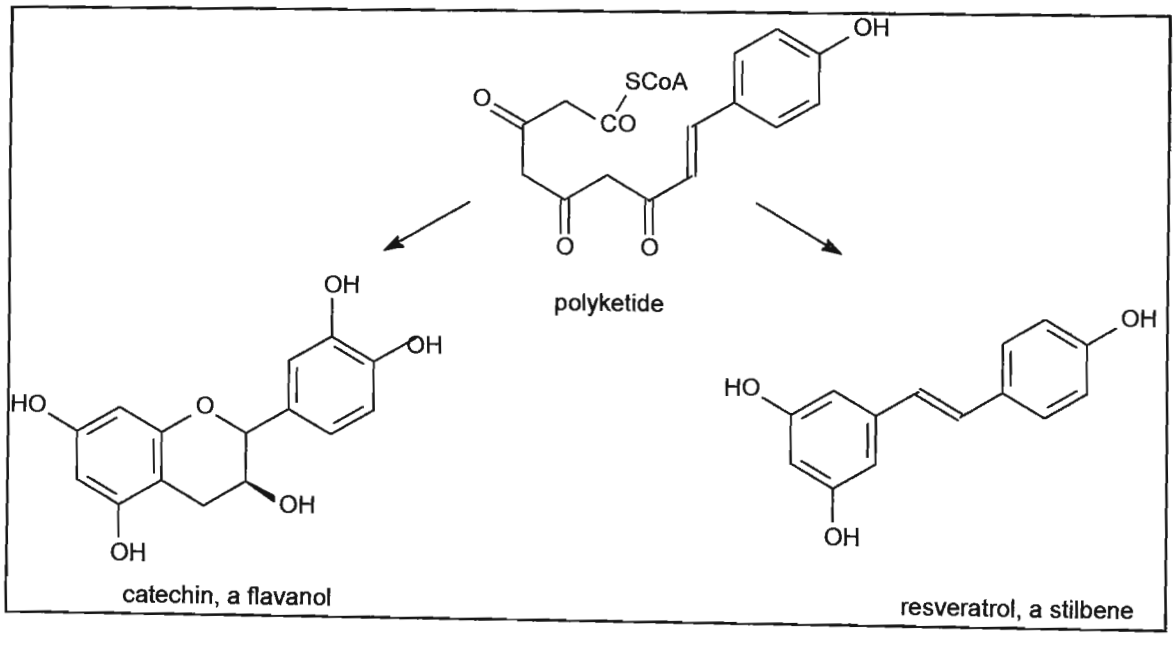


Figure 1.4.2 The relationship between stilbenes and flavones

It is interesting to note the common biosythetic link between proanthocyanidins such as catechin present in *Vitaceae*, and stilbenes such as combretastatin A1 in *Combretaceae*. The prostaglandins, spasmogens involved in the labour process, are also biosynthesized via the polyketide route.

Shikimic acid, first isolated from the Japanese plant "shikimino-ki" in 1885, is a key metabolite of pivotal importance in the biosynthesis of aromatic compounds and other related six-membered ring compounds (Clayden *et al* 2001). The starting materials for the biosynthesis of shikimic acid (Fig. 1.4.3) are phosphoenolpyruvate (PEP) and the four carbon sugar erythrose-4-phosphate, both intermediates in glucose metabolism. PEP is the nucleophile in an Aldol addition reaction with erythrose-4-phosphate. Prior to ring closure the C-5 hydroxyl is oxidised to a keto group which facilitates loss of the phosphate moeity. Ring closure occurs via a second Aldol reaction to form 3-dehydroquinic acid which is converted to the enol form, dehydrated, and finally reduced to shikimic acid as shown in the following scheme:

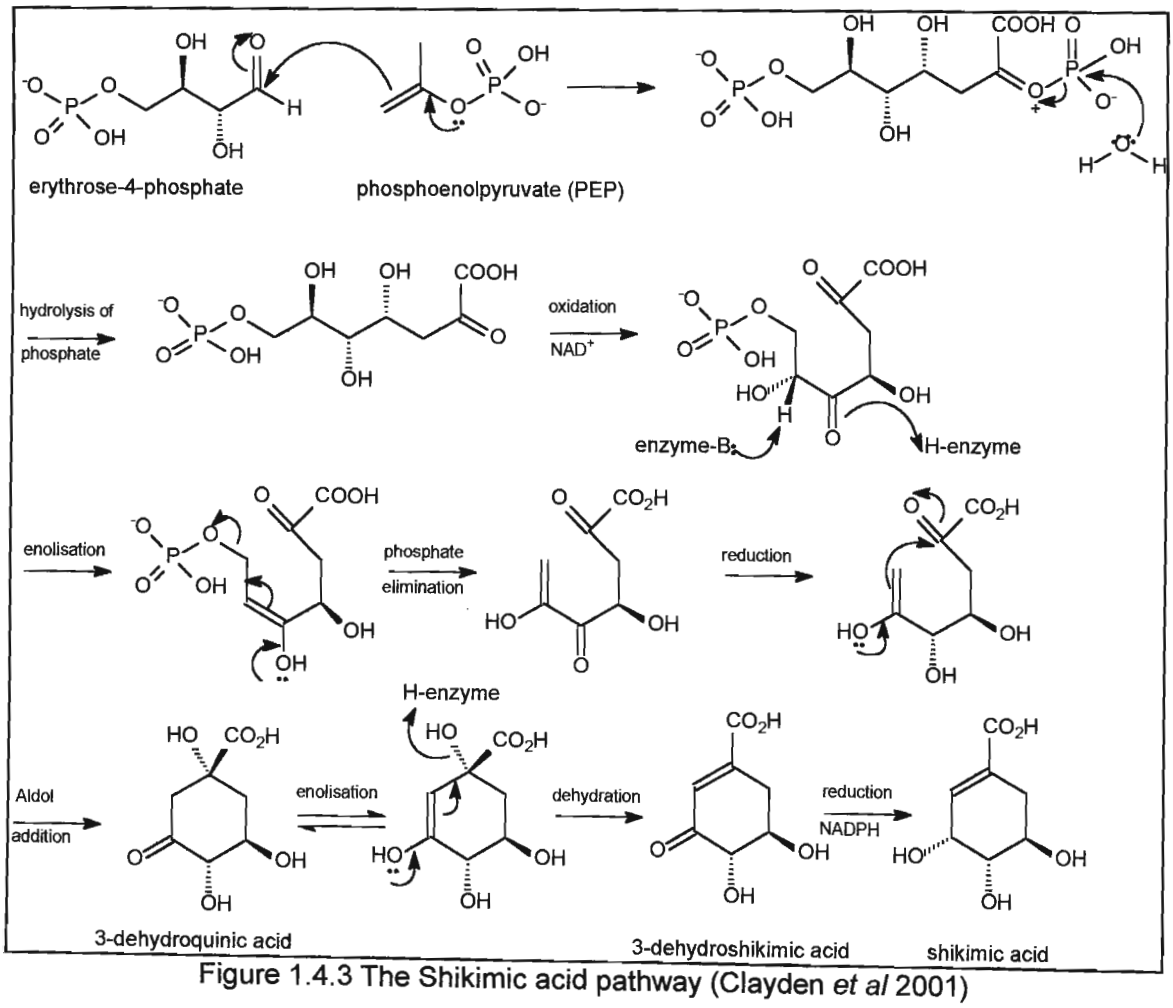
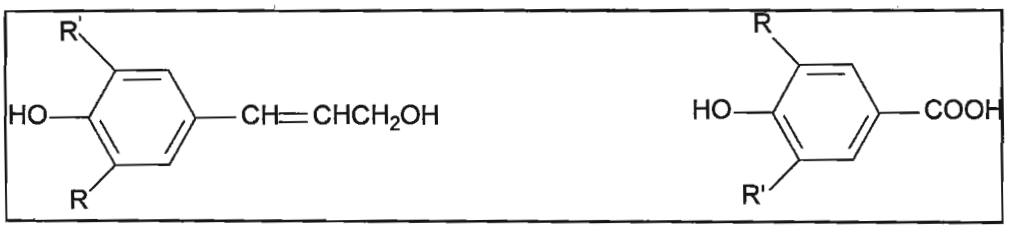


Figure 1.4.3 The Shikimic acid pathway (Clayden *et al* 2001)

Lignins and tannins

Lignins and tannins are the most abundant and widely distributed phenolic polymers found in higher plants. Lignins are so widespread that they could be considered to be universal, being present in far higher quantities than all other natural products except cellulose. Lignins (Latin lignum, "wood") are complex cross-linked polymers based on one or more C_9 phenylpropane monomers, so are classed as phenylpropanoids, and are the principal constituents of all woody tissue. Oxygen functions of the monomer are located on the terminal carbon of the side chain and also on the benzene ring para to the propyl side chain (Fig.1.4.4). The simplest monomer is p-hydroxy cinnamyl alcohol and the most common one is coniferyl alcohol. The third monomer, sinapyl alcohol, is found only in lignins which also contain coniferyl alcohol, and is particularly associated with angiosperms (Harborne,1964). Three phenolic acids (Fig.1.4.4), are found in plants which contain lignin, being broadly absent from those which do not contain lignin. These acids could be present as esters in the lignin polymer, or could also possibly arise from oxidative hydrolysis of the phenylpropanoid monomer double bond.



Lignin monomers:		Lignin-associated acids:	
p-hydroxy benzoic acid	R = R' = H	p-hydroxy cinnamyl alcohol	R = R' = H
coniferyl alcohol	R = H, R' = OCH_3	vanillic acid	R = OCH_3 R' = H
sinapyl alcohol	R = R' = OCH_3	syringic acid	R = R' = OCH_3

Figure 1.4.4 Lignin phenylpropanoid monomers and associated acids

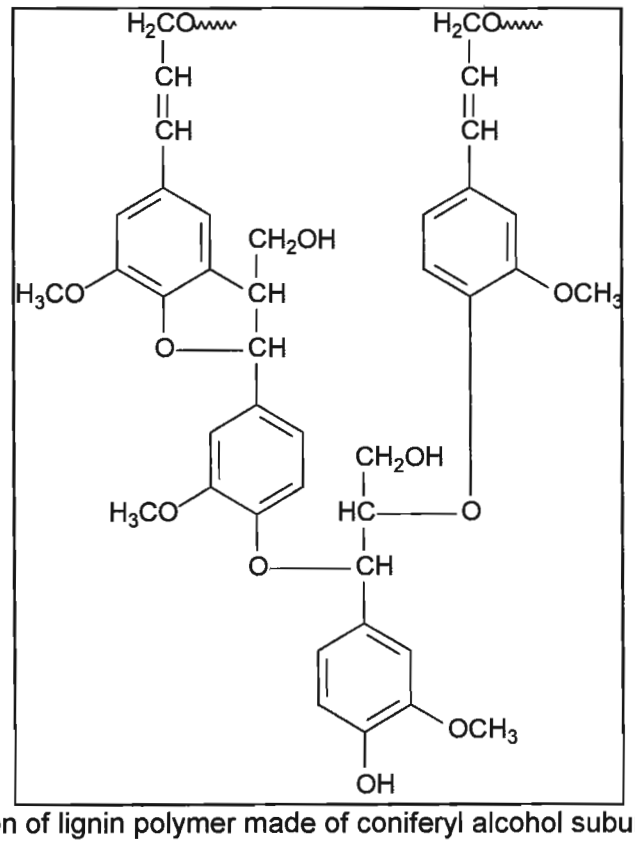


Figure 1.4.5 Section of lignin polymer made of coniferyl alcohol subunits (Mann 1994)

Lignin (Fig.1.4.5) is the major non-polysaccaride component of cell walls, which consist largely of cellulose, but other polysaccarides such as pectin, a polymer of D-galacturonic acid, and hemi-celluloses are also present (Campbell 1995, Carr and Cordell 1992). Cell wall components are cross-linked to lignin. A study by Grabber and co-workers in 1996 demonstrated how p-hydroxycinnamyl alcohols were polymerised via wall-bound peroxidases in maize cell walls to form dehydrogenation polymer-cell wall complexes. The hydrophobic nature of lignin and its attachment to structural polysaccahrides and proteins affects cell wall properties, increasing their strength and resistance to penetration by pathogens (Grabber *et al.* 1996).

Lignins and proanthocyanidins possibly have a common role in defence, even though there is no evidence that proanthocyanidins play a structural role in wood, as lignins do (Harborne 1994). Proanthocyanidins play a key role in as anti-oxidants in seedcoats and also play a defensive role against parasites. There is recent evidence that lignified dietary fibre reduces the incidence of gastrointestinal cancer in humans (Grabber *et al.* 1996).

Biosynthesis of lignin

The ultimate source of lignin and other aromatics in plants is carbon dioxide absorbed during photosynthesis. This has been demonstrated via radioactive labeling (Harborne, 1964), and

an enormous amount of carbon in the biosphere passes through this route. The key role of shikimic acid in the formation of lignins was first elucidated by Davis and associates in 1955. Shikimate is converted by a single biosynthetic pathway into the aromatic acids phenylalanine, tyrosine and tryptophan, the precursors to vast numbers of plant substances ranging from lignins to tannins, pigments, flavourants of spices, peptides, proteins and alkaloids (Mathews and van Holde 1990).

Phenylalanine and tyrosine are metabolised to cinnamic acid or 4-hydroxycinnamic acid via the action of specific lysases which remove ammonia. Non-specific free radical coupling of the alcohol analogues of these aryl-C_3 (phenylpropanoid) units produces tough and durable lignin polymers which represent about 50% of the structural material in woody plants (Mann, 1994).

The term lignan initially referred to a phenylpropanoid dimer with a C-8 to C-8' bond between monomers (Fig.1.4.6), but now also includes dimers with different linkages, and other classes of lignans such as flavanol lignans (Moss 2000).

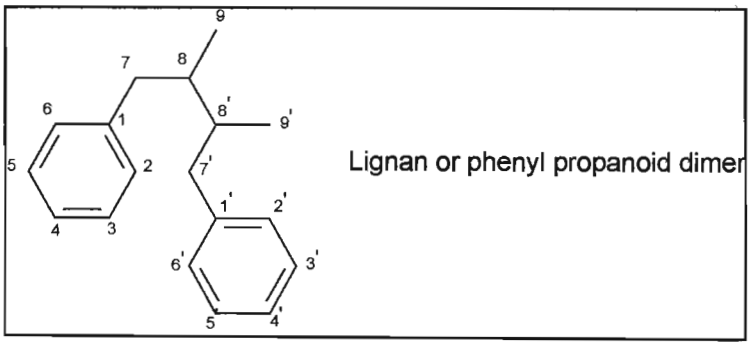


Figure 1.4.6 Lignan

Lignans often have significant pharmacological properties, including evidence of properties against cancer and HIV. Podophyllotoxin (Fig 1.4.7), from the plant *Podophyllum pelatum* is used to cure warts. Mayflower pilgrims found that New England Indians used the roots of this plant for that purpose. When podophyllotoxin was later isolated in the 1940's it was shown to be a powerful inhibitor of cell division by inhibiting the tubulin polymerase enzyme. Since the protein tubulin is an essential part of the fibres holding two daughter cells together during division, the process is interrupted. A derivative of podophyllotoxin, etoposide (Fig.1.4.8), is useful in cancer treatment, particularly for testicular teratoma and small lung cancer (Mann 1994). The low mortality from hormone-dependent cancer among Japanese people on traditional vegetarian diets has also been attributed to lignans, which could be converted in the intestine into hormone-like substances which act as cancer-protective agents (Aldercreutz *et al* 1991). In addition two lignans isolated from *Ipomoea cauica* strongly inhibited replication of the human immunodeficiency virus HIV-1 (Schroeder *et al* 1990).

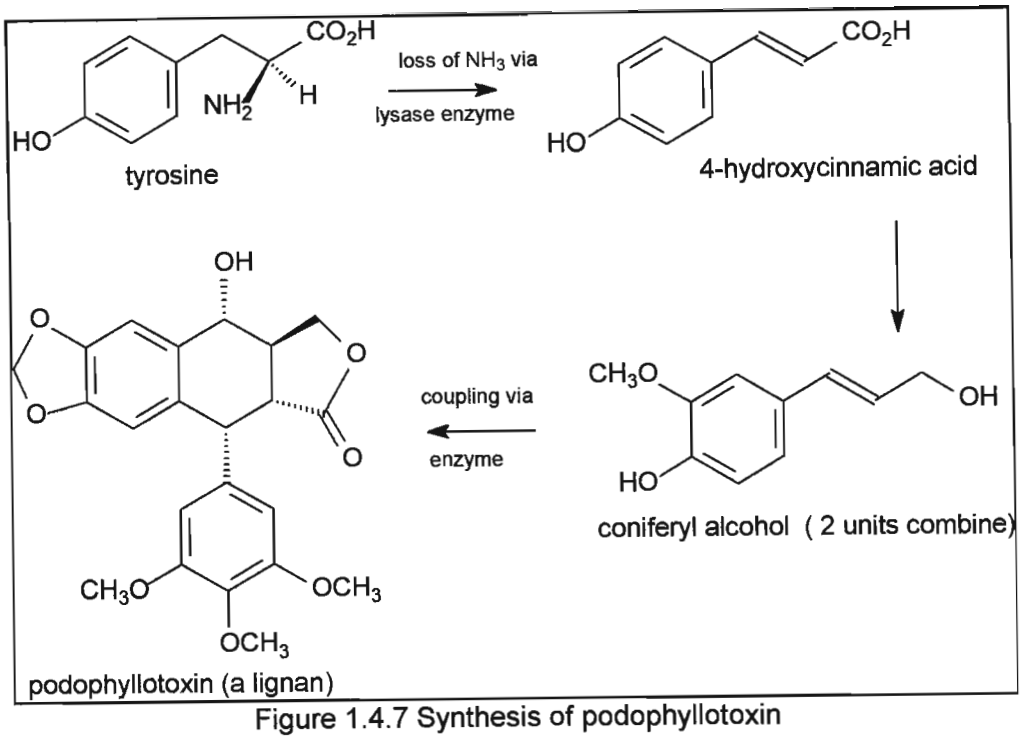


Figure 1.4.7 Synthesis of podophyllotoxin

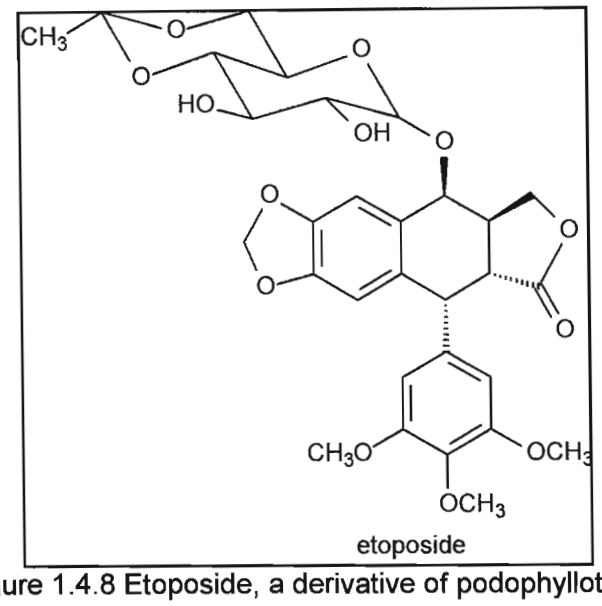


Figure 1.4.8 Etoposide, a derivative of podophyllotoxin

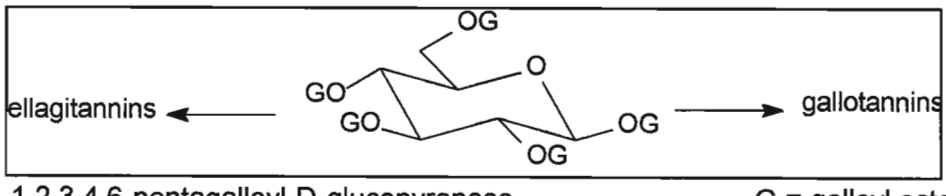
Tannins

These are complex polyphenols which are widely distributed secondary metabolites in the plant kingdom. They are broadly classified as:

- Condensed proanthocyanidins with the flavan-3-ol (catechin) nucleus
- Galloyl and hexahydroxydiphenoyl esters and their deivatives, the gallotannins and ellagitannins

Gallotannins and ellagitannins are the two main groups of hydrolysable tannins, and can be hydrolysed via acid or enzymes. However the non-hydrolysable tannins convert into more complex insoluble substances under these conditions.

Gallotannins are commonly occurring multiple esters of gallic acid (3,4,5-trihydroxybenzoic acid) with D-glucose. Ellagitannins are assumed to form by oxidative coupling of vicinal galloyl ester groups in a galloyl-D-glucose ester. The key biosynthetic intermediate is β-1,2,3,4,6-pentagalloyl-D-glucose which leads to ellagitannins as well as other gallotannins (Fig.1.4.9).

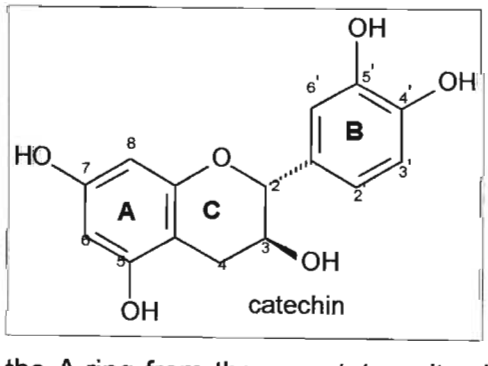


β-1,2,3,4,6-pentagalloyl-D-glucopyranose G = galloyl ester

Figure 1.4.9 Common intermediate for tannins.

Biosynthesis of condensed tannins

Condensed tannins can arise by the oxidative polymerisation of diphenylpropanoids such as catechins. Numerous studies have shown that diphenylpropanoids are formed by condensation of a phenylpropanoid unit with three acetate units. Wild cherry trees incorporated ^{14}C labelled cinnamic acid for the synthesis of the B-ring C_6-C_3 part of catechin. (Harborne 1964). It was also shown in 1962 that the A and B rings of tea catechins are formed from acetate and shikimic acid respectively.



The synthetic route has subsequently been elucidated further (Harborne 1994) and is now well established for proanthocyanidins of the 2,3-trans series such as catechin, and is shown below. Both precursors for the flavonoid skeleton are derived from carbohydrates. The B-ring is derived from a hydroxy-cinnamic acid ester, commonly 4-coumaryl-CoA, and the A-ring from three acetate units via malonyl-CoA. The key step of flavonoid synthesis (Fig.1.4.10) usually involves the condensation of 4-coumaroyl-coenzyme A, derived from L-phenylalanine, with three molecules of malonyl coenzyme A to form the C_{15} tetrahydroxychalcone intermediate. The subsequent steps include ring closure of ring C and an intermediate flavan-3,4-diol which is finally reduced to form catechin (Harborne 1994).

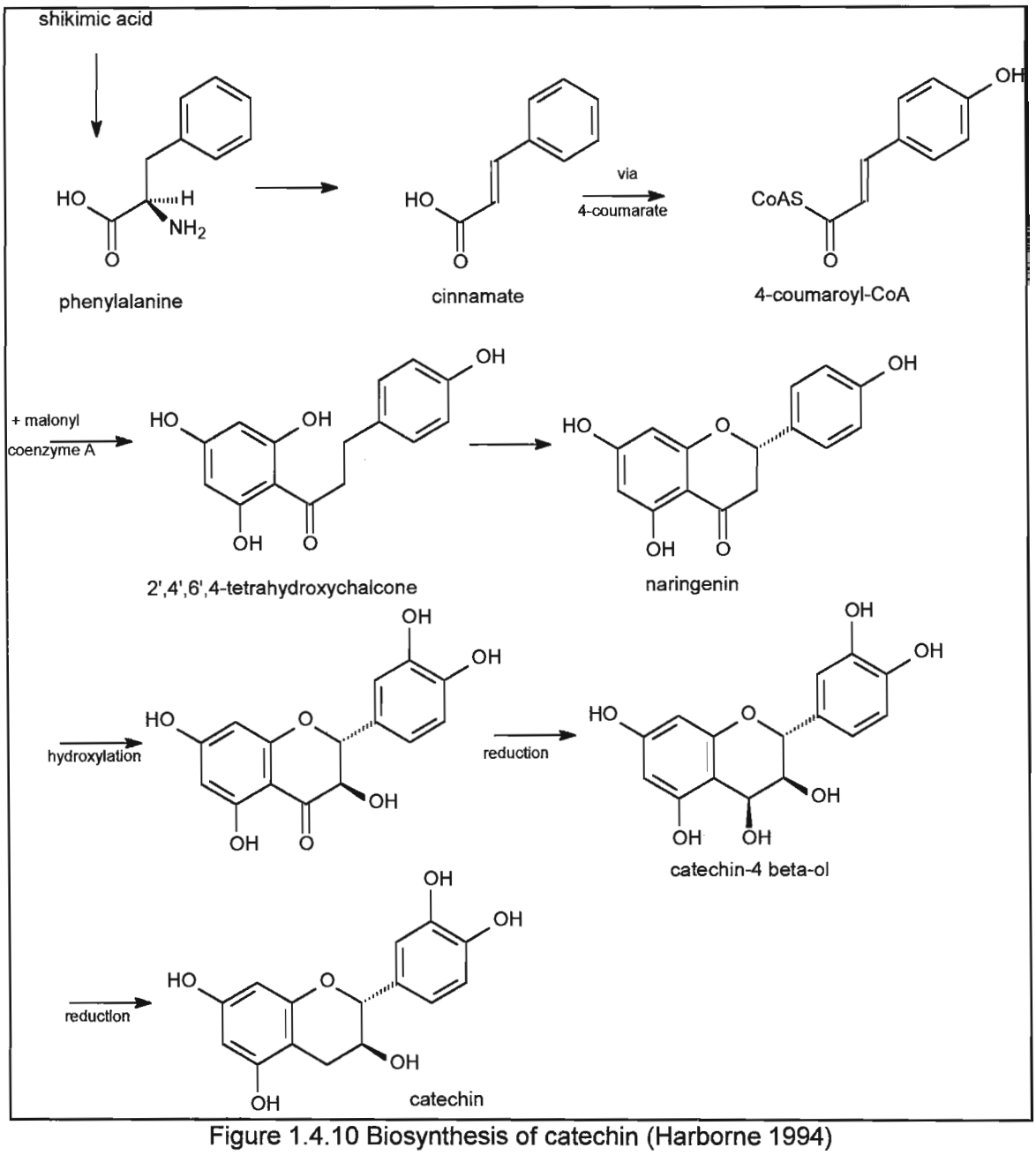


Figure 1.4.10 Biosynthesis of catechin (Harborne 1994)

CHAPTER 2

MATERIALS AND METHODS

2.1 INSTRUMENTS AND REAGENTS

The following techniques are typical of those used routinely throughout the course of this research. All general reagents were AR (Analytical Reagent) quality, and solvents for HPLC were all HPLC grade.

2.1.1 *Chromatography*

Thin layer chromatography

Qualitative thin layer chromatography (TLC) was performed on pre-coated aluminium-backed silica gel plates, layer thickness 0.2mm, using either Merck 5548 (silica gel 60 F_{254}) or Merck 5553 (silica gel 60) plates. The former were high performance plates for nano-t.l.c. Plates were not activated prior to use. Pre-coated 2mm thick silica gel 60 plates (Merck 5745) were used for preparative TLC.

Spray reagents for TLC

1. Anisaldehyde was used as a general purpose spray or dip: Anisaldehyde (0.5 cm^3), glacial acetic acid (10 cm^3) and conc. H_2SO_4 (5cm^3) were dissolved in methanol or ethanol (85cm^3). Plates were heated to ca. 100° for a few minutes after spraying or dipping the plate.

2. Aniline phthalate spray for carbohydrates: Aniline (0.93 g) and *o*-phthalic acid (1.66 g) were dissolved in water-saturated n-butanol (100 cm^3).

3. Bromocresol green spray for acids: Bromocresol green (0.04 g) was dissolved in 100 cm^3 ethanol and the pH adjusted to 6 with 0.1M NaOH. Acids appeared as yellow spots on a blue-green background.

4. Ferric chloride spray for phenols: A 1% aqueous $FeCl_3$ solution was used to detect phenols which frequently appeared as grey spots.

5. 2,4-dinitrophenylhydrazine spray for carbonyl compounds: 2,4-dinitrophenyl-hydrazine (0.4 g) was dissolved in 100 cm^3 of 2M HCl. Aldehydes and ketones appeared as orange spots on a pale yellow background.

Colour tests

1. Test for ester bond. Ten drops of water were added to a few milligrams of hydroxylamine hydrochloride in a test-tube, then 3 drops of sample for testing, 2 drops of ethanol and 3 drops of concentrated NaOH. The mixture was boiled, cooled, then 10 drops of 2M HCl were added. Universal indicator or litmus paper was used to confirm that the solution was acid, then 1-3 drops of 1% $FeCl_3$ were added. The development of a violet-red colour confirms the presence of an ester bond.

2. Molisch test for sugars. Two to three drops of a 1% ethanolic solution of 1-naphthol were added to 0.1 g of the compound for testing. Thereafter 2 cm^3 of conc. H_2SO_4 was added carefully down the side of the test-tube. A deep violet colour where the liquids met showed a carbohydrate was present.

3. Liebermann Burchard test for sterols. A few crystals (approximately 0.2 g) of the compound in a test-tube were dissolved in 3 cm^3 CH_2Cl_2. After the addition of 10 drops of acetic anhydride, and 3 drops c. H_2SO_4, the contents of the test-tube were shaken. Sterols with a double bond at C-5 produced a green, violet, blue or red colouration.

Column chromatography

Column chromatography was carried out using Merck 9385 silica gel, 230-400 mesh, particle size 0.040-0.063. The loading ratio was approximately 1:20 for the applied extract to silica gel, and silica gel was applied as a slurry in the solvent to be used. Components were dissolved in the mobile phase, but in certain cases where solubility was poor they were dissolved in another solvent, applied to a small amount of silica gel and the solvent evaporated. The small plug of silica gel was then applied to the top of the

prepared column. Components were eluted from silica gel columns in order of increasing solvent polarity, typically using the following, or similar, solvent mixtures:

hexane-ether	2:1
chloroform-methanol	20:1
hexane-ether	1:5
chloroform-methanol	10:1
chloroform-methanol	3:1
chloroform-methanol	1:1
methanol-water	1:1

The structures of the isolated components were elucidated using ^{1}H NMR, ^{13}C NMR, UV, IR, MS, GC/MS and HPLC spectroscopic methods. Unless otherwise specified, the purity of components isolated was greater than 95%.

2.1.2 Instruments

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian Gemini 300MHz spectrophotometer. TMS was used as internal standard for ^{1}H NMR spectra, and the central line of the deuterated chloroform triplet at δ 77.07 for ^{13}C NMR spectra.

IR spectra were recorded on a Pye Unicam SP3-200 infrared or a Nicolet Avatar 320 FT-IR spectrophotometer on samples dispersed in KBr discs. AA spectroscopy was carried out on a Pye Unicam SP9 spectrophotometer.

HPLC data were recorded on a Millipore Ion Chromatograph using a Lichrosorb RP 18 NC-04 column (particle size 5 μm, dimensions 250 × 4 mm ID); or a Thermo Separation Products (TSP) Spectraseries P100 HPLC using a Grom-sil 100 ODS-O AB column (particle size 5 μm, 250mm × 4mm ID) or a Hichrom C18-RP H5ODS column (250 × 4 mm ID) or a Wakosil C18-RP (250 × 4 mm ID) column. The detectors were a Spectraseries UV-106 254 nm fixed wavelength detector and a Shodex RI-71 detector. Sugar analyses were carried out on a Waters Carbohydrate Analysis HPLC column (125 Å 10 μm, 300 × 3.9 mm) or an Astec (Advanced Separation Technologies Inc.) Cyclobond II γ-cyclodextrin column (serial # A175-8), and peaks were detected with a Shodex RI-71 detector. Semi-preparative HPLC was carried out on a Bischoff 250x8 mm Prontosil 120-5-C18-AQ 5μm column.

GC/MS spectra were recorded on a Hewlett-Packard 5890 Series 11 Spectrophotometer with a 5971 Series Mass Selective detector. Silylation, if required for GC-MS, was carried out on 1-2 mg of freeze-dried sample by heating with bis(trimethylsilyl)acetamide (300 µl) in sealed vials at 60-80 °C for 1 h. Excess derivatising reagent was removed in a N_2 stream, and the sample was dissolved in $CHCl_3$ for GC/MS procedures.

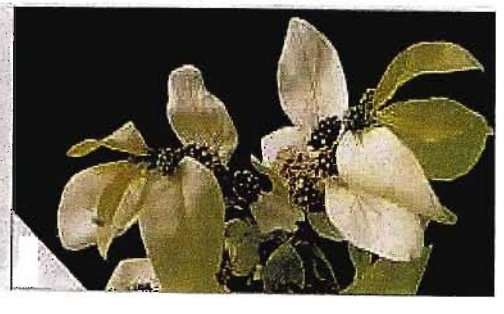
UV spectra were recorded on a Pye Unicam PU 8800 UV/VIS or a Varian Cary 50 Conc UV-visible spectrophotometer.

Mass spectral determinations were carried out using EI and FAB MS techniques on a VG 70-70E instrument.

Melting points, uncorrected, were determined on a Kofler hot stage apparatus.

2.2 *COMBRETUM KRAUSSII*

Trees with young leaves (white)



Young leaves and flowers

Winged fruit

Distribution in South Africa

Plant photos : *A Field Guide to Trees of Southern Africa*, 1983, Struik Publishers ; Map : *Medicinal Plants of South Africa*, 2000, 2nd ed., Briza Publications.

2.2.1 Background and uses

Combretum kraussii Hochst., commonly known as the forest bushwillow, is a shrub or medium-sized tree which grows up to 12 metres in height. Zulu name: *umdubu*. It is found in the North-Eastern and coastal regions of South Africa as shown in the distribution map above. It grows at medium altitudes down to sea level in evergreen forests, wooded valleys or open woodland, rocky hillsides, and also mountain grassland (van Wyk and van Wyk 1983). Creamy-white flowers appear in August to November. The 4-winged fruit becomes russet-red when mature and dries to a mid-brown in February to June.

Shrubs and trees of the 250 species of the genus *Combretum* represent over one third of the Combretaceae family , found in tropical and sub-tropical areas, and are well-known for various primitive medical treatments. Twenty-four African species have diverse applications such as heart, diarrhoea and worm remedies, wound dressings, treatment for the mentally ill, and scorpion stings, as well as infertility and bleeding after childbirth. Eight species of the Combretaceae family (genus *Terminalia* and genus *Guiera*) have a long-standing reputation amongst traditional healers as being effective in the treatment of cancer (Pettit *et al.* 1982; Pettit *et al.* 1988) as well as the Indian *Combretum latifolium*.

Dust from the sawn wood can prove irritating to the skin and is reported to have caused dermatitis on several occasions, and produced blisters between the toes of a child who walked barefoot on the sawdust (Watt and Breyer-Brandwijk 1962). Blisters on the hand and irritation inside the nostrils were also observed while working with a milled root sample during my research.

The Zulu regard *Combretum erythrophyllum* which is also listed as *umdubu*, together with *Combretum kraussii* (Cunningham 1988), as poisonous, but small doses of root are given to dogs as a fattening tonic. The tree has slightly positive antibiotic properties and yields a gum used for tanning. The root is a purgative (Watt and Breyer-Brandwijk 1962). Although the fruits of the genus *Combretum* are widely regarded as toxic, and are not consumed by wild animals or used by healers, there is only one case where the toxicity of the fruit has been clinically proven (Rogers *et al* 1996). The fruit is known as the "hiccup nut" because it produces persistent hiccupping when eaten.

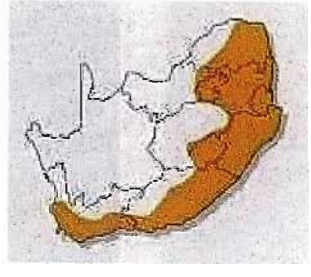
2.2.2 ***Plant material***

Combretum kraussii roots, 3 kg, were obtained from Pigeon Valley Nature Reserve, Durban, in June 1995 (voucher specimen no. 121751 at the National Botanical Institute Natal Herbarium). Most of the compounds isolated were from this batch of roots, although subsequent smaller specimens were obtained from the same source. All extracts were prepared by boiling plant material with approximately ten times the volume of solvent under reflux for about 1 h. The extract prepared with hot methanol, comprising 8.57% of material from 3 kg of dried and milled roots of *C. kraussii*, was separated by column chromatography. HPLC analysis of water and methanol extracts gave similar results.

2.3 *GUNNERA PERPENSA*

Whole plant (Photo B. Brookes)

Flower



Distribution in South Africa

Flower and map: *Medicinal Plants of South Africa*, 2000, 2nd ed., Briza Publications.

2.3.1 ***Background and uses***

Gunnera perpensa L. is one of six subgenera belonging to the family Gunneraceae which is essentially restricted to the Southern hemisphere. The genus *Gunnera* is subdivided into six subgenera based on its ample morphological diversity. It grows in the Southern hemisphere in the Eastern part of South Africa, and also in Malasia, Hawaii, Antarctic Islands and South America. It is usually associated with water and grows wild near river banks, often being referred to as the "river pumpkin". The shrub has dense, feathery

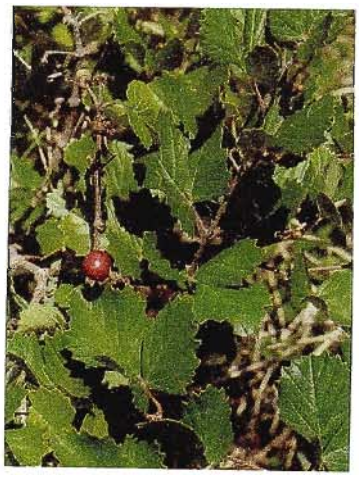
foliage and bears pink flowers on spikes (Pujol 1990). The Zulu, Xhosa and Sotho people of South Africa use the soft fleshy roots medicinally for a variety of purposes, such as treating diseases of the urinary tract, relief of rheumatic pains, to treat psoriasis, and as a remedy for impotence and barrenness (Watt and Breyer-Brandwijk). Root decoctions are commonly credited with many benefits associated with pregnancy and childbirth including good foetal development, a quick, easy labour, as well as expulsion of the afterbirth (Hutchings 1996). In addition to human usage, *Gunnera perpensa* preparations are also used in veterinary science to speed up labour in animals. There are apparently antiseptic and/or antibiotic properties associated with root decoctions as they are used to dress wounds and treat colds. The Xhosa people use water extracts to wash cows and sheep which have given birth. The strong antiseptic properties are credited with giving protection from tick bites and other parasites (Pujol 1990).

It is not surprising, considering the profusion of medicinal properties attributed to *Gunnera perpensa,* that it is amongst the most frequently cited species used by South African traditional healers in "*isihlambezo*" decoctions (Veale et al 1998).

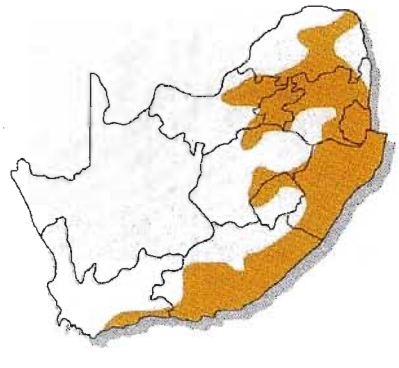
2.3.2 ***Plant material***

Gunnera perpensa roots were harvested at Silverglen Nature Reserve, Kwa-Zulu Natal and voucher specimen Brookes (1) NH was deposited at the National Botanical Institute, Durban. Roots, which are soft and fleshy, were chopped with a knife or shredded in a blender before extraction. Typical yields from 726 g roots harvested in November 1999 were 1.6% per dry root mass for the CH_2Cl_2-MeOH extract, and 12.9% for the water extract. HPLC analysis of water and methanol extracts gave similar results.

2.4 *RHOICISSUS TRIDENTATA*



Leaves with fruit and distribution map in South Africa.



Photos: *Medicinal Plants of South Africa*, 2000, 2nd ed., Briza Publications.

2.4.1 Background and uses

Rhoicissus tridentata (L.f.) Wild & Drumm. subsp. *cuneifolia* (Eckl. & Zehr.), N.R. Urton, a shrubby creeper in the Vitaceae family, is commonly known as the wild or bitter grape, with the Zulu name *isinwazi* or *umtwazi* (Hutchings 1996). The genus *Cissus* L. consists of climbing herbs or shrubs growing in tropical or warm regions.

Chopped root decoctions are frequently used to facilitate the delivery of a baby or to initiate labour if the onset is delayed. *Rhoicissus tridentata* is used in *isihlambezo* mixtures prescribed by traditional birth attendants to ensure the health of the mother and baby during pregnancy. Other typical ingredients are *Callilepis laureola* D.C., *Gunnera* perpensa L. and *Vernonia* natalensis. Zulu women also use root decoctions as an enema for painful menstruation (Watt and Breyer-Brandwyk 1962, Hutchings 1996). Tubers are used for sterility, epilepsy, renal complaints and cattle diseases (Pujol 1990).

Medicinal uses of *Rhoicissus tridentata* are also documented in various other parts of Africa. For example the Sotho use decoctions to treat colds and infertility, and Sotho children eat the astringent fruit. Tubers decoctions are taken by pregnant women in Botswana for abdominal distension. The Masai use unspecified plant parts as a nerve stimulant. In Tanzania deaths of swine from ingestion of tubers have been reported, and the toxicity of tubers has been confirmed in rabbits (Hutchings 1996). Tubers are also suspected of causing a human death due to acute paralysis of the central nervous system leading to respiratory paralysis (Watt and Breyer-Brandwyk 1962, Hutchings 1996).

2.4.2 Plant material

Plant material used by healers as "izinwazi" (Zulu), harvested in the Umbumbulu district, October 1996, was supplied by Protas Cele of the Traditional Healers Association, Umlazi, Kwa-Zulu Natal, and identified by the National Botanical Institute, Natal Herbarium, as *Rhoicissus tridentata* (L.f.) Wild and Drumm. Subsp. Cuneifolia (Eckl. and Zeyh) N.R. Urton. This species is routinely used by traditional healers in the Kwa-Zulu Natal area (personal communication, Protas Cele). Material obtained from Treasure Beach Grasslands in July 1995 was also identified as the same species, and voucher specimen number 086931 was deposited at the C.E. Moss Herbarium, Wits University.

Plant roots were typically milled or shredded and successively extracted by boiling for approximately 1 h in solvents of increasing polarity *i.e.* chloroform (or methylene chloride), methanol and water. Solvents were removed under vacuum below 50 °C or by freeze-drying. Typical yields, based on the dry root mass are: methylene chloride extract 0.5%; methanol extract 2.1%; water extract 3.8%. These yields were for roots harvested in the Umbumbulu area in October, 1996. However the mass of methanol extractable material is generally higher for roots harvested in mid-summer, and roots harvested in January 2000 from the same area produced 3.5% of freeze-dried methanol extract, based on the dry root mass. HPLC analysis of water and methanol extracts gave similar results.

2.5 UTERINE ACTIVITY TESTS

2.5.1 Introduction

All pharmacological tests related to uteroactivity, with the exception of preliminary tests described below, were carried out by Joy Veale and Lynn Katsoulis at Wits University Pharmacology Department, with the relevant ethical approval for animal experiments. Uterine tests in this research involved pharmacological screening of fractions obtained from plant extracts. The bioassay involved strips of rat uterine smooth muscle tissue for *in vitro* detection of uterotonic components.

Isolated animal tissues are frequently reported for bioassays and toxicity determinations in medicinal plant research. Apart from the testing of uterotonic compounds, isolated uterus has also been used to determine structure-activity relationships for biologically active molecules, and for the understanding of drug-receptor mechanisms (Thomas 1981). In an *in vitro* study the response to an agonist is proportional to both its concentration and the density of the relevant receptor sites. There are several major advantages with respect to *in vitro* analysis over *in vivo* experiments where the response to an agonist is influenced by many metabolic variables such as excretion of the drug being tested. Although certain pharmaco-kinetic changes can also occur *in vitro*, the probability is low, and therefore results are generally more reliable than those from *in vivo* analyses.

2.5.2 *Preliminary measurements of pharmacological activity*

Initial uterine response tests were carried out by Dr Des Raidoo at the Physiology Department, Medical School, University of Natal, Durban. The uterine muscle response was tested electrically 1 to 3 times for each plant species, as shown in the table below. Plant roots were obtained from Mr Govender's herbalist shop at 22 Commercial Rd, Durban. Roots were chopped, boiled for 30 min. in water, the solutions filtered and made up to 250 cm^3.

Root	Water %	Mass dry root per cm^3 of water solution mg/ cm^3	Uterine Response 1	2	3
"ugobo" *Gunnera perpensa*	65.5	36.8	N/R	100 cm^3 N/R	100 cm^3 N/R
"Umdubu" *Combretum kraussii*	24.7	33.1	N/R	100 cm^3 +	100 cm^3 +
"Isinwazi" *Rhoicissus tridentata*	82.7	32.0	N/R	-	100 cm^3 + 200 cm^3 +
"Ibhuma" *Typha latifolia*	41.4	16.1	-	-	200 cm^3 N/R or +
"Ishinga" *Asclepias fruticosa*	20.6	35.4	+	100 cm^3 + 100 cm^3 +	300 cm^3 +

N/R = no response + = positive response

Following these preliminary results, it was decided not to carry out further work on *Typha latifolia* and *Asclepias fruticosa*. Samples of *Typha latifolia* from the Botanical Gardens, identified by Anne Lambert, grew at the edge of the water and the water content of fresh samples was extremely high, usually over 90%. This meant that a very large amount of plant material was needed in order to have sufficient for tests, and the results were not conclusively positive. The plant had also become scarce so it was very difficult to obtain samples.

Although *Asclepias fruticosa* gave positive results, it is known to contain lethal cardiac glycosides, and for that reason could not be recommended for use by healers for pregnant women. In addition, it was clear by comparing roots sold as "ishinga" in Commercial Road with those obtained from Silverglen Nursery and identified as *Asclepias fruticosa*, that these were two different plants. Roots of *Asclepias fruticosa* are

hard and woody whereas those from the herbalist shop were soft, could be easily sliced, and were totally different in appearance. The poisonous nature of *Asclepias fruticosa* is known among the Zulu people, and I was even told by our laboratory cleaner that "those roots are dangerous" when I was working with them. I suspect that the original "ishinga" has been substituted by a less toxic equivalent.

Further work was restricted to the first three plants in the table, even though *Gunnera perpensa* "ugobo" did not look promising from the above lack of response. Preliminary investigations carried out by Joy Veale and Lynn Katsoulis at Wits University Pharmacology Department showed that *Gunnera perpensa, Combretum kraussi* and *Rhoicissus tridentata* extracts stimulated contractions of isolated uterine tissue in a dose-dependent fashion. This meant that all three chosen plants showed positive preliminary tests for uterine activity. Crude hot water root extracts of these three plants were prepared in the same way as used by traditional healers. The results of further tests on components isolated from the three plants are shown in Chapter 6.

It was also felt that five plants would have been too many for a comprehensive chemical and pharmacological study on each. *Combretum kraussi*, and *Combretum erythrophyllum* are both listed as "umdubu" used medicinally during pregnancy (Cunningham 1988). However it was established that the species of *Combretum* used locally in Kwa-Zulu Natal was *Combretum kraussi*, and not *Combretum erythrophyllum*, via identification of a sample provided by Protas Cele, a leading traditional healer in Umlazi.

2.5.3 Isolated organ preparations

The following experimental procedures were followed for all further pharmacological tests carried out at Witwatersrand University.

Virgin Sprague-Dawley rats weighing about 250 g were oestrogenized with stilboestrol (Maybaker SA) by intraperitoneal injection 24 h prior to euthanasia with CO_2 and dissection (Veale *et al.*1989; Kaido *et al* 1997).In more recent work the use of stilboestrol was discontinued, and it was replaced by oestrogen. The uterine horns were exposed and 1 cm sections were dissected from the central portion of each horn. The sections were trimmed of fat and opened longitudinally. Portions of uterus (1.5 cm) were mounted in 50 cm^3 organ baths containing Tyrode solution, and aerated with 5% CO_2 in oxygen. The temperature was maintained at 26 °C to decrease spontaneous contractility

(Kumagai *et al* 1952). An equilibration period of at least 30 min. was allowed, and during this period the bath was rinsed by upward displacement at least three times.

Isotonic contractions against a load of 1 g were measured and recorded electronically on a Metrohm Labograph Potentiometric Recorder (model E428). The organ was allowed to rest for at least 30 min. between drug challenges, and the bath was rinsed at least three times during this rest period. Cumulative dose-response curves were constructed. A control curve with the reference drug, acetylcholine hydrochloride (ACh) (Sigma), was obtained before every administration of the test extract. The organs were pre-treated for 5 min. with the herbal extract, or purified component, before cumulatively adding the reference drug, acetylcholine. The stock solution contained 2 g of dried plant extract in 15 cm^3 water solution, and the maximum dose administered was usually 0.7 cm^3. The maximal responses to the plant extract are expressed as a percentage of the maximal response to acetylcholine. All stock solutions were made up in Milli-Q double distilled water.

Extracts and purified components from roots of the three plants were generally used for uterine tests. However small-scale extractions of different plant parts using water and other solvents were also carried out for organ tests on crude extracts. For example *C. erythrophyllum* (Burch.) Sond. leaves and branches were collected in Magaliesburg, and leaves and branches of *C. kraussii* Hochst were collected from the Pretoria National Botanical Gardens for organ tests on crude aqueous extracts. Both species were harvested in March 1996. All plant material was dried away from direct sunlight, milled and boiled in water for 1 h to mimic the preparation techniques followed by local traditional healers. The infusion was lyophilised, then reconstituted in constant concentrations.

2.6 DETERMINATION OF NUTRIENTS IN PLANT ROOTS

Levels of various ions as well as simple sugars that could play a role as nutrients, or contribute to muscle contractility during pregnancy, were measured. Calcium, iron, sodium and phosphate ions were determined in ashed samples of plant roots as well as in solutions made from plant roots directly. These measurements were carried out for all three plant roots under investigation. Metal ions were determined via AA spectroscopy and phoshate levels via a colorimetric method. All solutions for these analyses were prepared using MilliQ water.

Ashing and sample preparation for metal ion analysis

Approximately 1 g samples of shredded roots were accurately weighed into crucibles and heated at 500 °C in a furnace for 30 min., then cooled in a desiccator. Thereafter 3 cm^3 of 1:1 conc.HNO_3:water was added and the solutions carefully boiled to dryness in a fumehood. The crucibles were then returned to the furnace for a further 30 min. After cooling, 10 cm^3 of 1:1 conc.HCl:water was added to each and the contents quantitatively transferred to 50 cm^3 volumetric flasks with water.

For water extraction followed by measurement of the ion content, 5 to 15 g of shredded plant roots were accurately weighed and extracted by boiling with water for about 30 min. The solution was then transferred to a 50 cm^3 volumetric flask, the roots washed three times, and then the solution made up to the mark. Three 10 cm^3 aliquots were pipetted into crucibles and ashed and prepared for AA measurements according to the above method for the roots themselves, except that solutions were finally transferred to a 10 cm^3 volumetric flask. The ashing procedure is necessary in order to remove organic material which could interfere with the spectrophotometric determination.

For AA measurements a 1000 ppm stock solution was prepared using the relevant Analar grade standard in MilliQ water, and this was diluted to produce solutions containing 2, 5, 10, 15 and 20 ppm as standards for the analysis. The following standards and wavelengths for the AA were used:

Analysis	Standard	Wavelength λ in nm.
Ca	$Ca(NO_3).4H_2O$	422.7
Fe	$(NH_4)_2Fe(SO_4)_2.6H_2O$	248.3
Na	NaCl	330.3
K	KCl	769.9

Each analysis was carried out in duplicate or triplicate using a Phillips AA hollow cathode lamp or a potassium lamp.

Phosphate analysis

A 1000 ppm stock solution was prepared by dissolving 4.390 g AnalaR grade $K_2H_2PO_4$ (99%) in MilliQ water acidified with 25 cm^3 3.5M H_2SO_4 and making up to 1000 cm^3 in a volumetric flask. Six standards, between 1 and 10 ppm, were prepared by diluting the stock solution. Two solutions were prepared for developing the colour for analyses. Solution A consisted of approximately 0.25 g of potassium antimony tartrate, $KSbO.C_4H_4O_6$, UniLAB, chemically pure (99.5%), which was dissolved in 100 cm^3 water in a 500 cm^3 volumetric flask. Concentrated H_2SO_4, 37.5 cm^3, was added slowly and the solution was cooled, then 3.2 g ammonium molybdate, chemically pure, dissolved in 75 cm^3 water was added and the solution made up to the mark with water. Solution A was stored in the refrigerator and Solution B made up only on the day of analysis. Solution B consisted of 0.1 g gelatine dissolved in 100 cm^3 hot water to which 0.1 g. of ascorbic acid was added and the solution mixed thoroughly and then 15 cm^3 of solution A was added as well. The colour was developed by taking 1 cm^3 of the standard or plant root solution and adding 4 cm^3 of water and 5 cm^3 of Solution B, then taking a reading on the UV/VIS spectrophotometer at 680 nm within 30-45 min. after addition of Solution B.

2.7 TOXICITY TESTS

Several methods for testing the toxicity of compounds are available such as *in vitro* vetinary screening, or *in vivo* animal or human experiments. However cytotoxicty testing using live cells in culture is frequently the modern method of choice, and was used for determining toxicity levels in this study which was carried out in order to establish whether the three plants chosen exhibited any significant toxic effects at a cellular level. There has recently been a move away from *in vivo* rodent models towards *in vitro* models for drug evaluation. Although *in vitro* methods cannot simulate the interaction of the tested component with the whole living organism, they do offer a quick and efficient means of establishing responses at a cellular level which can serve as an indicator for the response of the whole organism. The present study, using monkey vero and human fibroblast cell lines, established cell viabilty levels in the presence of varying concentrations of *Gunnera perpensa, Combretum kraussii* and *Rhoicissus tridentata* root extracts.

Cell viability tests were conducted by Mr T. Venketsamy in the Virology Department, Medical School, University of Natal, Durban, Kwa-Zulu Natal, and the Head of Department, Prof. A. N. Smith, offered valued procedural advice. Viability levels for

survival of both monkey vero and human fibroblast cells were established in the presence of the plant root extracts, and non-viable cells were counted after staining.

Experimental procedure

Plant roots were all harvested in the Durban area : *Gunnera perpensa* from Silverglen Nature Reserve in November 1999 ; *Combretum kraussii* from Pigeon Valley Nature Reserve in May 1998 and *Rhoicissus tridentata* from Umbumbulu in February 2000. The three commonly used species were identified either by staff at the nature reserve where they were grown or by submission of a specimen to the Natal Botanical Gardens Herbarium for identification. Extracts were freeze-dried and stored *at* –5 °C. Solutions containing 0.1% of freeze-dried plant extract dissolved in milli-Q water were further diluted for the tests.

Duplicate readings were obtained at each concentration of the plant extracts for monkey Vero cells. The threshold concentration which resulted in no monkey vero cell deaths at all was ascertained by triplicate readings. It was then established, via triplicate readings, that at the same concentrations there were also no human fibroblast deaths. Thus all zero cell death concentrations for vero cells also apply to fibroblasts (Results Chapter 8).

Vials containing 1 cm^3 of Human Fibroblast and 1 cm^3 of Monkey Vero cells were taken out of liquid nitrogen storage and thawed in a 37 °C waterbath. The contents were then transferred to a 75 cm^3 culture flask and diluted with Eagle Minimum Essential Medium (EMEM) obtained from BioWhittaker and 10% Fetal Bovine Serum (FBS) obtained from Delta Bioproducts. The flasks containing the diluted cells were incubated at 37 °C and thereafter read daily for percentage growth. Upon reaching 100% confluency, the cells were trypsinised, diluted with EMEM and 10% FBS and placed in 25 cm^3 flasks for the toxicity tests.

The plant extracts were diluted with EMEM and used to cover the cells in the 25 cm^3 flasks which were then incubated at 37 °C for 24 h. The cells were then washed with phosphate buffered saline at pH 7.2 and trypsinised using a 0.25% trypsin solution. The trypsinisation was stopped with 10% FBS in EMEM and a 1:1 dilution of the cells was made with a 0.4% tryphan blue solution which stains the dead cells. Both the total number of cells and the number of stained cells were counted using an Olympus light microscope. The percentage of non-viable cells was calculated from these data.

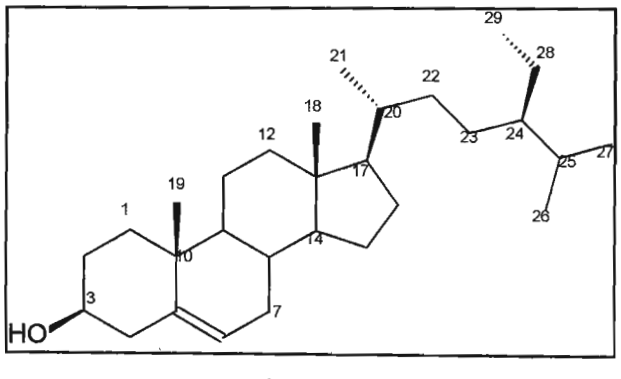
CHAPTER 3

METABOLITES FROM COMBRETUM KRAUSSII

Eleven components were isolated from *C. kraussii* root extracts by silica gel chromatography and nine of these were characterised as sitosterol, combretastatin, 3,3',4-tri-O-methylellagic acid lactone, combretastatin B-1, combretastatin A-1, 3,3'-di-O-methylellagic acid lactone, ellagic acid lactone, and a mixture of combretastatin B-1 2'-O-β-D-glucopyranoside and combretastatin A-1 2'-O-β-D-glucopyranoside. Components **10** and **11**, extremely polar ellagic acid glucosides, were not identified fully, and require further investigation. Ellagic acid derivatives and combretastatin were reported for the first time in *C. kraussii* (Brookes *et al.* 1999). Most of the compounds were isolated from a hot methanol extract of *C. kraussii* roots harvested from Pigeon Valley Nature Reserve in June 1995. Methanol was generally preferred to water for preparing root extracts because it is far more volatile and easier to remove. It was also found that even the most polar compounds, such as **10** and **11**, were soluble in methanol as well as water.

Compound 1 (*Sitosterol*)

Appendix: CI-MS, IR and ^{1}H NMR and ^{13}C NMR. Acetate: IR.



sitosterol

Physical data

The fraction eluted from a silica gel column with hexane-ether 2:1 produced 1.78 g (0.37%) of crude compound **1**, based on the dry plant mass. After evaporation of solvents, 15 cm^3 of methanol was added and the methanol-insoluble material was filtered off and recrystallised from methanol afforded colourless plates, m.p. 142-144 °C (lit. m.p.

138-139 °C for sitosterol, Chattergee *et al.* 1977), $[\alpha]_D^{25}$ –25.4 °. Compound **1** gave a violet spot R_f 0.44 on TLC in hexane-ether 1:1, identical to that of an authentic sitosterol standard, and produced a violet colour for the Liebermann-Burchard sterol test.

EI-MS m\z M^+ 414.3866 (414.3861 = $C_{29}H_{50}O$);

399	$M^+ - CH_3$
396	$M^+ - H_20$
381	$396 - CH_3$

IR (KBr disc): 3400; 2920, 2860; 1455; 1375 and 1060 cm^{-1}.

^1H NMR ($CDCl_3$) δ values: 0.66 (3H, s, H-18); 0.78 (3H, d, J = 7.1 Hz, H-27); 0.80 (3H, d, J = 7.9 Hz, H-26); 0.82 (3H, t, J = 7.2 Hz, H-29); 0.91 (3H, d, J = 3.2 Hz, H-21); 0.98 (3H, s, H-19); 3.50 (1H, m, H-3α); 5.32 (1H, m, H-6).

^{13}C NMR ($CDCl_3$) in ppm: Characteristic resonances at 140.77 (C-5), 121.77 (C-6) and 77.4 (C-3) for sitosterol. All other signals matched those of an authentic standard.

The monoacetate was prepared by heating in pyridine-acetic anhydride for 1 h at 100 °C. Recrystallisation from methanol gave a pure product, m.p.122-123 °C (lit.124-125 °C for sitosterol acetate, Briggs *et al.* 1961), M^+ 456.751 for $C_{31}H_{52}O_2$.

Discussion

Compound **1** gave a positive sterol test, and had an accurate mass of M^+ 414.3866, consistent with formula $C_{29}H_{50}O$ for a sterol. The IR spectrum showed O-H stretch for an alcohol, C-H stretch at 2920 and 2860 cm^{-1}, as well as C-O stretch at 1060 cm^{-1}, in the range for a secondary alcohol.

The position and multiplicity of the shielded ^1H NMR signals between δ0.66 to 0.98 corresponded well with those of sitosterol. In addition the characteristic multiplet at 5.32 indicated the deshielded C-6 alkene proton, and the multiplet at δ3.50 was assigned to the C-3α proton expected for a C-3 β-hydroxy sterol.

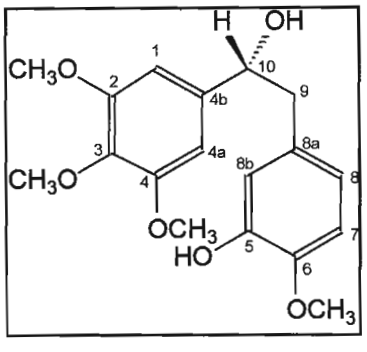
The acetate showed M^+ at 456.751, consistent with formula $C_{31}H_{52}O_2$ for a mono-acetate. Compound **1** was identified as sitosterol (24R-stigmasterol-5-en-3β-ol) by comparison of

optical rotation and IR, ^{1}H NMR and ^{13}C NMR spectra with literature data (Chattergee *et al.* 1977 and Briggs *et al.* 1961).

Sitosterol is an anti-lipidaemic agent widely distributed in higher plants. It usually occurs together with stigmasterol and campesterol as complex mixtures, and these closely related sterols are difficult to separate.

Compound 2 (*Combretastatin*)

Appendix: FAB-MS, IR, ^{1}H and ^{13}C NMR
Diacetate: FAB-MS, IR, ^{1}H and ^{13}C NMR



combretastatin

Physical data

Compound **2**, 0.52 g, (0.018% based on the dry root mass) eluted from the column with chloroform-methanol 20:1. A portion was recrystallised from diethyl ether to produce a pure specimen of colourless needles, as florets, R_f 0.56 in hexane:ether 1:10 (orange-brown spot), m.p. 135-137 °C. A further recrystallisation from ethyl acetate gave crystals m.p. 144-146 °C (lit. 130-131 °C for combretastatin, Pettit *et al.* 1982), $[\alpha]_D^{25}$ –1.3°.

FAB-MS:	334	M^+ for $C_{18}H_{22}O_6$
	317	M^+ - OH
	197	$C_{10}H_{13}O_4$
	154	base peak, $[197 - CHOH - CH_2 + H]^+$
	137	$C_8H_9O_2$

Accurate mass: 334.1450 (requires 334.1416 for $C_{18}H_{22}O_6$)

UV spectrum (methanol): 206, 225 (shoulder), 276 nm. A bathochromic shift was obtained on addition of NaOH. Methanol + NaOH: 211, 231(shoulder), 298 nm.

IR (KBr disc): 3490, 3420, 3020, 2970, 2940, 2900, 2830, 1600, 1510, 1120.

The ^{1}H NMR results are tabulated below, together with those of Pettit and coworkers.

Table 3.1 ^{1}H NMR data ($CDCl_3$) for **2** compared to those of combretastatin.

Chemical Shift for **2** (δ)	Chemical Shift for combretastatin (δ) (Pettit *et al.* 1982)	Assigned proton
2.02	2.12	1H, broad s, C-10-OH
2.85	2.91(2xdd)	1H, dd, J=8.7, J=14.4 Hz, H-9
2.89	-	1H, dd, J=4.6, J=14.4 Hz, H-9
3.82; 3.83,3.85	3.90	12H, s, 4xOCH_3
4.76	4.83	1H, dd, J=4.6 and 8.6Hz, H-10
5.61	5.72	1H, s, C-5-OH
6.56	6.64	2H,s, H-1 and H-4a
6.67-6.81	6.74-6.93	3H, m, H-7, H-8 and H-8a

Table 3.2 ^{13}C NMR data for combretastatin, compound **2** and compound **2** acetate.

Combretastatin (Pettit *et al.* 1982) ppm	Compound **2** ppm	Carbon No.	Compound **2** acetate ppm
-	-	7 acetate C=O	170.1 s
-	-	10 acetate C=O	169.0 s
153.3	153.2	2 and 4	153.1 s
145.8	145.7	5	149.8 s
145.5	145.5	6	139.4 s
139.7	139.6	3	137.6 s
137.4	137.2	4b	135.4 s
131.3	131.2	8a	129.4 s
121.0	121.0	7	127.8 d
115.6	115.5	8	124.0 d
110.8	110.7	8b	112.1 d
103.0	102.8	1 and 4a	103.6 d
75.5	75.5	10	76.6 d
60.9	60.9	3-OCH_3	60.8 q
56.2	56.1	2 and 4- OCH_3's	56.1 q
56.1	56.0	6- OCH_3	55.9 q
45.6	45.6	9	42.0 t
-	-	7 acetate-CH_3	21.2 q
-	-	10 acetate-CH_3	20.7 q

Acetylation of 0.10 g of **2** with acetic anhydride / pyridine at 90 °C for 6 h produced very little material after pouring into iced water. The solution was extracted with ethyl acetate

and the extract dried over anhydrous sodium sulphate to form 0.08 g of solid material. Recrystallisation from methanol/water, gave the diacetate as colourless needles, m.p. 93 - 95 °C, M^+ 418 for $C_{22}H_{26}O_8$, giving a brown spot R_f 0.67 in hexane-ether 1:5. The two acetate singlets appeared at δ 2.03 and δ 2.27 in the ^1H NMR spectrum.

Discussion

The C-9/C-10 bridge between the two aromatic rings is fragile, and was cleaved in the mass spectrometer to yield major fragments at m/z 197 for $C_{10}H_{22}O_6^+$ and 137 for $C_8H_9O_2^+$.

The ^1H NMR spectrum displayed a doublet of doublets at δ 2.87, being a classic example of an ABX system for the diastereotopic methylene H-9 protons which are vicinal to the chiral C-10 atom. This 8-peak system for these two H-9 protons is also apparent for the acetate of **2**. H-10 resonates as a double doublet at δ 4.74 and δ 4.77 with coupling constants of 4.6.Hz and 8.6 Hz. The aromatic protons at positions 1 and 4a for combretastatin appeared as a singlet at δ 6.56, and the three- proton multiplet at δ 6.67-6.81 was assigned to the 7, 8 and 8b protons. In addition the proton-decoupled ^{13}C NMR spectrum displayed signals at 45.58 and 75.45 ppm, which substantiated the presence of the C-9/C-10 substituted ethyl alcohol system.

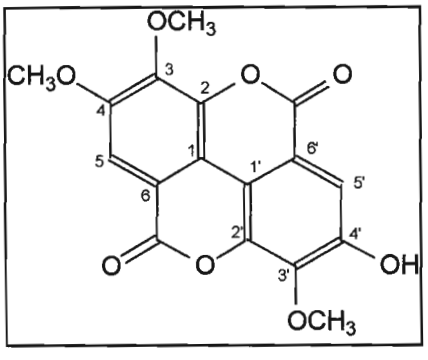
The di-acetate showed two acetate singlets appeared at δ 2.03 and δ 2.27 in the ^1H NMR spectrum, as well as four methoxy groups at approximately δ 3.8. The eight-peak system for the C-9 methylene protons adjacent to the C-10 chiral centre resonated at δ3.00 (dd at δ 2.93 and δ 3.07). The C-10 proton appears as a triplet in the spectrum of the acetate, due to overlap of the two central peaks, but the expected double doublet is clearly visible for the unacetylated compound. The aromatic protons at positions 1 and 4a are slightly upfield at δ6.42 after acetylation. Combretastatin diacetate was not reported by Pettit and coworkers (Pettit *et al.* 1982).

Compound **2** was identified as combretastatin on the basis of the excellent agreement between the IR, ^1H NMR , ^{13}C NMR, FAB-MS and optical rotation data with literature data for combretastatin (Pettit *et al.* 1982). Pettit and co-workers elucidated the structure of combretastatin, first isolated from *C. Caffrum* roots. At the time of isolation and identification from *C. caffrum* roots, nothing had been reported about the presence of combretastatin in other *Combretum* species.

Combretastatin (+/-)-1-(3,4,5-trimethoxyphenyl)-2-(3'-hydroxy-4'-methoxyphenyl) ethanol has notable biological activity, and its anti-cancer properties are discussed in Section 1.3.1. Its presence in *C. kraussii* is reported for the first time (Brookes *et al.* 1999).

Compound 3 (*3,3',4-Tri-O-methylellagic acid lactone*)

Appendix: IR, ^{1}H NMR. Acetate: IR, ^{1}H NMR.



3,3',4-tri-O-methylellagic acid lactone

Physical data

Compound **3**, an off-white solid, 0.23g (0.008% based on the dry root mass), eluted from the column with chloroform-methanol 20:1, was washed with methanol and recrystallised from dioxane to yield shiny colourless needles, m.p. 312-315 °C (lit.303-306 °C for 3,3',4-tri-O-methylellagic acid lactone, Pettit *et al.* 1987b). It was practically insoluble in most organic solvents but dissolved in aqueous sodium carbonate or ammonia giving a bright yellow solution. HPLC (reversed phase C-18 column) showed a peak at 4.4 min. in acetonitrile-water 60:40 at a flow rate of 1.0 cm^3/min.

FAB-MS: 344 M^+ HR-MS 344.0499 (344.0531 for $C_{17}H_{12}O_8$)
329 $M^+ - CH_3$

UV: λ_{max} (MeOH) 243, 385 (sh), 366 nm; (MeOH + NaOAc) 246, 390, 401 nm.

IR (KBr disc): 3430 (OH); 2960 (CH); 1750 and 1730 (lactone C=O); and 1608, 1578 and 1494 cm^{-1} (aromatic C=C).

^{1}H NMR (DMSO-d_6): δ 3.98, δ 4.03 and δ 4.05 3xs 3xOCH_3
δ 7.52 and δ 7.60 2xs H-5/H-5'

The corresponding signals for the ^{1}H NMR spectrum in C_5D_5N were δ 3.72, δ 4.01 and δ 4.06, (3xOCH_3); δ 7.69 and δ 7.91 (H-5'/H-5).

Acetylation of 0.10 g of **3** and recrystallisation from dioxane/hexane gave an acetate m.p. 286-292 °C, which showed a new signal in the NMR spectrum ($CDCl_3$) for the acetyl protons at δ 2.38. In addition it showed δ 4.04, 4.21, 4.27 (9H, 3xs, 3xOCH_3) and δ 7.70 and δ 7.88 (2x1H, 2xs, C-5/C-5' aromatic protons).

MS (m/z) for acetate: 386.1190 M^+ for formula $C_{19}H_{14}O_9$.
344, base peak for $C_{17}H_{12}O_8$

Discussion

Compound **3** had a slightly higher m.p. than that of 3,3',4-tri-O-methylellagic acid lactone (Pettit *et al.* 1987b), and showed a single peak on HPLC. It dissolved in aqueous sodium carbonate or ammonia giving a bright yellow solution, a characteristic of ellagic acid derivatives. The IR spectrum showed absorptions at 3430 cm^{-1} (OH); 2960 (CH); 1750 and 1730 (lactone C=O); and 1608, 1578 and 1494 (aromatic C=C).

The ^{1}H NMR spectrum in DMSO-d_6 displayed three singlets at δ 4.05, δ 4.03 and δ 3.98 for the three aromatic methoxyl groups, and the aromatic H-5'/H-5 protons resonated as one-proton singlets at δ 7.60 and δ 7.52 respectively. Similar chemical shifts were obtained for the ^{1}H NMR spectrum in C_5D_5N.

Evidence for a free hydroxyl group was also obtained by the easy acetylation of **3** to form the mono-acetate, which showed a new signal in the NMR spectrum ($CDCl_3$) for the acetyl protons at δ 2.38. In addition it showed δ 4.04, 4.21, 4.27 (9H, 3xs, 3xOCH_3) and δ 7.70 and δ 7.88 (2x1H, 2xs, C-5/C-5' aromatic protons). An accurate mass of 386.119 for the acetate was consistent with formula $C_{19}H_{14}O_9$. The mass spectral fragmentation pattern of the lactone and its acetate was the same, both showing a base peak at m/e 344 corresponding to the molecular ion M^+ of the parent compound

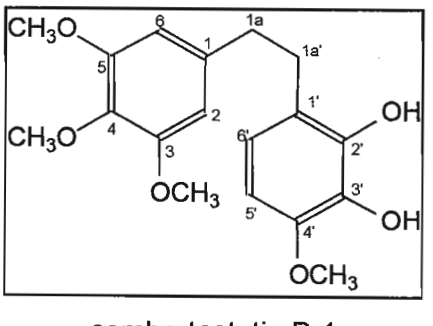
The IR, ^{1}H NMR and ^{13}C NMR data were identical to those published for 3,3',4-tri-O-methylellagic acid (Do Khac *et al.* 1990 and Jurd 1959). Comparison of the UV spectrum with literature data (Jurd 1959) enabled the positions of the methoxy groups to be unequivocally confirmed. It is only possible to distinguish between the closely similar isomers of tri-methylated ellagic acid by their UV spectra (Jurd 1959). A free hydroxy group in the 3 or 3' position is closer to the electron-withdrawing lactone carbonyl group,

and is hence more acidic, than a hydroxyl in the 4 or 4' position. Sodium acetate therefore selectively ionises these 3 or 3' hydroxyls, which are conjugated to the carbonyl group, whereas the 4 and 4' hydroxyls are unaffected. The intense absorption at 243 nm was not shifted appreciably on addition of NaOH or NaOAc, and no new band appeared at about 270 nm, thus clearly establishing that the free hydroxy group was in the 4'-position, and that **3** was therefore 3,3',4-trimethylellagic acid. The peaks at δ 7.54 and δ 7.48 are assigned to the C-5' and C-5 aromatic protons respectively.

Ellagic acid lactone form has antimutagenic and anticarcinogenic properties (Loarca-Pina *et al.* 1996) and therefore has potential health benefits for pregnant women. The precursor of ellagic acid is gallic acid, which is one of the most widely spread acids of dicotyledonous plants, occurring as the free acid, esters, amides and also as ellagitannins in a more or less complex combination with gallate and sugar residues. In contrast to the known widespread distribution of ellagic acid, simple alkyl ethers of the compound have rarely been reported (Chattergee *et al* 1977). The trimethyl ether of ellagic acid was reported in *C. caffrum* extracts (Pettit *et al.* 1987b) and is now also reported in *C. kraussii* extracts (Brookes *et al.* 1999).

Compound 4 (*Combretastatin B-1*)

Appendix: IR, ^{1}H NMR and ^{13}C NMR, DEPT. GC-MS for trimethylsilyl derivative.



combretastatin B-1

Physical data

The fraction eluted from a silica gel column with hexane-ether 1:5 had mass 0.173 g (0.007% based on dry root mass). It had t_r 4.39 min. on HPLC in acetonitrile:water (60:40) at 1.0 cm^3 min^{-1}, and co-eluted with a combretastatin B-1 standard. It was crystallised from ether-hexane to yield colourless needles, m.p. 99-101 °C. Compound **4** provided a distinctive intense cherry-red spot R_f 0.70 on TLC in hexane-ether 1:4.

UV (methanol): 235, 269 nm.

UV (methanol + NaOH): 250, 275 nm.

IR (KBr disc): 3540, 3350, 2940, 1635, 1590, 1510, 1465, 1280, 1120, 1120, 1209 and 905 cm^{-1}.

^1H NMR ($CDCl_3$: δ 2.84 (4H, m, $-CH_2CH_2-$); δ3.81 (3H, s, 4'-OCH_3); δ3.81 (6H, s, 3 and 5 –OCH_3); δ3.83 (3H, s, 4-OCH_3); δ 5.40 (2xOH, D_2O exchangeable, C-2'/C-3') δ 6.37 (1H, d, J=8.5 Hz, H-5'); δ 6.40 (2H, 2xs, H-2/H-6); δ 6.56 (1H, d, J=8.4Hz, H-6').

The ^{13}C NMR multiplicities were obtained from a DEPT spectrum, with results in Table 3.3.

Table 3.3 ^{13}C NMR data for compound **4** in $CDCl_3$

Carbon	Chemical Shift ppm	Carbon	Chemical Shift ppm
1	138.2 s	1'	121.5 s
2+6	105.4 d	2'	142.1 s
3+5	153.0 s	3'	136.1 s
4	132.3 s	4'	145.3 s
3,5-OCH_3	56.1 q	5'	103.4 d
4-OCH_3	56.1 q	6'	120.1 d
1a	36.7 t	4'-OCH_3	60.9 q
1'a	31.8 t		

Table 3.4 MS breakdown for trimethylsilyl derivative of **4**

m/z	Fragment
478	M^+ $C_{24}H_{36}Si_2O_6^+$
463	M^+ - CH_3
297	$C_{14}H_{23}Si_2O_3^+$ base peak
267	297 – 2xCH_3
209	267 – CH_3 – $COCH_3$
181	$C_{10}H_{13}O_3^+$
73	$Si(CH_3)_3^+$

Discussion

Compound **4** was crystallised from ether/hexane to yield colourless needles, m.p. 99-101 °C. There was no available literature comparison as Pettit and coworkers did not report a melting point for combretastatin B-1 because it was isolated as a colourless gum (Pettit *et al.* 1987a).

The UV and IR spectra of **4** suggested aromaticity, as a bathochromic shift was obtained with NaOH for the UV spectrum and C=C stretch was observed in the IR spectrum, as well as hydroxyl O-H stretch. Compound **4** gave a small molecular ion at 334 corresponding to formula $C_{18}H_{22}O_6$. Two major mass fragments at m/z 181 and 153 which were attributed to cleavage of a bibenzyl linkage to yield $C_{10}H_{13}O_3$ and $C_8H_9O_3$ fragments respectively. These results supported the presence of three methoxy groups on one aromatic ring and one methoxy and two hydroxy groups on a second aromatic ring.

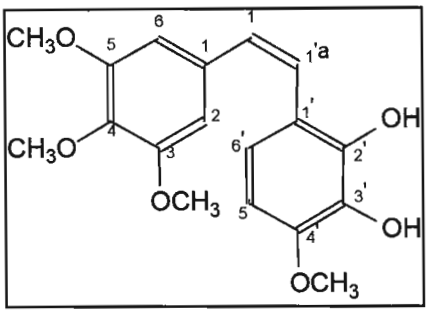
The ^1H NMR spectrum showed two chemically equivalent aromatic protons at δ 6.40 for the meta-coupled H-2/H-6 protons, and also two ortho coupled protons at δ 6.37 and δ 6.56 with J = 8.5 Hz, assigned to the H-5'/H-6' protons of the second ring. A four-proton multiplet at δ 2.84 was typical of the $-CH_2CH_2-$ of a bibenzyl system. Two equivalent aromatic C-2/C-6 signals were also observed in the ^{13}C spectrum at 105.4 ppm and those for C-3/C-5 at 153.0 ppm, deshielded by methoxy groups. The second aromatic ring showed six non-equivalent signals for the C-1' to C-6' carbons however. Four methoxy signals were apparent in the spectrum, two being magnetically identical.

The trimethylsilyl derivative of **4** showed a molecular ion at 478 for $C_{24}H_{36}Si_2O_6$, showing that two hydroxy groups had been silylated. An easy cleavage of the bibenzyl bond in the mass spectrometer produced the base peak fragment m/z 297 for $C_{14}H_{23}Si_2O_3^+$, and also the m/z 181 fragment for $C_{10}H_{13}O_3^+$ attributed to the other aromatic ring, which was also noted in the mass spectrum of **4** itself.

An authentic sample of combretastatin B-1 was also obtained from the NIH, USA, for spectral comparisons. HPLC, UV, ^1H NMR, ^{13}C NMR and IR data were identical to those of the combretastatin B-1 standard and literature data (Pettit *et al.* 1987a) except for a weak carbonyl absorption at 1635 cm^{-1} in the IR spectrum. This was probably due to partial oxidation of the catechol unit to the quinone form while grinding crystals of **4** with KBr under an IR lamp. Pettit *et al.* did not report this absorption, but ran a film for their IR spectrum, as they isolated combretastatin B-1 as a gum, as did Pelizzoni *et al.* in1993. The ^{13}C spectrum of **4** showed no evidence of a carbonyl group, however. The MS data for the trimethylsilylated derivative also support the assigned structure. Component **4**, identified as combretastatin B-1, $C_{18}H_{22}O_6$, has previously been reported in *Combretum caffrum* roots (Pettit *et al.*1987a) and *C. kraussii* seeds (Pelizzoni *et al.*1993). It is directly related the the stilbene combretastatin A-1 to which it has been converted via hydrogenation (Pettit *et al* 1987a).

Compound 5 (*Combretastatin A-1*)

Appendix: GC-MS for trimethylsilyl derivative.



combretastatin A-1

Physical data

Compound **5**, eluted from the column with hexane-ether 1:5, was present in mixtures together with **4**. Although compound **5** was not isolated pure, it was estimated to be present at a concentration of 0.005% based on the dry root mass. This was calculated from peak areas in HPLC results for mixtures of compounds **4** and **5** isolated by column chromatography. Compound **5** separated from **4** by TLC in butanone:$CHCl_3$ 1:1, showing a red spot R_f 0.88 for **4**, and a brown spot R_f 0.76 for **5**. It also separated by HPLC analysis with t_r 4.15 min. on HPLC in acetonitrile-water 60:40 at 1.0 cm^3 min^{-1} using the same conditions as for **4**, which had t_r 4.39 min. Compound **5** had the same mobility as an authentic sample of combretastatin A-1 for both TLC and HPLC analysis. The trimethylsilyl derivatives of **5** and **4** also separated on GC/MS analysis, the derivative of **5** having a shorter retention time 40.39 min. than that of **4** at 40.57 min.

MS (m/z):		
	476	M^+ base peak for $C_{24}H_{34}Si_2O_6$
	461	$M^+ - CH_3$
	446	$461 - CH_3$
	73	$Si(CH_3)_3^+$

Discussion

Compound **5**, eluted from the column with hexane-ether 1:5, was present in mixtures together with compound **4**. However it separated from **4** both by TLC, HPLC and by GC-MS analysis. Compound **5** had a lower R_f on TLC than **4**, and a shorter HPLC retention time than **4** eluted. In addition compound **5** had the same mobility as an authentic sample of combretastatin A-1 obtained from the NIH, USA for both TLC and HPLC analysis, and produced the same colour brown TLC spot with anisaldehyde reagent as the standard.

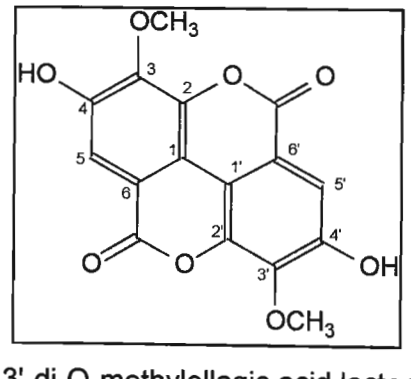
The trimethylsilyl derivative of **5** had a shorter retention time than that of **4** on GC/MS analysis. The base peak at *m/z* 476 for the molecular ion corresponding to formula $C_{24}H_{34}Si_2O_6$ indicated that two hydroxyl groups had been silylated. The formula of compound **5** was therefore deduced to be $C_{18}H_{20}O_6$ prior to silylation. The fact that the base peak was also the molecular ion is consistent with the stable highly conjugated aromatic stilbene structure of combretastatin A-1. By comparison combretastatin B-1 showed an easy fragmentation across the bibenzyl bond linking the two aromatic rings. Pettit and coworkers (Pettit *et al.* 1987a) found a similar mass spectral breakdown for combretastatin A-1 as was found for the silylated derivative of **5**. In both cases the molecular ion was at 100% abundance, being at m/z 476 for the silylated derivative and m/z 332 for combretastatin A-1. Only one other peak recorded by Pettit for $M^+ - CH_3$ (93.7%). The silylated derivative of **5** also showed a smaller peak at m/z 461 for $M^+ - CH_3$, as well as at 446 for $M^+ - 2xCH_3$.

Compound **5** was therefore identified as combretastatin A-1 on the basis of the above comparisons to MS literature data (Pettit *et al* 1987a) and to an authentic standard.

Although Pettit and coworkers extracted 77 kg of *C. caffrum* stemwood, they also isolated combretastatin A-1 in mixtures with combretastatin B-1. Although they purified enough combretastatin A-1 for identification and to show PS *in vivo* activity, discussed in Section 1.3.1, the quantities were insufficient for further biological tests. They therefore resorted to synthesis in order to obtain larger quantities, and to also confirm the structure proposed.

Compound 6 (*3,3'-di-O-methylellagic acid lactone*)

Appendix: IR



3,3'-di-O-methylellagic acid lactone

Physical data

Compound **6** was separated as almost colourless insoluble solid material from the fraction eluted from a column with chloroform/methanol (10:1) during the purification of compounds **8** and **9**. When this fraction was dissolved in methanol, compound **6** remained insoluble (0.10 g, 0.017% based on the dry root mass). It was insoluble in acetone, chloroform and methanol but dissolved in DMF and slightly in DMSO, m.p. *ca.* 280 °C (decomp.). Compound **6** had t_r 3.30min in acetonitrile-water 60:40 at 1 cm^3 min^{-1} (76.1%); t_r for trimethylellagic acid was 4.31 min. under the same conditions. It turned yellow in the presence of base, as noted for ellagic acid derivatives.

HRMS:		M^+ 330.0358 (Calc. for $C_{16}H_{10}O_8$: 330.0375)
MS (m/z):	330	M^+
	315	$M^+ - CH_3$
	300	$315 - CH_3$

UV: λ_{max} (MeOH) 238, 358nm; (MeOH + NaOAc) 239, 358, 402 (sh) nm.

IR (KBr disc): 3200, 1725, 1610, 1570, 1480 1345,1205, 1100, 980 and 910cm^{-1}

^{1}H NMR (DMSO-d_6):	δ 7.42 s	(2H H-5 and H-5')
	δ 4.02 s	(6H 2xOCH_3 for C-3 and C-3')
	δ 3.41 m	(HOD in DMSO-d_6)

Discussion

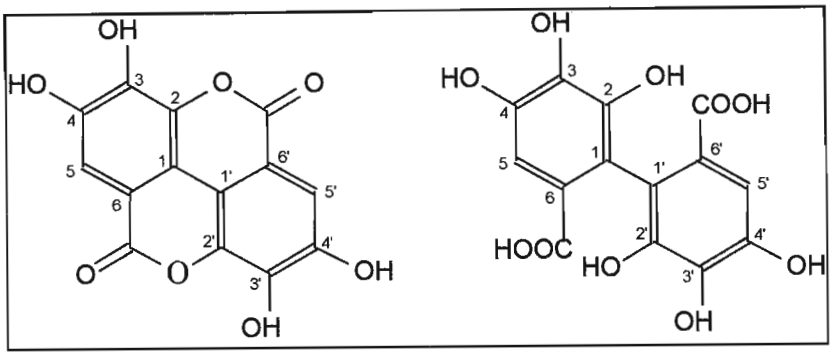
Compound **6** was separated as insoluble solid material from the fraction eluted from the column with chloroform-methanol 10:1. The melting point of approximately 280 °C (decomp.) was lower than that in the literature for 3,3'-di-O-methylellagic acid (338-340 °C, Do Khac 1990; 332-335 °C, Jurd 1959). However compound **6** was not pure by HPLC, and this would account for the lower melting point. The fact that compound **6** eluted earlier than 3,3',4-tri-O-methylellagic acid from a reversed phase C-18 column shows that it is more polar than trimethylellagic acid. The IR spectrum showed OH stretch at 3200, C=O stretch for a lactone at 1725 and C=C aromatic stretch at 1610 cm^{-1}.

The UV spectrum in methanol showed no significant shift of absorption on addition of sodium acetate, indicating that the free aromatic hydroxyls were not acidic enough for reaction with sodium acetate. These free hydroxyls were accordingly assigned to the 4 and 4' positions in the molecule. The free hydroxyl in trimethylellagic acid (compound **3**,) was assigned on the same basis (Jurd *et al.* 1959) as previously discussed.

The MS, ^{1}H NMR, IR and UV spectroscopic data all compared well with data in the literature (Do Khac 1990; Sato 1987), and compound **6** was accordingly allocated the structure 3,3'-di-O-methylellagic acid lactone, formula $C_{16}H_{10}O_8$.

Compound 7 (**7a** *Ellagic acid lactone*, **7b** *ellagic acid*)

Appendix: FAB-MS, IR for **7a**; IR for **7b**; FAB-MS, EI-MS and IR for **7a** acetate.



ellagic acid lactone **7a** ellagic acid open ring form **7b**

Physical data

Component **7** was obtained by hydrolysis of approximately 4 g of a mixture of components **10** and **11** for 3h at 90 °C with 1M HCl. This yielded 0.5 g of khaki-coloured material, which precipitated out of solution, and was filtered off. HPLC in acetonitrile-water 60:40 showed the following results:

1.55.min. (13.1%) **10**
2.07 min. (10.9%) **11**
2.48 min. (74.8%) precipitate from hydrolysis

The above results showed that hydrolysis was incomplete, as compounds **10** and **11** were still present in small amounts. However the major product of hydrolysis (**7a)** was washed with methanol, which caused a small amount (**7b)** to dissolve. After removal of methanol, both **7a** and **7b** were purified by recrystallisation from DMF. Both samples were pale khaki-yellow fine needles, m.p. >330 °C, but were shown to be different by IR spectroscopy. Both compounds gave a positive test for a phenol with $FeCl_3$, producing a dark green-black colour. An authentic ellagic acid lactone standard gave the same colour with $FeCl_3$. HPLC analysis of fresh root extracts did not reveal the presence of appreciable amounts of ellagic acid. It was commonly found in hydrolysates.

FAB-MS **7a** m/z: 303 $M^+ + H$ for $C_{14}H_6O_8$
154 $C_6H_5\text{-}C_6H_5^+$ from $M^+ - (2xCO_2 + 4xH) - (4xOH + 4H)$

IR **7a** (KBr disc): 3460, 3400, 1740, 1610, 1430, 1340, 1250, 1090, 1030 cm^{-1}

UV **7a** (MeOH): 241, 348 nm; (MeOH + NaOH) 239 (sh), 271, 330 (sh), 380 (sh) nm.

^1H NMR **7a**: (DMSO-d_6) δ 7.44 s and δ 3.54 br. s.

IR **7b**: 3550 (sharp); 3100 (broad); 1685; 1605; 1325; 1100; 1050 cm^{-1}
The IR spectrum of **7b** matched that of an authentic (Fluka) standard.

Component **7a** was also separated from a MeOH/CH_2Cl_2 (1:1) extract (room temperature), by elution from a silica gel column with $CHCl_3$/MeOH (1:1) and also identified by the above spectral characteristics.

Compound **7a** was acetylated with pyridine/acetic anhydride for 6 h on a steam bath then precipitated in ice/water. It showed the following MS data:

FAB-MS:	470	EI-MS:	-	M^+ for $C_{22}H_{14}O_{12}$
(m/z)	428	(m/z)	428	M^+ - CH_2=C=O
	386		386	428 - CH_2=C=O
	345		344	386 - CH_2=C=O
	307		-	
	302		302	$C_{14}H_6O_8^+$
	289		-	

An authentic ellagic acid (Sigma) standard was also acetylated and had an identical IR spectrum to that of **7a** acetate.

Discussion

Components **7a** and **7b** were obtained by hydrolysis of a mixture of components **10** and **11**. Both samples were pale khaki-yellow fine needles, m.p. >330 °C, and both gave a positive test for a phenol and there was also a bathochromic shift on addition of NaOH in the UV spectrum, indicating a phenol. However they were shown to be different by IR spectroscopy. A δ-lactone, *i.e.* six-membered ring, shows a carbonyl absorption between 1750-1730 cm^{-1} (Silverstein 1998). The IR spectrum of **7a** showed C=O stretch at 1740 cm^{-1} for a δ-lactone whereas **7b** showed C=O stretch at 1685 cm^{-1}, within the range for a free carboxylic acid carbonyl group *i.e.* 1680-1720 cm^{-1}.

The FAB-MS for **7a** showed M^+ + H at m/z 303 for the molecular ion corresponding to formula $C_{14}H_6O_8$. The significant ion at m/z 154 was assigned to a stable conjugated biphenyl fragment formed by loss of two carbon dioxide and four hydroxy moieties. The

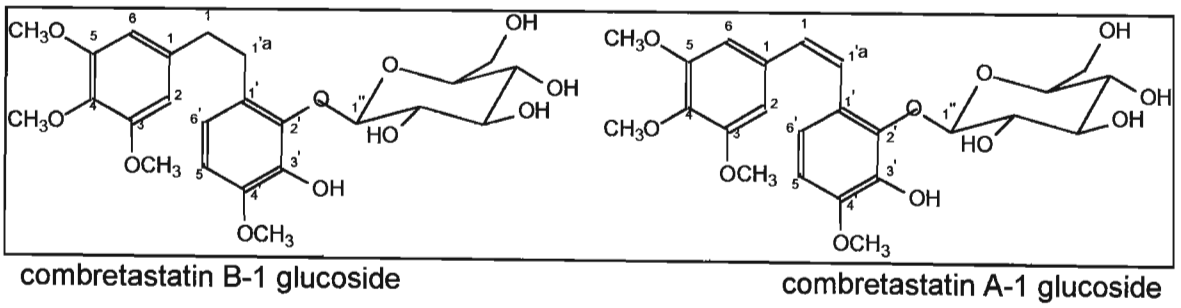
1H NMR spectrum of **7a** in DMSO-d_6 displayed signals at δ 7.436 and δ 3.543 corresponding to those of the H-5/H-5' protons and the four hydroxy groups of ellagic acid lactone respectively. The identity of **7a** as ellagic acid lactone was established by comparison of UV, IR and HPLC data with literature data (Pegel and Rogers 1968; Nawwar 1984) and an authentic ellagic acid lactone (Sigma) standard. Component **7b** had an identical IR spectrum to that of an authentic ellagic acid open open chain form (Fluka) standard.

The tetra-acetate of **7a** showed a molecular ion at m/z 470 in the FAB-MS spectrum representing $C_{22}H_{14}O_{12}$ for ellagic acid lactone tetra-acetate. The mass spectrum also showed sequential loss of acetate moieties to form the parent ion at m/z 302 for ellagic acid lactone form. In addition an authentic ellagic acid lactone standard was acetylated and the IR was identical to that of **7a** acetate. On the basis of these results **7a** was identified as ellagic acid lactone form and **7b** as the related open ring form. Ellagic acid was found predominantly in hydrolysates of root extracts. The possibility that it arises from the breakdown of comounds **10** and **11** is discussed later.

Ellagic acid and its derivatives, reacting in a tautomeric quinoid form, can be oxidised in air, resulting in further hydroxylation and intermolecular linking. This instability of ellagic acid and derivatives is the probable explanation for the difficulty experienced in trying to purify components **10** and **11**, which are derivatives of ellagic acid. Ellagic acid lactone was obtained from various hydrolysates of *C. kraussii* fractions. It was also isolated in a free state from an unhydrolysed methanol-methylene chloride 1:1 root extract. Its presence in *Gunnera perpensa* extracts is discussed in Chapter 4.

Compounds 8 and 9 (*Combretastatin B-1 and A-1 glucosides*)

Appendix: FAB-MS and ^{1}H NMR



combretastatin B-1 glucoside combretastatin A-1 glucoside

Physical data

A mixture of these compounds was eluted from the column with chloroform/methanol (10:1) as a dark brown gum, 14 g, in 2.14% yield based on the dry plant material. It was re-chromatographed on silica-gel to yield a chromatographically homogeneous substance by TLC, giving a single dark grey-brown spot R_f 0.44 in ether-chloroform-methanol 2:2:1. It also gave a single peak on HPLC analysis.

FAB MS m/z:
519 $M^+ + Na^+$ for combretastatin B-1 glucoside
517 $M^+ + Na^+$ for combretastatin A-1 glucoside
494 combretastatin A-1 glucoside
333 base peak $[C_{18}H_{22}O_6 - H]^+$ combretastatin B-1 residue
331 $[C_{18}H_{22}O_6 - H]^+$ combretastatin A-1 residue

Chemical shifts for the ^1H NMR spectrum are presented in Table 3.5.

Table 3.5 ^1H NMR ($CDCl_3$) results for **8** and **9** mixture.

Chemical Shift (δ)	Assigned proton in combretastatin A-1 or B-1	
6.66, 1H, d, J=8.6 Hz	H-6'	B-1
6.48, 1H, d, J=8.2 Hz	H-5'	B-1
6.41, 1H, d, J=11.3 Hz	-CH=CH-	A-1
6.36, 2H, s	H-2/H-6	B-1
6.30, 2H, s	H-2/H-6	A-1
4.65, 1H, d, J=7.6 Hz	H-1''	A-1
4.42, 1H, d, J=7.2 Hz	H-1''	B-1
3.75, 3H, s	$-OCH_3$	B-1
3.73, 3H, s	$-OCH_3$	B-1
3.71, 6H, s	H-2/6-OCH_3	B-1
3.70, 3H, s	$-OCH_3$	A-1
3.56, 6H, s	H-2/6-OCH_3	A-1
3.19, br. s	OH's	A-1/B-1
2.90, 4H, m	$-CH_2CH_2-$	B-1

Hydrolysis of **8** and **9** (5.5 g) was carried out in methanolic 2M HCl for 6 h under argon. Methanol was then evaporated and the mixture was extracted with ethyl acetate yielding a solid material (2.2 g) comprising two major components. By comparison with standards, these were determined to be combretastatin B-1 **4** (approximately 50%), combretastatin A-1 **5** via TLC and HPLC. A third minor component was visible on TLC. Combretastatin B-1 was obtained as colourless crystals from a hydrolysate. HPLC analysis of the water layer after hydrolysis using a reversed phase C-18 column showed the presence of glucose, t_R 9.594 min in acetonitrile-water 80:20 at 1.0 cm^3 min^{-1}, which co-eluted with an

authentic glucose standard. The presence of glucose after hydrolysis was also shown by TLC.

Discussion

Compounds **8** and **9** were isolated as a mixture which was chromatographically homogeneous by TLC and HPLC analysis.

Hydrolysis of **8** and **9** yielded two major components. By comparison with authentic standards, these were determined to be combretastatin B-1 **4** (approximately 50%), combretastatin A-1 **5** and a third minor component, possibly a phenanthrene resulting from ring closure during hydrolysis. HPLC analysis of the water layer after hydrolysis showed the presence of glucose. The presence of glucose after hydrolysis was also shown by TLC.

The presence of both glucosides was shown by the peaks in the FAB MS at 519 and 517 corresponding to $M^+ + Na^+$ for combretastatin B-1 and A-1 glucosides respectively. The molecular ion at 494 in the FAB MS represents combretastatin A-1 glucoside. Combretastatin B-1 glucoside should exhibit M^+ at 496 but this is absent, probably due to conversion via loss of two hydrogen atoms during MS analysis to form combretastatin A-1 glucoside which contains a very stable conjugated aromatic system. The base peak at m/z 333 represents loss of glucose, leaving the combretastatin B-1 residue $[C_{18}H_{22}O_6 - H]^+$. Correspondingly the smaller peak at 331 represents the combretastatin A-1 residue $[C_{18}H_{22}O_6 - H]^+$ after loss of the glucose moiety. The peak at m/z 181 is due to fragmentation of the 2-O-β-D-glucoside of combretastatin B-1 at the bibenzyl bridge, which was also noted for combretastatin B-1, to form $C_{10}H_{13}O_3^+$.

The ^1H NMR data in Table 5 clearly show that the compound **8** and **9** mixture consists mainly of combretastatin A-1 and B-1 glucosides. The doublet at δ6.66, J=8.7 Hz for the combretastatin B-1 glucoside H-6' is clearly distinguishable (lit. δ6.68, Pelizzoni *et al.* 1993), even though some of the other H-5' and H-6' signals overlap in that area. One of the doublets for the combretastatin A-1 glucoside –CH=CH- group at δ6.41, J=11.3 Hz, is also easily discernable, although the downfield coupled proton is obscured. The two singlets at δ6.36 and δ6.30 arise from the equivalent H-2/H-6 protons of combretastatin B-1 and A-1 glucosides respectively. The relative intensities of these two signals show that the ratio of combretastatin B-1 to combretastatin A-1 glucosides is approximately 1.4 to 1.0 in the mixture. The doublets at δ 4.65, J=7.6 Hz, and δ 4.42, J=7.2 Hz, are

attributed to the H-1'' anomeric glucose protons of combretastatin A-1 and B-1 glucosides respectively, and are typical of β-glucoside linkages. The methoxy groups on the equivalent C-3/C-5 positions appear at δ3.71 and δ3.56 for combretastatin B-1 and A-1 glucosides respectively, in good agreement with literature values of δ3.74 and δ3.55 (Pelizzoni 1993). The broad singlet at δ3.19 is attributed to the free hydroxyls, and the four-proton multiplet at δ2.90 is due to the $-CH_2CH_2-$ group of the bibenzyl bridge in combretastatin B-1 glucoside.

Both combretastatin B-1 and combretastain A-1 glucosides were previously identified in *C. kraussii* seeds (Pelizzoni *et al.*1993) and compound **8** and **9** mixture showed signals and multiplicities which matched those of these two glucosides apart from the free hydroxyl resonance which is commonly variable. From consideration of the ^1H NMR analysis as well as FAB-MS, and also acid hydrolysis results, the mixture of **8** and **9** was identified as an approximately equal mixture of the 2'-*O*-β-D-glucopyranoside of combretastatin B-1 and the 2'-*O*-β-D-glucopyranoside of combretstatin A-1 by comparison to literature data (Pelizzoni 1993).

Compounds 10 and 11

Appendix: IR. Methylated **10+11** Fr.7 FAB-MS.

Physical data

These phenolic glycosides, at present not fully characterised, are the most polar substituents isolated from *C. kraussii* roots and were both obtained as khaki-yellow powders from a hot water extract of roots previously extracted with methanol. A partial purification was obtained as follows. Compound **10** was separated first from the concentrated water extract by addition of methanol/acetone (1:1). From the concentrated mother solution after removal of **10**, further addition of methanol/acetone (1:1) precipitated **11**. HPLC in acetonitrile-H_2O 60:40 at 1.0 cm^3 min^{-1} showed a retention time of 1.59 min for **10** and 2.20 min for **11**.

Water and methanol both are suitable for the extraction of these polar components. For example a 1:1 MeOH/CH_2Cl_2 extract prepared at room temperature showed a major HPLC peak (72%) at 3.21 min., in acetonitrile-H_2O 60:40 at 1.0 cm^3 min^{-1}, attributed to compound **10**. The same peak (3.16 min., 98%) was present in the HPLC of a hot water

extract of roots that had previously been extracted with hot methanol. Conditions for these two consecutive HPLC runs were identical. Components **10** and **11** were also eluted as a mixture from silica gel columns. Chromatography of 53 g of a hot methanol extract of 583 g (dry mass) of roots yielded a major fraction, Fr. 25-46, with mass 30.7 g (60% of the methanol extract and 5.3% based on the dry root mass) consisting of approximately 62% of a **10/11** mixture. Solvents used for elution were $CHCl_3$-MeOH 1:1, methanol, and finally MeOH-acetic acid 5:1. HPLC showed that ellagic acid lactone was also present in the fraction. TLC in butanol-acetic acid-water-chloroform 6:2:2:1 showed one predominant green-yellow spot on the origin with anisaldehyde reagent.

Acid hydrolysis of the **10/11** mixture released ellagic acid lactone and open forms (**7a** and **7b**), discussed previously, and also glucose, which was identified by TLC and HPLC in acetonitrile-water 80:20 at 1 cm^3/min. using authentic standards. The sugar released on hydrolysis had R_t 9.41, co-eluting with a glucose standard with R_t 9.34 min. Xylose, fructose and sucrose had R_t 6.76, 8.27 and 13.78 min. respectively under the same conditions. There was no free glucose prior to hydrolysis.

It was frequently observed that water solutions of **10** and **11** underwent an easy natural hydrolysis at room temperature, with the more polar **10** converting to **11**. This hydrolysis also occurred in solutions of HPLC mobile phases such as 80:20 acetonitrile : water, shown below:

	Component **10** R_t min.	Component **11** R_t min.
Fresh solution	2.396 (42%)	2.587 (45%)
Solution 6 days old	-	2.525 (100%)

All attempts to fully purify compounds **10** and **11** on silica gel or Sephadex LH 20 to obtain a pure enough sample for structural elucidation were unsuccessful. Semi-preparative HPLC was also unsuccessful.

Because of their instability, which resulted in extracts darkening and becoming gummy very readily, methylation was attempted in order to stabilise the compounds, followed by repeated silica gel column chromatography to purify them. Per-methylation of 3.0 g of the **10/11** mixture with 2.7 g NaH and 46g cm^3 MeI in 100 cm^3 DMSO was carried out for 4 h (Bindra *et al.* 1988 and Cuicano and Kerek 1984) under argon, then the product precipitated in ice/water. The dried product was a mustard coloured powder (2.1 g), and 1.5 g of this material was purified on silica gel. Fraction 2, eluted with 10:1 CH_2Cl_2-

acetone, was precipitated from CH_2Cl_2 solution with hexane to give 0.18 g of material containing a major compound producing a green spot, R_f 0.59 on TLC in CH_2Cl_2-acetone 30:1. However it was not pure so 0.060 g was re-chromatographed. The bulk of the material, Fraction 7, 0.042 g, eluted with CH_2Cl_2-acetone 30:1, showed two green spots R_f 0.52 and 0.35 on TLC in CH_2Cl_2-acetone 30:1, as well as other minor impurities. Fraction 7 showed molecular ions at m/z 1366 and 1410 on FAB-MS analysis. Tetra-methylellagic acid was found as a minor product in the earlier column fractions. It gave a blue-white UV visible spot, R_f 0.86 in CH_2Cl_2-acetone 50:1, identical to that of an authentic standard.

Further attempts to purify the products of methylation via column chromatography to enable spectral identification were unsuccessful. A third column for Fr.7 gave low recoveries and much of the material was more polar than the starting material. Silica gel appears to cause de-methylation or degeneration of the material, and efforts in this area were not continued. Acetylation followed by column chromatography was also unsuccessful.

Discussion

It was noted on many occasions that natural hydrolysis of components **10** and **11** occurred in solution with the more polar **10** converting to **11** at room temperature. This suggests that the two compounds are related. A similar phenomenon was observed for the 2-component phenolic glucoside mixture isolated from *Gunnera perpensa* extracts, and is discussed in Chapter 4.

It was also shown by TLC and HPLC using authentic standards that glucose was released as a result of acid hydrolysis of these two compounds. Seeing there was no free glucose prior to hydrolysis, it was evident that **10** and **11** are related glucosides. The fact that ellagic acid was also released by acid hydrolysis of components **10** and **11** (discussed for ellagic acid **7**) indicates that these two compounds are ellagic acid glucosides, with **10**, the more polar component, having more than one molecule of glucose present. Both **10** and **11** are more polar than ellagic acid itself on HPLC.

Per-methylation followed by silica gel column purification gave a sample with molecular ions at m/z 1366 and 1410 on FAB-MS analysis. The ^1H NMR spectrum was similar to that of *Gunnera perpensa* methylated fractions, but signals in the aromatic area indicated

that the sample was not completely pure. There were a large number of methoxy signals in the δ4.0 area, consistent with polyhydroxy compounds.

Tetra-methylellagic acid was found as a minor product in the earlier column fractions of methylated **10+11** samples and identified by TLC comparison to an authentic standard.

Further attempts to purify the products of methylation via column chromatography to enable spectral identification were unsuccessful and caused degeneration of the methylated samples. Purified methylated samples were sent to experts in the field of carbohydrate chemistry at Cape Technikon and Imperial College, Biochemistry Department, however structures could not be fully elucidated. Acetylation followed by column chromatography was also unsuccessful.

Although not fully characterized, it is proposed from the results of hydrolysis and the high molecular weights that these two compounds are ellagic acid glucosides, with compound **10** containing at least one more glucose moiety that compound **11**. Further research is required to obtain a full structural elucidation.

CHAPTER 4

GUNNERA PERPENSA

Seven compounds, **12** to **18**, were isolated from *Gunnera perpensa* roots with 5 of these being novel to the species at the time of identification. These are 3,3'4'-tri-O-methyl ellagic acid lactone, ellagic acid lactone, 1,1'-biphenyl-4,4'-diacetic acid, p-hydroxy-benzaldehyde and Z-methyl lespedezate. Two phenolic glucosides **17** and **18** have been partially characterized.

Auto-oxidation is extremely facile for *Gunnera* extracts, and also for the sliced roots themselves. It was noted on many occasions that pale yellow or beige extracts turned dark brown or even blackish over a period of time and were sticky and extremely difficult to purify. Freshly sliced roots rapidly turn a reddish colour and then darken to dark brown/black over a period of days. Extracts are also highly reactive with metals, sometimes turning metal spatulas black, and also towards $FeCl_3$ solution, which caused a very dark blue precipitate, rather than a colouration, to form when added to Gunnera extracts. These reactions could be due to the presence of pyrogallol (suggestion made by Examiner, 2005).

A preliminary TLC investigation in $CHCl_3$:MeOH 9:1 of the major components in a methanol extract of *Gunnera perpensa* roots showed only two components, one giving a dark green spot on the origin with anisaldehyde reagent, and a second less polar component (green spot) in lower concentration R_f 0.14. In a more polar system, butanol-acetic acid-water-$CHCl_3$ 6:2:2:1, the major component had R_f 0.08 (green spot) with 4 other constituents at R_f 0.41, 0.64, 0.79 (green spots) and 0.96, the least polar component giving a violet spot.

Compound 12 (3,3'4'-tri-O-methyl ellagic acid lactone)

Appendix: Spectra identical to those of compound **3** from *Combretum kraussii*

Physical data

The hot methanol extract of 3130 g roots with harvested in May 1995 at Silverglen Nature Reserve, produced 147 g of a brown powder after removal of methanol. Further

extraction of the same roots with hot water produced 38 g of a light khaki powder, after solvent removal. The hot methanol extract was re-extracted with $CHCl_3$ and then butanone. The $CHCl_3$ extract produced colourless crystals of **12**, approximately 0.3g (0.057% based on the dry root material) insoluble in acetone and methanol, but soluble in DMSO and DMF. A portion of compound **12** was recrystallised from DMF to produce 0.0658 g of material m.p. 302 - 304°. A very small amount of **12** in water produced a yellow solution on addition of one drop 10% Na_2CO_3 solution, or NaOH solution, or ammonia solution. The accurate mass of **12** was 344.0517 (344.0530 for $C_{17}H_{12}O_8$).

EIMS:	344	100%	M^+
	329	20%	M^+-15 (CH_3)
	301	12%	329-28 (CO)
	286	16%	301-15 (CH_3)

UV:	Methanol:	241 (major) and 361 nm.
	Methanol + NaOAc:	243 and 394 nm
	Methanol + NaOH:	247 and 393 nm.

IR (KBr disc): 3440 (O-H); 2970, 2680 (C-H); 1755, 1730 (C=O); 1615, 1580 (C=C); 1365 (O-H bend); 1120, 1095 cm^{-1} (C-O).

^1H NMR (DMSO-d_6): δ 3.98, δ 4.03, δ 4.05, 3xs, 9H, H-4', H-3', H-3 3xOCH_3; δ 7.50, s, 1H, H-5'; δ 7.58, s, H-5.

Table 4.1 ^{13}C NMR chemical shifts for **12** in DMSO-d_6

Carbon	Shift, ppm	Carbon	Shift, ppm
1	111.0	1'	111.9
2	141.0	2'	141.5
3	140.3	3'	140.3
4	153.0	4'	153.8
5	107.5	5'	111.8
6	112.5	6'	113.5
7	158.4	7'	158.6
3-OMe	61.0	3'-OMe	61.4
		4'-OMe	56.8

Acetylation of 0.07 g of **12** with 1 cm^3 pyridine and 0.5 cm^3 acetic anhydride for one h on a steam bath was carried out followed by standing overnight at room temperature. The acetate was then precipitated from ice/water and recrystallised twice from acetone to yield colourless needles m.p. 274 - 276° (lit. for trimethylellagic acid 262 °C, Do Khac *et*

*al.*1990; 264-265 °C, Sheshadri and Vasishta 1962). The acetate had R_f 0.88 on TLC in butanone:$CHCl_3$ 1:1, giving a violet spot with anisaldehyde reagent. The R_f of **12** itself in the same system was 0.59.

MS (m/z): M^+ 386.0637 corresponding to formula $C_{19}H_{14}O_9$ (requires 386.0579)

344 $M^+ - CH_2=C=O$

329 344 - CH_3

301 329 - CO

286 301 - CH_3

Discussion

Compound **12** was purified as colourless crystals from a hot methanol root extract. The yellow solution produced when an aqueous solution of sodium carbonate or sodium hydroxide was added to a solution of **12** is characteristic of an ellagic acid derivative. The UV spectrum indicated that the free hydroxyl was in the 4-position as discussed for trimethyl ellagic acid isolated from *Combretum kraussii.* The ^1H NMR spectrum of **12** showed the presence of three methoxy groups, and two aromatic protons. The ^1H NMR spectrum of the acetate showed one acetate group in addition to the three methoxy groups. Comparison of UV, IR, ^1H NMR, ^{13}C NMR, MS and melting points of **12** and its monoacetate with literature data confirmed that it was 3,3',4'-tri-O-methylellagic acid lactone (Do Khac *et al.*1990, Bindra *et al.*1988; Sheshadri and Vasishta 1962). It was also isolated from *Combretum kraussii* (Chapter 3) and has antihaemorrhagic properties (Do Khac *et al.*1990). The presence of this compound in *Gunnera perpensa* is reported for the first time.

Compound 13 (Ellagic acid lactone)

Appendix: Spectra are identical to those of compound **7a**.

Physical data

Approximately 20 g of the combined hot methanol and hot water extracts of roots harvested in May 1995 at Silverglen Nature Reserve was hydrolysed with 150 cm^3 1M HCL for 3 h at 90°. The mother liquor was evaporated and a small quantity of methanol added, resulting in the formation of yellow crystals, 0.24 g (0.33% based on the dry root mass). These were recrystallised using 8 cm^3 DMF to yield fine orange-yellow crystals

0.21 g R_f0.32 in ethyl acetate:pyridine:water:methanol 80:20:10:5, white spot on darker background with anisaldehyde reagent. Compound **13** was more polar than **12** on TLC and had m.p. >320 °C (m.p. ellagic acid, lactone form >360 °C, Buckingham1994). It gave a positive test for phenol (blue colour) with $FeCl_3$.

EIMS M^+ 302.0075 ($C_{14}H_6O_8 \equiv 302.0062$).

UV (MeOH): 248 nm (major) 356 nm. Addition of NaOH caused bathochromic shift of major absorption to 277nm.

IR (KBr disc): 3400 (broad OH); 2970, 2920 (C-H stretch); 1725 (C=O); 1620 (C=C), 1345 (O-H bend) and 1030 cm^{-1} (C-O stretch).

^1H NMR (DMSO-d_6): δ 7.45 (s); δ 3.43 (broad OH signal at which shifted to approximately δ 4.2 on addition of D_2O).

The acetate was prepared by heating 0.10 g of **13** with pyridine/acetic anhydride for 5 h at 90 °C to produce 0.12 g of a pale cream-yellow powder with m.p. 316-320 °C.

IR acetate (KBr disc): 3060, 1780, 1740, 1605, 1185, 1165 cm^{-1}.

Discussion

Compound **13**, obtained after the acid hydrolysis of the combined hot water and hot methanol extracts, displayed many features suggesting it could be ellagic acid. It showed a carbonyl absorption at 1725 cm^{-1}, typical of a lactone. A δ-lactone *i.e.* six-membered ring, shows a carbonyl absorption between 1750-1730 cm^{-1}. However unsaturation α to the C=O reduces this absorption frequency (Silverstein 1998). There is unsaturation in the α-position to the C=O group for ellagic acid, which is probably the reason for the slight decrease in frequency observed. Apart from a few minor interfering signals due to DMF and DMSO-d_6 solvent impurities, there was only one proton signal at δ 7.45 in the ^1H NMR spectrum which was assigned to the two identical ellagic acid protons at the 5 and 5' positions, and an OH signal at δ 3.43.

Acetylation of **13** produced a pale yellow-cream powder which had an identical IR spectrum as an acetylated authentic ellagic acid lactone standard.

Compound **13** was identified as ellagic acid, lactone form (4,4',5,5'6,6'-hexahydroxydiphenic acid 2,6,2',6'-dilactone) by comparison of UV, IR, ^{1}H NMR, mass spectral and melting point data with those in the literature (Hathway 1956) and an authentic standard. It was also isolated from *Combretum kraussii* (Chapter 3).

Ellagic acid is a dimeric condensation product of gallic acid, which is very reactive, as shown below. The biphenyl, hexahydroxydiphenic acid, can also convert to the dilactone via ring closure with loss of two moles of water. In this work a small amount of hexahydroxydiphenic acid was isolated from *Combretum kraussii* together with the major ellagic acid lactone form. Ellagic acid has antimutagenic and anticarcinogenic properties. It was found to significantly inhibit the potent mutagen aflatoxin B_1 using a *Salmonella* microsuspension assay (Loarca-Pina *et al* 1996).

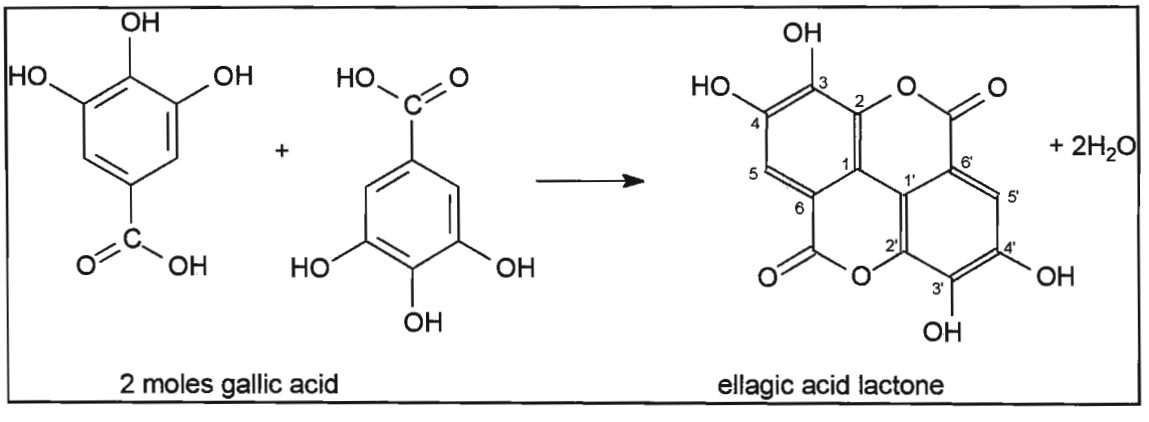
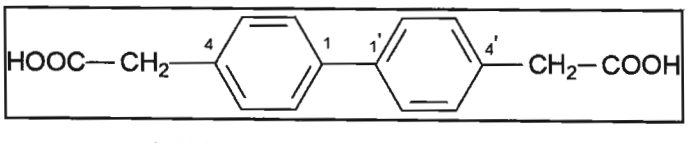


Figure 4.1 Conversion of gallic acid to ellagic acid lactone

Compound 14 (1,1'-biphenyl-4,4'-diacetic acid)

Appendix: **14** IR and ^{1}H NMR.



1,1'-biphenyl-4,4'-diacetic acid

Physical data

Compound **14** was separated after hydrolysis of combined methanol and water extracts of roots harvested at Silverglen Nature Reserve in May 1995. The ethyl acetate extract after hydrolysis was purified on a silica gel column and compound **14** was eluted with hexane:ether 1:2. It was soluble in ether, acetone and methanol, and was recrystallised

from ether/hexane to yield 0.08 g colourless needles (0.11% based on dry root mass) m.p.155 - 158°, giving a pink/red spot R_f 0.27 on TLC in hexane:ether 1:1. Compound **14** was more polar than **15** which had R_f 0.65 on the same TLC plate (pale mauve spot). HPLC of **14** in acetonitrile:water 60:40 showed a peak at 2.41 min. at a flow of 1 cm^3/min. using a reversed phase C-18 column and a UV detector at 254 nm. It gave a negative test for aldehydes and ketones with 2,4 – dinitrophenylhydrazine reagent. When a little of **14** was dissolved in water-methanol and tested with pH paper, the pH was found to be 2, indicating that it was an acid.

The mass spectrum showed M^+ 270.0903 ($C_{16}H_{14}O_4$ = 270.0892)

UV (MeOH): 231 nm (major); 282nm.

IR (KBr disc): 3250 cm^{-1} broad OH stretch; 1700 cm^{-1} C=O stretch; 1605, 1590 and 1510 C=C stretch; 1400 O-H bend and 1210 C-O stretch.

The ^1H NMR (CD_3OD) showed the following signals:

δ 3.52 s	4H	2xCH_2	
δ 6.76 d	4H	J = 8.6 H_z	H-2, H-6, H-2', H-6'
δ 7.12 d	4H	J = 8.5 H_z	H-3, H-5, H-3', H-5'

Discussion

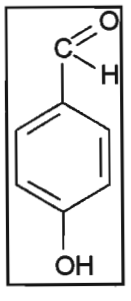
Compound **14** was isolated after hydrolysis of mixed *Gunnera perpensa* extracts followed by purification on a silica gel column. The IR spectrum displays typical features of aromatic acids *e.g.* C-O stretch in the region of 1320-1210 cm^{-1} and O-H bend at 1440-1395 cm^{-1}. The ^1H NMR spectrum for **14** is very simple and suggests a symmetrical biphenyl compound with two identical sets of ortho-coupled protons. It also indicates that the carboxylic acid groups are not directly attached to the para positions of the aromatic rings. This conclusion is based on the signal at δ 7.12 for the 3/5 and 3'/5' protons. This signal should be further downfield if the carboxylic acid groups were directly attached to C-4 and C-4' respectively. For example p-hydroxybenzaldehyde the C-3/5 protons are at δ 7.809 due to the deshielding effect of the carbonyl group. Thus the isomer bibenzyl-4,4'-dicarboxylic acid, involving a $-CH_2-CH_2-$ group between the two aromatic rings and carboxylic acid groups directly attached in both para positions was ruled out as a possible structure. In addition **14** had m.p. 155-158 °C whereas bibenzyl-4,4'-dicarboxylic acid has

m.p. > 320 °C (Heilbron 1969). Compound **14** was therefore assigned the structure 1,1'-biphenyl-4,4'-diacetic acid by comparison of MS, UV, IR and NMR with literature data (Gotthardt and Pflaumbaum 1987; Cassidy *et al* 1991).

The biphenyl nucleus present in **14** is also a feature of ellagic acid biosynthesis, where hexahydroxydiphenic acid is a precursor. It is surmised therefore that 1,1'-biphenyl-4,4'-diacetic acid is likely to arise from a common biosynthetic pathway to that of ellagic acid. This is the first report of 1,1'-biphenyl-4,4'-diacetic acid in *Gunnera perpensa*.

Compound 15 (p-hydroxybenzaldehyde)

Appendix: **15** IR and ^{1}H NMR.



p-hydroxy benzaldehyde

Physical data

Compound **15** was present in large amounts after hydrolysis of various *Gunnera perpensa* extracts. For example approximately 20 g of a combined hot methanol and hot water extract was hydrolysed in 150 cm^3 1M HCl for 3 h at 90°. After hydrolysis extraction with EtOAc was carried out, and a dark brown gum was obtained after solvent removal. This gum was treated with cold methanol that caused a yellow precipitate, compound **13**, to form. The residue contained 90% of compound **15** by HPLC analysis, R_t 3.45 min., in 95:5 acetonitrile:water at 1 cm^3/min. using a γ-cyclodextrin column and a UV detector.

The EtOAc extraction was purified by silica gel chromatography. The fraction eluted with hexane:ether 1:2 was crystallized from $CHCl_3$ to yield colourless crystals m.p. 116 - 119° (115 - 116° for p-hydroxybenzaldehyde, Buckingham1994). These crystals showed R_t 2.85 min. (99.3%) on HPLC using a reversed phase column with 60:40 acetonitrile:water at 1 cm^3/min. with a UV detector at 254 nm. An alternative γ-cyclodextrin column for

HPLC showed one major peak (98.2%) at 3.45 min. in mobile phase 95:5 acetonitrile : water. TLC of **15** produced a pale mauve spot R_f 0.65 in hexane:ether 1:1.

On a separate occasion, while purifying another extract after hydrolysis, 1:3 EtOAc : $CHCl_3$ was also found useful for eluting p-hydroxybenzaldehyde, initially as a reddish-brown solid. After re-purification on a second column, and recrystallisation from water, a pure white solid was obtained which had the typical pleasant, sweet caramel fragrance, hard to describe, but similar to that of vanilla essence/mowed grass/mild antiseptic/mild tobacco, which characterizes this aglycone. The same fragrance has been noted in *Combretum kraussii* hydrolysates as well.

Silica gel chromatography seems to have a detrimental effect on this compound, as well as other compounds isolated from *Gunnera* extracts, because fractions in contact with silica gel for long periods deteriorate and become dark, gummy and difficult to recover from columns. Sephadex LH-20 as well as cellulose were investigated as alternatives to silica gel, but ultimately silica gel gave better separations.

A few crystals of **15** were dissolved in hexane:ether 1:4 and a drop of 2.4-dinitrophenyl-hydrazine was added. An immediate red precipitate formed indicating that **15** was an aldehyde.

FAB-MS: M^+ 122.0370 $C_7H_6O_2 \equiv$ 122.0368

UV (methanol): 247 (major), 358 nm (minor)

IR (KBr disc): 200cm^{-1} broad OH, C=O stretch at 1665cm^{-1} and C=C stretch at 1590cm^{-1}.

^{1}H NMR (CD_3OD): δ 9.80 1H, s, aldehyde H
δ 7.81 2H, d, J=8.7Hz aromatic (C-2 and C-6)
δ 6.95 2H, d, J=8.6Hz aromatic (C-3 and C-5)

A comparison of HPLC analyses of *Gunnera perpensa* extracts of roots using different solvents showed that p-hydroxybenzaldehyde was present in all of them, as shown below, but that the chloroform extract contained the greatest amount. A mobile phase of 80:20 acetonitrile:water at 0.5 cm^3/min. was used and a UV detector at 254 nm, giving a peak at 4.8 min. for p-hydroxybenzaldehyde.

Extract	Compound **15**
	%
Chloroform	84
Ether	55
Acetone	10
Methanol	13
Water	13

Discussion

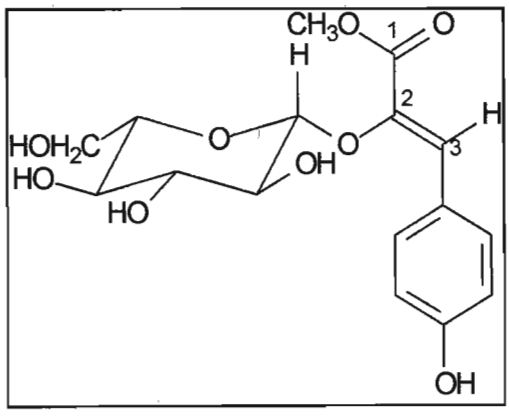
Initially when the aglycone compound **15** was isolated and characterized from methanol extracts, it was not known that it arose from the two phenolic uteroactive glucosides **17** and **18**. This fact was established later when the fraction containing compounds **17** and **18**, was hydrolysed.

The common aglycone, compound **15**, was found in various extracts, and also produced by natural hydrolysis as well as acid hydrolysis of fractions. It is closely related to the lignin monomers discussed in Section 1.4. It was purified from the methanol extract as well as other extracts after hydrolysis. Extraction with ethyl acetate after hydrolysis and column purification on silica gel yielded p-hydroxy benzaldehyde, identified by melting point and spectral characteristics. This aglycone is generated in abundance over a period of time in extracts, and sometimes precipitates out of solution in water extracts, which undergo a natural hydrolysis, accompanied by gas evolution on standing at room temperature for a few days.

The molecular ion for **15** at m/z 122.0370 was in excellent agreement with 122.0368 required for $C_7H_6O_2$. The two sets of doublets observed in the ^1H NMR spectrum of **15** indicated two identical sets of ortho coupled protons on the C-2/C-6 and C-3/C-5 positions of an aromatic ring system. From the melting point, IR, UV and ^1H NMR spectral characteristics **15** was identified as p-hydroxybenzaldehyde. This is a major product obtained from chemical hydrolysis of various water or methanol extracts, and was also released from the two major glucosides purified from extracts. It also formed readily by natural hydrolysis of various extracts on standing in solution at room temperature, and has not been reported previously in *Gunnera perpensa*. In addition it was identified by HPLC in *Gunnera perpensa* extracts using five different solvents, but was present in the greatest amounts in the ether (55%) and chloroform extracts (84%).

Compound 16 (Z-methyl lespedezate)

Appendix: **16** EI-MS, IR, ^{1}H NMR; **16** acetate FAB-MS, IR, ^{1}H NMR ($CDCl_3$ and CD_3OD), HMBC, COSY and ^{13}C NMR.



Z-lespedezic acid methyl ester

Physical data

The phenolic glucoside **16** was obtained by silica gel column chromatography of a hot methanol extract of 4 kg of roots harvested at Silverglen Nature Reserve in April 1996. Roots were chopped and extracted twice by refluxing in methanol, after prior extraction with cold ethanol. The extract was concentrated under vacuum and applied to the column. Compound **16** (0.13 g, 0.019% based on the dry root mass) was eluted from the column with $CHCl_3$:MeOH 1:3 and purified by evaporation of solvents and addition of methanol which caused brilliant needle-like colourless crystals to separate, m.p. 290°, R_f 0.72 in $CHCl_3$:MeOH 5:1, producing a green spot with anisaldehyde reagent. Compound **16** did not dissolve well in $CHCl_3$ or MeOH, but dissolved better in acetone.

FAB-MS m/z:	M^+	absent
	180	$C_6H_{12}O_6^+$
	163	$C_9H_7O_3^+$
	134	
	106	

UV (MeOH): 221 (small) 315 nm (major).

UV (methanol + NaOH): 233 (small), 348 nm (major).

IR (KBr disc):3540cm^{-1}, free OH; 3250cm^{-1} broad OH; 1700cm^{-1}, C=O;1620 and 1600cm^{-1}, C=C.

^{1}H NMR (DMSO-d_6): δ 7.61 (2H) d J=8.8 Hz

δ 6.80 (2H) d J=8.7 Hz

δ 6.72 (1H) s

δ 5.17 (1H) d J=7.8 Hz, anomeric sugar proton

δ 4.96, 5.52 and 5.81 broad singlets (disappeared with D_2O)

δ 3.2 to 4.1 sugar protons obscured by DMSO peak.

Compound **16** acetate

Compound **16** (0.1 g) was acetylated at 100° in pyridine/acetic anhydride for 2 h, then recrystallised from MeOH as colourless needles m.p. 176 - 178°. TLC in hexane:ether 1:5 gave a green spot R_f 0.69.

FAB-MS:	589.1	$M^+ + Na^+$
	331.1	glucose tetra-acetate
	169.1	base peak
	109.1	glucose tetra-acetate fragment

IR (KBr disc): 2950cm^{-1} C-H; 1750cm^{-1} C=O (broad); 1650, 1600cm^{-1} C=C; 910 cm^{-1} C-H bend.

Glucose penta-acetate was prepared as a standard for comparison to **16** acetate by acetylating glucose with pyridine/acetic anhydride, and the ^{1}H NMR spectrum was run.

1H and ^{13}C NMR spectra were run for the penta-acetate of **16**, as well as HMBC, COSY and NOE spectra, in order to establish the structure of the compound. Results are tabulated in Table 4.2 in the discussion section.

Discussion

The EI-MS of 16 did not produce a molecular ion, but showed breakdown fragments at 180, corresponding to glucose, as well as at 163, 134, and 106. The ion at 163 probably corresponds to the p-coumaryl fragment (Fig.4.2) after loss of glucose and a methyl group. It was previously established that the uteroactive fraction released p-hydroxybenzaldehyde (compound 15) on hydrolysis, and that this could have arisen from

oxidative hydrolysis of the alkene double bond of a phenyl propanoid such as p-coumaric acid (Section 1.4).

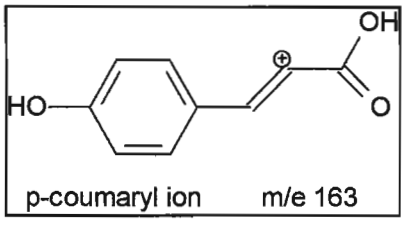


Figure 4.2

The UV spectrum of compound **16** showed a major absorption at 315 nm with similarities to compounds like p-coumaric acid which has absorptions at 227 and 310 nm in ethanol. On addition of NaOH a bathochromic shift of 33 nm was observed in the UV spectrum of **16**, comparable to the 25 nm bathochromic shift for p-coumaric acid with NaOH in methanol. The UV λ_{max} of **16** is consistent with a trans system for a conjugated acid such as p-coumaric acid. Due to the superior conjugation of the trans system the UV maxima are consistently at longer wavelengths and of higher ε_{max} values than those of the corresponding cis isomers. For example λ_{max} for trans-4-methoxy ethyl cinnamate is 310 nm whereas it is 305 nm for the corresponding cis isomer (Bloomfield 1961). The trans configuration of the double bond of **16** was subsequently confirmed by further spectral evidence.

The strong carbonyl absorption at 1700cm^{-1} in the IR spectrum of **16** indicates an aryl α, β- unsaturated carboxylic acid which displays a carbonyl absorption at approximately 1710 – 1680cm^{-1}. Conjugation with the alkene double bond delocalises the π electrons of both systems, reducing the double bond character of the carbonyl group, and therefore giving rise to an absorption at lower frequency than that of benzoate esters *i.e.* 1730-1715 cm^{-1} as illustrated:

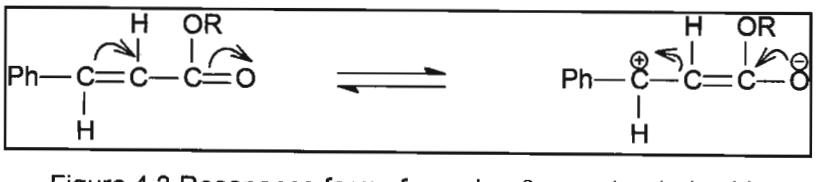


Figure 4.3 Resonance forms for aryl α, β- unsaturated acids

The ^{1}H NMR spectrum indicates a para disubstituted aromatic system with two identical sets of ortho-coupled protons (Table 4.2). Apart from the anomeric C-1 proton doublet at δ5.171 the glucose protons were mainly obscured by the DMSO peak, but were subsequently clearly observed in the spectrum of **16** after acetylation. Three hydroxy

signals, which decreased in size on D_2O addition, were also observed in the spectrum of **16**.

The structure of **16** was elucidated by means of 1D and 2D NMR as well as other spectral data. The molecular ion was absent in the EI MS of **16**, suggesting an easy breakdown of this molecule, with a mass fragment at 180 being attributed to glucose. The molecular ion appeared as a small peak at 589.1 in the FAB-MS spectrum of the penta-acetate of **16**. It was originally suspected that **16** might contain nitrogen on the basis of the odd molar mass, but subsequently it was realised that this peak represented $M^+ + Na^+$ (23), and therefore the actual molar mass is 566 for the penta-acetate of **16**, corresponding to a formula of $C_{26}H_{30}O_{14}$. The molar mass of **16** itself on this basis is 356, corresponding to a formula of $C_{16}H_{20}O_9$. The easy fragmentation observed for **16** was also apparent in the MS of **16** penta-acetate where the ion at 331.1, corresponding to the glucose tetra-acetate moiety, had an intensity over 6 times greater than that of the molecular ion. The base peak at m/z 167 and significant peak at m/z 109 are typical fragments of the glucopyranose tetra-acetate moiety, as established with an authentic acetate standard.

Table 4.2 NMR spectral data for **16** penta-acetate

Carbon	^{13}C shift ppm $CDCl_3$	^{1}H shift ppm CD_3OD	J value Hz	HMBC Correlations	COSY Correlations
1	163.8	-	-	H-3β -OCH_3β	-
4'	151.3	-	-	H-3'/5'α; H-2'/6'β	-
2	139.7	-	-	H-3α; H-1"β	-
3' + 5'	131.9	7.83 d	8.79	H-2'/6'β; H-3β	H-2'/6'
1'	130.3	-	-	H-3'/5'β	-
3	125.6	7.11 s	-	H-2'/6'β	-
2' + 6'	121.5	7.08 d	8.72	H-2'/6'β; H-3'/5'α	H-3'/5'
1"	99.1	5.41 d	7.88	H-2"α	H-2"
3"	72.7	5.34 t	9.43	H-2"α; H-4"β	H-2"/4"
2"	72.0	5.19 t	8.79	H-3"α	H-1"/3"
4"	71.5	5.06 t	9.71	H-3"α	H-3"/5"
6"	68.3	4.13 dd	12.36/4.67	H-6"	H-5"/H-6"
		3.99 dd	12.27/2.38	H-6"	H-5"/H-6"
5"	61.5	3.84 m	-	H-4"α	H-4"/6"
C-1-OCH_3	52.3	3.84 s	-	-	-
C-6"-$COCH_3$	170.5	2.28 s	-	CH_3α H-6"β	-
C-2"-$COCH_3$	170.2	2.00 s	-	CH_3α H-6"β	-
C-3"-$COCH_3$	169.6	2.00 s	-	CH_3α H-6"β	-
C-4"-$COCH_3$	169.4	1.99 s	-	CH_3α H-6"β	-
C-4'-$COCH_3$	169.1	1.95 s	-	CH_3α H-6"β	-

The ^{1}H NMR spectra of **16** penta-acetate differed slightly in $CDCl_3$ and CD_3OD, with the methoxy group on C-1 separating from the sugar 5" proton in $CDCl_3$ but not in CD_3OD. In

addition the alkene proton separated better from the nearby aromatic protons in $CDCl_3$. However CD_3OD was found a more useful solvent than $CDCl_3$ for resolving sugar signals in the δ 5 region of the spectrum.

There is a significant downfield shift of H-2', H-3' and H-4' sugar protons on acetylation and these obscure the anomeric axial H-1' proton in the ^1H NMR spectrum in $CDCl_3$. For the glucose penta-acetate standard in $CDCl_3$, the H-1' doublet appears at δ5.695, significantly downfield from H-2', H-3', H-4' protons. The aromatic ring in **16** is therefore presumably joined via the C-1' glucose hydroxyl, and is therefore not as far downfield as when the C-1' hydroxyl is acetylated. Spectra of **16** penta-acetate and that of a 1,2,3,4,6-penta-O-acetyl-β-D-glucopyranose standard, prepared from glucose, were compared while assigning signals in **16** spectra.

^1H-^1H COSY (Correlation spectroscopy) spectra show off-diagonal peaks for all protons that have significant coupling (Silverstein and Webster 1998). From the cross peaks one can deduce which protons are close to each other in the molecule. Through COSY correlations all the assignments for the glucose protons were clearly made.

HMBC (Heteronuclear multiple bond coherence) spectra show long-range ^1H-^{13}C correlations, *i.e.* 2-bond and 3-bond proton-carbon couplings. Although 2-bond correlations are almost always shown, 3-bond correlations are sometimes absent. This technique is valuable for showing connectivities beyond those protons directly attached to carbon, so is useful for structural elucidation.

The nuclear Overhauser effect (NOE) is extremely useful for working out the connectivities of protons. These "through space" interactions between protons that are close to each other can be detected even between protons that are not actually coupled (Silverstein 1998). Nuclei are irradiated with a much weaker irradiation in a NOE experiment than that used for spin decoupling. If a proton is coupled therefore, it remains at least partially coupled. The weak irradiation increases the population of the higher energy level in the nearby non-irradiated proton, causing an increase in signal intensity. This signal enhancement is usually less than 20%, but the NOE difference experiment increases the sensitivity however by subtracting the conventional ^1H spectrum from the specific proton-irradiated spectrum. Only the enhanced signals remain, and effects between nuclei up to 4 A^o apart are detectable. The technique is particularly useful for

distinguishing between isomers, and this was indeed found to be the case when establishing the configuration about the alkene bond in compound **16**.

Irradiation of the ester $-OCH_3$ group at δ 3.84 enhanced the glucose δ 5.41 doublet for the H-1' axial proton, and vice versa on irradiation of the H-1' of glucose. The alkene proton singlet at δ 7.03 was not affected by irradiation of either the anomeric glucose H-1' or the methoxy group, leading to the conclusion that neither was in proximity to this alkene proton, and that glucose is therefore trans to the alkene proton *i.e.* the Z-isomer. This was the expected configuration based on the fact that the biogenetically linked naturally occurring acids such as E-p-coumaric acid have this configuration (Fig.4.4) which equates to the Z isomer of **16** because of the higher priority oxygen replacing hydrogen for **16**. The Z and E isomers are shown in Fig. 4.4 and the proximity of the glucose anomeric proton is clear for the E isomer. A molecular model was built and also supported these designations for the stereochemistry about the double bond.

The conjugated alkene-carbonyl system was deduced to be in the s-cis (single bond-cis) planar conformation as shown in Fig.4.4. The α,β-unsaturated keto system tends to be planar, unless steric hindrance prevents this, and may adopt the s-cis or s-trans conformation. If it had been in the s-trans conformation, the methoxy group would have been in close proximity to the alkene proton at δ7.03, and therefore shown NOE enhancement. However this was not the case. Well-known phenyl propanoid acids such as p-coumaric acid commonly adopt the s-cis conformation.

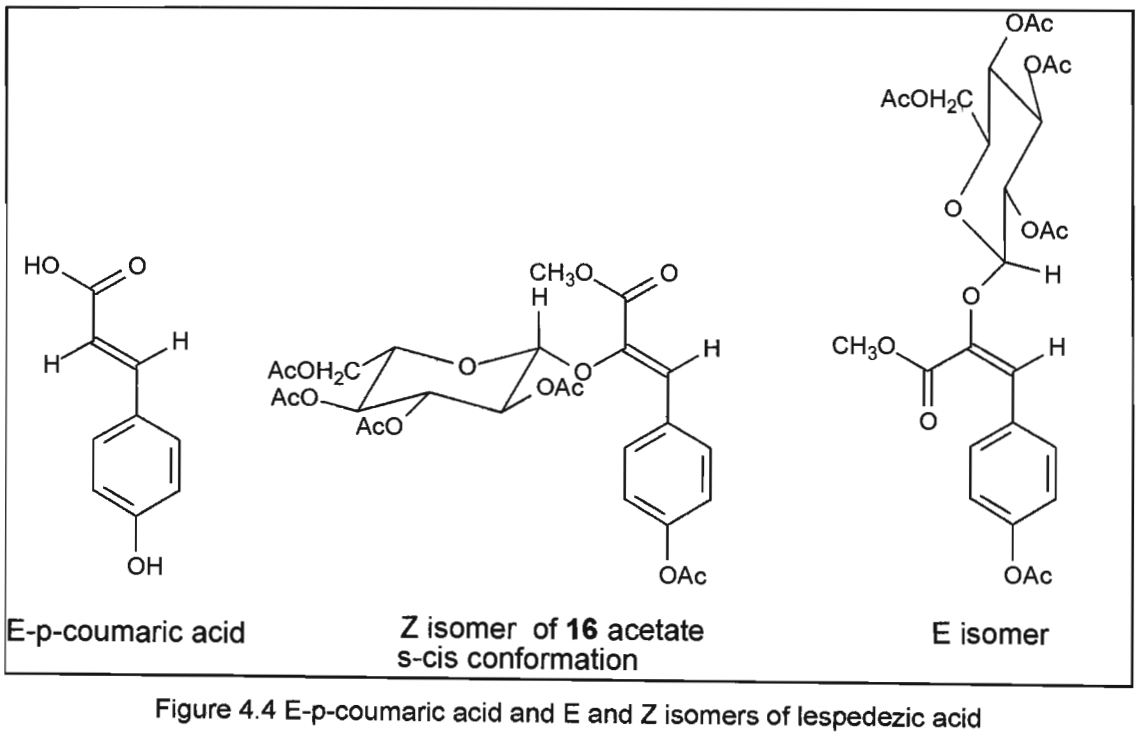


Figure 4.4 E-p-coumaric acid and E and Z isomers of lespedezic acid

On the basis of the described spectral evidence, the structure of **16** was unequivocally confirmed, and matches literature data for lespedezic acid methyl ester (Shigemori *et al* 1990) or the Z isomer of 2-O-β-D-glucopyranosyl-3-[p-hydroxyphenyl]-propenoic acid methyl ester. The potassium salt of lespedezic acid, isolated from *Lespedeza cuneata*, has recently-discovered biological activity, being responsible for the leaf-opening mechanism in nyctinastic plants (Ohnuki 1998). It is interesting to note that the glucose moiety is required for this activity, and that in the evening potassium lespedezate is deactivated by enzymatic hydrolysis with β-glucosidase to yield 4-hydroxyphenylpyruvate and glucose. This then allows the leaf-closing chemical, potassium D-idarate, to dominate.

Compound **16**, or Z-methyl lespedezate, is a derivative of p-coumaric acid which is commonly found in both woody and herbaceous monocotyledons. *Gunnera mandicata* contains p-coumaric acid, and in fact ellagic acid, caffeic acid and p-coumaric acids have been isolated in several *Gunnera* species. In woody plants caffeic acid often accompanies p-coumaric acid, whereas in herbaceous plants ferulic and sinapic acids are frequently found with it (Harborne 1964). This derivative of p-coumaric acid, Z-lespedezic acid methyl ester, purified in 1997, initially proved difficult to characterise. The structure was finally fully elucidated in January 2004, as described above, and had not previously been reported in *Gunneraceae* at the time. However the presence of Z-methyl lespedezate in *Gunnera perpensa* was subsequently reported in an independent investigation concerning the contractility of uterine muscle (Drewes *et al.* 2004). In the same study a related major component, the phenylpropanoid Z-venusol, was identified, which induced a state of continuous contractility of the uterus. Z-venusol can be converted to methyl lespedezate by boiling methanol. In my own investigation methyl lespedezate, a minor component, was not subjected to uterine tests because it was not obtained in sufficient amounts. Uterine tests were also not reported for methyl lespedezate by Drewes and co-workers, possibly because it was also obtained in small quantities.

Compounds 17 and 18 (phenolic glucosides)

Appendix: **17+18** IR; **17+18** methylated Fr.9 FAB-MS, ^{1}H and ^{13}C NMR.

Physical data

Compounds **17** and **18** together typically made up well over 50% of methanolic or aqueous root extracts by HPLC analysis using a UV detector. For example a hot methanol extract contained 32% compound **17** and 66% compound **18**. The high amounts of these two compounds was also confirmed by weighing a water extract after freeze-drying, and is described on p. 84. After prior removal of less polar components with CH_2Cl_2/MeOH, compounds **17** and **18**, present as the remaining water-soluble constituents, comprised 12.2% of the plant material based on the dry root mass.

Gunnera perpensa roots, 4 kg, harvested April 1996, were blended in a food processor and extracted for 2 days at room temperature with ethanol, followed by 2 extractions by refluxing with boiling methanol. The concentrated hot methanol extract was chromatographed on silica gel using hexane:ether, $CHCl_3$:MeOH in ratios of increasing polarity, MeOH:HOAc 5:1 then 10:1, and finally water. Compounds **12**, **16** and glucose eluted prior to compounds **17** and **18** as outlined briefly below.

The fraction eluted with $CHCl_3$:MeOH 1:1 showed evidence of colourless crystals on evaporation of solvents m.p. 296 - 298°. HPLC revealed one major peak at 5.46 min., co-eluting with a trimethyl ellagic acid standard 5.48 min. in 60:40 acetonitrile:water, 1cm^3/min. The IR spectrum was also identical to that of the trimethyl ellagic standard. This fraction was identified as trimethylellagic acid lactone, compound **12**, previously identified in a chloroform extract.

The following fraction, containing compound **16**, was eluted with 1:3 chloroform-methanol and has been described. Next followed a white gum, eluted with methanol, which gave a negative $FeCl_3$ test. HPLC analysis using the Carbohydrate Analysis column revealed one major component at 9.4 min. which was identified as glucose by co-elution with an authentic standard. An amount of 7.15 g of this gum was also acetylated with 10 cm^3 pyridine and 25 cm^3 acetic anhydride at 90 °C for 2 h. The acetate was precipitated in ice/water, dried and 2.6 g was purified on a Si gel column. It was eluted with hexane:ether 1:5 and recrystallised from cold methanol, m.p. 142 – 146 °C. A glucose acetate standard had mp. 149 - 152°. Both the purified acetate from the column and the glucose acetate standard had identical R_f values of 0.55, giving green spots on TLC in hexane:ether 1:5.

All further fractions, eluted with methanol, methanol-acetic acid 5:1 and water, yielded mixtures of the two most polar compounds in the extract, compounds **17** and **18**, both phenolic glucosides which are water-soluble. HPLC analyses for these fractions are shown in Table 4.3. It was clear from the quantities obtained that the two compounds present in all fractions from 39 – 59 were the major constituents of the extract.

Table 4.3 HPLC at $1cm^3$/min, in 60:40 acetonitrile:water, reversed phase C-18 column with UV detector at 254nm.

Fraction	R_t min.	%
39-44	1.73	75.2
	2.34	24.8
54-52	1.81	71.3
	2.42	28.5
53-57	1.78	82.5
	2.34	17.5
58-59	1.74	54.7
	2.33	45.3

IR (KBr disc): 3400 (very large), 1705, 1600, 1330, 1170, 1050, 870 cm^{-1}.

The approximate quantity of **17** and **18** present was established by extracting roots from Silverglen Nature Reserve harvested in November 1999. Roots, 726.4 g, water content 84.7%, were blended then extracted at room temperature overnight with 1.5 litres 1:1 CH_2Cl_2-CH_3OH. After filtering they were re-extracted with 1.0 litre of the same solvents for two nights, followed by two consecutive 1.0 litre extractions with water at room temperature overnight. After filtering and removing CH_2Cl_2-CH_3OH under vacuum on a rotary evaporator and removing water by freeze-drying, the following results were obtained:

	Mass (g)	% of total mass extracted	% of dry root mass
CH_2Cl_2-MeOH extract	1.78	11.6	1.6
Water extract	13.53	88.4	12.2
Total mass extracted	15.31		

The water extract, a dark brown powder, was composed mainly of glucosides, containing 75% of compound **17**, based on HPLC analysis. Further attempts to purify **17** and **18** enough for full spectral analysis were unsuccessful. In addition acetylation and methylation were also carried out followed by repeated column chromatograpy in order to purify them, using various methods described for compounds **10** and **11** from *Combretum kraussii* extracts. The following is an example of one of these.

A sample of 7.5 g of the **17/18** mixture was methylated by refluxing in 600 cm^3 dry acetone with 50 g K_2CO_3 and 70 cm^3 $(CH_3)_2SO_4$ for five days. The product was purified on a silica gel column and Fr. 7-10, eluted with 5:1 CH_2Cl_2-acetone, was recrystallised from CH_2Cl_2-hexane to produce 0.7 g of a yellow powder with a negative $FeCl_3$ test. This material (0.4 g) was re-chromatographed and Fr.9, eluted with 30:1 CH_2Cl_2-acetone, gave 40.3 mg of yellow shiny leaflets. It gave a single green spot, R_f 0.48, on TLC in 30:1 CH_2Cl_2-acetone and had m.p. 180-181 °C. The bulk of the material from the column, eluted with 10:1 CH_2Cl_2-acetone, was more polar than Fr.9 on TLC, and showed a hydroxyl peak in the IR spectrum. Fr.9 showed one major peak (68%) at 6.73 min. on HPLC in 80:20 acetonitrile-water, and a second peak at 6.32 min. (22%). FAB-MS analysis showed a molecular ion at m/z 1320, the ^{13}C NMR spectrum showed 60 carbons, and the ^1H NMR spectrum showed a large number of $-OCH_3$ signals in the δ 3.6 to δ 4.3 region. The integrals for the aromatic protons were not whole numbers however, confirming that this, as well as other methylated or acetylated fractions, were not suitable for spectroscopy.

Hydrolysis of *Gunnera perpensa* extracts:

1. Natural Hydrolysis

It was noticed that several different *Gunnera* extracts underwent a fairly easy natural hydrolysis on standing in solution at room temperature. Gas evolution and natural precipitation of a slimy precipitate frequently accompanied the initial stages of water extraction. The following natural hydrolysis (Table 4.4) at room temperature in 60:40 acetonitrile-water mobile phase illustrates this facile interconversion.

Table 4.4 Natural hydrolysis at room temperature of mixture of compounds **17** and **18** in 60:40 acetonitrile:water. Flow for HPLC 1 cm^3/min., UV detector at 254 nm.

Sample	Compound **18**		Compound **17**		Compound **15**	
	R_t min.	%	R_t min.	%	R_t min.	%
Fresh solution	1.59	83.7	1.91	16.3	4.42	0.04
Solution after a few days	1.59	68.0	1.91	25.5	4.54	5.37

Storage of this solution at room temperature in a sealed vial for one month led to the complete disappearance of the first two polar components, leaving the major remaining compound with R_t approximately 4.5 min. (98% by HPLC analysis). This phenolic aglycone was identified by HPLC and TLC as compound **15**, p-hydroxybenzaldehyde, previously described.

In addition to the phenolic components, it was also shown by TLC and HPLC that glucose, not present in a significant amount in the fresh extract, was also released on hydrolysis. A root extract, prepared with hot methanol, released a large colourless crystal after storage for a month at room temperature. This crystal had R_f O.27 in ethyl acetate : pyridine : water : methanol 80:20:10:5, identical to that of a glucose standard. In addition it had R_t 9.60 min. on HPLC in 80:20 acetonitrile - water at 1 cm^3/min. on a Carbohydrate Analysis column, co-eluting with a glucose standard.

2. Acid hydrolysis of **17** and **18**

An amount of 1 g of the mixture of compounds **17** and **18** was heated with 10 cm^3 conc. HCl at 100° for 4 h in a test tube, then neutralized by passing through Amberlite IRA – 400 resin and extracted with ethyl acetate. Pale beige crystals separated from the ethyl acetate extract, after standing and evaporation, having R_t 4.76 min. (91%) on HPLC in 80:20 acetonitrile-water, and co-eluting with a standard of p-hydroxybenzaldehyde. HPLC analysis of the aqueous phase showed that the 2 major polar components, as shown in the Table above, had both disappeared and a new peak had developed with R_t 4.51 min. in 60:40 acetonitrile:water at 1 cm^3/min. The aqueous phase after hydrolysis showed a green spot R_f 0.21 with anisaldehyde spray in 6:2:2:1 butanol-acetic acid-water-chloroform. A glucose standard had the same R_f in this system, whereas the **17/18** mixture before hydrolysis contained no free glucose.

A bonded phase γ-cyclodextrin column developed for sugar analysis by Prof. Armstrong of Iowa State University gave an excellent separation of compounds in *Gunnera perpensa extracts*, and was found useful for monitoring hydrolysis results. For the best separations the organic modifier should be 65 to 95%, (Armstrong and Jin 1989) so three acetonitrile-water systems were investigated as mobile phases for the γ-cyclodextrin column. The amount of water in the mobile phase has a marked effect on the retention time of the phenolic glucosides, with increased amounts of water resulting in shorter retention times. For example compound **16** has a retention time of 3.4 minutes in 85:15 but 4.6 min. in 95:5 acetonitrile-water at a flow rate of 1 cm^3/min. using a detector at 254 nm. The more polar Compound **17** has R_t 3.6 and 5.3 min. respectively, in the same two systems.

The separation of a column fraction containing the three glucosides, which was eluted with $CHCl_3$-MeOH and isolated as a brown gum, is shown below (95:5 acetonitrile-water):

3.45 min (1.1%)	**15**
4.72 min. (8.9%)	**16**
5.49 min. (64.1%)	**17**
5.99 min. (24.1)	**18**

Hydrolysis of the above fraction (0.97 g) was carried out in 5 cm^3 methanol, 4 cm^3 water and 1 cm^3 conc. HCl in a sealed vial for 3 h at 100 °C. This resulted in the complete disappearance of compounds, **16**, **17** and **18** and the formation of glucose, with R_t 3.63.min. in 85:15 acetonitrile-water at 195 nm, identical to that of a glucose standard. Fructose and sucrose standards had R_t 6.87 and 10.80 min. respectively under the same conditions. The only other compound detected after hydrolysis was the aglycone **15** which was detected at 254nm.

Discussion

Compounds **17** and **18** were found to be major components in the roots, and together they typically made up over 90% of aqueous extracts by HPLC analysis.

Results from both natural hydrolysis in various solutions and chemical hydrolysis of the active phenolic glucosides **17** and **18** by heating with HCl or H_2SO_4 showed that both compounds converted to a common aromatic aglycone, p-hydroxybenzaldehyde as well as glucose. This indicates that they are related glucosides.

A HPLC comparison of various extracts prepared using chloroform, ether, acetone, methanol and water as solvents showed that all extracts had a common component at R_t approximately 4.8 minutes in 80:20 acetonitrile-water (0.5 cm^3/min). This was shown to be p-hyroxybenzaldehyde, released after hydrolysis of the biologically active glucoside fraction. The non-polar chloroform and ether extract contained the greatest amount of this component *i.e.* 84% and 55% respectively. The polar phenolic glucosides are extracted in greater amounts by polar solvents such as water, as expected. The water extract, after freeze-drying, accounted for 12.2% of material dissolved per dry root mass, whereas the methylene chloride-methanol extract contained only 1.6%. The major chemical components in *Gunnera perpensa* extracts are the polar water-soluble glucosides and the aqueous extracts from these plants, used by traditional healers, would therefore contain significant quantities of these glucosides. The most polar compound, a glucoside, **18**, decreases in quantity in solution to form the less polar compound, glucoside **17**. This natural hydrolysis also yields more of the phenolic aglycone, **15**, with R_t approximately 4.5 mins.

The large amount of free glucose obtained from a typical silica gel column, prior to elution of the glucosides, suggests that glucose could be the sugar associated with the phenolic aglycone in the plant. Possibly both glucose and the aglycone are released from extracts as they hydrolyse naturally in solution. This natural hydrolysis process was shown to occur readily in extracts.

Compound **17** could be partially purified from brown gummy mixtures by adding a small amount of ethanol or methanol and grinding/macerating after warming the mixture first. Dark material went into solution and loose, friable particles could be separated to give a beige powder 96% pure by HPLC analysis, but the NMR spectrum was unsatisfactory. Silica gel chromatography was not successful in completely separating compounds **17** and **18**.

A bonded phase γ-cyclodextrin column developed for sugar analysis (Armstrong and Jin 1989) gave an excellent separation of compounds in *Gunnera perpensa* extracts. For the best separations the organic modifier should be 65 – 95%, so three acetomitrile:water systems were investigated as mobile phases for the γ-cyclodextrin column. The amount of water in the acetonitrile mobile phase has a marked effect on the retention time of the phenolic glucosides, with increased amounts of water resulting in shorter retention times.

For example compound **16** has a R_t 3.4 min. in 85:15, but 4.6 min. in 95:5 acetonitrile-water. Compound **17** has R_t 3.6 and 5.3 min. respectively in the same two systems.

The methanol extract, a brown gum, of *Gunnera perpensa* roots chromatographed on 200 g of silica gel. Non-polar components were eluted with hexane-ether 1:3 and then the solvent was changed to $CHCl_3$-MeOH mixtures of increasing polarity, and finally water. Fractions 17-19 and 20 – 27, eluted with 1:1 and 1:3 $CHCl_3$-MeOH respectively, contained one major component **17** by HPLC, with **18** also being present. All subsequent fractions also contained these components right up to Fr.49, eluted with 5:1 methanol-water (1% HOAc). The silica gel from the column was later washed with water in a beaker and yielded a dark olive-green solution that was filtered and evaporated. This material still contained the major polar component found in earlier fractions indicating the difficulty in removing the polar *Gunnera perpensa* components from silica gel.

Compound **17** was slightly more polar than a glucose standard, having R_f 0.53 whereas glucose had R_f 0.56 in methanol-acetic acid 5:1 on TLC. Both showed a green spot with anisaldehyde reagent. Compound **16** (Z-methyl lespedezate) however was less polar than glucose on TLC in ethyl acetate-pyridine-water-methanol 80:20:10:5, having R_f 0.71, whereas glucose had R_f 0.21. Both compounds showed green spots with anisaldehyde reagent.

The IR spectrum of compound **17/18** mixtures showed a massive broad hydroxyl absorption at 3400 cm^{-1} which obscured the C-H stretch absorption. In addition C=O and C=C stretch absorptions were evident at 1705 and 1600 cm^{-1} respectively, similar to those of compound **16**, Z-methyl lespedezate.

Methylation and acetylation of the glucoside mixture was carried out in order to purify the two components, but was only partially successful. An example of a partially purified methylated derivative was Fr. 9 which was chromatographed twice, and eluted from the second silica gel column with 30:1 CH_2Cl_2-acetone. It gave a single green spot, R_f 0.48 in 30:1 CH_2Cl_2-acetone and had m.p. 180-181 °C. However it was only 68% pure by HPLC and also appeared to be impure on ^1H NMR spectroscopy. The ^1H NMR spectrum also showed a large number of $-OCH_3$ signals in the δ 3.6 to δ 4.3 region. The ^{13}C NMR spectrum showed 60 carbons, and FAB-MS gave a molecular ion at m/z 1320. Further research is required to purify these two compounds sufficiently for full structural elucidation. From present results they are probably higher glucosides of Z-lespedezic acid methyl ester.

CHAPTER 5

RHOICISSUS TRIDENTATA

Components **19** to **38** were isolated from *Rhoicissus tridentata* extracts which were found to contain a high proportion of flavonoid constituents, a common feature in the Vitaceae family. Eleven proanthocyanidin monomers and dimers (Fig.5.1) and gallic acid were identified, and a high proportion of polymeric proanthocyanidins, known for their astringent properties, was also found to be present. A hydrogel of glucose was partially characterised. The non-polar components identified were sitosterol and sitosterolin, γ-sitosterol, oleanolic acid, lupen-3-one and taraxastananol. All 19 compounds identified are novel in this species which has had virtually no prior chemical investigation.

5.1 Colorimetric determination of total proanthocyanidin content

Physical data

A deep red colour developed when a water extract of roots was heated in acidic alcoholic solution in a sealed vial, which indicated the presence of proanthocyanidins. This fact was used to develop a method to determine the relative amounts of proanthocyanidins in various extracts, and also in milled root samples. The method is adapted from that recommended by the U.S.A. Grape Seeds Methods Evaluation Committee (http://www.activin.com).

Methanol and ethanol were each investigated as solvents for the acid hydrolysis, but reaction was found to be faster in methanol. Hydrolysis was initially carried out at 80 °C on 2.4 mg of freeze-dried methanol extract of roots harvested in January 2000 in the Umbumbulu area of Kwa-Zulu Natal. The methanol solution of the extract after addition of 0.3 cm^3 conc. HCl was in a sealed vial. Results are shown below.

Table 5.1 Hydrolysis of root extract in methanolic HCl

Hydrolysis time min.	Absorbance at 540 nm
30	1.65
45	2.22
60	2.58
90	2.80
120	2.89

Hydrolysis of 2.4 mg of the same extract under the same conditions but using ethanol instead of methanol as solvent produced lower absorbance readings and was incomplete even after 2 h. By raising the temperature to 90-95 °C hydrolysis in methanol was complete in 1 h, with no subsequent further increase in absorbance.

Approximately 10 mg quantities of plant extracts of material harvested from various sites in the Johannesburg area were heated with 0.3 cm^3 conc. HCl and 4 cm^3 methanol in sealed vials at about 95 °C for 1 h. The absorbance maximum at 540 nm was then recorded using a Cary UV-visible spectrophotometer and results were reproducible within 2%. For 15 different extracts of plants collected from various sites and in different seasons in the Johannesburg area the absorbance per gram of extract varied from 918 to 40. Examples are shown below.

Table 5.2 Proanthocyanidin colour test for different samples collected in the Johannesburg area.

Plant part	Sample	Season	Abs./g	Sample	Season	Abs./g	Sample	Season	Abs./g
Roots	-	-	-	C	spring	752	G	summer	247
Branches	M	autumn	154	C	spring	805	G	summer	626
Leaves	M	autumn	40	-	-	-	G	summer	179

The same procedure was also used directly on finely milled root samples, except that 50 mg quantities of roots were used. Roots were harvested in January and July 2000 in the Umbumbulu area Kwa-Zulu Natal. After hydrolysis the samples were cooled, filtered, the volume adjusted to 5 cm^3, and the absorbance measured as shown below.

Table 5.3 Colour development using milled roots directly

Season	λ_{max}	Absorbance per g dry roots
Summer (January)	530 nm	73.5
Winter (July)	532 nm	37.1

Discussion

Complex oligomeric proanthocyanidins (OPC's) have for many years presented a formidable analytical challenge. Recently, after three years of investigation, the Grape

Seeds Methods Evaluation Committee, which falls under the auspices of the National Nutritional Foods Association, USA, proposed certain methods for the quantification of OPC's in a White Paper in 2001 (http://www.activin.com). The two recommended methods for estimating the relative amounts of oligomers are the Porter and Procyanidolic methods. These involve a colorimetric measurement after HCl/butanol or HCl isopropanol hydrolysis of proanthocyanidin dimers or larger polymers, which generates the intensely coloured cherry red anthocyanidin chromophore. In addition HPLC was recommended in the same White Paper in 2001 for identifying proanthocyanidins, and is discussed in Section 5.2.

The method developed during this research was similar to those recommended above, and involved heating the extract with methanolic hydrochloric acid. It provided a rapid and reliable means of estimating the relative proanthocyanidin content of extracts, or even the finely milled root samples themselves. Hydrolysis occurred more rapidly in methanol than in ethanol. Porter and co-workers have also noted that the thiolysis reaction, used for cleavage of complex procyanidins, proceeded more readily in methanol than in ethanol as solvent (Porter *et al.*1991).

The colour absorbance after hydrolysis, measured at 540 nm, varies tremendously and is influenced by factors such as the region of sample collection, season of collection and the plant part extracted. For 15 different extracts form plants collected in the Johannesburg area the absorbance per gram of extract varied from 918 to 40. Results agreed within 2% for duplicate analyses. For comparable samples the richest sources of proanthocyanidins were roots or branches, with leaves always being a poor source. This fact suggests that branches could be an alternative plant part to roots for healers, thus avoiding sacrificing the whole plant when harvesting for medicinal purposes. Many medicinal plant species in Africa are threatened with extinction due to over-harvesting, so this option should be explored further.

The absorbance measured after developing the red colour using milled roots collected in Kwa-Zulu Natal in summer (January) was 73.5 and that for roots harvested in winter (July) from the same area was only 37.1. Absorbance readings are expressed per gram of dry roots. Uterine activity is greatest for extracts of plants collected in the summer and autumn seasons indicating that the concentration of uteroactive chemicals is highest in these warmer seasons (Katsoulis *et al.* 2002). These results infer that proanthocyanidins are possibly uteroactive, and this was in fact confirmed by *in vitro* tests on the

proanthocyanidin Fraction A (Section 6.3). There is also great variation in uterine activity depending on the geographical area of harvesting plants (Katsoulis 1999).

5.2 Separation of proanthocyanidin Fraction A containing 19-30

Appendix: MALDI-TOF MS, UV, HPLC (1), HPLC (2) Alpha Labs. All spectra for methanol extract.

Physical data

Column chromatography on silica gel of 66g of a methanol extract of roots harvested in July 1995 (winter) from Treasure Beach Grasslands yielded a major component Fr. A, eluted with methanol, and further purified by dissolving in methanol and precipitating with ether to give 21 g (1.21% based on the dry root mass) of a reddish-brown powder after drying. Fraction A gave a positive phenolic test with $FeCl_3$, and produced a single fairly long reddish-brown spot R_f 0.73 with anisaldehyde reagent on TLC in 9:1 methanol : acetic acid. Red wine, known for its proanthocyanidin content, gave a similar spot R_f 0.70 on TLC IR (KBr disc) for Fr. A showed a large OH absorption at 3000-3700 cm^{-1} and C=C stretch at 1530 and 1615 cm^{-1}, but showed no carbonyl absorption. A 0.01% solution of the freeze-dried methanol extract in methanol acidified with HCl gave a UV absorption maximum at 280 nm which is characteristic of proanthocyanidins. Fraction A showed a single peak at 3.53 min. on HPLC at a flow-rate of 0.5 cm^3/min. in 60:40 acetonitrile : water using a UV detector at 254 nm.

Sugars with a mass of 21 g, were eluted from the column with chloroform : methanol 1 : 1, immediately before Fraction A. This sugar fraction was recrystallised from methanol/acetone, gave a negative $FeCl_3$ test, and showed a green spot with anisaldehyde reagent having R_f 0.38 on TLC in chloroform-methanol 1:1. HPLC analysis of this fraction showed one major peak at 13.8 min. using a Carbohydrate Analysis column, flow rate 0.5 cm^3/min. in acetonitrile:water 80:20. It was identified as glucose, R_t 13.6 min. by comparison to an authentic standard.

A sample of 1.2 mg of the freeze-dried methanol extract was tested for the presence of ellagitannins (Bate-Smith 1972) by dissolving in 2 cm^3 50% aqueous methanol and adding 0.16 cm^3 6% acetic acid and 0.16 cm^3 6% sodium nitrite solution in a cuvette. Nitrogen was bubbled through the cuvette and it was sealed. No red colour developed within 1 h and no absorbance maximum was detected in the 600 nm region.

Acetylation of Fraction A

Silica gel column chromatography was unsuccessful for further purification of Fraction A, and also caused degradation/oxidation of the material. Because of the instability and difficulty in separating the proanthocyanidin fraction, acetylation and methylation were investigated as a means of stabilizing this fraction.

Three methods of acetylation were investigated. Firstly an amount of 2 g of the dried methanol extract was acetylated with acetic anhydride/pyridine for 5 h at 90 °C and precipitated in ice/water to yield 1.9 g of acetylated material. Two further methods involved refluxing in THF/DABCO/acetic anhydride, or alternatively standing overnight at room temperature in DMF/DABCO/acetic anhydride. The latter method was found to be the best system for complete acetylation without degeneration of the product. Ice/water precipitation of the acetate was best achieved by rapid magnetic stirring while adding the acetylated product. Typical quantities for acetylation were 1 g freeze-dried extract, 40 cm^3 dry DMF, 1 g DABCO and 30 cm^3 Ac_2O. The acetate was dissolved in $CHCl_3$ and precipitated by the addition of butanol to produce a cream powder m.p. approx. 280 °C (charring). However if ether was present in solvents for recrystallisation this caused degeneration of the acetate. The acetate gave a single beige spot R_f = 0.53 on TLC in CH_2Cl_2/ethyl acetate 1:1.

A proton NMR spectrum showed the acetate to be a complex mixture with broad peaks, so attempts were made to purify it via silica gel columns using CH_2Cl_2 or $CHCl_3$ for elution, and also via semi-preparative HPLC in 1:1 AN-MeOH or AN-MeOH 60:40. Silica gel caused degeneration of the acetate. Semi-preparative HPLC showed four major acetate fractions in mobile phase 1:1 acetonitrile:methanol at a flow of 2 cm^3/min. Yields of the four compounds were small and NMR spectra were poorly resolved in the aromatic area. Several further unsuccessful attempts were made to purify the unstable acetate mixture by HPLC but this route was then abandoned.

Methylation of Fraction A

Several methods were investigated for methylation. Refluxing in dry acetone/MeOH/K_2CO_3/CH_3I for 1 h caused degeneration and was unsuitable. The best

method (Bindra *et al.*1988; Cuicano and Kerek 1984) involved per-methylation at room temperature. The following were typical reaction quantities:

Methanol extract	0.08 g
DMSO dry	10 cm^3
NaH	0.24 g
CH_3I	2 cm^3

NaH (stored under oil) was washed with hexane and dried in a N_2 stream prior to weighing. The extract and the NaH were each dissolved in 5 cm^3 DMSO then the two solutions were combined and left at room temperature for 10 minutes. After cooling in ice to control effervescence, the CH_3I was added and the flask flushed with N_2 and stirred at room temperature for 1 h. A sample of glucose was per-methylated under the same conditions as a control. Ice-water precipitation of the product gave a better result than chloroform extraction. The product was re-crystallised from ethyl acetate/hexane to yield a cream-beige powder which gave a grey-brown spot with anisaldehyde reagent R_f 0.65 in 10:1 $CHCl_3$:methanol, and gave a negative $FeCl_3$ test showing that methylation was complete. Glucose also showed one major spot R_f 0.85, and no unmethylated material. Attempts to further purify the Fraction A methylated material via semi-preparative HPLC or silica gel chromatography were unsuccessful however, as for the acetate.

It was noted that paper chromatography of the acetylated and methylated products gave better spots with less streaking than TLC plates. Possibly a cellulose column or thick paper chromatography could be a better way to purify these compounds because they are sensitive to various conditions and can be unstable on silica gel, standing long periods in air, standing in solution, heat, ether, THF, *etc.*

Amberlite and Sephadex chromatography

Seeing that silica gel degraded Fraction A Amberlite XAD7 (BDH) and Sephadex LH-20 (Fluka) were therefore investigated for purification of the proanthocyanidin fraction. Three roots, total mass 2665 g, moisture content 36.45%, harvested in January 2000 (mid-summer) in the Umbumbula district were shredded and extracted with 3.5 litres of chloroform at room temperature overnight then filtered. The roots were subsequently extracted with 3 litres of methanol which was boiled for a few minutes then left at room temperature for two nights before filtering and evaporating the solvents under vacuum below 50 °C. This yielded 0.97 g of brown, oily solid from the chloroform extract and 58.76 g of shiny reddish-brown flakes from the methanol extract after freeze-drying. The

phenolic compounds extracted by methanol are also water-soluble, but methanol is a more efficient solvent for their extraction from plant material.

Initially a column separation using 60 g Sephadex LH-20 for 2 g of the methanol extract was attempted using ethanol, ethanol:water 9:1, methanol, and finally acetone:water 4:1 for elution. A major portion of the methanol extract (52%) could only be eluted with acetone:water 4:1, indicating that polymeric material was present. Alpha Laboratories California estimated this oligomeric proanthocyanidin content for the same methanol extract to be 55%, confirming the high polymeric content. Several Sephadex columns were run but separations were unsatisfactory. Separations were poor, and major compounds in the extract, with approximately 6 min. and 10 min. retention times, were present in many different fractions.

Amberlite XAD7 was effective in giving a crude preliminary separation of 0.81g of freeze-dried methanol extract, and the following components were eluted:

A Sugars and salts (26%) with 0.1% aqueous HCl.

B Proanthocyanidin material (20%) with 40:60 or 60:40 methanol:water containing 0.1% HCl.

C Polymeric material (54%) with methanol containing 0.1 % HCl.

Excluding sugars and salts, proanthocyanidin material constitutes 74% of the methanol extract, based on results from the Amberlite XAD7 separation.

Column Chromatography using Sephadex LH-20 was only partially successful in further purifying this mixture. The proanthocyanidin component (B above, 0.129 g) was subjected to Sephadex chromatography and fraction 8, eluted with ethanol:water 1:1, yielded 4.2 mg of a fairly pure component on the basis of HPLC, and could therefore be readily identified by TLC and HPLC.

Identification of Compounds **19-30**:

Several HPLC systems were investigated for following the progress of column chromatography seeing that neither the 60:40 acetonitrile:water system previously used or the 80:20 acetonitrile: aqueous 0.01M H_3PO_4 system, useful for separating ellagic acid

type compounds, was successful for resolving the components in the methanol extract. Finally the isocratic system of 1:4 methanol :1% aqueous acetic acid (Porter *et al* 1991) at 1 cm^3/min. was found to give the best separations for proanthcyanidins, using a Hichrom reversed phase C-18 column and a 254 nm fixed wavelength detector. Fr.8, was identified as (+)-catechin by co-elution with a pure standard in three different mobile phases with decreasing water content, and correspondingly shorter retention times (Table 5.4). Fr. 8 solution was also spiked by adding a few crystals of (+)-catechin standard which gave a single larger peak at the same retention time. In addition, on TLC in 6:3:1 toluene-acetone-formic acid, both Fr. 8 and (+)-catechin gave an orange-red spot with anisaldehyde reagent having R_f 0.73.

Table 5.4 Retention times for HPLC of Fr.8 in three mobile phases.

Component	Retention time (minutes)		
	Mobile phase 1	Mobile phase 2	Mobile phase 3
(+)-catechin	16.691	10.038	5.758
Fraction 8	16.758	9.920	5.885

Mobile phase 1: 1:4 methanol:1% aqueous acetic acid
Mobile phase 2: 1:3.5 methanol:1% aqueous acetic acid
Mobile phase 3: 1:3 methanol:1% aqueous acetic acid

The Porter system showed approximately 25 components in Fraction A. Ten compounds, common in Vitaceae, were identified in the methanol extract by co-elution with the relevant standard on HPLC in the Porter mobile phase (Table 5.5). Agreement between the retention time for the unknown compound and the standard was generally within 0.1min. Several runs were carried out in order to match a particular unknown compound with a standard. The concentration of standards was approximately 1 mg/cm^3. The percentage for each compound in Table 2.3 was determined by comparison of the peak area count of the authentic standard, of known concentration, to that of the corresponding peak in Fraction A.

Table 5.5 HPLC of proanthocyanidins in methanol extract. Mobile phase: 1:4 methanol : 1% aqueous acetic acid, flow 1 cm^3/minute.

Compound	R_t min.	Quantity %	Identity
19	7.0	1.04	(-)-epigallocatechin
20	7.4	0.54	(+)-gallocatechin
21	10.3	0.61	Procyanidin B3
22	15.3	0.88	Procyanidin B4
23	16.8	2.03	(+)-catechin hydrate
24	18.5	1.79	(+)-mollisacacidin
25	22.5	0.88	(+)-epicatechin
26	33.8	0.10	fisetinidol-(4α-8) catechin
27	35.6	0.33	(-)-fisetinidol
28	50.9	0.24	fisetinidol-(4β-8)catechin

Compounds with retention times longer than 20 minutes were also run with less water in the system to give shorter, more reproducible retention times and are reported in Table 5.6. The mobile phase was 1:3 methanol:1% aqueous acetic acid. Three peaks were obtained for the epicatechin standard in both mobile phases, but only the longest retained peak, assumed to be a hydrate, is reported in Table 5.5, whereas all three are shown in Table 5.6 below.

Table 5.6 HPLC of proanthocyanidins in methanol extract. Mobile phase: 1:3 methanol : 1% aqueous acetic acid, flow 1 cm^3/minute.

Component	R_t standard (min.)	R_t for compound in methanol extract (min.)
(+)-epicatechin	3.9 7.0 13.3	3.8 6.9 13.4
fisetinidol-(4α-8) catechin	8.1	7.9
fisetinidol-(4β-8)catechin	9.9	9.8
(-)-fisetinidol	11.1	11.0

In addition HPLC analysis at 280 nm by Alpha Laboratories, California showed four common monomers: catechin (0.85%), epicatechin (0.17%), gallic acid (0.20 %) **29** and epicatechin-3-O-gallate (0.08%) **30**. The latter two standards were not available for my analyses. The former two results confirmed my findings, although percentages found by Alpha Laboratories were slightly lower.

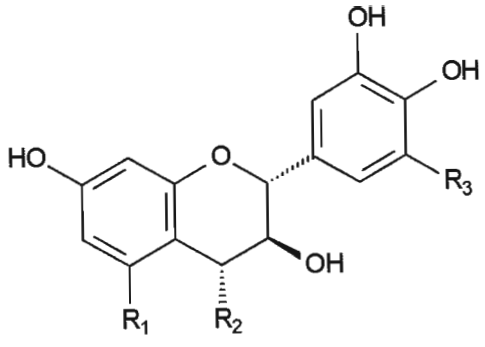
A MALDI-TOF mass spectrum of the methanol extract also showed several molecular ions in the m/z range greater than 1000. These were at m/z 1075.39, 1149.52, 1193.49 and 1345.45.

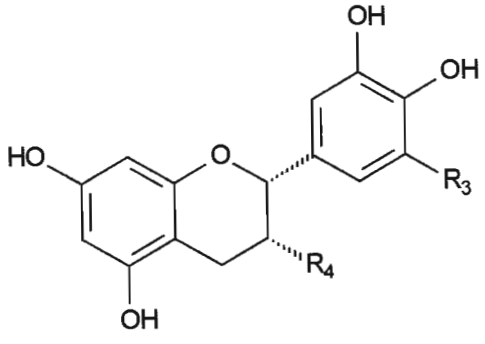
Discussion

A partially successful separation of the freeze-dried methanol extract was achieved by prior treatment with Amberlite XAD7, used to purify anthocyanins (Yoshitama et al 1992), followed by Sephadex LH-20 chromatography. Amberlite XAD7 removed sucrose and glucose and gave a rough separation of monomers and larger polymeric molecules. This was followed by HPLC identification of proanthocyanidins with the aid of authentic standards. Optical rotations were not run on any of these compounds because they could not be isolated in a pure state. The (+) or (-) stereochemical designations indicated in Fig. 5.1 therefore correspond to those of the standards, which are common in *Vitaceae* family.

HPLC is a recommended method for estimation of the relative amounts of monomers, oligomers and polymers (http://www.activin.com, Porter *et al* 1991, Darne and Glories 1988, da Silva *et al.* 1991, Goldberg *et al.* 1996, Yoshitama *et al.* 1992) and was found useful for identifying 11 proanthocyanidin monomers and dimers in Fraction A as well as gallic acid. Results for HPLC of components in the methanol extract of *Rhoicissus tridentata* are shown in Table 5.5. Catechin was found in the highest amount, comprising 2% of the methanol extract. It is assumed that the longest retained peak for epicatechin corresponds to a hydrate by analogy with the catechin hydrate standard which has a long retention time. The peak with the shortest retention time is presumably the anthocyanidin cation. Interestingly the three peaks observed for epicatechin are in different proportions for the standard and the methanol extract. For the standard the longest retained peak was in the highest amount, while for the methanol extract the peak with the shortest retention time was present in the greatest amount. It was also noted that when changing the mobile phase by altering the water content, the order of elution of components sometimes also changed.

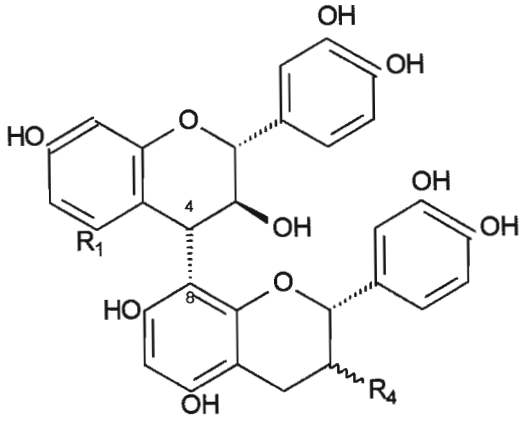
Structures of compounds identified in Fraction A are shown in Figure 5.1.

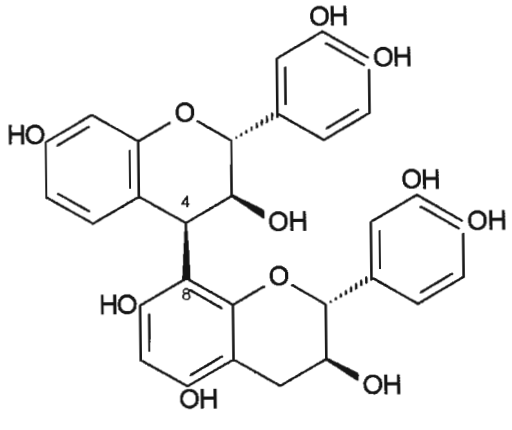




	R_1	R_2	R_3
catechin	OH	H	H
gallocatechin	OH	H	OH
fisetinidol	H	H	H
mollisacacidin	H	OH	H

	R_3	R_4
epicatechin	H	OH
epigallocatechin	OH	OH
epicatechin-3-O-gallate	H	O-gallate





	R_1	R_4		
catechin (4α-8)catechin (procyanidin B3)	OH	β-OH	fisetinidol	(4β-8)catechin
catechin (4α-8)epicatechin (procyanidin B4)	OH	α-OH		
fisetinidol (4α-8)catechin	H	β-OH		

FIGURE 5.1: Proanthocyanidin monomers and dimers in Fraction A

Proanthocyanidins are amongst the most powerful antioxidants in nature and have well documented health benefits for the heart, cardiovascular system and immune system. These findings support the traditional healers' claims that "isinwazi" promotes good health during pregnancy. The polymeric proanthocyanidins, or condensed tannins, also produce astringent effects (Czochanska et al 1980) which can account for their stimulatory action on uterine tissue. Polymeric proanthocyanidins can be distinguished from hydrolysable tannins, which contain gallate or hexahydroxybiphenyl ester moieties. The latter exhibit a carbonyl absorption in their infrared spectra which is absent for the former compounds. The absence of a carbonyl absorption in the IR spectrum of the uteroactive Fraction A eliminates the possibility of hydrolysable tannins being present in significant amounts. In addition a colorimetric test for ellagitannins (Bate-Smith1972) was negative. This led to the conclusion that the proanthocyanidins are major spasmogens in this plant species, as shown via uterine tests in Chapter 6.

This polymeric or tannin material represents the major portion, 52%, of the methanol extract and was eluted from Sephadex LH-20 with acetone-water 4:1 (Czochanska et al 1980). In addition Amberlite XAD7 resin was used to separate sugars and salts, monomers and polymeric material (54%) with methanol containing 0.1 % HCl. Alpha Laboratories, California also found a similar oligomeric proanthocyanidin content of 55% for a sample of the same material. Excluding sugars and salts, proanthocyanidin polymers therefore constitute 74 % of the proanthocyanidin fraction, based on the Amberlite XAD7 separation.

Acetylation and methylation of the methanol extract

Several methods of acetylating or methylating the freeze-dried methanol extract were investigated because of the instability and difficulty in separating this proanthocyanidin fraction. The best method for acetylation involved standing the extract overnight at room temperature in DMF/DABCO/acetic anhydride, and TLC showed that the reaction was complete. However the ^{1}H NMR spectrum showed that the acetate to be impure and silica gel column chromatography as well as semi-preparative HPLC were unsuccessful for obtaining pure acetylated compounds from the mixture. The acetate mixture also degenerated readily under a variety of conditions.

Per-methylation (Cuicano and Kerek 1984) of the extract was successful, but again further purification by silica gel column chromatography as well as semi-preparative

HPLC was not, and the product mixture tended to be unstable. Further attempts to purify the mixture via acetatylated or methylated derivatives were abandoned.

A MALDI-TOF (Matrix Assisted Laser Desorption/Ionisation Time of Flight) mass spectrum was run on the methanol extract, and this revealed several molecular ions in the m/z range over 1000. The MALDI is a gentle procedure mainly used for large biomolecules. The sample is dispersed in a matrix (2,5-dihydroxybenzoic acid was used for the methanol extract), then desorbed from a metal surface and ionized with a laser beam. The high-boiling viscous solvent matrix minimizes the amount of vibrational excitation, resulting in less destruction of the ionized molecules (Silverstein and Webster 1998). Fast atom bombardment (FAB) mass spectrometry is based on the same principle, except that a beam of zenon or argon is used for ionization. FAB is also a "soft" technique which usually enables the molecular ion to be preserved.

The molecular ions detected at m/z 1075.39, 1149.52, 1193.49 and 1345.45 were in the tetramer range for procyanidins. An example is epicatechin-(4β→8)$_3$-epicatechin which showed $[M-H]^-$ at 1153 (Porter *et al* 1991).

5.3 Compound 31 from Hydrogel Fraction B

Appendix: IR for **31**, IR for acetate after hydrolysis of **31**.

Physical data

Fraction B was obtained by water extraction of roots which had been previously extracted with methylene chloride and methanol. All extractions were carried out at room temperature. Roots, 1109 g, harvested October 1996 in Umlazi, were extracted for 3 days with 9 litres CH_2Cl_2 to produce 2.9 g of dry extract after solvent removal. Further extraction with 6 litres CH_3OH for 4 days produced 14.9 g of dry extract. Finally water extraction for 3 days yielded 133 g of Fraction B, a brown syrup after evaporation of water. Stirring of this syrup with methanol or acetone produced 14.4 g of an almost white gummy precipitate as compound **31**, which shrank remarkably while drying to yield 3.2 g (0.45% of the dry root mass) of dry white solid material m.p. 260 °C (charring/decomp.). Inorganic salts, mainly KCl were identified in methanol or acetone solutions after separation of compound **31**.

IR (KBr disc): 3400 (br. Large OH), 2940, 1740, 1640, 1100 cm^{-1}

Solutions of this material were viscous and jelly-like, and tended to block Millipore filters. Compound **31** gave a positive Molisch colour test for sugars, and negative colour tests for phenols and sterols. It remained on the origin on TLC in methanol-acetic acid 10 : 1 or 5:1, giving a pale green spot with anisaldehyde reagent, whereas sucrose gave a green spot R_f 0.64 in methanol- acetic acid 10 : 1. It also remained on the origin on descending paper chromatography in butanol : acetic acid : water 4 : 1 : 5, producing a pale brown spot with aniline phthalate spray, whereas sucrose produced a brown spot, R_f 0.21. Addition of an iodine solution to a solution of Fraction B gave no colour change whereas a 1% starch solution gave an immediate blue colour on addition of iodine. A test with ammoniacal $AgNO_3$ on **31** was negative whereas glucose gave a positive silver mirror showing the presence of a free reducing aldehyde group.

Hydrolysis of 1 g of Fraction B in 50 cm^3 of 2M HCl for 3 h at 90 °C yielded only glucose, which was identified by HPLC, TLC and also by acetylation. The hydrolysis solution showed only one product by HPLC analysis on a Carbohydrate Analysis column with R_t = 13.62 min. which was identified as glucose via an authentic standard having R_t = 13.69 min. in 80:20 acetonitrile:water, 0.5 cm^3/min. TLC of the hydrolysis solution in methanol:acetic acid 5:1 produced one green spot with anisaldehyde reagent R_f = 0.73, identical in colour and R_f with that of glucose.

Solvents were evaporated completely and the solid obtained was recrystallisated from water/acetone to give a white solid which was filtered off and a pale beige gum, 0.29 g, after evaporating the remaining solution. The dried white solid hydrolysis product was acetylated with pyridine/acetic anhydride and recrystallised from methanol to yield white crystals, m.p. 118-120 °C, identified as α-D-penta-O-acetylglucopyranose m.p. 119-120 °C (Heilbron 1969). TLC in hexane-ether 1:5 showed a brown-green spot, R_f 0.56, identical to that of glucose penta-acetate.

IR of **31** acetate (KBr disc): 2930, 1740 (strong), 1360, 1215 (strong) and 1030 cm^{-1}. The major absorptions corresponded well to those of a β-D-penta-O-acetylglucopyranose standard.

Discussion

Fraction B, a hydrogel polymer of glucose, stimulated uterine contractions over the full

range of acetylcholine concentrations (Section 6.4.3). Although the structure is not fully elucidated, it is different to the amylose starch polymer which has α-1, 4-glucoside linkages. Various hydrogels, such as the natural gels lining the intestine and uterus, play a direct role in muscle contraction. Cervical mucous, for example, is a complex hydrogel which is important in sperm transport. (www.das.psu.edu). A common property of all gels is the ability to undergo abrupt changes in volume in response to small changes in external conditions, *e.g.* chemical or electrical changes. These can cause the gel to swell or shrink as much as 1000 times. The rates of contraction and consequent forces generated by certain synthetic gel fibres approximate those of human muscle (www.ai.mit.edu). These contractile materials are therefore the subject of investigation with regard to their potential as "artificial muscle" in robotic devices.

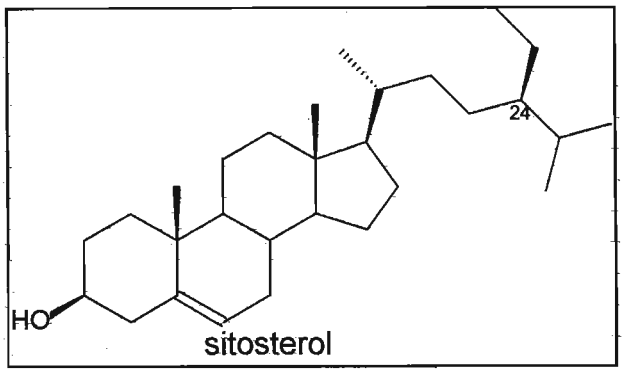
Addition of acetyl choline to uterine tissue in the presence of hydrogel B is probably the chemical stimulus leading to gel contraction and enhancement of uterine contractility. However a polymer such as Fraction B is unlikely to be absorbed through the intestinal wall, so should not reach the uterus when administered orally in medications. A water-soluble glucoside from *Cissus quadrangularis*, also from the Vitaceae family, is reported as non-toxic when administered orally to mice, rats and guinea-pigs. However it caused convulsions and death within 5 minutes of intravenous administration. (Hutchings 1996). Possibly this glucoside is similar to the polymeric Fraction B, and is hydrolysed to some extent during digestion, whereas it might over-stimulate smooth muscle tissue when entering the blood-stream directly. Fraction B warrants further investigation.

5.4 Non-polar sterol, triterpenoid and fatty alcohol fraction

Four compounds with structural similarities to the oestrogens were identified in *Rhoicissus tridentata* extracts. These were **32** sitosterol, **33** sitosterolin (sitosterol glucoside), **34** γ-sitosterol, and **35** oleanolic acid, discussed in this section. In addition to these, two further triterpenoids were identified via a GC-MS analysis of the chloroform extract, but were not isolated from the extract in a pure state.

Compound 32 (sitosterol)

Appendix: IR, ^{1}H and ^{13}C NMR spectra were identical to those of compound **1**, *C. kraussii*, and are not presented for that reason.



sitosterol

Compound **32** $C_{29}H_{50}O$, 0.15 g (0.009% of the dry root mass) was eluted with hexane : ether 1:1 from the same silica gel column used to isolate the proanthocyanidin Fraction A. It was recrystallised from hot methanol yielding a white powder, m.p. 143-144 °C (Merck Index 136-141 °C for sitosterol). It gave a positive Liebermann-Burchard test, producing a dark violet colour which changed to green after 30 minutes. It gave a dark violet spot with anisaldehyde reagent on TLC in hexane : ether 2:1, R_f 0.35, identical with that of an authentic sitosterol standard.

^1H and ^{13}C NMR ($CDCl_3$) : These were identical to those of compound **1** isolated from *Combretum kraussii*, and an authentic sitosterol standard.

IR (KBr disc) : 3400, 2940, 2880, 1650, 1460, 1380 and 1060 cm-1

Acetylation of 0.10g of **32** carried out by heating for 1 h at 60 °C in 1.5 cm^3 pyridine and 1.5 cm^3 acetic anhydride. The reaction mixture poured into iced water yielding crystals which, after washing well with water and drying produced 0.09 g of the mono-acetate, m.p. 118-120 °C, R_f 0.72 (dark violet spot) in hexane-ether 10 :1.

IR (KBr disc) : 2920, 2850, 1730, 1465, 1375, 1260 and 1040 cm^{-1}.

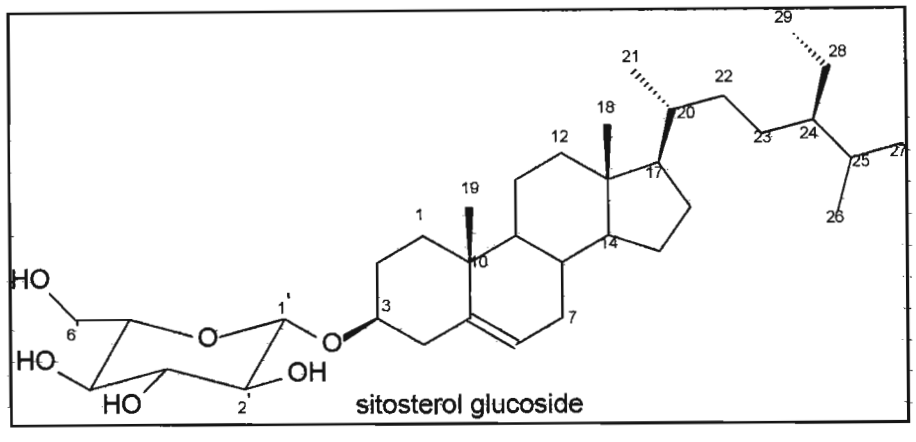
Discussion

Compound **32** was eluted with hexane-ether 1:1 from the column used to purify Fraction A, and it gave a positive Liebermann-Burchard test for a sterol. The m.p., TLC, R_f, IR and ^{13}C NMR data confirmed the identity of **32** as sitosterol (24R-stigmast-5-en-3-β-ol) by comparison with an authentic standard.

The acetate of compound **32** m.p. 118-120 °C was also identical to literature data (Chattergee *et al.* 1977). It was also identical by m.p., IR and R_f to the acetate of Compound **1** isolated from *Combretum kraussii,* and also identified as sitosterol acetate.

Compound 33 (sitosterol glucoside or sitosterolin, sitosterol-3-O-β-D-glucopyranoside)

Appendix: NC-MS, IR, ^1H and ^{13}C NMR for **33**.
IR and ^1H NMR for acetate of **33**.



sitosterol glucoside

Physical data

Compound **33** was obtained from 15 g of a chloroform extract of shredded roots and branches of *Rhoicissus tridentata* harvested from Treasure Beach in July 1995. It was eluted from a silica gel column with 5:1 chloroform:methanol to yield 0.4 g (0.013% based on the dry root mass) of solid material. Recrystallisation from chloroform/hexane yielded colourless plates, 0.16 g, m.p. 306-310 °C (decomp.) which compared fairly well to that of Matida *et al* who found m.p. 302-305 °C for sitosterol glucoside (Matida *et al.*1996). Compound **33** gave a positive Liebermann-Burchard test, producing a deep violet colour which also changed to green slowly, as with sitosterol itself. It gave a deep violet spot, R_f 0.54, with anisaldehyde reagent on TLC in chloroform : methanol 7:1 which had identical R_f to that of an authentic standard of sitosterol glucoside, $C_{35}H_{60}O_6$.

EIMS showed a mass fragment at 414.3844 $[M - C_6H_{10}O_5]^+$ (414.3861= $C_{29}H_{50}O$ for sitosterol). This corresponds to loss of the glucose moeity during fragmentation. The molecular ion at 576 for $C_{35}H_{60}O_6$ was absent.

IR (Kbr disc) : 3200, 2740, 2670, 1620, 1500, 1360 and 904 cm^{-1}

The signals for the sugar protons (asterisk) and certain characteristic sitosterol protons in the ^1H NMR spectrum of **33** are shown below (C_5D_5N):

δ 5.20 1H, d, J = 4.8 Hz	H-6
δ 4.92 1H, d, J = 7.7 Hz	H-1'
δ 4.43 1H, dd, J_1 = 11.8 Hz, J_2 = 2.4 Hz	H-6'
δ 4.27 1H, dd, J_1 = 11.8 Hz, J_2 = 5.1 Hz	H-6'
δ 4.15 2H, m	H-3' and H-4'
δ 3.92 1H, m	H-5'
δ 3.85 2H, m	H-2' and $H\text{-}3_{ax}$
δ 2.59 1H, dd, J_1 = 13.4 Hz, J_2 = 2.7 Hz	$H\text{-}4_{eq}$
δ 2.33 1H, dd (appears as t)	$H\text{-}4_{ax}$
δ 0.79 3H, s	H-19
δ 0.51 3H, s	H-18

The tetra-acetate of **33** had a sharp m.p. at 168-170 °C which compared well to literature data for sitosterol glucoside tetra-acetate *i.e.* 165-167 °C (Matida *et al* 1996) and 169-171 °C (Carvalho and Seita 1993).

IR (KBr disc) : 2930, 2850, 1740, 1360, 1220, 1040 cm^{-1}

The acetate showed following characteristic signals in the ^1H NMR spectrum :

δ 5.34, 1H, d, J=4.9 Hz	H-6
δ 5.18, 1H, t, J = 9.5 Hz	H-3'
δ 5.05, 1H, t, J = 9.6 Hz	H-4'
δ 4.93, 1H, t, J = 8.8 Hz,	H-2'
δ 4.57, 1H, d, J = 8.1 Hz	H-1'
δ 4.24, 1H, dd Hz, J_1 = 12.2 Hz J_2 = 4.9	H-6'
δ 4.09, 1H, dd, Hz, J_1 = 12.2 Hz J_2 = 2.4	H-6'
δ 3.66, 1H, m	H-5'
δ 3.47, 1H, m, J = 2.6 Hz	H-3α
δ 1.98, δ2.00, δ2.03 and δ2.05	$4xCOCH_3$

δ 0.97 3H, s	H-19 CH_3
δ 0.78-δ0.91 9H	4xCH_3
δ 0.65, 3H, s	H-18 CH_3

Discussion

This EIMS fragment at 414.3844 $[M - C_6H_{10}O_5]^+$ corresponds to loss of the glucose moeity during fragmentation to yield the sitosterol residue ($C_{29}H_{50}O$ for sitosterol requires 414.3861). The molecular ion at 576 for $C_{35}H_{60}O_6$ was absent, as also found by Negrute and co-workers (Negrute *et al.*1989) and other researchers for the mass spectrum of sitosterolin, indicating and easy fragmentation of the glucoside.

The ^1H NMR spectrum for sitosterolin exhibited the characteristic doublet for the axial hydrogen on the anomeric carbon for a β-glucoside linkage at δ 4.92, J= 7.7 Hz. The structure of **33** as sitosterolin were assigned by comparison of spectral and other data to literature data and those of an authentic standard. The ^1H NMR (C_5D_5N) and ^{13}C ($CDCl_3$) spectra matched those of the standard and those of Negrute *et al.* There is some disagreement in the literature regarding the assignment of chemical shifts for sugar protons of sitosterol glucoside. There is however evidence of strong geminal coupling for the two H-6' protons at δ 4.43 and δ 4.27, J = 11.8 Hz, supporting the allocations given here for **33**. These agree well with those of Negrute *et al* , but not with those of Matida *et al* who place the H-6' protons further upfield. Geminal coupling, for the two sterol H-4 protons also shows a double doublet for the equatorial and axial protons. The upfield resonance is poorly resolved however and appears as a triplet with J_2 unrecorded.

Table 5.7 ^{13}C Chemical shifts for **33** and Sitosterolin (Negrute *et al.*1989) in $CDCl_3$ in ppm

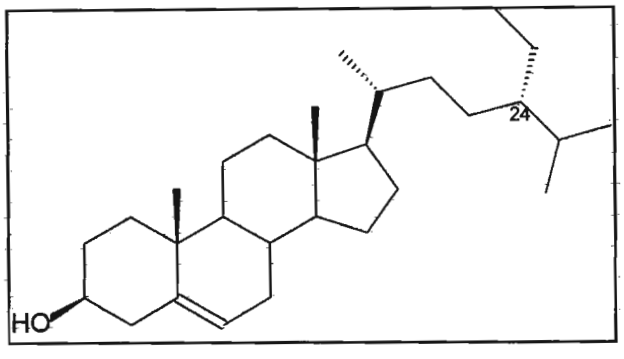
Carbon	**33**	Sitosterolin
1	37.3	37.5
2	30.1	30.2
3	78.3	78.5
4	39.2	39.3
5	140.7	140.9
6	121.7	121.9
7	32.0	32.2
8	31.9	32.0
9	50.2	50.3
10	36.7	36.9
11	21.1	21.3
12	39.8	39.9
13	42.3	42.5
14	56.7	56.8
15	24.3	24.5
16	28.4	28.5
17	56.1	56.2
18	11.8	12.0
19	19.0	19.2
20	36.2	36.4
21	18.8	19.0
22	34.0	34.2
23	23.2	23.4
24	45.9	46.0
25	29.3	29.4
26	19.8	20.0
27	19.2	19.4
28	26.2	26.3
29	12.0	12.2
1'	102.4	102.5
2'	75.2	75.3
3'	78.4	78.6
4'	71.5	71.6
5'	77.9	78.1
6'	62.7	62.8

The ^1H NMR data for the acetate of **33** also correlate well with those of Matida and co-workers (Matida *et al.*1996) and Carvalho and Seita (1993). Results for the ^{13}C NMR of **33** are compared to those of sitosterolin (Negrute *et al.*1989) in Table 5.7 above. All chemical shifts in the ^1H NMR spectrum of **33** are approximately δ 0.1 upfield from literature data (Negrute *et al.*1989 and Matida *et al.*1996), which is most likely due to the setting of the instrument with regard to the TMS signal.

The structure of **33** was accordingly designated as sitosterol 3-O-β-D-glucoside via physical data and spectral evidence which was compared to literature data, and also by comparison to an authentic standard.

Compound 34 (γ-sitosterol)

Appendix : **34** IR ; Acetate **34** IR and GC-MS (NIST library match, 130000 spectra).



γ-sitosterol

Physical data

Compound **34** was purified from a silica gel column of the 1:1 $CHCl_3$:CH_3OH extract of branches of young plants from Treasure Beach Grasslands, July, 1995. It was eluted with $CHCl_3$:CH_3OH 3:1 to give 1.8 g of material which was purified by dissolving in ethyl acetate and to give a small quantity, 0.09 g (0.008% of the dry root mass) of a pale beige powder m.p. 100-108 °C. It was more polar that sitosterol, remaining on the origin on TLC in benzene-chloroform 1:5 whereas sitosterol had R_f 0.31 in this system. It produced a violet spot with anisaldehyde reagent R_f 0.45 in $CHCl_3$:CH_3OH 10:1 and a positive sterol test, giving a green colouration.

IR (KBr disc): 3420 broad, 2920, 2850, 1600, 1460, 1120 cm^{-1}.

EIMS: 452.1 (45%); 396.1 for $C_{29}H_{48}$ (16%); 57 (base peak).

Acetylation of 0.06 g **34** in 2 cm^3 pyridine/2 cm^3 acetic anhydride for 3 h on a steam bath then precipitation in ice/water gave 0.06 g of acetate when dried. This was further purified by boiling with charcoal in chloroform/acetone solution to give 0.04 g of white solid after solvent removal, m.p. 134-138 °C, with R_f 0.33 (violet spot with anisaldehyde reagent) on TLC in hexane-ether 1:1.

IR (KBr disc): 2940, 2870, 1760, 1470, 1380, 1240, and 1060 cm^{-1}

High temp. EIMS: 414; 396.4 (base peak) for $C_{29}H_{48}$
Low temp. EIMS: 453; 453 452.4 (42%); 57.1 (base peak)

The acetate of **34** was identified as γ-sitosterol by GC-MS analysis of the peak at 22.6 min. which showed a mass fragment at 414 for $C_{29}H_{50}O$ and was matched via the GC-MS NIST library of 130000 spectra to γ-sitosterol. The conditions for the GC were: Injector 250 °C, column 50 °C for 2 min., temperature ramp 20 °/min. until 300 °C which was held for 20 minutes. The GC-MS was run at the University of Kwa-Zulu Natal.

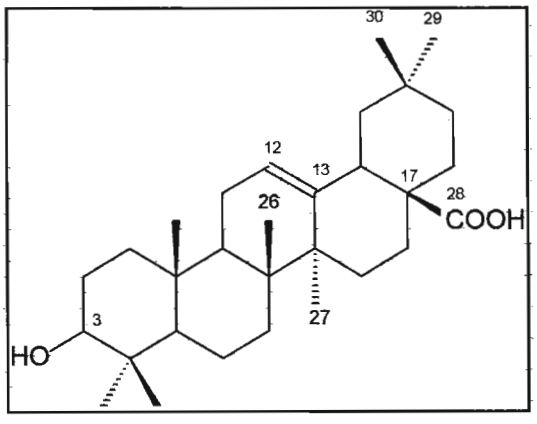
Discussion

Compound **34** was purified from a silica gel column of the 1:1 $CHCl_3$:CH_3OH extract of branches of young plants. The IR spectrum was similar to that of sitosterol, but there were slight differences on TLC. The high temperature EIMS for the acetate of 34 showed a base peak at 396.4 for $C_{29}H_{48}$ (sterol-H_2O) and a small peak at 414 for $C_{29}H_{50}O$, a sterol fragment. However the mono-acetate molecular ion at 456 was absent, probably due to easy loss of the acetate group. The mass spectra for 34 and its acetate were very similar, both having fragments at m/z 452.1 (45%) and 452.4 (42%) respectively. These could be due to M^+ - H - 39(K^+) for the sterol fragment in each case.

γ-sitosterol was identified by GC-MS. The only difference between sitosterol (24R-stigmast-5-en-3-β-ol) and γ-sitosterol (24S-stigmast-5-en-3-β-ol) is the orientation at the chiral C-24.

Compound 35 (3β-hydroxyolean-12-en-28-oic acid)

Appendix: **35** CI-MS, IR, ^{1}H NMR; Acetate **35** NC-MS, EI-MS, IR, ^{1}H and ^{13}C NMR.



3β-hydroxyolean-12-en-28-oic acid

Physical data

Compound **35** was obtained from 15 g of a chloroform extract of shredded roots and branches of *Rhoicissus tridentata* harvested from Treasure Beach in July 1995. It was eluted from a silica gel column with 20:1 chlororform/methanol and crystallised from the concentrated mother liquor by addition of hexane to yield approximately 100 mg (0.003% of the dry root mass) of colourless needles, m.p. 240-250 °C (decomp.). On TLC in hexane/ether 1:2 it produced a violet spot with anisaldehyde reagent, R_f 0.60, showing that **35** was slightly more polar than sitosterol, R_f 0.64. It gave a positive Liebermann-Burchard test for sterols, producing a dark violet colour. The IR spectrum showed a broad OH absorption at approximately 3440 cm^{-1}; 2940, 2860 cm^{-1} C-H stretch; 1690 cm^{-1} C=O. The mass spectrum (EI) showed the following fragments:

456.2 [M^+]	$C_{30}H_{48}O_3$
248.1 (248.1793)	$C_{16}H_{24}O_2$=248.1776
203.1	$C_{15}H_{23}O$ (248.1 – COOH)
189.1	$C_{14}H_{21}$

The ^1H NMR spectrum in $CDCl_3$ showed the following features for an olean-12-ene derivative:

δ 0.7-1.3	7xs, 7xCH_3
δ 2.80	dd, 1H, J_1=13.5 Hz; J_2=4.1 Hz, H-5α
δ 3.20	dd, 1H, J_1=10.5 Hz; J_2=4.8 Hz, H-3α
δ 5.26	t, J=3.4 Hz, H-12

Acetylation of 70 mg of **35** in 1.5 cm^3 pyridine and 1.5 cm^3 acetic anhydride for 1 h at 60

°C produced 60 mg of the acetate, precipitated by ice/water. After column purification, being eluted with carbon tetrachloride-methylene chloride 1:1, the acetate had m.p. 138-144 °C (decomp.), and gave a violet spot R_f 0.66 on TLC in hexane-ether 2:1. The IR spectrrum (KBr disc) showed no OH absorption, 2939, 2860 cm^{-1} C-H stretch;1730 cm^{-1} C=O; 1690 C=O, and 1240 cm^{-1} C-O. The ^1H NMR spectrum in $CDCl_3$ showed the following features:

δ 0.7-1.1	7xs, 7xCH_3
δ 2.80	dd, 1H, J_1=13.3 Hz; J_2=3.8 Hz, H-5α
δ 4.47	dd, 1H, J_1=7.93 Hz; J_2=7.94 Hz, H-3α
δ 5.25	t, poorly resolved,1H, H-12

Methylation of **35** was carried out using diazomethane generated from nitrosomethyl urea and base. The ^{13}C NMR spectrum in $CDCl_3$ showed the ester methyl signal at 51.6 ppm and the carboxylic acid carbon at 178.4 ppm.

Discussion

Compound **35** was identified as an olean-12-ene derivative on the basis of the ^{13}C chemical shifts for the alkene carbon atoms as well the mass spectral fragmentation. The alkene double bond in olean-12-enes typically shows ^{13}C chemical shifts at approximately 122 and 145 ppm and **35** exhibits resonances in this area, at 122.6 and 143.6 ppm (Table 5.8). In addition mass fragments of 248 and 203 (248-COOH) are consistent with a retro Diels-Alder mass fragmentation in ring C of an olean-12-ene derivative (Rogers and Subramony 1988). The major mass fragments are shown in Fig. 5.1.

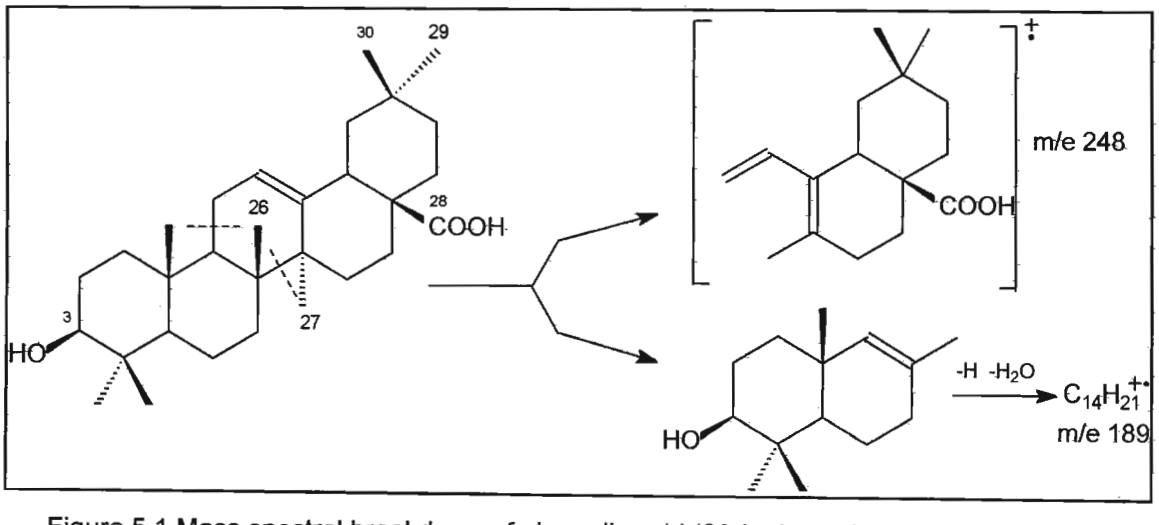


Figure 5.1 Mass spectral breakdown of oleanolic acid (3β-hydroxyolean-12-en-28-oic acid)

The carbon bearing the hydroxy group in **35** has exactly the same chemical shift as the C-3 β-OH of β-amyrin. Confirmation of the β orientation of this hydroxy group is provided by coupling constants of J_1=10.5 Hz and J_2=4.8.Hz for the axial proton on C-3. For a β-OH there is strong coupling (8-14 Hz) of this axial proton to the viscinal axial proton, on C-2, at a dihedral angle of 180°. For an α-OH the dihedral angle for viscinal protons is only 60°, with correspondingly weaker coupling of 1-7 Hz for both J_1 and J_2. In addition acetylation of **35** caused an upfield shift for C-2 of 3.5 ppm which is completely consistent with acetylation of an adjacent C-3 OH (Silverstein and Webster 1998). This causes a downfield shift for the C-3 resonance of about 2 ppm but an upfield shift for a β C-2 of 2.5-4.5 ppm, as observed.

Table 5.8 ^{13}C data for 35 and related compounds ($CDCl_3$)

Carbon	**35**	**35** methyl ester	Oleanolic acid	β-amyrin	**35** acetate
1	38.4	38.4	38.5	38.7	38.0
2	27.1	27.2	27.4	27.1	23.6
3	79.0	79.0	78.7	79.0	80.9
4	38.7	38.7	38.7	38.8	37.7
5	55.2	55.2	55.2	55.3	55.3
6	18.3	18.3	18.3	18.5	18.1
7	32.5	31.9	32.6	32.8	32.4
8	39.2	39.3	39.3	38.8	39.2
9	47.6	47.6	47.6	47.7	47.6
10	37.1	37.0	37.0	37.6	37.0
11	23.4	23.4	23.1	23.6	23.4
12	122.6	123.4	122.1	121.8	122.5
13	143.6	143.8	143.4	145.1	143.6
14	41.6	41.6	41.6	41.8	41.6
15	27.7	27.7	27.7	26.2	27.6
16	25.9	23.1	23.4	27.0	25.9
17	46.5	46.7	46.6	32.5	33.0
18	40.9	41.3	41.3	47.4	46.5
19	45.6	45.9	45.8	46.9	40.9
20	29.7	30.7	30.6	31.1	45.8
21	33.8	33.8	33.8	34.8	33.7
22	32.4	32.3	32.3	37.2	29.7
23	28.1	28.1	28.1	28.2	28.0
24	15.5	15.6	15.6	15.5	16.6
25	15.3	15.3	15.3	15.6	15.4
26	17.1	16.8	16.8	16.9	17.1
27	22.9	25.9	26.0	26.0	22.8
28	183.5	178.4	181.0	28.4	30.6
29	30.7	33.1	33.1	33.3	183.9
30	23.6	23.6	23.6	23.7	23.5

Two reviews of ^{13}C NMR data, each for about 400 pentacyclic triterpenoids, (Agrawal and Jain1992; Mahato and Kundu 1994), did not reveal an exact match for **35**. There is excellent agreement however between ring A and B resonances of both β-amyrin and oleanolic acid and those of **35**. The possibility that **35** was an urs-12-ene rather than an olean-12-ene was also considered. The former class of compounds has one methyl group at the 19 β position and one at C-20 instead of both at C-20 as for the oleananes. This equatorial methyl at C-19 is close to the double bond and causes C-12 to be deshielded by approximately 2 ppm and C-13 to be shielded by about 5 ppm in ^{13}C spectra. Urs-12-enes are therefore characterised by C-13 resonances around 138-139 ppm, which is clearly not the case for **35** which cannot therefore be an urs-12-ene. The possible sites for the COOH group in **35** are carbons 26, 27, 28, 29 or 30 because the mass spectral breakdown shows that the carboxylic acid group is on ring C, D or E. The carboxylic acid function for 3β-hydroxy olean-12-en-30-oic acid is at 179.4 ppm which is not in agreement with that of **35**, found at 183.5 ppm. The carboxylic acid function for 3α-hydroxy olean-12-en-28-oic acid at 183.9 ppm was however in close agreement with that of **35**, suggesting that the carboxylic function of **35** could be on C-28. There are no instances of the carboxylic acid function being at C-26, possibly for steric reasons. The chemical shifts of carboxylic acid functions can be affected by slight changes in pH, so methylation was carried out to clarify the position of the carboxyl function. Methylation of **35** showed an upfield shift of the carboxylic acid carbon to 178.4 ppm, which correlated well with that of C-28 in methyl oleanolate. Literature data also showed conclusively that the carboxylic acid function was not on C-27, C-29 or C-30 (Agrawal and Jain1992; Mahato and Kundu 1994). In addition the other ^{13}C NMR resonances were in good agreement with those of methyl oleanolate, so **35** was accordingly unambiguously identified as oleanolic acid.

Oleanane triterpenoids, the largest group of triterpenoids, are an important group of naturally occurring compounds, and many of them are pharmacologically active. Oleanolic acid (3β-hydroxy-12-oleanen-28-oic acid) is a very widely distributed aglycone and occurs as glycosides in cloves, sugar beet, *etc.* All naturally occurring oleananes have five six-membered fused rings with trans fusion except for rings D and E which are cis fused. The oleanane skeleton also has eight angular methyl groups. There are six sub-groups, all having a common characteristic of a gem-dimethyl group at C-20, but the site of the remaining six methyl groups varies. Ring methylene and angular methyl groups may be hydroxylated or further oxidised (Agrawal and Jain 1992 ; Mahato and Kundu 1994).

Compound 36 (20(29)-lupen-3-one)

Appendix: **36** GC-MS and NIST library match (147000 spectra)

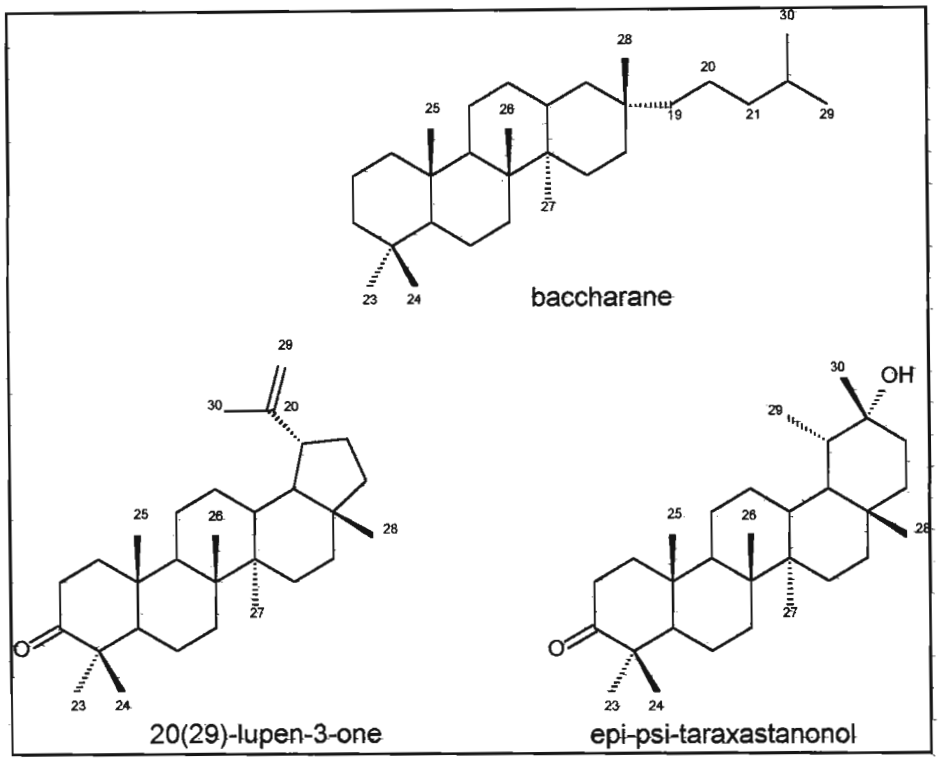


Figure 5.2 Relationship of **36** 20(29)-lupen-3-one and **37** 20-epi-ψ-taraxastananol to baccharane

Physical data

Compound **36** was identified via GC-MS analysis of a chloroform extract but was not isolated from the extract. Compound **36** with GC R_t = 24.26 min., showed the following GC-MS ions, using ammonia chemical ionisation :

442.4042	$M + NH_4^+$	($C_{30}H_{52}NO$ requires 442.4049)
425.3784	$[M + H]^+$	($C_{30}H_{49}O$ requires 425.3783)
409	$[M - CH_3]^+$	
205		
189.19	$C_{14}H_{21}^+$	
109		

The above data give M^+ 424 and formula $C_{30}H_{48}O$.

Compound **36** was established as 20(29)-lupen-3-one by a GC-MS NIST library search of 147000 spectra, carried out at Iowa State University.

Discussion

The identity of **36** was established via a GC-MS analysis of the chloroform extract using the ammonia chemical ionization technique, which clearly established the ammonium ion adduct of the molecular ion at m/z 442.4042 with $C_{30}H_{52}NO$ for this adduct. The molecular ion itself was visible at 425.3784 $(M + H)^+$ for $C_{30}H_{49}O$, establishing the formula for **36** as $C_{30}H_{48}O$. The NIST library search matched the spectrum to that of 20(29)-lupen-3-one.

The ion at m/z 189.19 for $C_{14}H_{21}^+$ represents fragmentation of the A and B rings from the rest of the molecule as in **35**, 3β-hydroxyolean-12-en-29-oic acid.

Lupenone has been identified in several plant species, one being the Korean mushroom, *Daedaleopsis tricolor*, which has proven powerful antibiotic activity. The bioactivity was subsequently attributed to 20(29)-lupen-3-one, purified from this mushroom species (Kim *et al.*2001). An *in vitro* study showed that lupenone exhibited a broad spectrum of antibiotic activity against yeast, fungi and bacteria. In addition it displayed antioxidant activity comparable to the widely-used anti-oxidant α-tocopherol or vitamin E.

Compound 37 (20-epi-ψ-taraxastananol)

Appendix: **37** GC-MS and Wiley library match (329171 spectra)

Physical data

Compound **37** was obtained from the same chloroform extract as **36** and was also not isolated from the extract. GC-MS analysis of the peak R_t = 25.24 min. gave m/z:

424.3660 $M^+ - H_2O$ $C_{30}H_{48}O$ requires 424. 3705

205.20

189.19 $[C_{14}H_{21}]^+$

109.11

M^+ at 442 for $C_{30}H_{50}O_2$ was absent due to loss of H_2O.

A Wiley library search of 329171 spectra, carried out at Iowa State University, matched the spectrum of **37** to that of 20-epi-ψ-taraxastananol.

Discussion

Compound **37**, present in the same chloroform extract as **36**, was matched to 20-epi-ψ-taraxastananol by GC-MS analysis via a Wiley library search.

The molecular ion at m/z 442 corresponding to formula $C_{30}H_{50}O_2$ was absent due to loss of water. However accurate mass analysis of the fragment at 424.3660 ($C_{30}H_{48}O$ requires 424. 3705) confirmed the formula of this ion after water loss. The ion at m/z 189.19 for $C_{14}H_{21}^+$ represents fragmentation of the A and B rings as shown in Fig. 5.1 for **35**, 3β-hydroxyolean-12-en-29-oic acid, and this ion is also present in the GC-MS spectrum of **36**.

Lupane, oleanane and taraxastane triterpenoids arise from the same baccarane precursor (Fig.5.2). Ring closure via the baccarane side-chain gives a 5-membered ring E for the lupanes, but a six-membered ring for the oleananes and taraxastanes. Loss of a proton followed by a methyl migration in the oleanane precursor leads to the taraxastane skeleton. The three triterpenoids identified in *Rhoicissus tridentata*, lupen-3-one, 3β-hydroxyolean-12-en-28-oic acid and 20-epi-ψ-taraxastananol, are thus biogenetically related through their common precursor.

Triterpenoids are well known for their anti-inflammatory properties. A bioassay-guided fractionation of an extract of marigold (*Calendula officinalis* L., Asteraceae) flowers showed that triterpenoids are shown as the most important anti-inflammatory principles of this medicinal plant. The mono-ols psi-taraxasterol, lupeol, taraxasterol, and beta-amyrin are less active than the free diol, faradiol, which is the most relevant principle for the activity of the drug, present as faradiol monoester in this species (Della Loggia *et al.* 1994).

Compound 38 (triacontanol)

Appendix: **38** CI-MS, IR, ^{1}H NMR; Acetate **38** CI-MS, EI-MS, IR, ^{1}H NMR.

$CH_3(CH_2)_{28}CH_2OH$ triacontanol

Physical data

This compound was eluted with $CHCl_3$-MeOH 5:1 from a silica gel column of a 1:1 $CHCl_3$-MeOH extract of branches harvested from Treasure Beach Grasslands in July 1995. Recrystallisation from hot MeOH yielded a white powder, 0.58 g, m.p. 93-95 °C, which gave negative results for sterol, $FeCl_3$ and Br_2 tests showing that it was not a sterol, phenol and had no unsaturated bonds. TLC in hexane-ether 2:1 showed a blue-violet spot R_f 0.38 whereas sitosterol had R_f 0.23 on the same plate.

IR (KBr disc): 3400 (br.OH), 2920, 2840 (C-H), 1470, 1460, 1055, 730 and 715 cm^{-1}

^1H NMR ($CDCl_3$): δ 3.62 2H, t; δ 1.57 m; δ 1.26 m; δ 0.87 3H t

EIMS m/z:	438	tiny peak (no %)	M^+
	420	4%	$[M - H_2O]^+$
	392	6%	$[420 - CH_2]^+$
	57	100%	$C_4H_9^+$
	43	84%	$C_3H_7^+$

Accurate masses for the 420 and 392 peaks were:

420.4678 $C_{30}H_{60}$ requires 420.4695

392.4403 $C_{28}H_{56}$ requires 392.4382

Prior to this spectrum another FAB-MS was run, but the highest mass fragment was m/z 289, so clearly the molecular ion was not captured.

Acetate of **38**

The acetate was prepared by heating 0.2 g of **38** for 3 h at 90 °C in 2 cm^3 pyridine and 2 cm^3 acetic anhydride, followed by precipitation in ice-water. The dry white solid product was recrystallised from acetone to yield 0.1 g of colourless crystals after drying in a dessicator over P_2O_5 with m.p. 78-80 °C. TLC in hexane-ether 2:1 showed that acetylation was complete giving a single violet spot with anisaldehyde reagent of R_f 0.9, and no un-acetylated **38**.

IR (KBr disc): 2900, 2815, 1730, 1465, 1360, 1230, 1035, 720 and 710 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ 4.03 (2H, t, J = 6.8 Hz, H-2); δ 2.02 (3H, s, Ac. CH_3); δ 1.59 (m) δ 1.23 (m); δ 0.857 (3H, t, J = 6.7 Hz, H-30).

The peaks at δ 1.593 and δ 1.231 were large and did not give accurate whole numbers of protons for the integration, but the upfield peak showed around 60 protons.

Two mass spectra were run for the acetate of **38**:

EIMS (Spectrum 1):	480	tiny peak, no % listed	
	452.3	5%	
	139.1	13%	
	97.1	75%	
	57.1	100%	
	43.1	71%	
EIMS (Spectrum 2):	480	12%	M^+
	452	42%	$[M^+ - CH_2CH_3 + H]$
	83	100%	base peak

An accurate mass on the 480 and 452 ions from spectrum 2 showed:

480.4913 ($C_{32}H_{64}O_2$ requires 480.4906)

452.4604 ($C_{30}H_{60}O_2$ requires 452.4593)

Discussion

Compound **38**, m.p. 93-95 °C, isolated from a $CHCl_3$:MeOH extract of branches from young plants at Treasure Beach Grasslands, gave negative colour tests for sterols, phenols and alkenes. TLC showed it was less polar than sitosterol. The m.p. of **38** was higher than that of an early literature report (78-81 °C, Arthur *et al.* 1965) of a $C_{30}H_{62}O$ long chain alcohol isolated from a widely used Chinese medicinal plant *Acacia confusa* Merr., together with sitosterol, lupeol and taraxerol. The structure was unknown at that stage. However a 2003 report by CCA Biochemical Co. Ltd. (www.ccabio.chemnet.com/en/product/00000031.html) listed an m.p. of 85-90 °C for triacontanol, which is in closer agreement with that found for **38**.

The IR spectrum showed a broad OH absorption at 3400 cm^{-1}, very strong C-H stretch at 2920 and 2840 cm^{-1} and C-H bend at 1470 and 1460 cm^{-1}. The C-O stretch at 1055 cm^{-1} is within the range (1050 – 1085 cm^{-1}) for a primary saturated alcohol (Silverstein and Webster 1998).

Compound **38** presented a simple ^1H NMR spectrum with most proton signals appearing between δ 2.4 and δ 0.8 indicating a long aliphatic chain. The triplet at δ 0.87 was attributed to a terminal methyl group, and the large multiplets at δ 1.26 and δ 1.57 to methylene groups. The triplet at δ 3.62 was assigned to the two C-1 protons, deshielded by the alcohol hydroxyl. An R-CH_2OH group usually resonates at about δ 3.4 (Silverstein and Webster 1998).

Although **38** was clearly a long-chain alcohol form spectral data of the compound itself and its mono-acetate, the molecular ion was not evident in several mass spectra, with only breakdown fragments being captured. Sequential loss of two 28 mass units (2xCH_2 each) followed by loss of many 14 mass units (CH_2 groups) is evident in the spectrum, indicating a very long chain of methylene groups. The base peak at m/z 57 represents $C_4H_9^+$ at the end of the chain. The ion at 420 (4%) gave accurate mass 420.4678 corresponding to $C_{30}H_{60}$. Although the tiny peak at 438 was not reflected in the recorded abundances, it was thought to be the molecular ion, with m/z 420 reflecting $M^+ - H_2O$ for the alcohol.

Acetylation of **38** with pyridine/acetic anhydride produced colourless crystals after recrystallisation from acetone, m.p. 78-80 °C. TLC in hexane-ether 2:1 showed reaction was complete with no un-acetylated material being present. The IR also showed that the OH absorption at 3400 cm^{-1} was completely absent and the strong absorption at 1730 cm^{-1} for the acetate ester bond showed that **38** was fully acetylated. Acetylation caused a downfield shift for the C-1 protons in the ^1H NMR spectrum from δ 3.62 to δ 4.03 due to deshielding by the acetate group on the C-1 hydroxyl.

A GC-MS spectrum of the acetate in order to match it to a library spectrum showed only mass fragments, with the highest corresponding to a 26-carbon alcohol.

Two EIMS spectra run on different instruments both showed ions at 452 for [$M^+ - CH_3CH_2$ + H]. However the acetate molecular ion at 480 (12%) was only strong enough to reflect

in the abundances in Spectrum 2, although a tiny peak is evident at 480 in Spectrum 1. Accurate mass analysis of the 452 and 480 fragments in Spectrum 2 showed 452.4604 corresponding to $C_{30}H_{60}O_2$ and 480.4913 corresponding to $C_{32}H_{64}O_2$. The latter represents the mono-acetate molecular ion so the formula of **38** itself is therefore $C_{30}H_{62}O$.

From the physical and spectral evidence, compound **38** is therefore identified as triacontanol, structure $CH_3(CH_2)_{29}OH$.

Triacontanol, or melissyl alcohol, is a fatty alcohol, a common member of this group being the 18 carbon 1-octadecanol, or stearyl alcohol. The acute oral LD_{50}, female mouse was 1.5 g/Kg, male mouse 8 g/Kg. Although aliphatic alcohols do occur naturally in the free form, it is more common to find them as wax esters or glyceryl ethers. Although triacontanol appeared at first to be a rather mundane, or even boring-looking molecule, it has interesting physiological properties. Firstly it has been known as a naturally occurring plant growth hormone since 1975. For example it increases tomato yields by 30% when applied at strengths of 0.01-0.10 ppm, and is marketed in garden products (www.ccabio.chemnet.com/en/product/00000031.html). It also has cholesterol-lowering properties. A mixture of hexacosanol (C26), octacosanol (C28) and triacontanol (C30), three higher primary aliphatic alcohols from sugar cane (*Saccharum officinarum*, L.), exhibited cholesterol-lowering effects in rabbits, by affecting the rate-controlling enzyme. Octacosanol also suppressed lipid accumulation in rats fed on a high-fat diet, and inhibited platelet aggregation (www.cyberlipid.org/simple/simp0003.htm).

The fact that compound **38,** triacontanol, is a plant growth hormone was probably the reason it was found in quite a large quantity, compared to other compounds, in young actively growing branches.

CNS depressant effects of sterols and polyphenols

Acetylcholine is a neurotransmitter for signals throughout the neural network. Whether acetylcholine is stimulatory or inhibitory depends on the class of receptor to which it binds *i.e.* either nicotinic acetylcholine receptors or muscarinic acetylcholine receptors (Mathews and van Holde 1990).The passing of a signal, in the form of an action potential, involves cholinergic synapses which link nerve cells and which also make connections to muscle cells. When acetylcholine binds to nicotinic receptors it causes voltage-gated calcium channels to open, and this leads to a sequence of events for passing on the

action potential. By contrast when acetylcholine binds to muscarinic receptors, the effect can be inhibitory.

Certain toxic substances (antagonists) can inhibit cholinergic synaptic transmission by blocking the acetylcholine receptors *e.g.* the protein toxins in some snake venoms such as cobratoxin. Other toxins such as nicotine (agonists) lock the ion channels open. Some inhibitors of cholinergic synaptic transmission are medically useful as muscle relaxants when used in carefully regulated doses.

Rhoicissus tridentata root extracts are reported to cause CNS depressant paralysis and respiratory arrest (Veale *et al.*1992). The leaves of *Byrsonima crassifolia* cause CNS depressant and hypotensive effects (Bejar *et al.*1995). These contain 6 triterpenes, 2 sterols (sitosterol and sitosterolin), 6 flavanoids and 6 amino-acids, methyl gallate and a sulphonoglycolipid. There are many similar or identical compounds in *Byrsonima crassifolia* to those found in *Rhoicissus tridentata,* but the CNS depressant effect in these two species has not been linked to any particular compound/s (Bejar *et al.*1995 and Veale *et al.*1992). However in a search for the CNS active ingredients in ethanol extracts of the root bark of *Alangium plantaifolium*, five compounds were isolated (Zhu 1998), two being sitosterol and sitosterol glucoside, also found in *Rhoicissus tridentata* extracts. Sitosterol and a second compound, 5β, 6β-dihydroxycyclohex-2-en-1-ol 1-β-glucoside, were able to bind directly to muscarinic receptors with consequent inhibition of nerve signal transmission. This supported the finding that roots of *Alangium* species showed significant muscle relaxant effects in the dog.

In addition sitosterol and sitosterol glucoside were able to interact with control compounds and cause large changes in their binding activities to five types of receptors. For example both of these compounds had the same effects on adenosine 1 receptors which resulted in a 14-fold increase in their binding activity. Sitosterolin also increased the binding activity of two subtypes of 5HT1 (5-hydroxytryptamine) receptors. It increased the binding activity of a mesulergin standard with 5HT1A by 22-fold (Zhu 1998). These data supported findings that the ethanol extract of *Alangium* species potentiated the hypnotic effect of phenobarbitone, but that the extract itself produced no hypnotic effects. These compounds have also the potential therefore for indirect action on certain substances in the CNS.

Another factor to consider which could influence the CNS is that plant tannins or polyphenols generally show non-specific binding to proteins and may also affect receptor

binding (Zhu 1998). Such compounds are major constituents in *Rhoicissus tridentata* extracts.

It is clear therefore that there are several polar and non-polar compounds in *Rhoicissus tridentata* extracts with the capacity to affect the CNS both directly by binding to the muscarinic receptors, or indirectly by increasing the binding activity of other compounds, and consequently enhancing their effects.

There is likely to be a complex synergistic or interactive effect between the various plant ingredients, such as sterols, sterol glucosides and polyphenols, which could explain the observed CNS depression caused by crude extracts of *Rhoicissus tridentata*.

CHAPTER 6

EFFECTS OF CHEMICALS ON THE UTERUS

6.1 *BIRTH-RELATED CHEMICALS AND OVERVIEW OF LABOUR*

Levels of oestrogens and progesterone are far higher for pregnant women than for non-pregnant women. During pregnancy these levels increase steadily up to term, and determine when the uterus is ready for labour to commence. Progesterone inhibits uterine contractility during pregnancy, therefore helping to prevent expulsion of the foetus. On the other hand oestrogens (oestrone, oestradiol and oestriol) have a definite tendency to increase the degree of uterine contractility (Fig.6.1).

Both progesterone and oestrogen increase progressively throughout most of pregnancy, but from the 7th month onward oestrogen secretion continues to increase while progesterone secretion remains constant or in many cases shows a slight decrease. This increased oestrogen to progesterone ratio towards the end of pregnancy has been postulated to be at least partly responsible for the increased contractility of the uterus. In contrast to the decrease in progesterone production towards the end of pregnancy, there is a marked rise in oestrogen levels from about the 16th week onwards. Although levels of all three oestrogens are raised, oestrone and oestradiol increase by about 100-fold whereas oestriol increases by over 1000-fold and accounts for over 90% of the total oestrogens being excreted at the end of pregnancy. In fact, oestriol levels for pregnant women at term reach approximately 20000 μg per 24 h period, as measured in the urine. This contrasts markedly with the monthly maximum during the menstrual cycle for non-pregnant women of approximately 25 μg oestriol per 24 h period (Fotherby 1975). The biosynthesis of these large amounts of oestriol are attributed to the combined action of the foetus and the placenta, and decreased levels of this essential oestrogen are a useful index of fetoplacental function. Women with high oestriol levels at 34 to 36 weeks of pregnancy tend to go into labour earlier than those with lower oestriol levels at that time. Decreased oestriol excretion, measured in the urine or plasma, is associated with reduced foetal viability or placental insufficiency, and low values can suggest foetal death.

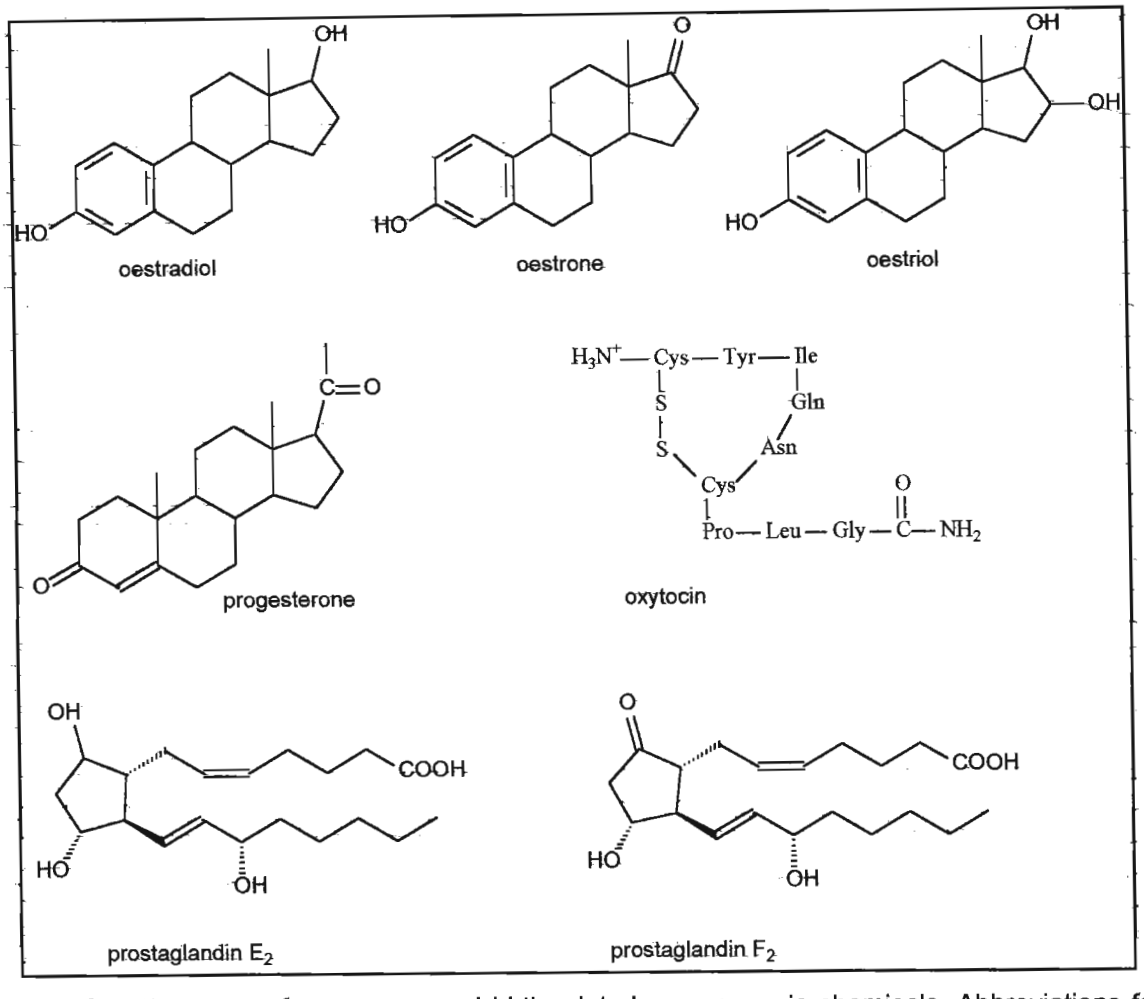


Figure 6.1 Structures of pregnancy and birth-related spasmogenic chemicals. Abbreviations for amino-acids : Ile = ilenine ; Tyr = tyrosine ; Cys = cysteine ; Gln = glutamine ; Pro = proline and Gly = glycine.

The factors which initiate labour are complex, with the foetus itself playing an important role. There is an interplay between different hormones acting in synergy, such as oxytocin, cortisol and the prostaglandins. Oxytocin, a polypeptide hormone containing 9 amino-acids, induces labour in pregnant women by causing uterine contraction, and also controls the subsequent release of milk. There are several reasons for believing that it is important in increasing the contractility of the uterus near term, one being that the uterine muscle increases its oxytocin receptors, and therefore its responsiveness to oxytocin, during the latter few months of pregnancy. At term the number of oxytocin receptors is great enough to cause contraction of the smooth muscle in the uterine wall in the presence of small amounts of oxytocin produced towards the end of pregnancy. The foetus descends towards the cervix which stretches, sending nerve impulses to the hypothalamus, and this in turn leads to the release of more oxytocin by the posterior pituitary gland, this being the only positive feedback cycle in the body. An increase of foetal hormones, namely oxytocin, cortisol and prostaglandins, with the latter being

released in foetal membranes in high concentrations at term, can also increase the intensity of the uterine contractions. (Guyton and Hall, 1996). Prostaglandins F_2 and E_2 have direct effects of contractility. It is noteworthy that the concentration of arachidonic acid, a precursor of the prostaglandins, is six times higher after the onset of labour than at term, prior to the initiation of labour (Seitchik and Chattkof 1976). This supports the biosynthetic relation between the compounds, and their collective crucial role in the onset and maintenance of labour. The cumulative effect of these spasmogenic hormones, both from the uterus and the foetus, lead to stronger contractions which culminate in the birth of the baby.

The thalidomide tragedy, which resulted in serious birth deformities made the medical community ultraconservative regarding medication for pregnant women. Today the use of medication by pregnant women in first world countries is strictly monitored and regulated by health professionals. The oxytocic agents approved for use in obstetrics to induce or augment labour or to control post-partem bleeding are: oxytocin, prostaglandin F_2 , prostaglandin E_2 and the alkaloid ergometrine (9,10-Didehydro-*N*-(2-hydroxy-1-methylethyl)-6-methylergoline-8-carboxamide). Ergometrine shows a structure-activity relationship to several other compounds including oestradiol (www.kurchii.h1.Ru/Mono.html). These drugs used in obstetrics are all capable of causing uterine hyperstimulation in sufficiently high doses so are therefore potentially toxic. Because of this well known fact they are only administered in regulated doses by trained medical staff.

6.2 *BIOISOTERISM OR "LOOK-ALIKES" FOR NATURAL BIRTH-RELATED CHEMICALS*

The concept of chemical isoterism was first developed by Langmuir in 1919 when he related similarities in physico-chemical properties of various chemicals to similarities in their electronic structures (Foye *et al.* 1995). Langmuir's research involved mainly elements, inorganic molecules, ions and small organic molecules. However the concept was subsequently expanded to include biologically active molecules, and named bioisoterism, which is the foundation of modern drug design. In developing new or improved therapeutic agents, the underlying principle of chemical "look-alikes" bringing about similar physiological responses is utilised, with a well-known example being the development of birth-control pills by slightly modifying the structures of known female hormones so that they could be taken orally. Molecular modifications cannot be too great

if the new drug is to react at the same receptor site as the original drug, to bring about the desired response.

Many derivatives of stilbene, which is considerably more stable as the trans isomer, are potent oestrogenic substances and are used therapeutically. One of the most important of these synthetic oestrogens is diethylstilboestrol (α,α'-diethyl-4,4'-stilbenediol) which is significantly cheaper than naturally occurring oestrogens and yet can produce all the same effects (Foye *et al.* 1995). Trans diethylstilboestrol has a 10 times greater oestrogenic activity than the cis isomer. The distance between the OH groups in trans stilbestrol closely approximates the distance between the OH groups in oestradiol. It is interesting that the fused four-ring steroid nucleus is not required for this oestrogenic activity, an example of cyclic versus non-cyclic bioisoterism. Various stilbenes as well as sterols similar to oestrodiol were isolated from the three plants studied, and their activity on the uterus are discussed in Section 6.4.

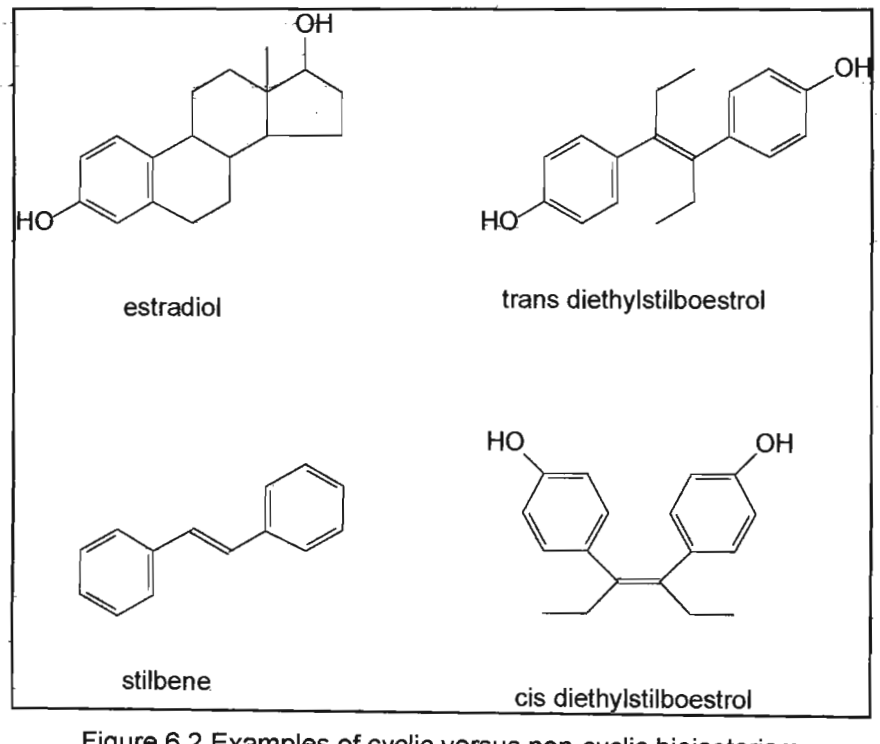


Figure 6.2 Examples of cyclic versus non-cyclic bioisoterism

6.3 *REVIEW OF UTEROACTIVE CHEMICALS ISOLATED FROM PLANTS*

Chemical metabolites may act on uterine smooth muscle in a variety of ways, which are briefly outlined here. Besides the reproductive tract, smooth muscle tissue is also found in the blood vessels, intestines, and urinary tract. The response to a chemical agonist is influenced by the concentration of the agonist, and also the density of its receptor sites. A drug may stimulate muscle contraction by increasing the permeability of cell membranes

to calcium ions, or by acting directly on receptor sites or contractile proteins. The effects of calcium and potassium ions on muscle are discussed in Chapter 7, as these ions were present in root extracts. A variety of chemical forces may contribute to the binding of a drug to the receptor, including ionic bonds, hydrogen bonds, and van der Waals attractive forces. Specific stereochemical requirements must be met for a drug to be an agonist, and once it has bonded to a receptor, a biological response may then occur. Molecules which can adopt the conformation required for binding at a receptor site may act as agonists or antagonists to the action of the receptor. Antagonists may have the correct stereochemistry to bind, but lack a key functional group to trigger the receptor response.

Acetyl choline (ACh, Fig.6.3) is a neurotransmitter and a natural agonist for cholinergic nerves or cholinergic receptors, and it affects all nerves that control skeletal muscles (Mc Murray 1996). It is therefore frequently chosen as a standard cholinergic receptor agonist for *in vitro* experimental work on smooth muscle.

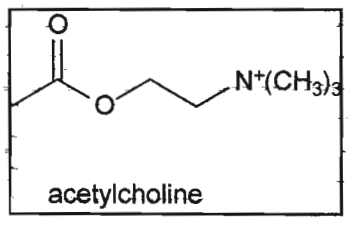


Figure 6.3

Neurotransmitters, produced at nerve endings in the brain, are chemical messengers in the central nervous system (CNS), and can be influenced by drugs. For example nicotine and atropine exert biochemical effects at ACh synapses in the CNS, and therefore alter the ACh activity. The role of ACh together with calcium and potassium ions in bringing about muscle contraction in discussed in Section 7.1.

Many herbal remedies are documented which strengthen and tone uterine smooth muscle tissue. Many of the plants used for such remedies contain phytoestrogens which structurally or functionally mimic mammalian oestrogens *i.e.* exhilibit bioisoterism (Ososki and Kennelly 2003). Phytoestrogens are therefore considered to play an important role in preventing cancer, heart disease, menopausal symptoms and osteoporosis. Since oestrogens influence the functioning of reproductive tissues, many women use phytoestrogens as an alternative to hormone replacement therapy. Isoflavones are the most well known phytoestrogens which are reviewed by Ososki and Kennelly, with examples being daidzein or 4',7-dihydroxyisoflavone and genistein or 4',5,7-

trihydroxyisoflavone (Fig.6.4) and their glycosides. The relative position of the 4' and 7 hydroxy groups approximates that in oestradiol, and is very likely to account for their oestrogenic activity, as also noted for trans diethylstilboestrol in Section 6.2.

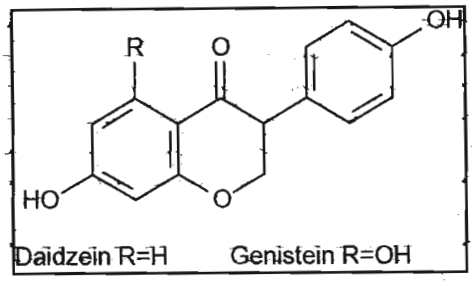


Figure 6.4 Phytoestrogens

Certain lignans also show oestrogenic activity, as well as stilbenes such as resveratrol, which is found in a variety of plants.

Many herbal formulae which strengthen and tone the smooth muscle of the uterus are documented world-wide. An example is Squaw Vine, and the effects are attributed to bitter glycosides, tannins and saponins (www.alternativescentral.com/phf16a-squawvine.htm). The Bayberry or *Myrica cerifera* containing miscellaneous tannins, phenols, resins and gums also has an excellent general effect on the uterus during pregnancy (www.holistic-online.com/Herbal-Med/_Herbs/h75.htm). Tannins, known for their astringent properties, are common constituents of such remedies and are also found in Raspberry leaves, *Rubus idaeus*, used since ancient times and reported as being safe and beneficial throughout pregnancy (Doran 1994). The action on the uterus has been attributed to the alkaloid fragrine, a water-soluble oxytocic chemical with the capacity to both stimulate and relax the uterus, producing a regulating effect. Other constituents of raspberry leaves are tannins of gallic and ellagic acid.

In South Africa results from a study of three of the most commonly used plants in *isihlambezo* mixtures showed that they had direct uterotonic activity (Kaido et al 1997). Extracts of *Agapanthus pentanisia*, *Pentanisia prunelloides* and *Gunnera perpensa* all augmented the response of the uterus to the standard agonist oxytocin. The extracts all had a greater effect on uterine smooth muscle than on ileum preparations. While the first two augmented the response of the ileum to low doses of acetylcholine, *Gunnera perpensa* had no direct effect on the ileum preparation at all. The maximal response of both uterus and ileum was inhibited by the plant extracts, except for *Agapanthus pentanisia* on uterus.

Prior to the current research very little work had been done in this country in the field of identifying the active chemicals in pregnancy-related traditional medicines. Colleagues Joy Veale and Lynn Katsoulis undertook in-depth pharmacological studies of the uteroactivity of *Clivia miniata* and *Agapanthus africanus* (Veale 2001) and *Rhoicissus tridentata* (Katsoulis 2000) respectively. A chemical investigation of *Ekebergia capensis, Clivia miniata* and *Grewia occidentalis,* all frequently used in traditional medicines in South Africa, revealed the presence of five compounds with uterotonic properties (Sewram 1997). These were oleanonic acid (3-oxo-12-oleanen-28-oic acid) and 3-*epi*-oleanolic acid (3-α-hydroxy-12-oleanen-28-oic acid) from *Ekebergia capensis*, linoleic acid and 5-hydroxymethyl-2-furancarboxaldehyde from *Clivia miniata,* and 3-(4-hydroxy-3-methoxyphenyl)-2-propenal (coniferaldehyde) from *Grewia occidentalis*, which also contained oleanolic acid. All five compounds (Fig.6.5) induced an agonistic muscle response using isolated guinea pig uterine tissue. It was also established through the use of receptor blockers, that the stimulatory effects were mediated through the cholinergic receptors. Acetylcholine was the standard agonist, and atropine was used as a cholinergic receptor blocking agent. Prostaglandins, which are powerful spasmogens, are biosynthesized from the C_{20} unsaturated fatty acid arachidonic acid, which is in turn formed from the two essential C_{18} fatty acids linoleic and linolenic acids (Campbell 1995). Linoleic acid was isolated in much higher quantities (1 g) than the four other compounds described above. The fact that prostaglandins can be biosynthesised *in situ* in extremely short times, in my opinion, could explain the observed action of linoleic acid on isolated uterus.

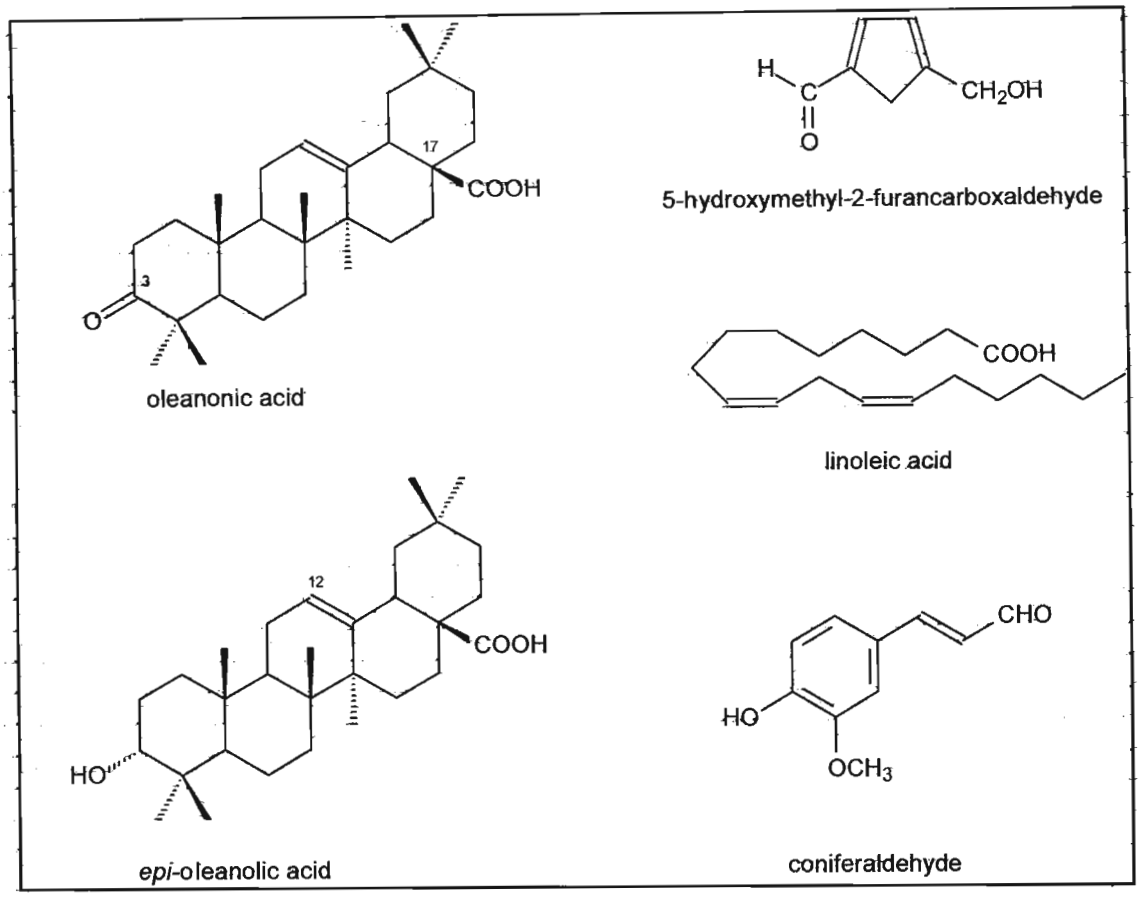


Figure 6.5 Uteroactive compounds from *Ekebergia capensis, Clivia miniata* and *Grewia occidentalis.*

6.4 Uteroactivity of chemicals isolated in the current research

All of the uteroctive compounds isolated in this study are regarded as secondary metabolites, and an overview of these is presented in Chapter 1. Those spasmogenic compounds which stimulated uterine tissue to the greatest extent were found to be aromatic in nature, being either phenols or phenolic glucosides. Such compounds are synthesized via the polyketide and shikimic acid pathway. Those compounds which exhibited oestrogenic effects, sensitising uterine tissue so that it was more responsive towards the spasmogens, were steroidal in nature. Such compounds are generally synthesised via the isoprenoid route.

Preliminary investigations were carried out on crude hot water extracts of plants, prepared in the same way as used by traditional healers. Results showed that extracts of all three plant roots stimulated dose dependent contractions of isolated uterine tissue. The graphical results of uterine tests for extracts and purified fractions from the three plants (Figures 6.6 to 6.12) are now presented, followed by discussion of these results.

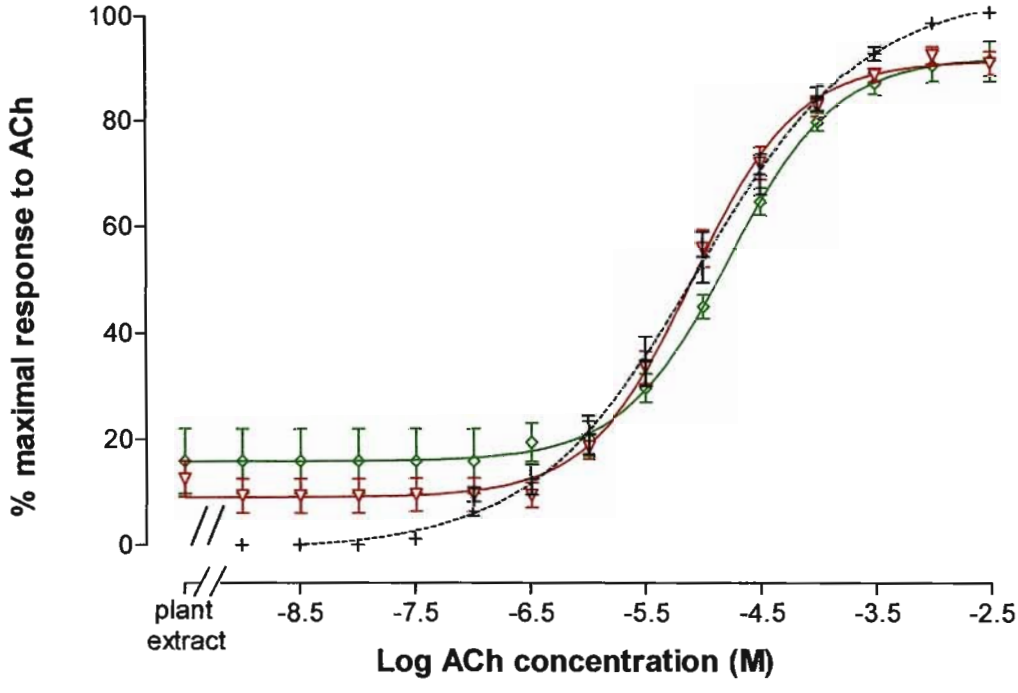


Figure 6.6 The effect of crude aqueous *C.kraussii* leaf (▽) and branch (◇) extracts on the isolated uterine response to acetylcholine. The organs were treated with the crude extracts 5 min. before the cumulative additions of acetylcholine. Each point represents the mean of 4 replicates.

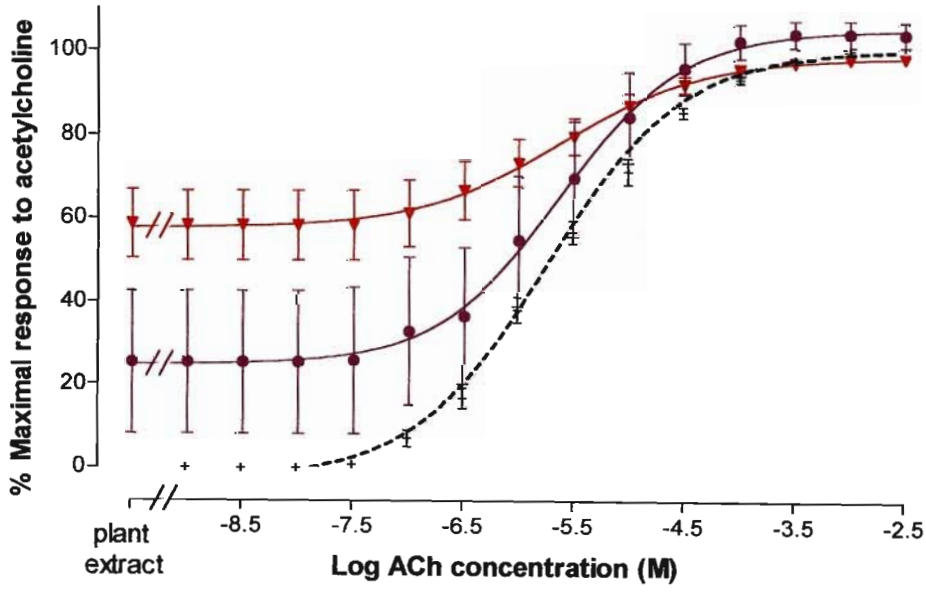


Figure 6.7 Dose response curves of *C. kraussii* root fractions which augment the response of isolated uterus to ACh without altering the maximal response to ACh. The organs were incubated with 1.3mg/ml of ▼ compound **11** (n=12) and ● compound **10** (n=3) 5 min. before the cumulative addition ofACh (+; n=9).

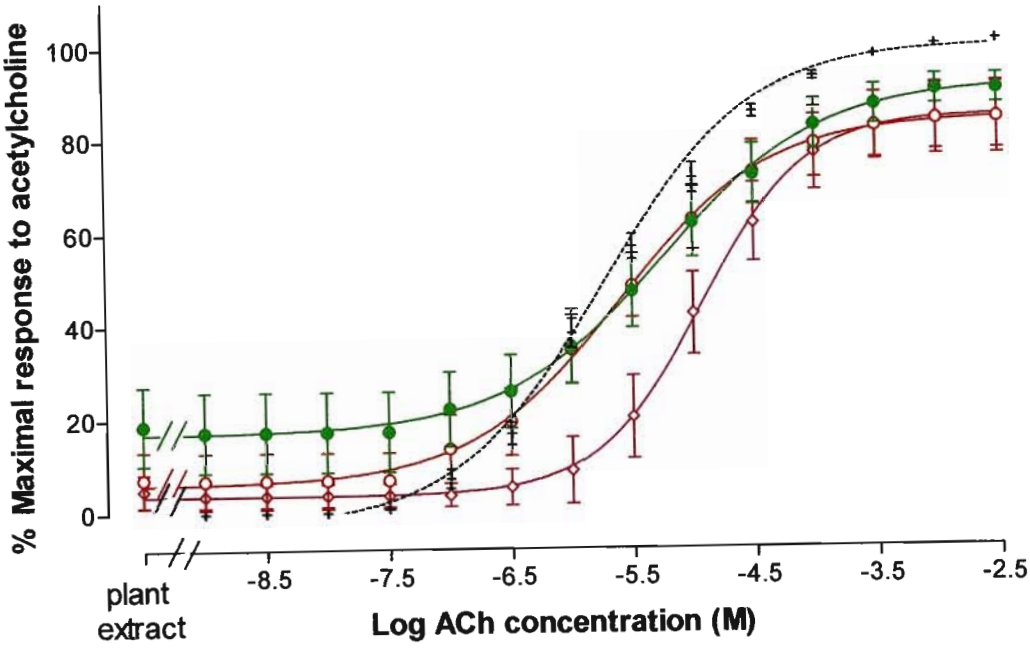


Figure 6.8 Dose response curves of fractions of *C. kraussii* roots which augment the initial response of isolated uterus to ACh and inhibit the maximal response to ACh. The organs were incubated with $1.3x10^{-3}$ g/ml of the following fractions 5 min. before the cumulative addition of Ah: ● **8+9** (n=12); ○ **4+5** (n=9) and ◇ **1** (n=6). The curves are compared to ACh alone (+ n=9).

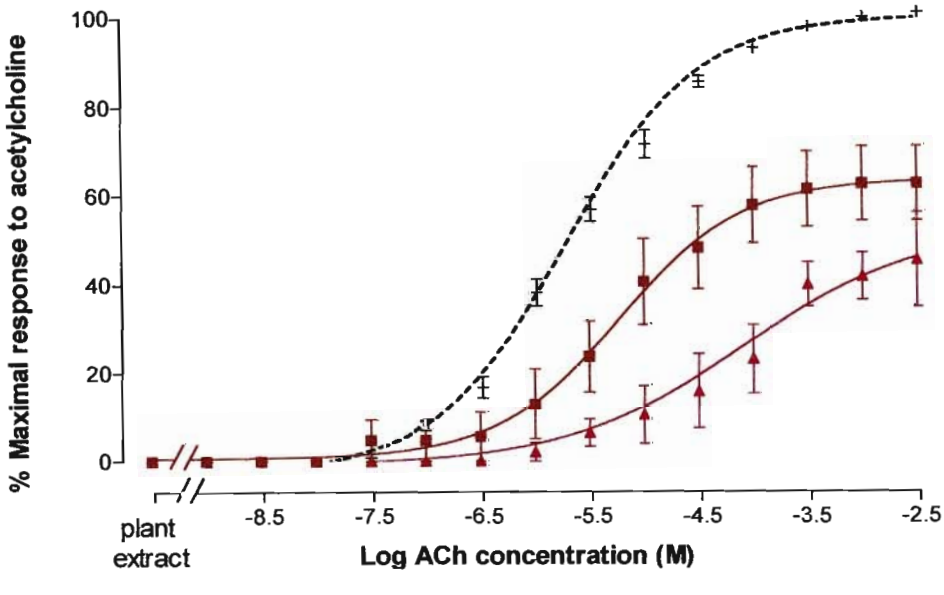


Figure 6.9 Dose response curves of fractions of *C. kraussii* roots which do not alter the initial response of isolated uterus to ACh but inhibit the maximal response to ACh. The organs were incubated with $1.3x10^{-3}$ g/ml of the following extracts 5 min. before the cumulative addition of ACh: ■ hot methanol extract (n=9) and ▲ methylene chloride:methanol extract (n=5). The curves are compared to ACh alone (+ n=9).

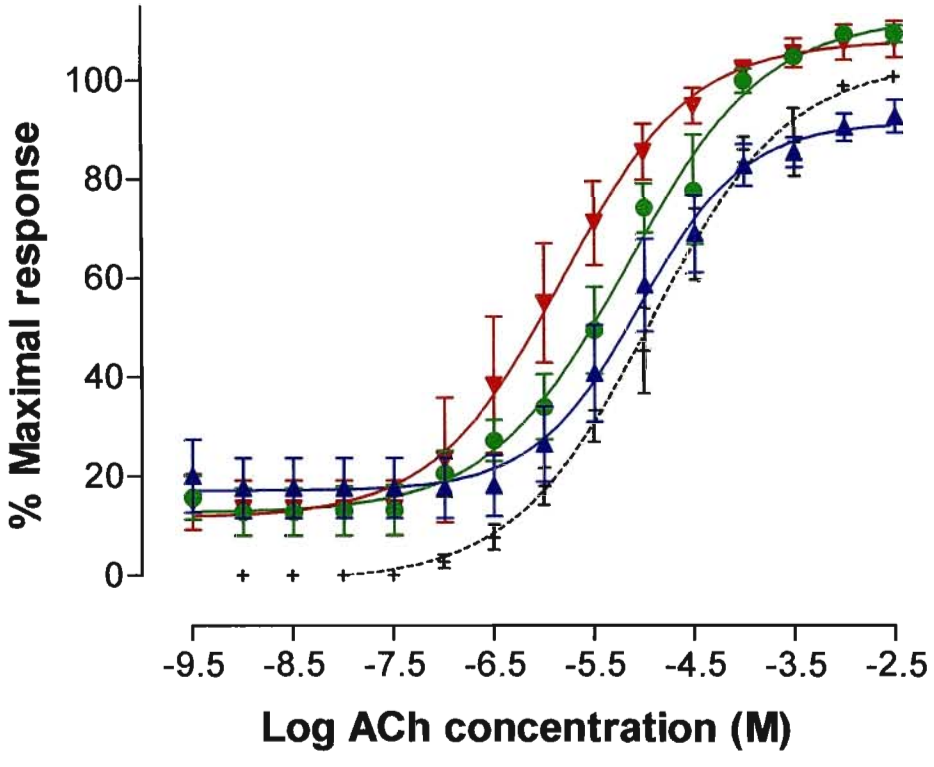


Figure 6.10 Dose response curves of fractions of *Gunnera perpensa* which augment the response of isolated uterus to acetylcholine. The organs were incubated with 1.3 mg cm^{-3} of ▼ compounds 17 and 18; ● water extract and ▲methanol extract.

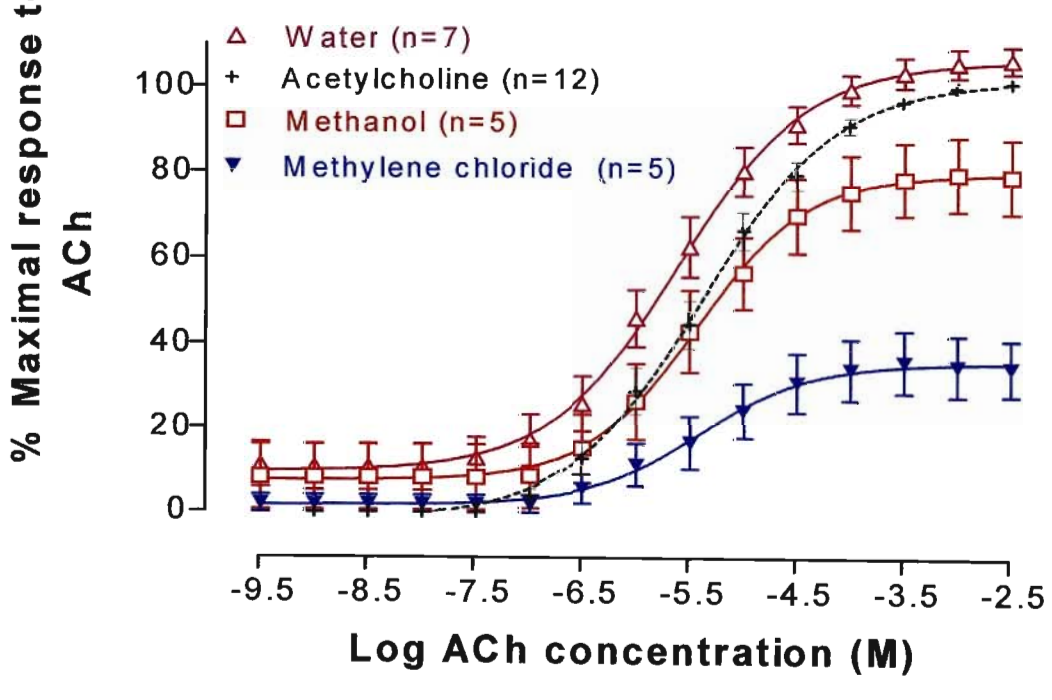


Figure 6.11 Dose response curves for extracts of *Rhoicissus tridentata* roots prepared by successive extraction with methylene chloride, methanol and then water. Isolated uterus was incubated with a solution containing 1.3 mg cm^{-3} of each extract 5 minutes before the cumulative addition of acetyl choline alone.

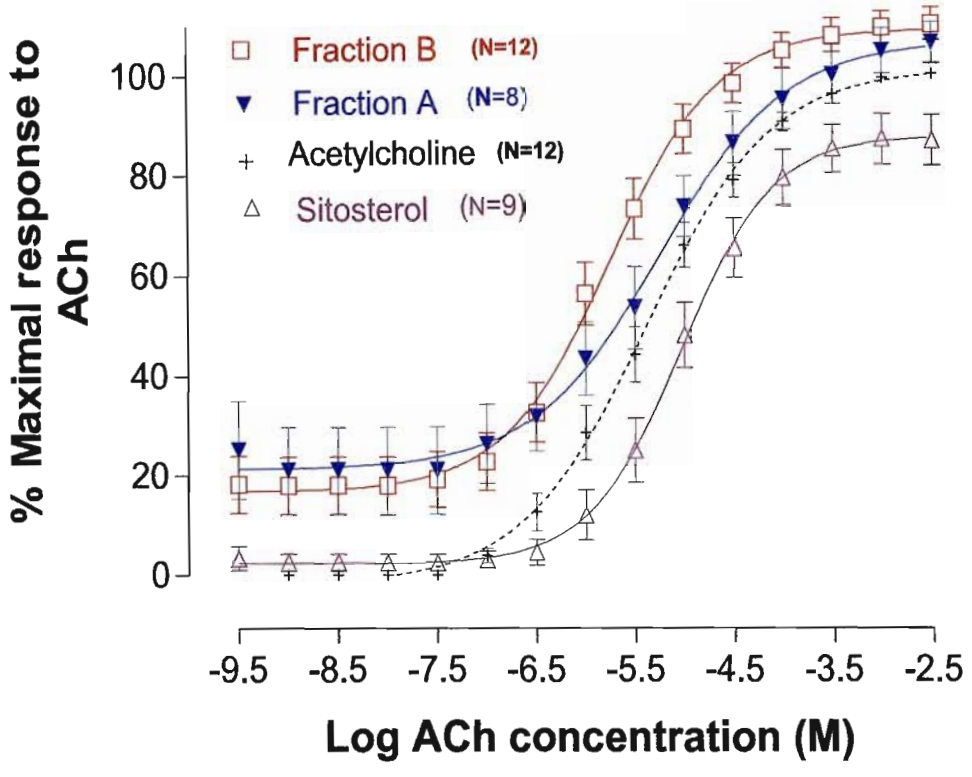


Figure 6.12 Dose response curves for *Rhoicissus tridentata* fractions A, proanthocyanidins, B, hydrogel and sitosterol. Isolated uterus was incubated with a solution containing 1.3 mg cm^{-1} of each plant fraction 5 minutes before the cumulative addition of acetylcholine. The curves are compared to acetylcholine alone.

6.4.1 *Combretum kraussii*

Preliminary investigations on crude aqueous extracts established that there was no significant difference in the size of the contractions stimulated by either *C. erythrophyllum* or *C. kraussii* extracts. Both species are known as *umdubu* and used in *isihlambezo* decoctions (Cunningham 1988), although *C. kraussii* is the species chosen locally in Kwa-Zulu Natal by healers. The direct activity of various crude extracts augmented the initial response to cumulative additions of acetylcholine, and the maximal response to acetylcholine was inhibited in all cases as illustrated for *C. kraussii* leaf and branch extracts (Figure 6.6). Branch extracts had a slightly greater stimulatory effect on uterine tissue than leaf extracts. Similar effects were obtained for *C. erythrophyllum* extracts.

The fractions isolated from *C. kraussii* roots displayed three distinct effects on the response of the isolated uterus to acetylcholine. The first group, phenolic glycosides compounds **10** and **11**, stimulated contractions of isolated uterine muscle directly which is illustrated by the first points on the curve, representing the response to plant fractions (Figure 6.7). A crude separation of **10** and **11** was achieved with solvents. Compound **10** was separated first from the concentrated water extract by addition of methanol/acetone (1:1). From the concentrated mother solution after removal of **10**, further addition of methanol/acetone (1:1) precipitated **11**, isolated as a khaki-yellow powder. HPLC in acetonitrile:H_2O (60:40 at 1.0 cm^3 min^{-1}) gave a retention time of 1.59 min for more polar **10** and 2.20 min. for **11**.

The contractions stimulated by both compound **10** and **11** were considerably larger than those caused by the crude hot water extracts, and neither fraction had an inhibitory effect on the maximal response to acetylcholine. Although the structures of **10** and **11** are not fully elucidated, it is proposed from existing evidence that they are ellagic acid glucosides. These two glucosides had a notable effect on uterine muscle, enhancing the reponse by up to 60% compared to that of acetylcholine alone. The fraction of **11** isolated as a powder gave the best response (Fig.6.7), whereas another sample of **11** isolated as a gum only enhanced the response of the uterus by about 40% enhancement. Presumably the gum was less pure than the powder.

The second group (Fig.6.8) compounds **1** (sitosterol), **4+5** (66% combretastatin B-1 and 22% combretastatin A-1) and **8+9** (approximately 1:1 mixture of combretastatin B-1 and A-1 glucosides) exhibited a range of direct uterotonic activity and also inhibited the maximal response to acetylcholine (Figure 6.3). This response indicates that although the

initial response is additive, at higher concentrations of acetylcholine the extracts inhibit the organ's response to acetylcholine in a non-competitive manner. This group of compounds are chemical « look-alikes » for oestrogens, and therefore possibly also sensitize the uterus in a similar way to the oestrogens themselves. The stilbene stilboestrol, discussed Section 6.2, was in fact commonly used as a synthetic oestrogen to sensitize uterine tissue prior to *in vitro* experiments, but has been recently replaced by oestradiol (Veale 1999). There is a noticeable difference between the effects of the **8** and **9** glucoside mixture and that of **4** and **5** combretastatin B-1/A-1 mixture, with the glucoside mixture augmenting the response of the uterus to a greater extent. Sitosterol (**1**) only showed a minimal stimulatory effect at low concentrations, being less than that of combretastatin B-1.

The third group (Fig.6.9), comprising the hot methanol and 1 :1 methanol:methylene chloride extracts, had no direct stimulatory effect on the uterine muscle and depressed the maximal response to acetylcholine (Figure 6.4). These results infer that these extracts act as non-competitive antagonists, and that they could be interfering with the activation of uterine cholinergic receptors in some way. The methanol and methanol:methylenechloride extractions make use of solvents not accessible to traditional healers who use hot water extracts. The action of these two extracts differs from the hot water extracts, in that they do not directly stimulate uterine contractions and have a marked inhibitory effect on the maximal response to acetylcholine. This fraction contains phytoestrogens such as sitosterol.

Preincubation of the organs with compounds **3** (trimethyl ellagic acid) and **7** (ellagic acid) did not alter the uterine response to acetylcholine *i.e.* these two compounds neither stimulated contractions directly nor inhibited the maximal response to acetylcholine. It is interesting to note that ellagic acid, and also trimethyl ellagic acid, have no noticeable effect on the uterus whereas the water-soluble glucosides of ellagic acid, **10** and **11**, have a marked effect. Possibly one reason is the very poor solubility of ellagic acid in water. Cardiac glycosides, drugs which strengthen heart muscle, consist of a steroid aglycone which is rendered soluble by a chain of sugar molecules, and the effects of **10** and **11** could be influenced by a similar phenomenon. However another factor could possibly be the direct involvement of the sugar moeity in binding to the receptor site. This is the case for the digitalis drug-receptor complex, which involves attachment of the lactone ring to site A, the steroid body to site B and the sugar residue closest to the steroid to site C of the receptor. This interaction of the sugar with site C on the receptor greatly increases the stability of the complex (Thomas 1981). Cardiac glycosides in nature contain 1 to 4

sugar moeities commonly drawn from 8 different sugars including D-digitoxose and D-glucose. Numerous cardiac glycosides have in fact also been chemically synthesised in the form of their β-D-glucosides for pharmacological testing (Wiesner *et al.* 1985). The water-soluble uteroactive glucosides described in this study might also have enhanced receptor binding via the glucose moeity. It was also noted, for example, that glucosides **8** and **9** exhibit greater uterotonic activity than their aglycones **4** and **5**. Although the aglycones in glucosides **8** and **9** are combretastatins, which are not steroidal in nature, many stilbene derivatives have oestrogenic properties as discussed in Section 6.2. In order to demonstrate this suggested role of glucose in increasing receptor binding a pharmaco-kinetic study of the purified active glucosides would need to be carried out in the future.

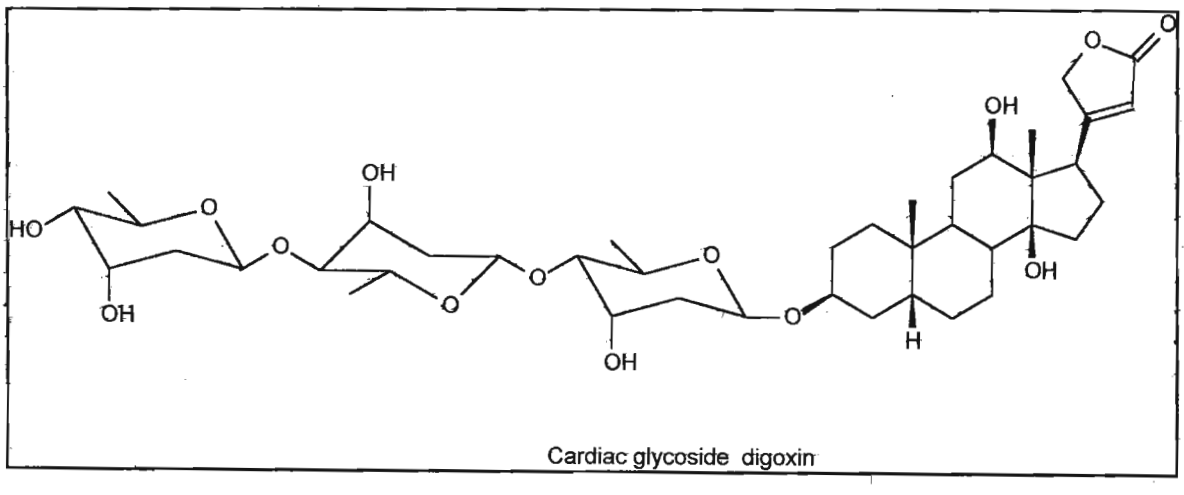


Figure 6.13 Cardiac glycoside

Extracts of the white and purple foxglove, *Digitalis purpurea and D. lantana*, have been used for over 3000 years to treat heart failure. Digoxin, one of the active plant ingredients, which are often collectively referred to as digitalis, is still amongst the top most frequently prescribed drugs (Hogue 1995). Digoxin (Fig 6.13) belongs to a group of cardiac glycosides, known as cardenolides, characterised by a 5-membered unsaturated lactone ring on one side of a steroid nucleus, and a carbohydrate moiety joined to the 3-β OH. Digoxin contains a chain of three β-linked digitoxose sugar residues in this position. In small doses it strengthens heart muscle, but in large doses it is a powerful heart toxin (Solomons 1992, Mc Murray 1996). Cardiac glycosides inhibit the membrane-bound enzyme that regulates Na^+/K^+ exchange, and thereby cause an increase in the intensity of the heartbeat. On membrane depolarisation intracellular Na^+ levels rise, leading to an action potential, which triggers the influx of Ca^{++} ions. Elevated intracellular Ca^{++}

concentrations result in a series of biochemical events that ultimately increase the force of myocardial contraction. However heartbeat irregularities frequently occur in patients taking digoxin, so the dosage must be carefully controlled.

6.4.2 *Gunnera perpensa*

Compounds **17** and **18** showed the greatest spasmogenic effect on uterine muscle tissue as shown in Figure 6.10. These compounds are more polar than compound **16**, Z-methyl lespedezate, and results of hydrolysis indicate that they are higher glucosides of this compound.

Hydrolysis of the most uteroactive fraction in *Gunnera perpensa* yielded p-hydroxybenzaldehyde, which is closely related to a lignin monomer. It has already been established that oxidation of lignin with alkaline nitrobenzene yielded p-hydroxybenzaldehyde, vanillin and syringaldehyde via cleavage of the double bond of the propyl side chain (Harborne 1964). It is probable that p-hydroxybenzaldehyde, obtained by hydrolysis of the active *Gunnera* fraction, is formed via oxidative hydrolysis of a phenylpropanoid double bond such as that in p-hydroxy cinnamyl alcohol. This type of hydrolysis is discussed in Chapter 1.

There is a close relationship between p-hydroxybenzaldehyde and the principal ingredients of *Filipendula Ulmaria (L.) Maxim*, or "meadowsweet" (Trickey 2003). Flower extracts of this plant have a high tannin content, various medicinal properties, and are prescribed during pregnancy. The flowers and plant have an almond-like odour, faintly reminiscent of methyl salicylate. The taste is astringent and bitter. Amongst the known active ingredients of the flowers are phenolic glycosides, salicylaldehyde (75%) anisaldehyde (2%) and methyl salicylate (1.3%). The phenolic glycosides yield salicylate aglycones which contribute to the anti-inflammatory and diuretic action of *Filipendula* extracts. Extracts of *Filipendula* flowers increased the tone of isolated strips of smooth muscle taken from guinea pig ileum, as well as that from rabbit intestine and uterus. Although this plant has no known toxic effects, and is considered safe in pregnancy and lactation, alcohol and water extracts of the flowers have shown a central nervous system depressant effect in mice. Topical application of an ointment prepared from *Filipendula* flowers to the cervix was found effective in the treatment and prevention of uterine cervical cancer for 67% of the patients tested.

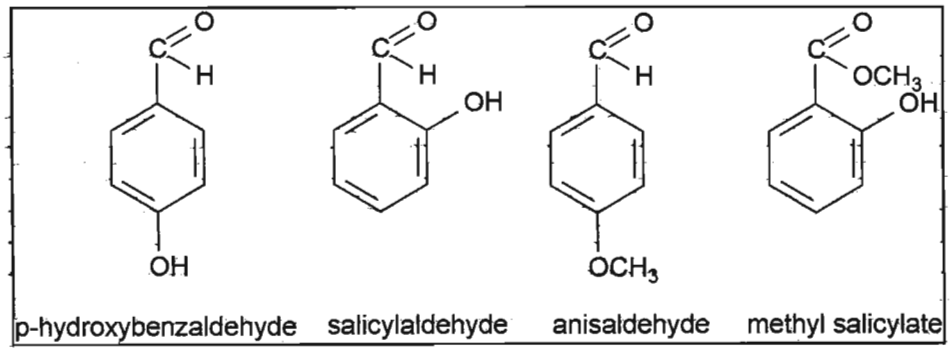


Figure 6.14 Bioactive compounds from *Gunnera perpensa* and *Filipendula Ulmaria*.

6.4.3 *Rhoicissus tridentata*

The response of the uterus to extracts prepared with different solvents is seen in Figure 6.11. Roots harvested in October in Kwa-Zulu Natal were successively extracted with methylene chloride, methanol and finally water. The phenolic compounds extracted by methanol are also water-soluble, but methanol is a more efficient solvent for their extraction from plant material. It is noteworthy that the water extract, once the non-polar components have been removed, only enhances spasmogenic activity over the whole range of concentrations tested, with no inhibition of the maximal response to ACh at all. Two purified water-soluble fractions, Fraction A and Fraction B, accounted for the overall activity of aqueous extracts (Fig. 6.12)

Fraction A, compounds **19** to **30**, contains eleven proanthocyanidin monomers and dimers, as well as gallic acid (Section 5.2). Figure 6.12 illustrates the stimulatory effect this fraction had on rat uterine tissue compared to that of sitosterol, also found in *C. kraussii* extracts. The proanthocyanidins found in Fraction A consist of flavanol units (Section 1.3.3) which are structurally similar to the related isoflavones, the most well-known class of phytoestrogens (Section 6.3). Phytoestrogens functionally mimic mammalian oestrogens, and this could account for the notable uteroactivity of Fraction A.

Proanthocyanidins are amongst the most powerful antioxidants in nature and have well documented health benefits. The polymeric proanthocyanidins, or condensed tannins, also produce astringent effects (Czochanska et al 1980) which can account for their stimulatory action on uterine tissue. There are many references to the excellent general effect of tannins on female organs and the uterus during pregnancy (Section 6.3).

The hydrogel Fraction B, compound **31**, stimulated uterine contractions over the full range of ACh concentrations (Figure 6.12). Although the structure is not fully illucidated,

Fraction B gave no colour with iodine solution, so is clearly different to the amylose starch polymer which has α-1, 4-glucoside linkages. Various hydrogels, such as the natural gels lining the intestine and uterus, play a direct role in muscle contraction. Cervical mucous, for example, is a complex hydrogel which is important in sperm transport (www.das.psu.edu). A common property of all gels is the ability to undergo abrupt changes in volume in response to small changes in external conditions, *e.g.* chemical or electrical changes. These can cause the gel to swell or shrink as much as 1000 times. Rates of contraction and consequent forces generated by some synthetic gel fibres approximate those of human muscle (www.ai.mit.edu). These contractile materials are the subject of recent investigation, with potential as "artificial muscle" in robotic devices.

Addition of acetylcholine to uterine tissue in the presence of hydrogel B is probably the chemical stimulus leading to gel contraction and enhancement of uterine contractility. It is my opinion, however, that a polymer such as Fraction B is unlikely to be absorbed through the intestinal wall, so should not reach the uterus when administered orally in medications. A water-soluble glucoside from *Cissus quadrangularis*, also from the Vitaceae family, is reported as non-toxic when administered orally to mice, rats and guinea-pigs. However it caused convulsions and death within 5 minutes of intravenous administration. (Hutchings 1996). Possibly this glucoside is similar to Fraction B, and is hydrolysed to some extent during digestion, whereas it might over-stimulate muscle tissue when entering the blood-stream directly. Fraction B warrants further investigation.

Sitosterol showed minimal effect on the uterus (Fig.12) as did its glucoside, for which an almost identical curve was obtained. There is some disagreement in the literature about sitosterol, which is reported to exert oestrogenic effects, possibly even causing abortion (Neuwinger 1996). However sitosterol and its glucoside are also claimed to be inactive on uterine tissue or inhibit spasmogens (Neuwinger 1996, Bejar *et al*, 1995, Dasgupta and Pandev 1970, Khan and Schoeb 1985). Results in this research suggest that the concentration of acetylcholine used for tests is the deciding factor as to whether there is a slight stimulation or inhibition. Possibly at higher acetyl choline concentrations there is competition for receptor sites.

Although compound **35**, oleanolic acid, was not isolated in sufficient quantities for uterine tests, closely related compounds *epi*-oleanolic acid and oleanonic acid from *Ekebergia capensis* have uterotonic properties (Section 6.3). The presence of oleanolic acid in *Rhoicissus tridentata* extracts should therefore contribute positively to the overall uteroactivity of the extracts.

CHAPTER 7

NUTRIENT METABOLITES

The role of nutrient metabolites, calcium and potassium ions, commonly found in plant extracts, is considered in regard to muscle contraction in Section 7.1. In Section 7.2 the concentrations of nutrients that could be dietary supplements found in the root extracts is presented.

7.1 *ROLE OF CALCIUM AND POTASSIUM IONS IN MUSCLE CONTRACTION*

Muscle contraction involves a complex series of inter-related steps, as in all biological processes, and is brought about by changes in the configurations of proteins in the muscle cell. Calcium (Ca) ions play a vital role in this regulatory process (Becker *et al* 1996) and when an action potential is generated via a neurotransmitter, calcium ions are released to bring about muscle contraction. When calcium levels are lowered again the muscle relaxes. Potassium (K) ions are also involved in the process, which is briefly outlined below.

Muscle cell fibres, or myofibrils, consist of repeating units made up of alternating thick and thin parallel filaments. The thick filament contains the protein myosin, and the thin filament consists mainly of the protein actin. In muscle contraction these two filaments slide over one another (Campbell 1995; Mc Murry and Castellion 1996). The mechanism for this movement is provided by myosin, principally a fibrous protein, but which has globular portions which project from the thick filament and attach as cross-bridges to actin to form an actomyosin complex. Once cross-bridges between the thick and thin filaments are present, each globular projection tilts (the "power" stroke) then detaches from the actin site and moves to a new site. The energy required for the muscle contraction process is provided by the hydrolysis of ATP.

Muscle cells have the ability to raise and lower calcium levels quickly in the cytosol around the myofibrils. Calcium's activity is linked to the two other proteins in the thin filament, namely troponin C and tropomyosin. Effectively these two proteins unblock the binding sites on actin so these sites are then accessible to myosin globular heads. For most cells in their resting state the calcium ion concentration in the cytosol within the cell is maintained at low levels of about 10^{-4} mM by the action of calcium pumps. At calcium

ion levels of less than 10^{-4} mM tropomyosin blocks the binding sites on the actin filament, and prevents their interaction with myosin. Thus the cross-bridge due to the formation of the actomyosin complex is inhibited, and the muscle is relaxed. The calcium ion concentration in the extra-cellular fluid is about 1.2 mM, or more than 10000 times higher than in the cytosol. As a result when calcium channels in the cell wall open in response to various stimuli, calcium ions rapidly enter the cell. Calcium ions released from intracellular stores can also assist in raising calcium levels at this stage. At concentrations greater than 10^{-3} mM calcium ions trigger a conformational change in troponin C, then in tropomyosin. It is this conformational change in tropomyosin that unblocks the actin site and initiates muscle contraction involving movement of the myosin filament with respect to the actin filament.

Potassium ions also play a role in muscle contraction and nerve conduction because of their effect on the membrane potential of the cell (Becker *et al*, 1996). An impulse is transmitted along a nerve cell by the exchange of positive and negative ions across the cell membrane. Cells at rest normally have an excess of negative charge inside and an excess of positive charge outside the cell. This is called the resting membrane potential. Muscle cells exhibit electrical excitability *i.e.* certain stimuli trigger a rapid sequence of changes in the membrane potential; this is known as action potential. Action potentials arise at neuromuscular junctions when acetyl choline (ACh) is released from the presynaptic membrane of the motor neuron. When ACh binds to its receptors in the postsynaptic membrane the gated ion channels of the receptor open to allow sodium ions across the membrane (Mc Murry and Castellion 1996). During an action potential the membrane potential changes from negative values (resting potential) to positive values and then back to negative values again in little over a millisecond. The anions in the cytosol are mainly macromolecules such as proteins, RNA and other molecules not present outside the cell. These negatively charged molecules cannot pass through the cell wall and are the main reason that cells develop a negative resting potential. In the cytosol potassium ions serve as counter-ions for these trapped anions. Cells exhibit a potassium ion gradient, having a higher potassium ion concentration inside the cell than in the extra-cellular fluid, where sodium is the dominant cation. This concentration gradient is maintained by active transport across the cell membrane due to the action of the sodium-potassium pump. This results in a K^+ ion concentration of approximately 100 mM in the cytosol, roughly 20 times higher than that in the extra-cellular fluid, which is only about 5 mM. The corresponding Na^+ ion concentrations are approximately 10 mM in the cytosol and 140 mM in the extra-cellular fluid (Mathews and von Holde 1990).

Once the neurotransmitter ACh has done its work in initiating an action potential it rapidly disengages from the receptor so that the postsynaptic neuron is ready to receive another impulse.

7.2 *NUTRIENT METABOLITES ISOLATED FROM RHOICISSUS TRIDENTATA, COMBRETUM KRAUSSII AND GUNNERA PERPENSA.*

The results of measurement of ion concentrations are shown below, and percentages are expressed per dry root mass for both tables. The moisture contents of the roots were:

Rhoicissus tridentata: 22.2%; *Gunnera perpensa*: 81.0%; *Combretum kraussii*: 13.0%.

Table 7.2.1 Nutrient ions from whole ashed root samples.

Root	Calcium %	Iron %	Sodium %	Phosphate %
Rhoicissus tridentata, Treasure Beach, Nov.'96	2.4	1.6	0.3	0.3
Gunnera perpensa, Silverglen, Apr. '96	0.4	0.4	1.5	0.4
Combretum kraussii, Pigeon Valley, June '95	0.7	0.1	0.3	0.1

Table 7.2.2 Nutrient ions in aqueous solutions of root samples (%)

	Calcium	Iron	Phosphate	Potassium
Rhoicissus tridentata	0.02	<0.01	0.04	0.54
Gunnera perpensa,	0.04	0.02	0.12	<0.01
Combretum kraussii	0.06	<0.01	0.04	<0.01

Results varied according to the source of each plant root *e.g.* a *Gunnera perpensa* sample obtained from a Herbal Shop at 22 Commercial Rd, Durban had only 0.1% Ca^{++} ions in the whole ashed root compared to 0.4% in the sample from Silverglen Nursery. There is a marked drop in the concentrations of all ions in aqueous solution compared to

those where the whole root sample was ashed. This could be due to compounds of low solubility containing the relevant ion, or incomplete extraction. The roots in these analyses were shredded in a garden shredder. Milling the roots might possibly result in more efficient extraction, however traditional healers generally chop roots, and do not mill them.

Crystals of KCl separated and were identified from *Rhoicissus tridentata* extracts and the counter-ion was established by Volhard titration to be chloride for one of the isolated crystal samples which contained 90% K^+, determined via AA. A subsequent measurement showed 0.54% K^+ ions or 1.04% KCl in an aqueous root extract, expressed as a percentage of dry roots harvested in the Umlazi area, Kwa-Zulu Natal, in October 1996. Although levels of potassium ions were relatively high in extracts of this plant, they were negligible in *Combretum kraussii and Gunnera perpensa* extracts. The concentration of calcium in the *Rhoicissus tridentata* ashed root sample was also higher than that of the other two plants.

Calcium and potassium ions and muscle contractility

Calcium and potassium ions play a crucial role in muscle contractility, as discussed in Section 7.1, so the high levels of these ions might contribute directly to enhancing the reponsiveness of uterine muscle tissue. They can be used as standard agonists in isolated organ work, for example by increasing their concentrations in the physiological salt solutions (Veale J 1999). Their concentrations in the organ baths during uteroactivity tests were therefore considered with this in mind. However results from isolated organ tests indicate that the concentrations of these two ions in several *isihlambezo* extracts studied fall below those necessary to stimulate uterine muscle contraction directly. Two such studies are shown below.

In a study of *Rhoicissus tridentata* the concentrations of Ca^{++} and K^+ ions in an extract of roots harvested in Umlazi, Kwa-Zulu-Natal, added to the organ baths, were found to be 0.15 mM and 0.96 mM respectively (Katsoulis 2000). The maximal volume of plant extract added to the organ bath was 1.3 cm^3 in 50 cm^3 Tyrode solution. Therefore, after dilution in the organ bath, the resultant Ca^{++} and K^+ ion levels would only have been 0.0039 mM and 0.0250 mM respectively, and were considered too low to have any direct effect on the isolated smooth muscle preparation, as established with standard ion solutions. These levels are also far below those found in plasma, previously mentioned in Section 7.1 This indicates that contractions were stimulated by pharmacolgically active ingredients within

the plant extract, and not by the direct action of ions in the organ bath solution (Katsoulis 2000). Calcium and potassium ion levels in three other plants, *Agapanthus africanus, Pentanisia prunelloides and Gunnera perpensa,* used in pregnancy-related herbal remedies, were also measured in the organ baths (Kaido *et al* 1997). The concentrations were also found to be too low to cause contraction of the isolated uterus tissue.

Extracts of two of the three plants in the current research therefore show no direct effect on isolated uterus according to the above studies. Although *Combretum kraussii* extracts were not tested it is unlikely that they would exert a direct effect either, because ion levels were comparable and low in aqueous extracts of all three plants, as seen in Table 7.2.2. However the presence of these ions in healers' decoctions of the three plants studied possibly contributes to the overall uterine contractility, in conjunction with the effects of the uteroactive chemicals.

Nutrient metabolites in the diet

Pregnant women often take dietary supplements in order to ensure good foetal development. The recommended daily intake of minerals for the maintenance of good nutrition in the U.S.A., for example, is calcium 900 mg, phosphorus 900 mg and iron 12 mg (Mc Murry and Castellion 1996). Recent evidence suggests that the greatest effects of supplementation occur when this is given in the first 3 months of pregnancy, or preferably before conception. It has even been suggested that a health-promoting diet should be followed for up to six months before conception, to maximize benefits. However there is controversy about certain supplements, for example iron, that is presently recommended in much obstetric care. A low iron status could merely indicate a generally inadequate diet. It is felt that by focusing on specific nutrients an imbalance might result, and it would be better to improve the diet as a whole. Therefore a modern trend is not to recommend increasing the amount of certain nutrients such as calcium or iron by supplementation (Barasi 1997).

Isihlambezo is generally given in the last trimester of pregnancy, so considering the above information, it probably does not play a significant role in terms of nutrients for the developing foetus. Also, levels of nutrients were much lower in extracts than in ashed plant material, as seen in Tables 7.2.1 and 7.2.2, so would not be present in medicinal solutions in significant amounts.

In addition to the dissolved ions determined in this section, the chemical investigation of *Combretum kraussii, Gunnera perpensa* and *Rhoicissus tridentata* uncovered a wealth of compounds with documented benefits for good health, which are briefly outlined here.

Sugars, found in all three species of plant, could be a source of energy and nutrition, particularly for women on a poor diet. Glucosides and some free glucose were present in extracts, with free glucose being notable in *Rhoicissus tridentata.*

Stilbenes and bibenzyls in the combretastatin category in *Combretum kraussii* are used as cancer therapeutic drugs. Triterpenoids, found in *Rhoicissus tridentata* have anti-inflammatory and molluscicidal properties. The phenolic constituents in *Gunnera perpensa* play a defensive role against various pathogen populations, which is in line with the strong antiseptic and antibiotic properties described for the traditional medicine *ugobo*. Ellagic acid derivatives, found in both *Combretum kraussii* and *Gunnera perpensa*, have anti-mutagenic, anti-carcinogenic and anti-haemorrhagic properties.

Rhoicissus tridentata was found to have an abundance of proanthocyanidins, amongst the most potent anti-oxidants in nature. They have documented health benefits for the heart, cardiovascular system and immune system, and also give protection against unfavourable lipid profiles.

Sitosterol and sitosterolin, found in *Combretum kraussii* and *Rhoicissus tridentata*, have an apparent stimulatory effect on the immune system, and a prophylactic effect on a variety of diseases (Pegel 1997). The fact that diets rich in fruit and vegetables promote health by reducing the risk of cancer, cardiovascular disease, diabetes and other common ailments, is well established via epidemiological studies. Besides the common disease-preventing agents found in plants, such as vitamins, many studies also report that plant constituents such as sitosterol may contribute to the observed health-promoting effect of plant diets.

CHAPTER 8

CYTOTOXICITY STUDIES

Introduction

This study was carried out to establish the toxicity of *Gunnera perpensa, Combretum kraussii* and *Rhoicissus tridentata* extracts. Aqueous extracts of these three species of plant roots are commonly used in pregnancy related traditional medicines or *isihlambezo*, and therefore have health implications for both the developing foetus and mother.

Approximately 80% of Zulu and Xhosa women in South Africa use traditional medications during pregnancy therefore a knowledge of the toxicity of plants commonly chosen by traditional healers for pregnant black women is of vital importance in assessing the potential impact they might have on the health of both the developing foetus and mother. Great care clearly needs to be exercised in the administration of drugs to pregnant women. Although the primary function of *isihlambezo* is to ensure adequate foetal growth and well-being and promote general maternal health and a easy labour, there have been some reports which raise concern about health aspects related to certain of the 60-90 plant species used in *isihlambezo* mixtures.

Rhoicissus tridentata extracts have been linked to a number of fatalities (Watt and Breyer-Brandwyk 1962 ; Brandt and Muller 1995). *Rhoicissus tridentata* extracts administered to rabbits caused death, although solutions were injected directly into the bloodstream, which is not the mode of administration of traditional medicines, which are taken orally (Hutchings 1996). The death of swine in Tanzania has also been attributed to ingestion of *Rhoicissus tridentata* tubers (Hutchings 1996). In a survey of approximately 4000 patients hospitalised in Ga-Rankuwa Hospital due to poisoning, *Rhoicissus tridentata* was cited as one of the toxic species in the traditional medicines category of poisoning. Central nervous system toxic effects such as respiratory failure were observed as well as gastro-intestinal and renal problems (Brandt and Muller 1995).

The fruits of the genus *Combretum* are widely regarded as toxic and are not consumed by wild animals or used by healers. However there is only one case in 1970 where the toxicity of the fruit has been clinically proven There has also been a more recent report in 1996 that five women in Zimbabwe died after vaginal insertion of material from *Combretum erythrophyllum*, from the same genus of the Combretaceae family as *Combretum kraussii* (Rogers and Verotta 1996). A cytotoxicity study of the fruit of 15 *Combretum* species showed that certain non-aqueous extracts were toxic to the brine

shrimp, with 4 species exhibiting toxicity. (Verotta and Rogers 1997). Acidic triterpenoids and their glycosides were identified in these extracts by chromatography. The butanol extract from *Combretum kraussii* was one of the toxic species. However the organic solvent butanol is not used by healers, who extract plants with boiling water. There are no reports to my knowledge concerning any toxicity related to *Gunnera perpensa* preparations. A survey in the 1980's by the National Cancer Institute uncovered an interesting series of stilbenes and bibenzyls from *C. caffrum* with notable cytotoxicity against various cell lines and therefore great pharmceutical potential. Several of these compounds were also found in the related species *C. kraussii* (Brookes *et al* 1999; Rogers and Verotta 1996).

There are many reports of plant toxicity in addition to those discussed above. For example lycorine is a toxic alkaloid present in *Clivia miniata*, a species frequently chosen for *isihlambezo* mixtures. Atropine and related alkaloids in *Datura stramonium* or « stinkblaar » are toxic due to their muscarinic receptor-blocking action (Brandt and Muller 1995). The toxic effects of 16 of the 93 species used in *isihlambezo* mixtures is discussed in Chapter 1, as well as their adverse effects on the birth process, such as uterine hyperstimulation (Veale 1992). One of these 16 toxic species, *Callilepis laureola*, has resulted in many deaths, particularly among black children, with reported symptoms of liver necrosis, hypoglycaemia and renal failure (Wainwright *et al* 1977). This plant was the topic of previous research (Brookes 1979) in which four toxic atractylosides, two being novel, were uncovered, as well as two novel thymol derivatives (Brookes *et al* 1983; Brookes *et al* 1985). *Callilepis laureola* was consequently banned by the Department of Health from use as a herbal medicine, but despite this it is still cited in *isihlambezo* decoctions (Veale 1992). An enzyme immunoassay was developed for the detection of atractyloside in tissues and physiological fluids, using an ELISA system (Bye *et. al.* 1990).

This study was carried out in order to establish whether the three plants chosen exhibited any significant toxic effects at a cellular level. There has recently been a move away from *in vivo* rodent models towards *in vitro* models for drug evaluation. Although *in vitro* methods cannot simulate the interaction of the tested component with the whole living organism, they do offer a quick and efficient means of establishing responses at a cellular level which can serve as an indicator for the response of the whole organism. The present study, using monkey vero and human fibroblast cell lines, established cell viabilty levels in the presence of varying concentrations of *Gunnera perpensa, Combretum kraussii* and *Rhoicissus tridentata* root extracts (Brookes and Smith 2003).

Results and discussion

The two types of cell lines used were monkey vero cells, known to be sensitive to toxins, and human fibroblasts. Cells were exposed to aqueous extracts of freeze-dried plant material at concentrations ranging from 500 μg/ml to 8μg/ml. Cell viabilty levels were established via a colorimetric assay involving staining of non-viable cells with tryphan blue reagent (Table 8.1 and Figures 8.1 and 8.2). At comparable concentrations *Gunnera perpensa* extracts produced the least and *Rhoicissus tridentata* extracts the most cell deaths for both types of cells. The threshold for zero cell deaths occurred for monkey vero cells at the following concentrations : *Gunnera perpensa* 250 μg/ml ; *Combretum kraussii* 67μg/ml ; *Rhoicissus tridentata* 8 μg/ml. At these concentrations it was found that 100% of human fibroblast cells also survived. These results indicated that none of the extracts could be considered toxic for human consumption (personal communication, Prof. A. N. Smith, Head of Virology Department, University of Natal).

The morphology of cells observed under the microscope differed for the three plants. Dead cells which had been exposed to *Gunnera perpensa* extracts appeared normal, whereas those exposed to *Combretum kraussii* extracts exhibited cytopathic effects. Those in contact with *Rhoicissus tridentata* extracts showed vaculations and crenations indicating that the cells were producing lysosomes.

In order to establish whether the concentrations of ingredients in the healers' solutions were comparable to these non-toxic levels, the amounts of dissolved solids in typical medicinal solutions were calculated for each chopped or milled plant root, as well as the estimated concentration after dilution in the bloodstream, assuming a blood volume of 4545 ml (Table 8.2). The dose of these medications is one tablespoon, three times daily or 45 ml total per day, so there is a dilution factor of approximately 100 on entering the blood stream. Compounds are considered to be cytotoxic when they kill 50% or more cells. It is therefore evident, when taking into account dilution in the bloodstream, that all three plant extracts should be regarded as completely non-toxic, as their concentrations fall below those resulting in zero cell deaths. Furthermore another roughly 10-fold dilution would occur throughout the various organs and tissues, leading to the conclusion that these plant extracts should be considered non-toxic at a cellular level.

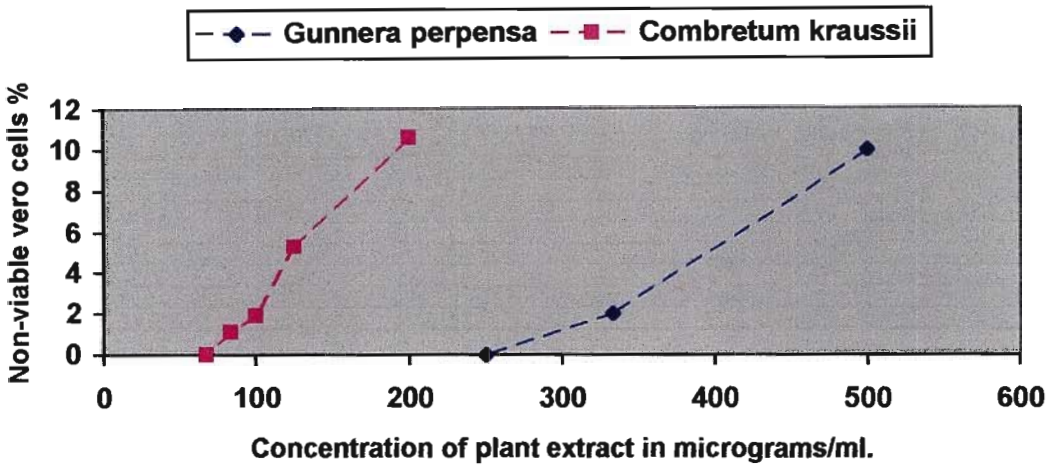


Figure 8.1. Viability of monkey vero cells exposed to varying concentrations of Gunnera perpensa and Combretum kraussii root extracts.

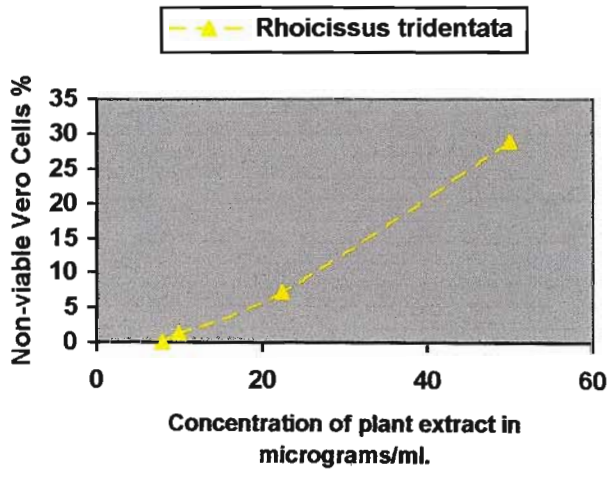


Figure 8.2. Viability of monkey vero cells exposed to varying concentrations of Rhoicissus tridentata root extract.

Table 8.I. Cytotoxic effects of plant root extracts against the vero cell line.

Plant root	Concentration µg/ml	Non-viable vero cells %
Gunnera perpensa	500	10.0
	333	2.0
	250	0
Combretum kraussii	200	10.6
	125	5.3
	100	1.9
	83	1.1
	67	0
Rhoicissus tridentata	50	28.9
	23	7.1
	10	1.2
	8	0

Table 8.2. Concentration of plant extracts in healer's solution and in bloodstream.

Plant Root	Typical concentration in healer's solution µg/ml	Zero toxicity level µg/ml	Estimated daily dose in bloodstream µg/ml
Gunnera perpensa	464	500	4.6
Combretum kraussii	900	67	8.9
Rhoicissus tridentata	617	8	6.1

None of the three plants tested is considered toxic in terms of human consumption based on these results, but it is recommended that *Rhoicissus tridentata*, which caused the most cell deaths, be used medicinally with caution. In addition to these cell viability results, it has also been found that *Rhoicissus tridentata,* together with *Clivia miniata* "umanyimi" and *Agapanthus africanus* "ubani", significantly augment the initial response of the uterus to oxytocin, and therefore must be considered to have the potential to cause uterine hyperstimulation and its associated toxicity (Veale *et al* 1998). Since concentrations of uteroactive ingredients in *Rhoicissus tridentata* increase in the summer and autumn seasons and there are also significant regional differences in its uterotonic activity

(Katsoulis 1999 ; Katsoulis *et al* 2002), traditional healers need to be aware of these variations in the light of possible uterine hyperstimulation, and alter dosages accordingly.

The vast amount of research by pharmaceutical companies regarding the safety of drugs for pregnant women is in stark contrast to the paucity of knowledge about herbal remedies, even though the latter are used by the majority of women in South Africa. An international workshop on the use and safety of medicinal herbs recommended that these therapeutic herbs should be tested, like other pharmceuticals, to characterise their acute and chronic toxicities (Matthews *et al* 1999). Herbal medicines are used world-wide by millions of people and some countries such as China and the U.S.A. monitor the use of these medicines, as discussed in Chapter 1. Scientific investigation and regulation of traditional medicines, as well as formal governmental recognition of their valuable role in health-care, would greatly improve the effectiveness and status of the centuries-old rich heritage of traditional medicine practice in South Africa.

CONCLUSIONS

The thirty-eight compounds isolated serve to remedy the lack of knowledge concerning the chemicals present in *Combretum kraussii*, *Gunnera perpensa* and *Rhoicissus tridentata*, widely used in pregnancy-related traditional medicines. The majority of compounds are reported for the first time in the species concerned, and many showed uterotonic properties, with proanthocyanidins and phenolic glucosides stimulating uterine muscle to the greatest extent. A method was developed for the rapid determination of the relative amounts of proanthocyanidins, as these vary seasonally. The most active components in all three species stimulated contractions of isolated uterine muscle directly, and showed no inhibition of the maximal response to acetylcholine. It is proposed that the observed activity of glucosides in this study might possibly be explained in a similar way to that of digitalis on heart muscle tissue. The sugar moiety, and also the steroid section of digitalis, are directly involved in binding to the receptor site, and this greatly stabilises the drug-receptor complex. Pharmaco-kinetic studies would need to be carried out in future to establish whether the digitalis drug-receptor complex is similar to those of the glucosides isolated in this research.

Ellagic acid glucosides have the greatest uterotonic activity in *Combretum kraussii*. Combretastatin B-1 and A-1 mixtures were moderately stimulatory, with the combretastatin B-1 and A-1 glucoside mixture enhancing contractility to a greater extent than the free aglycone mixture. Several bioactive compounds, such as ellagic acid and the combretastatins, have structure-activity relationships to natural oestrogens. Extracts using non-polar solvents, had no direct stimulatory effect on the uterine muscle and depressed the maximal response to acetylcholine, indicating that they act as non-competitive antagonists. Compounds in *Gunnera perpensa* with the greatest uteroactivity are higher glucosides of Z-methyl lespedezate, with partially elucidated structures. The uterotonic hydrogel in *Rhoicissus tridentata* has similarities to the natural gels lining the uterus, which play a direct role in muscle contraction. The uteroactive proanthocyanidin fraction contained 74% polymeric material, known for astringent effects. The uterotonic nature of this fraction could be explained by the fact that it consists of flavanol units which are structurally related to the isoflavones, the most well-known class of phytoestrogens, which functionally mimic mammalian oestrogens. Structures of certain uterotonic compounds were not fully elucidated, and further research is required in this area.

Cytotoxicity tests using two different cell lines indicated that the three species are safe for human consumption, supporting healers' claims regarding *isihlambezo* medications. It is

recommended that *Rhoicissus tridentata* be used with caution however, because of some reported toxic effects due to uterine hyper-stimulation. There is a seasonal variation in the uteroactive proanthocyanidins in this species, which should be considered in regard to the preparation of herbal medicines. *Rhoicissus tridentata* preparations are also reported to cause CNS depressant paralysis. This is possibly due to the action of sterols such as sitosterol or sitosterolin and proanthocyanidins, which are known to affect nerve signal transmission and receptor binding.

The concentrations of dissolved ions regarded as beneficial during pregnancy such as calcium, iron and phosphate were determined in extracts. The concentrations of calcium and potassium are regarded as too low to directly cause contraction of the isolated uterine muscle tissue. However the presence of these ions in healers' decoctions of the three plants studied possibly contributes to the overall uterine contractility, in conjunction with the effects of the uteroactive chemicals.

In addition to the dissolved ions, found present in low levels, the chemical investigation of *Combretum kraussii, Gunnera perpensa* and *Rhoicissus tridentata* uncovered a wealth of compounds with documented benefits for good health. Sugars, found in all three species of plant, could be a source of energy and nutrition, particularly for women on a poor diet. Glucosides and some free glucose were present in extracts, with free glucose being notable in *Rhoicissus tridentata*.

Rhoicissus tridentata was found to have an abundance of proanthocyanidins, amongst the most potent anti-oxidants in nature. They have documented health benefits for the heart, cardiovascular system and immune system, and also give protection against unfavourable lipid profiles. The phenolic constituents in *Gunnera perpensa* play a defensive role against various pathogen populations, which gives support to reports of strong antiseptic and antibiotic properties described for the traditional medicine *ugobo*. Ellagic acid derivatives, found in both *Combretum kraussii* and *Gunnera perpensa*, have anti-mutagenic, anti-carcinogenic and anti-haemorrhagic properties. Several combretastatins identified in *Combretum kraussii* are credited with potent anti-cancer properties. Phytosterols such as sitosterol and its glucoside sitosterolin, found present *Combretum kraussii* and *Rhoicissus tridentata*, are reported to boost the immune system, and in fact are marketed commercially in this capacity. Triterpenoids, found in *Rhoicissus tridentata* have anti-inflammatory and molluscicidal properties.

Collectively the above-mentioned classes of compounds could be of great benefit for the mother and developing foetus. Results validate traditional healers' claims that *isihlambezo* decoctions promote foetal and maternal good health and a quick and easy labour. Because of the potential impact of *isihlambezo* on both the foetus and mother, and the fact that millions of women in this country use these medications, it is recommended that further investigation of *isihlambezo* herbal medicines is undertaken. Standard dosages according to species, toxicity and seasonal variation of components could consequently be recommended by the Traditional Healers' Association in order to increase the safety and efficacy of these medications.

APPENDIX: SPECTRAL DATA

Combretum kraussii

1	Sitosterol	CI-MS, IR, ^{1}H NMR, ^{13}C NMR. Acetate: IR.	160
2	Combretastatin	FAB-MS, IR, ^{1}H NMR, ^{13}C NMR. Acetate: FAB-MS, IR, ^{1}H NMR, ^{13}C NMR.	165
3	Trimethylellagic acid lactone	IR, ^{1}H NMR. Acetate: IR, ^{1}H NMR.	173
4	Combretastatin B-1	IR, ^{1}H NMR, ^{13}C NMR, DEPT, GC-MS.	177
5	Combretastatin A-1	GC-MS.	182
6	Dimethylellagic acid	IR.	183
7a	Ellagic acid lactone	FAB-MS, IR. Acetate: FAB-MS, EI-MS, IR.	184
7b	Ellagic acid open form	IR.	189
8+9	Combretastatin B-1/A-1 Glucosides	FAB-MS, ^{1}H NMR.	190
10+11	Ellagic acid glucosides	IR. Methylated Fr.7: FAB-MS.	192

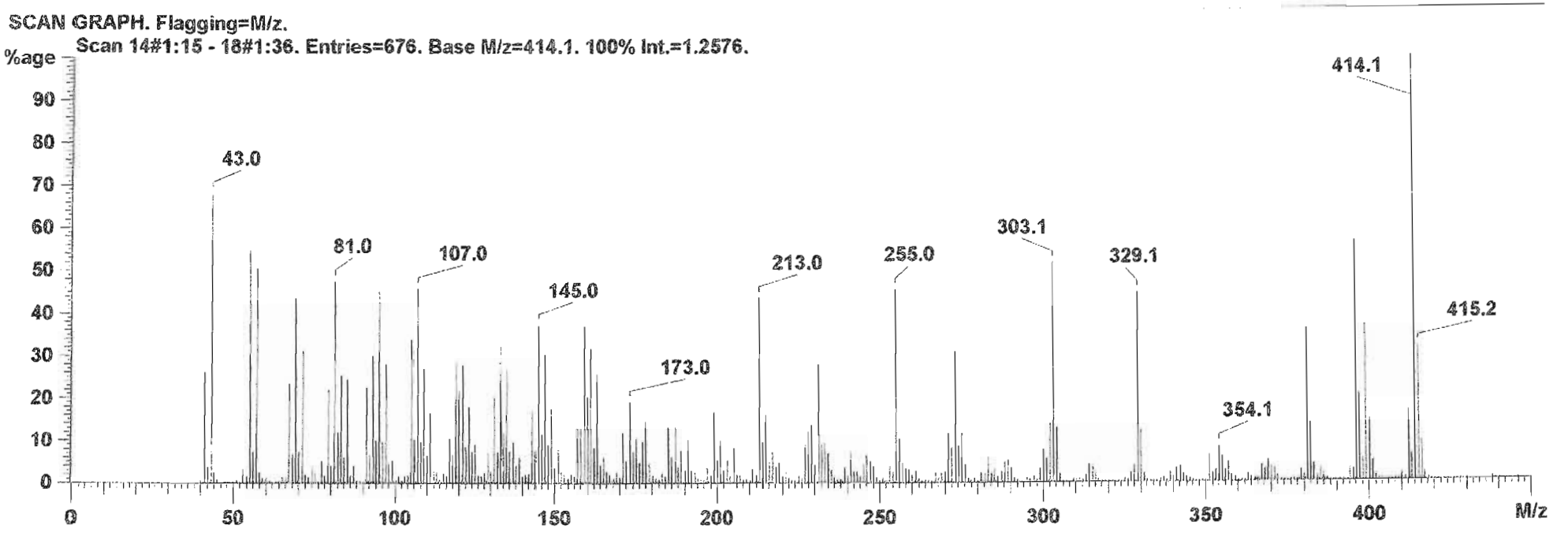
Gunnera perpensa

12	Trimethylellagic acid lactone	Spectra as for compound **3**.	
13	Ellagic acid lactone	Spectra as for compound **7a**.	
14	1,1'-biphenyl-4,4'-diacetic acid	IR, ^{1}H NMR.	194
15	p-hydroxybenzaldehyde	IR, ^{1}H NMR.	196
16	Z-methyl lespedezate	EI-MS, IR, ^{1}H NMR. Acetate: FAB-MS, IR, ^{1}H NMR ($CDCl_3$ and CD_3OD), HMBC, COSY, ^{13}C NMR.	198
17+18	Higher glucosides of Z-methyl lespedezate	IR. Methylated Fr.9: FAB-MS, ^{1}H NMR, ^{13}C NMR.	208

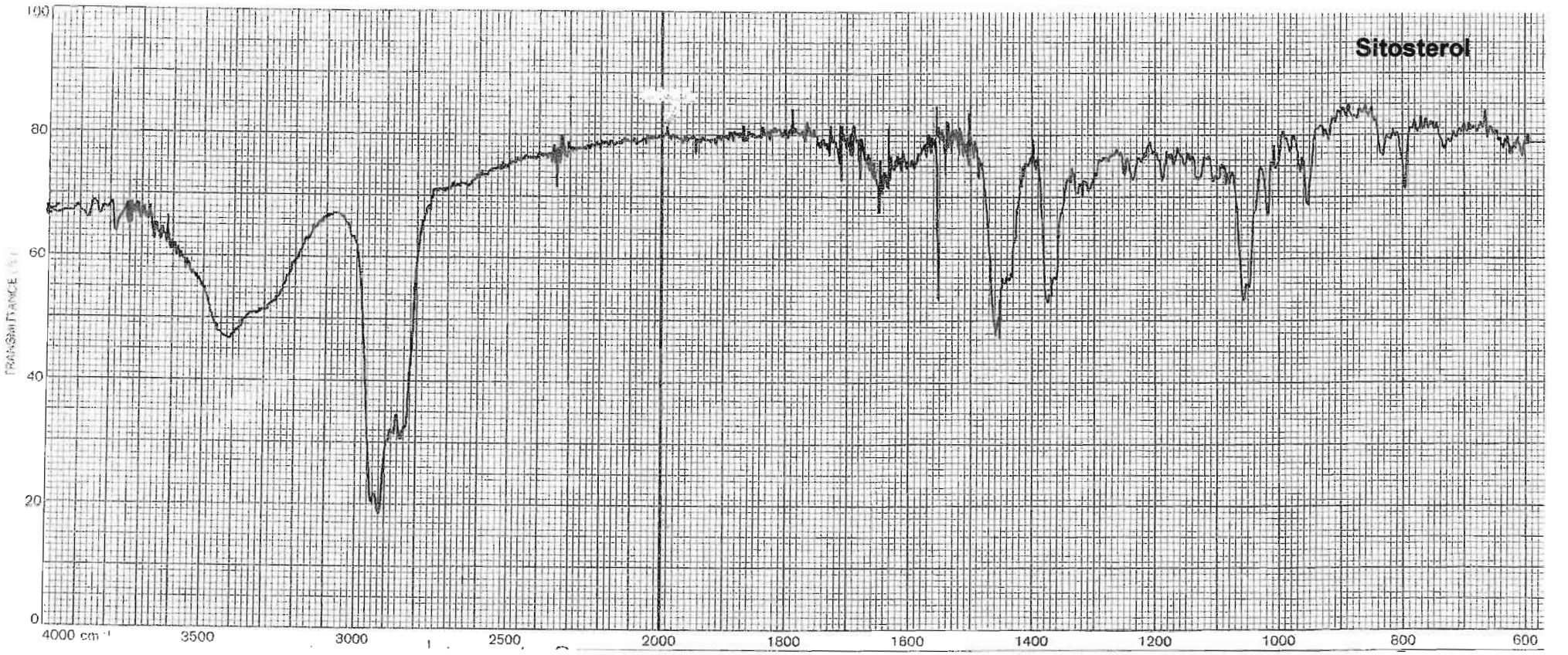
Rhoicissus tridentata

19-30	Proanthocyanidin Fr.A	MALDI-TOF MS, UV, HPLC (1), HPLC (2) Alpha Labs. All spectra for methanol extract.	212
31	Hydrogel Fr.B	IR. Acetate (after hydrolysis): IR.	216
32	Sitosterol	Spectra as for compound **1**.	
33	Sitosterolin	NC-MS, IR, ^{1}H NMR, ^{13}C NMR. Acetate: IR, ^{1}H NMR.	218
34	γ-sitosterol	IR. Acetate: IR, GC-MS.	224
35	oleanolic acid	IR, ^{1}H NMR. Acetate: NCMS, EI-MS, IR, ^{1}H NMR, ^{13}C NMR.	227
36	lupen-3-one	GC-MS, Library match.	234
37	20-epi-ψ-taraxastananol	GC-MS, Library match.	236
38	triacontanol	CI-MS, IR, ^{1}H NMR. Acetate: CI-MS, EI-MS, IR, ^{1}H NMR.	238

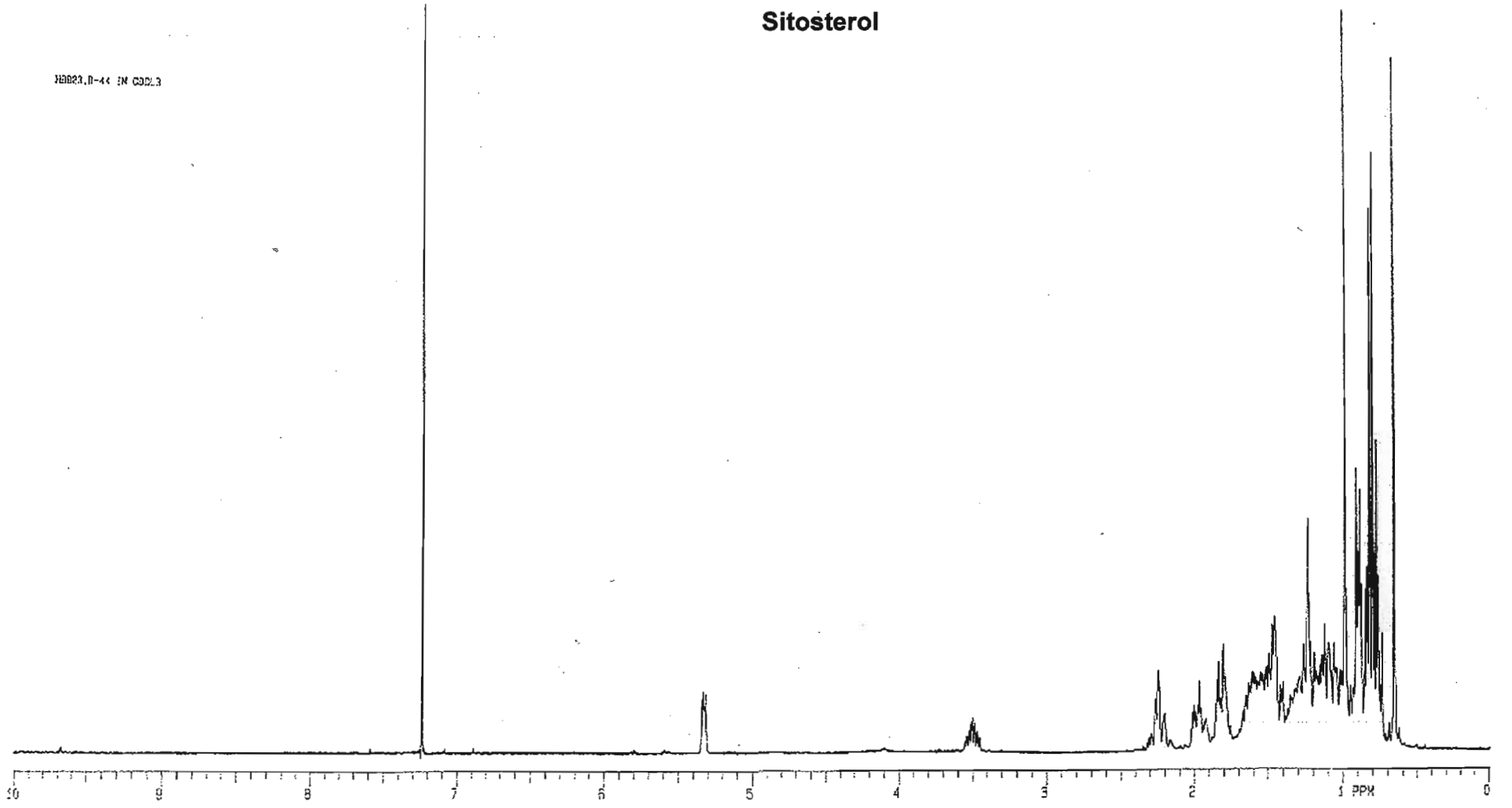
Sitosterol



1 CI-MS


Sitosterol
100
80
60
40
20
0
4000 cm⁻¹
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600

1 IR

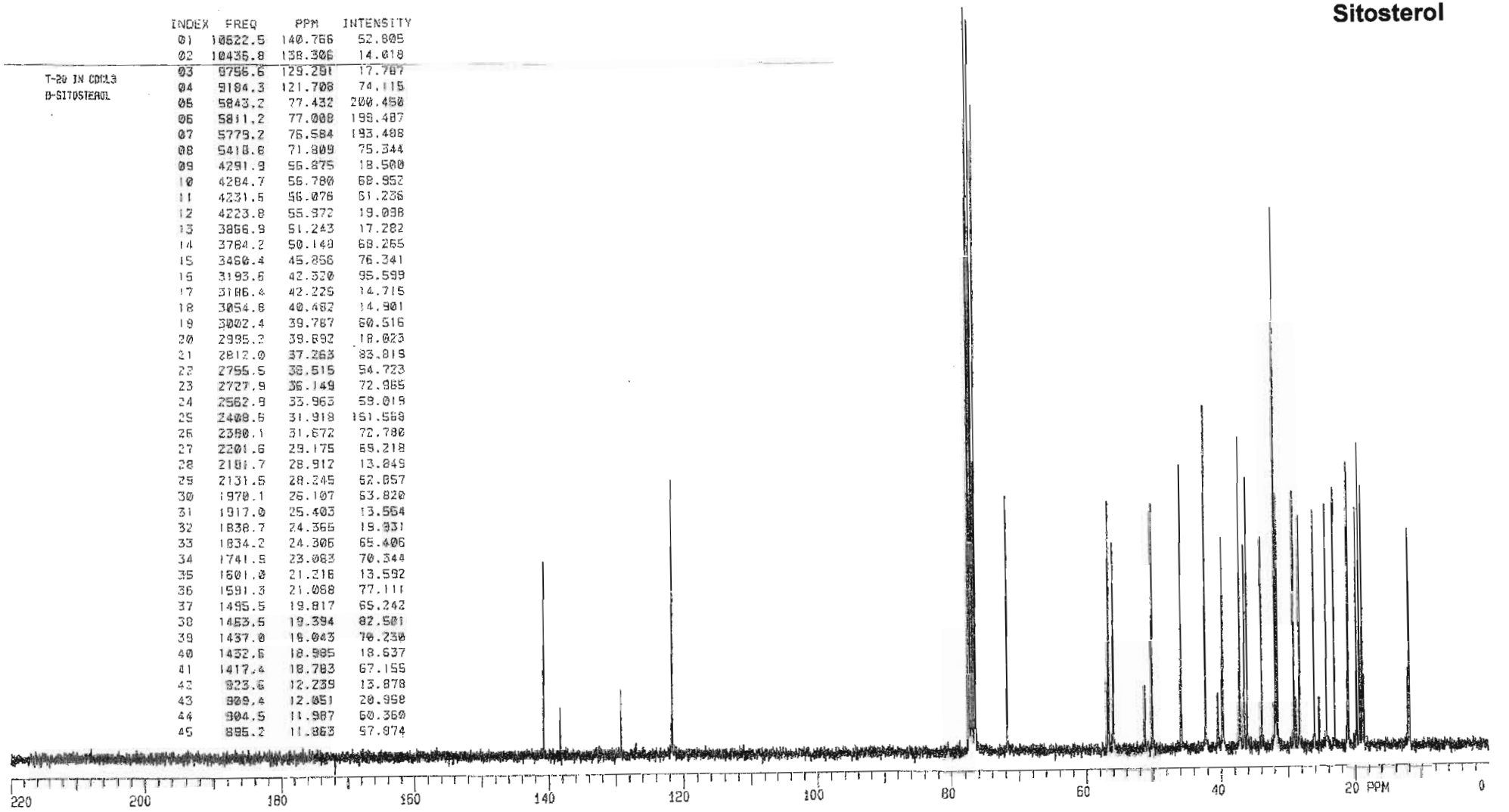


1 H-1 NMR

Sitosterol

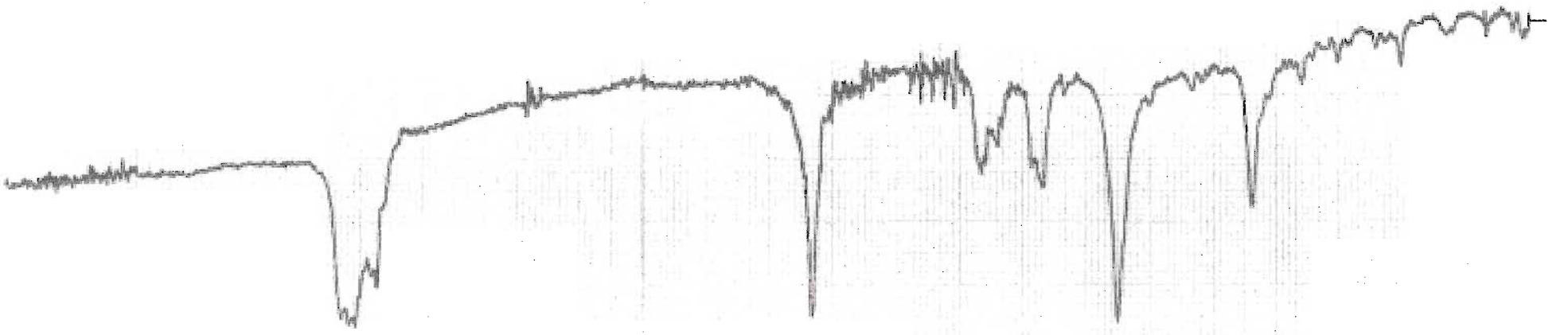
T-20 IN CDCL3
B-SITOSTEROL

INDEX	FREQ	PPM	INTENSITY
01	10522.5	140.766	52.805
02	10436.8	139.306	14.018
03	9756.6	129.291	17.787
04	9184.3	121.708	74.115
05	5843.2	77.432	200.450
06	5811.2	77.008	198.487
07	5779.2	76.584	193.488
08	5418.8	71.809	75.344
09	4291.9	56.875	18.500
10	4284.7	56.780	68.952
11	4231.5	56.076	51.236
12	4223.8	55.972	19.038
13	3866.9	51.243	17.282
14	3784.2	50.140	68.265
15	3460.4	45.856	76.341
16	3193.6	42.320	95.599
17	3186.4	42.225	14.715
18	3054.8	40.482	14.901
19	3002.4	39.787	60.516
20	2985.2	39.592	18.023
21	2812.0	37.263	83.819
22	2755.5	36.515	54.723
23	2727.9	36.149	72.965
24	2562.9	33.963	59.018
25	2408.5	31.918	161.568
26	2380.1	31.572	72.780
27	2201.6	29.175	65.218
28	2181.7	28.912	13.849
29	2131.5	28.245	62.857
30	1970.1	26.107	63.820
31	1917.0	25.403	13.564
32	1838.7	24.365	19.831
33	1834.2	24.305	65.406
34	1741.5	23.083	70.344
35	1601.0	21.216	13.592
36	1591.3	21.088	77.111
37	1495.5	19.817	65.242
38	1463.5	19.394	82.501
39	1437.0	19.043	70.230
40	1432.6	18.985	18.637
41	1417.4	18.783	67.155
42	923.6	12.239	13.878
43	909.4	12.051	20.998
44	904.5	11.987	60.360
45	895.2	11.863	57.974



1 C-13 NMR

Sitosterol acetate



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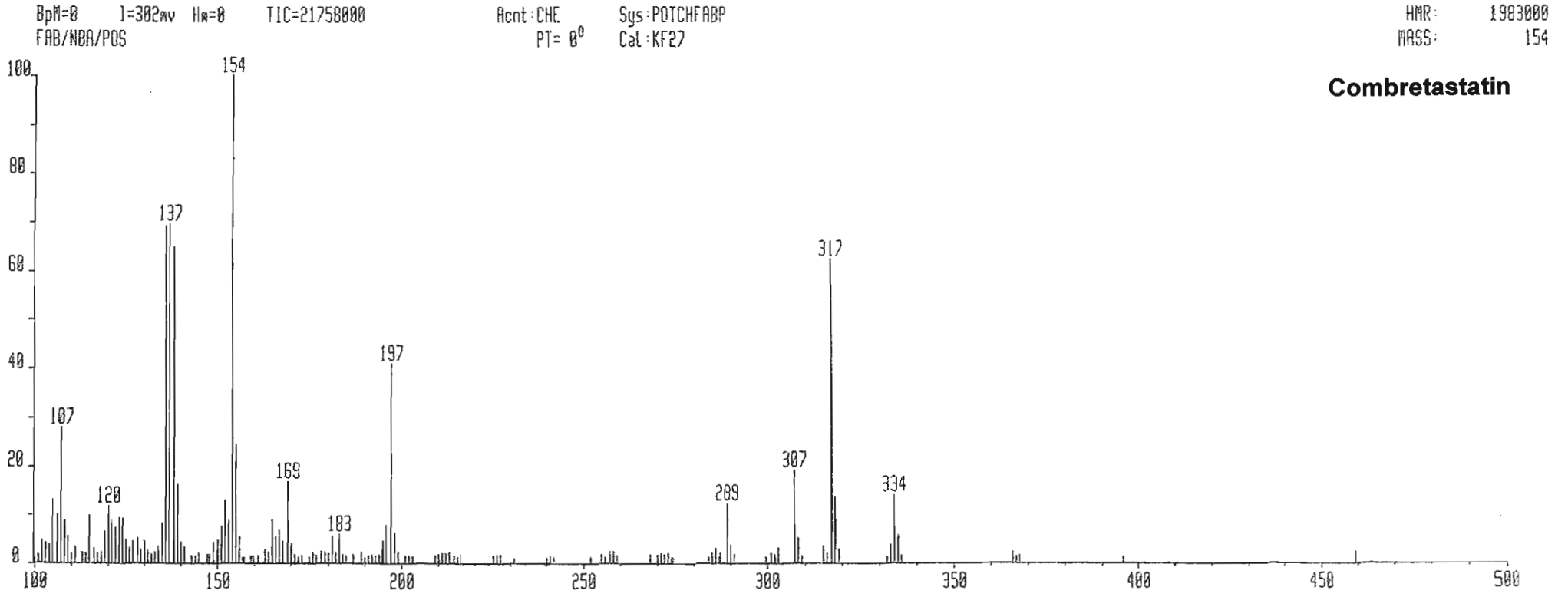
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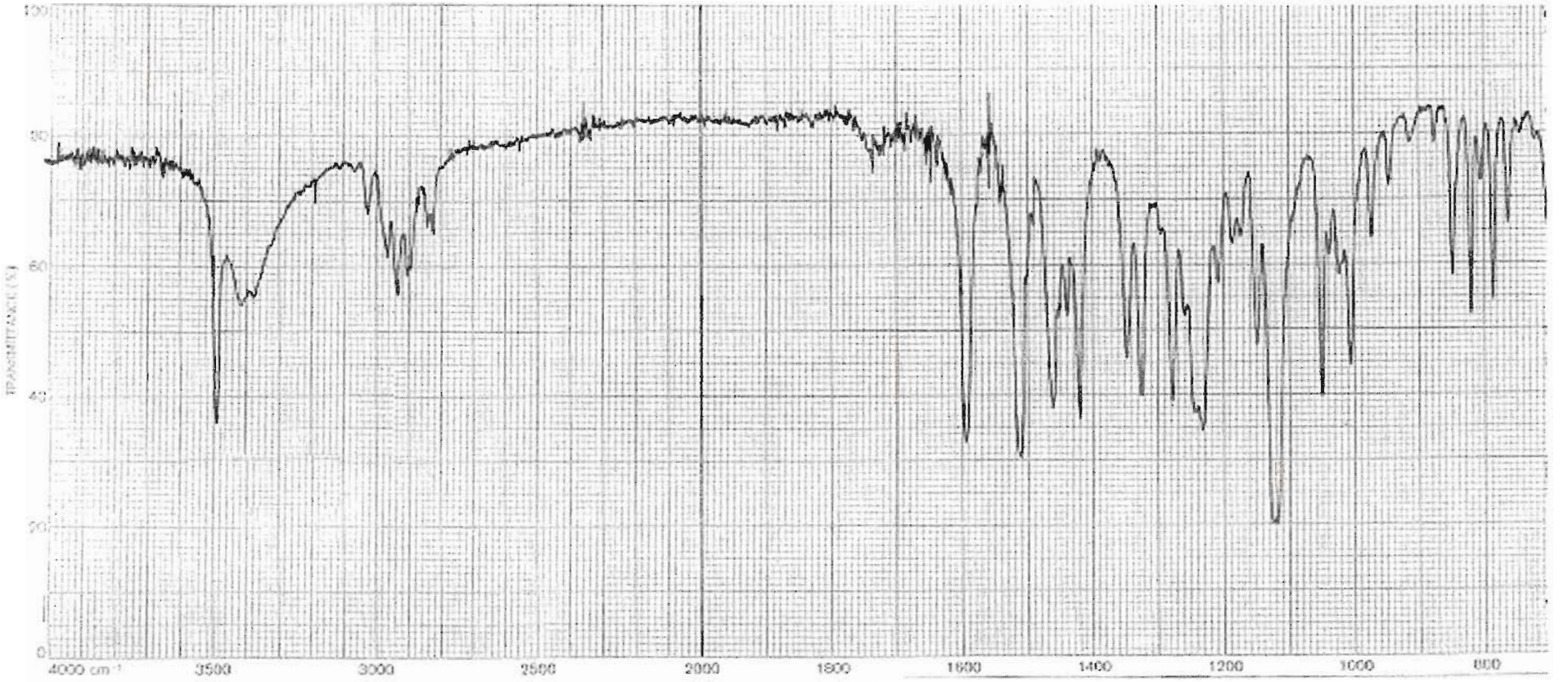
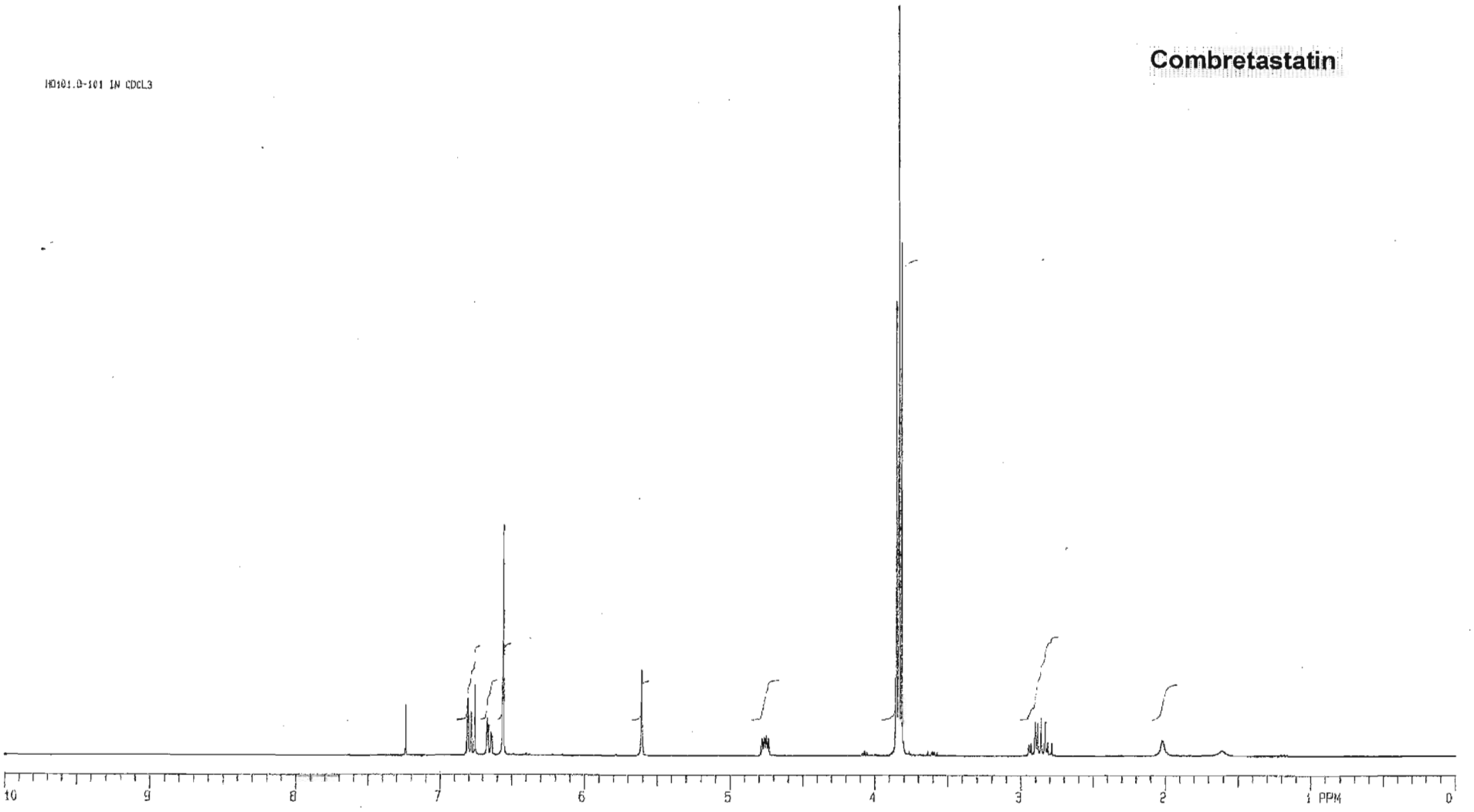
FAB/NBA/POS
Acnt:CHE
PT= 0°
Sys:POTCHFABP
Cal:KF27
HMR:
MASS:
1983000
154
Combretastatin
100
80
60
40
20
0
154
137
317
197
107
169
307
334
120
289
183
100
150
200
250
300
350
400
450
500

2 FAB-MS

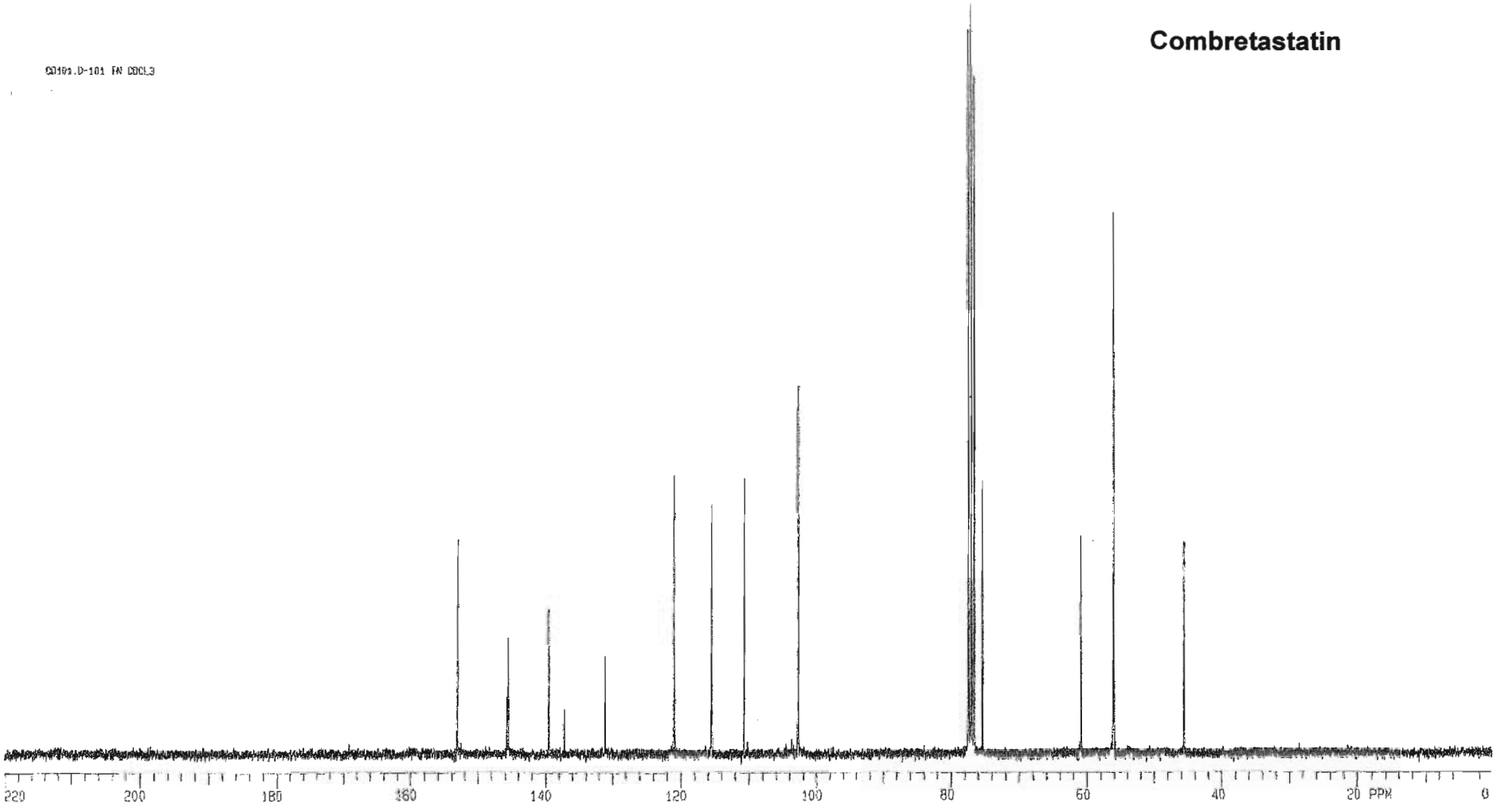
Combretastatin



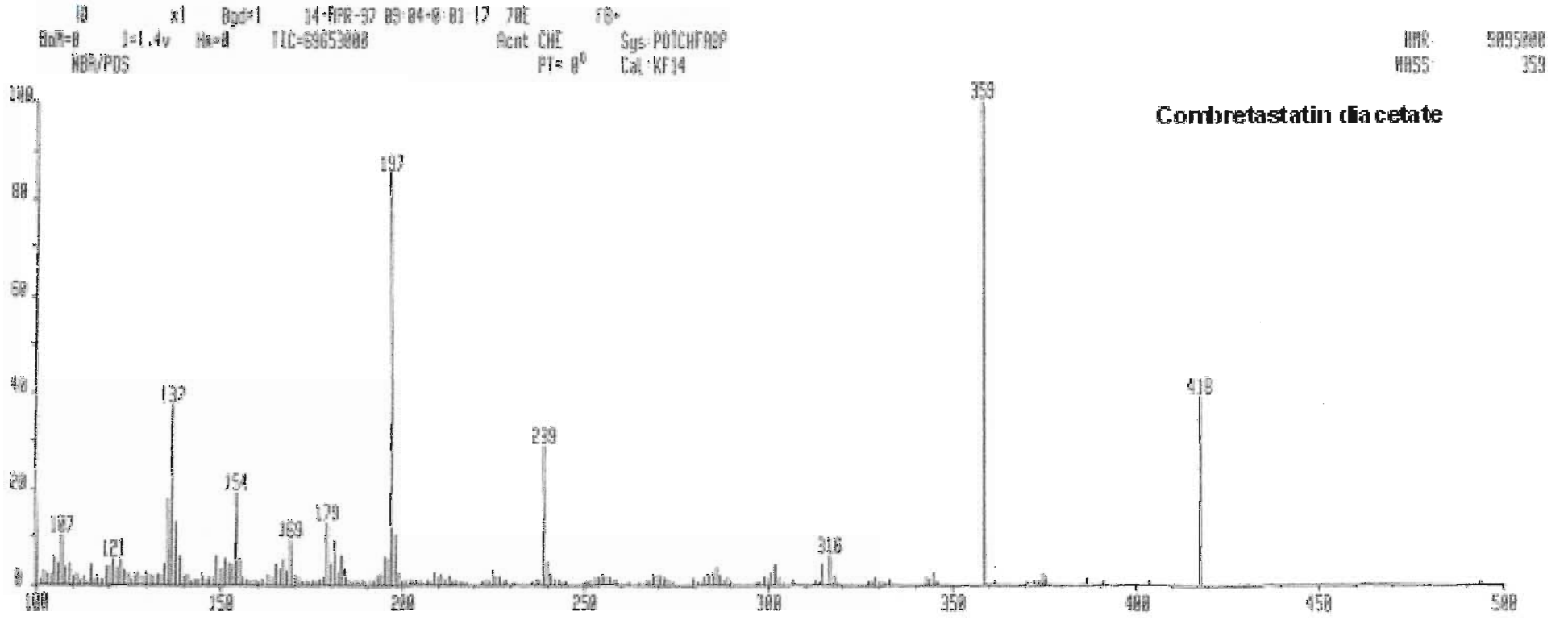
2 IR



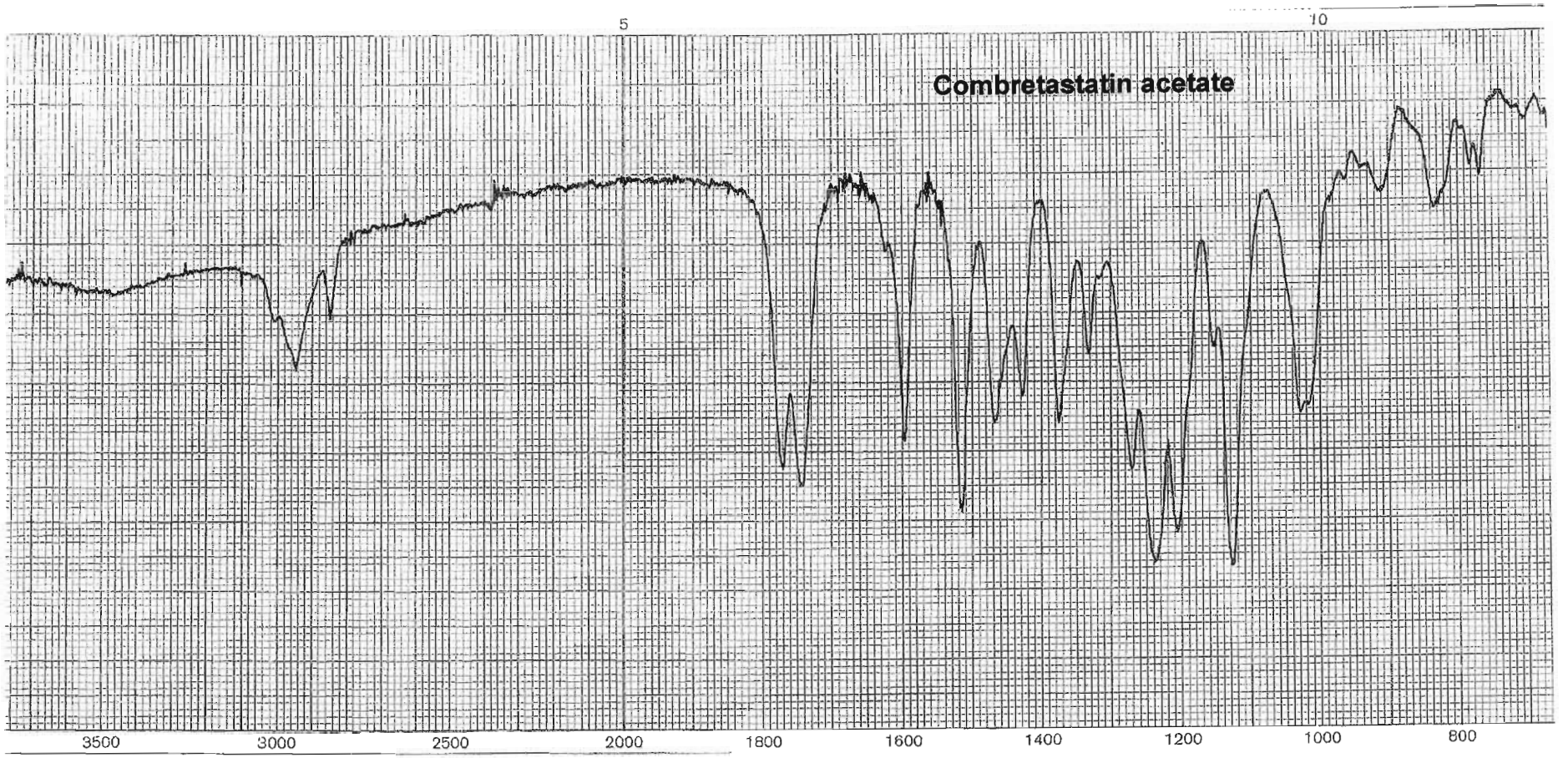
2 H-1 NMR


Combretastatin
CD193.D-101 FN CDCL3
220
200
180
160
140
120
100
80
60
40
20 PPM
0

2 C-13 NMR

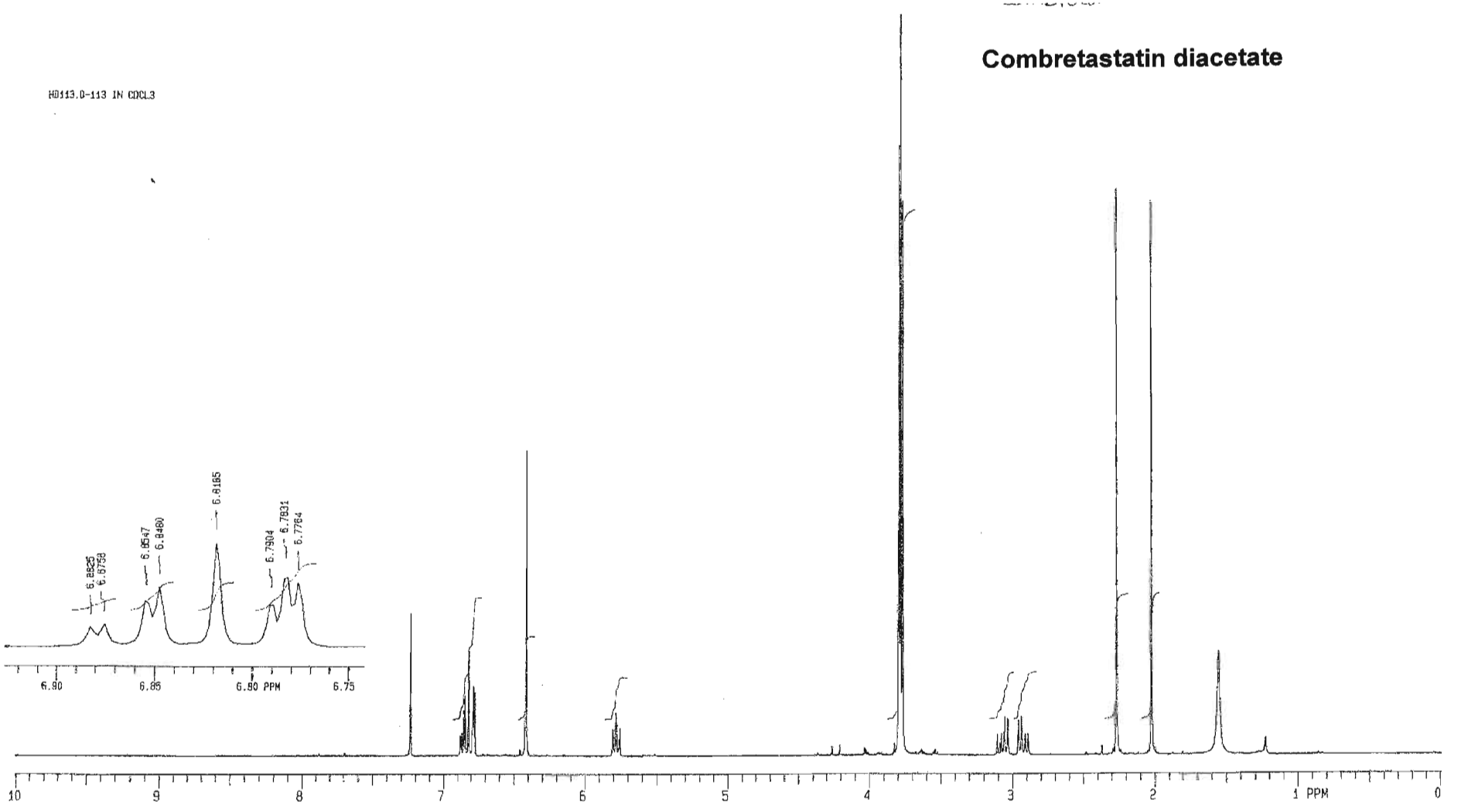


2 Acetate Fab-MS


Combretastatin acetate
5
10
3500
3000
2500
2000
1800
1600
1400
1200
1000
800

2 Acetate IR

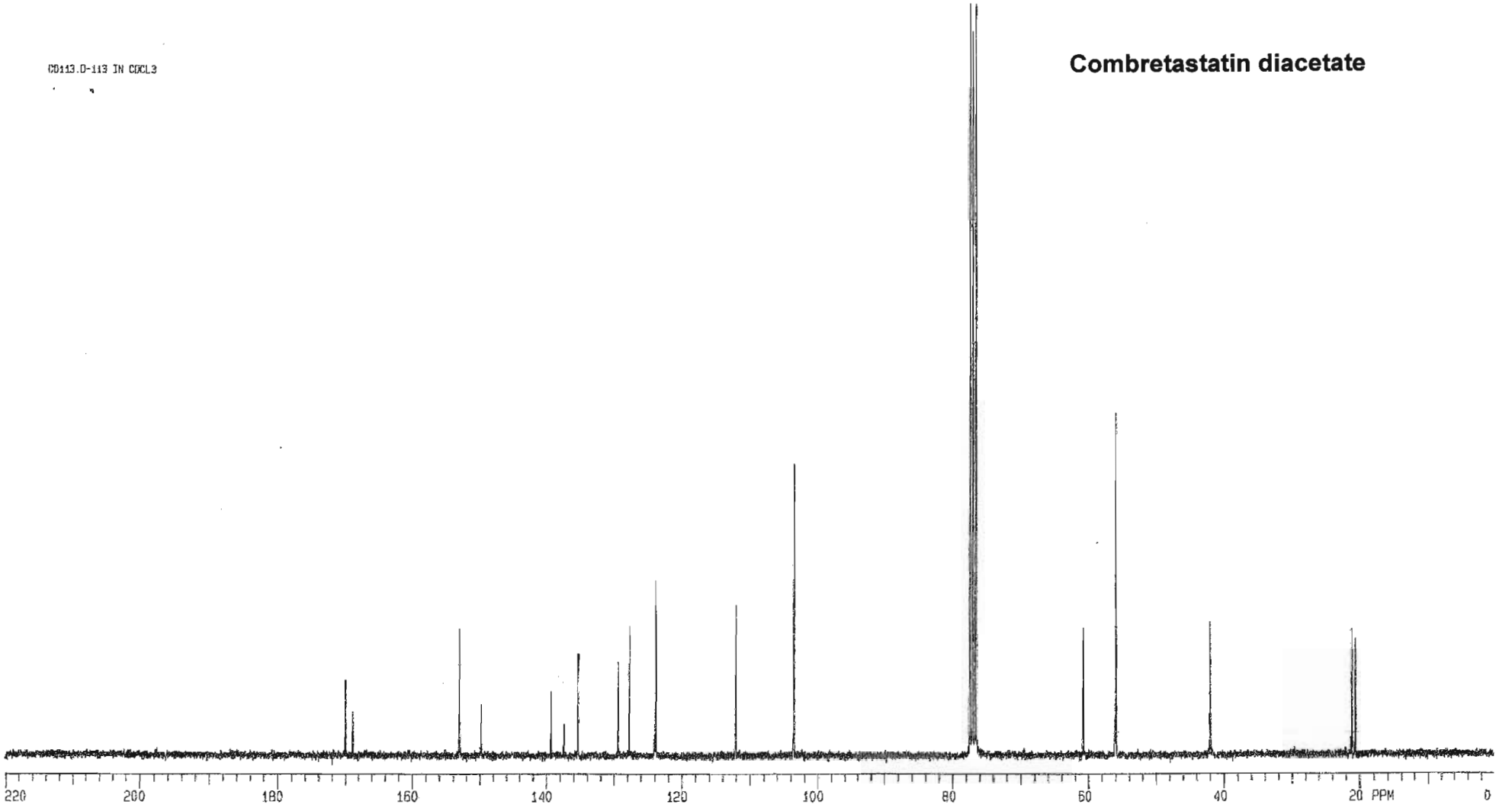
Combretastatin diacetate



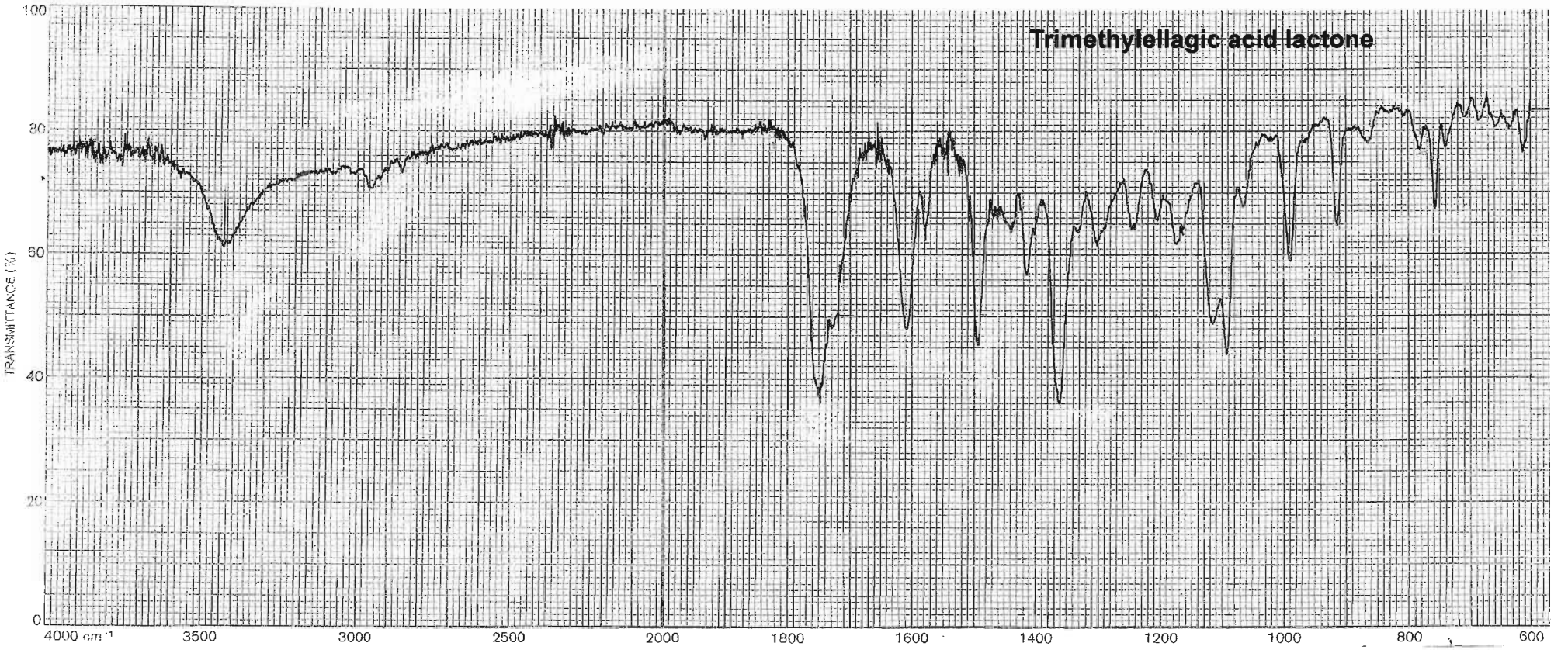
2 Acetate H-1 NMR

CD113.D-113 IN CDCL3

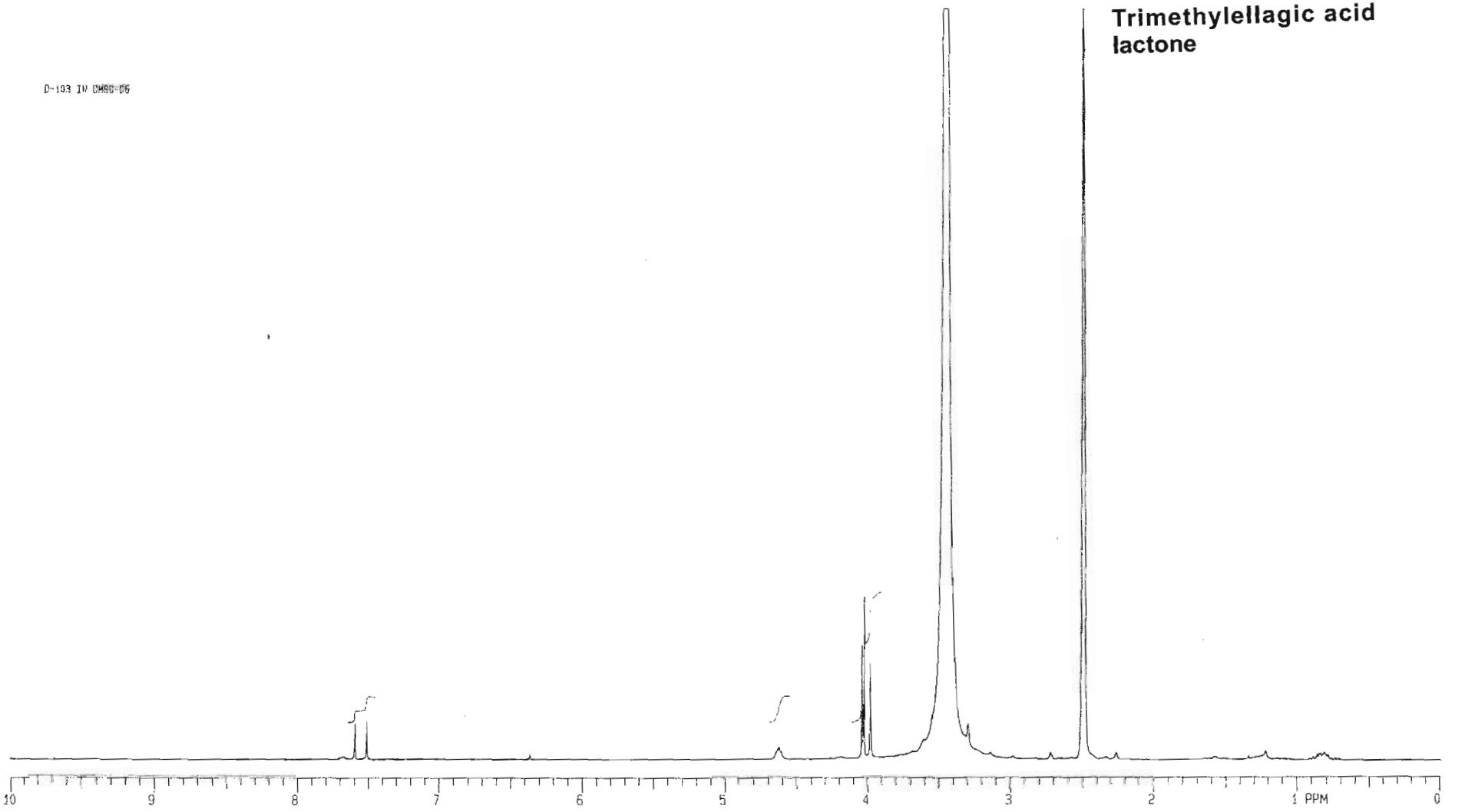
Combretastatin diacetate



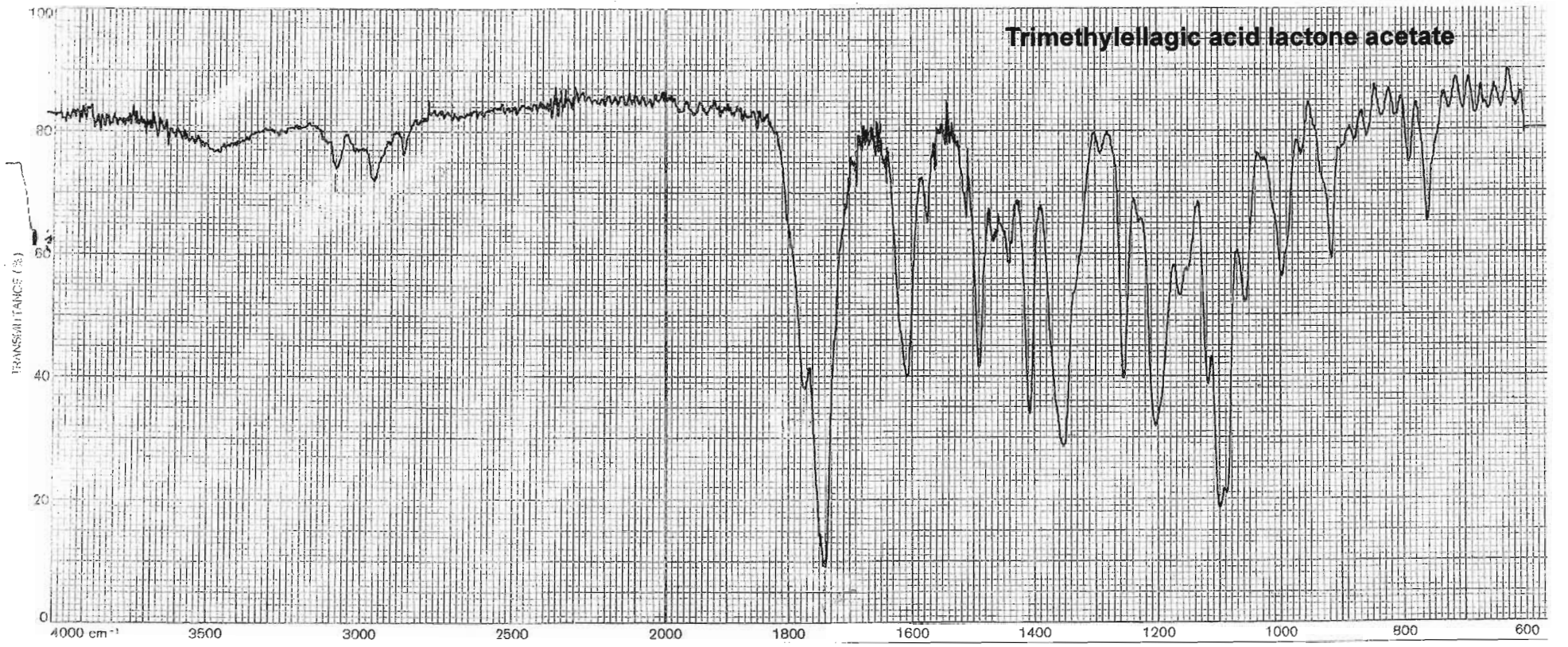
2 Acetate C-13 NMR

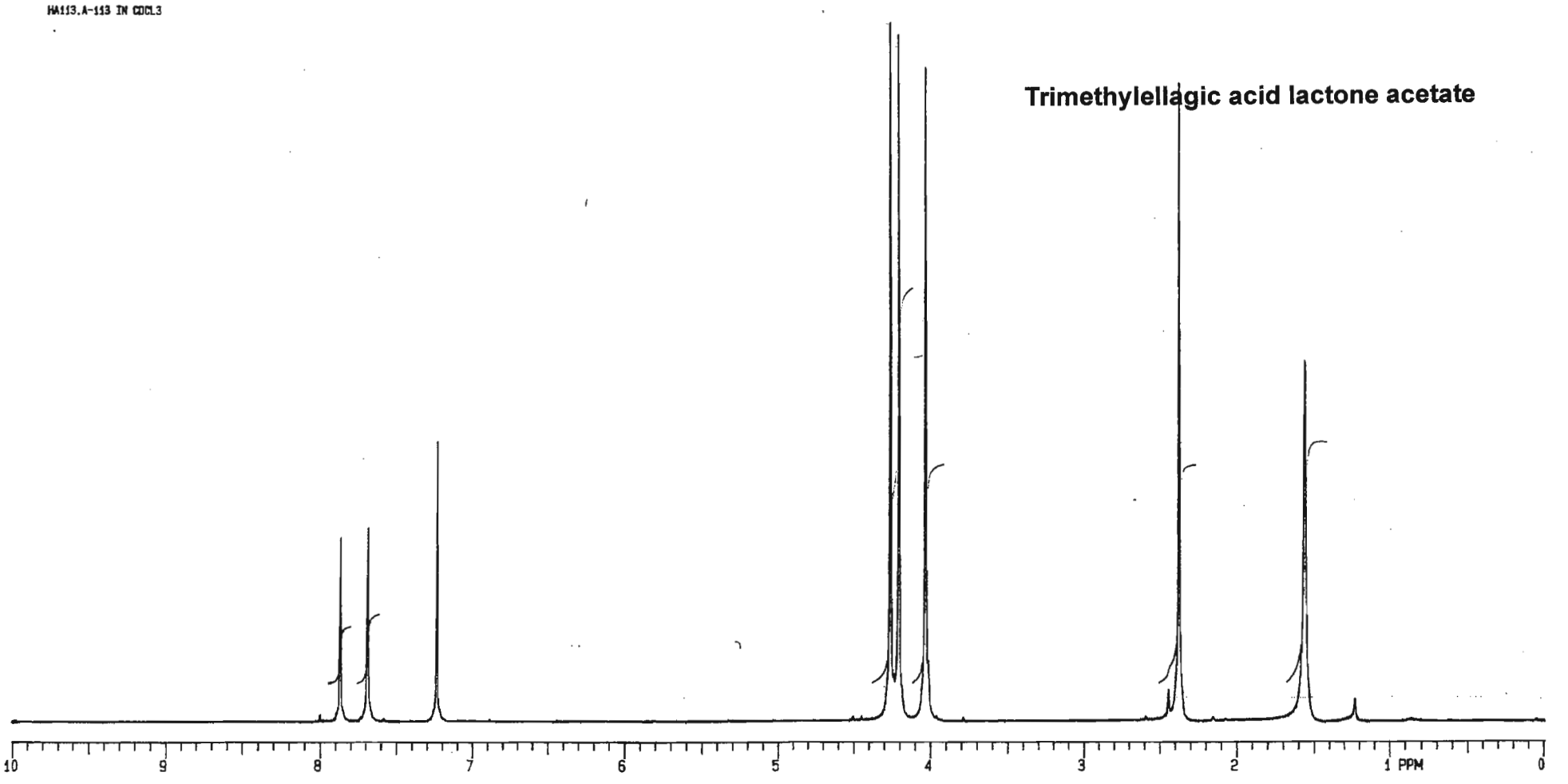

Trimethylellagic acid lactone
TRANSMITTANCE (%)
100
80
60
40
20
0
4000 cm⁻¹
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600

3 IR

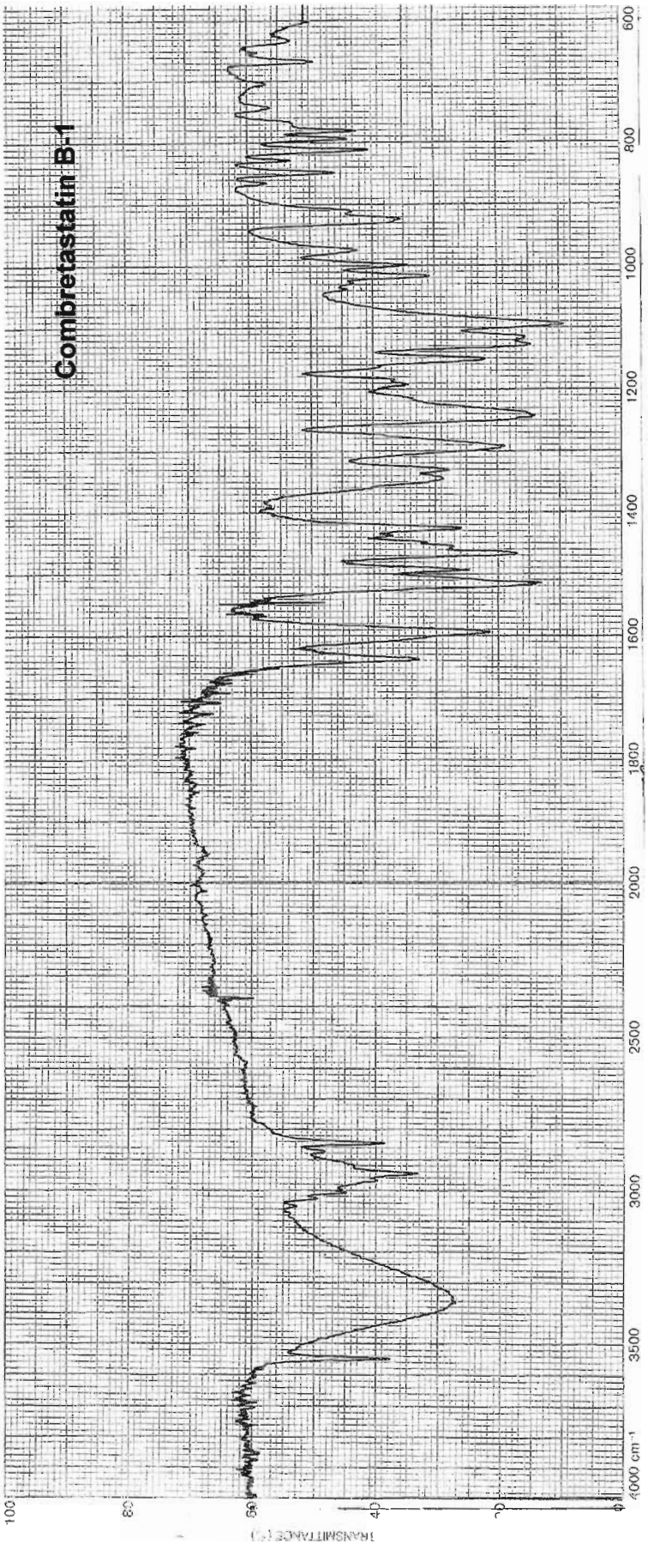

Trimethylellagic acid lactone
D-193 IN DMSO-D6
10
9
8
7
6
5
4
3
2
1 PPM
0

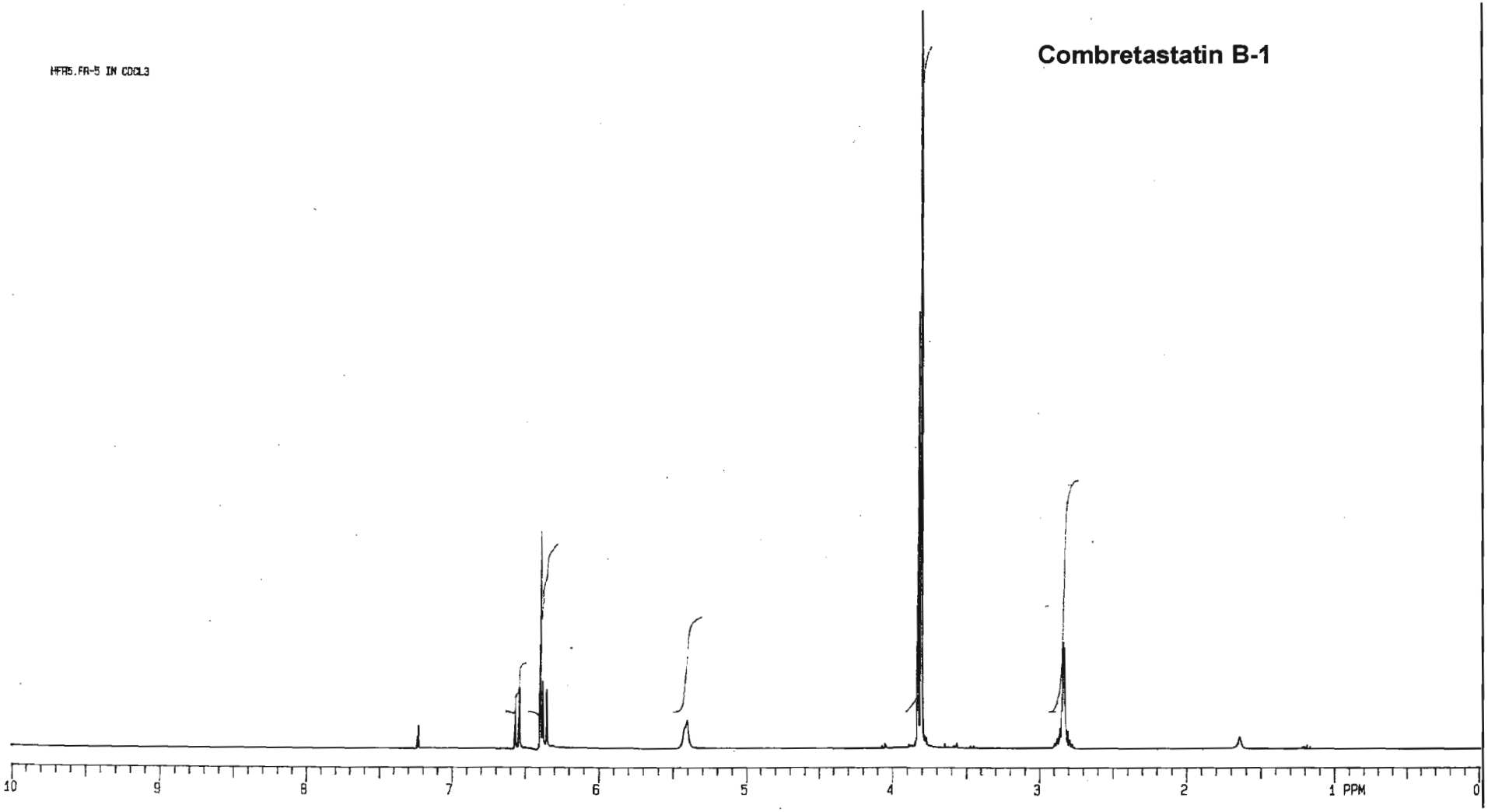
3 H-1 NMR


Trimethylellagic acid lactone acetate
100
80
60
40
20
0
4000 cm⁻¹
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600

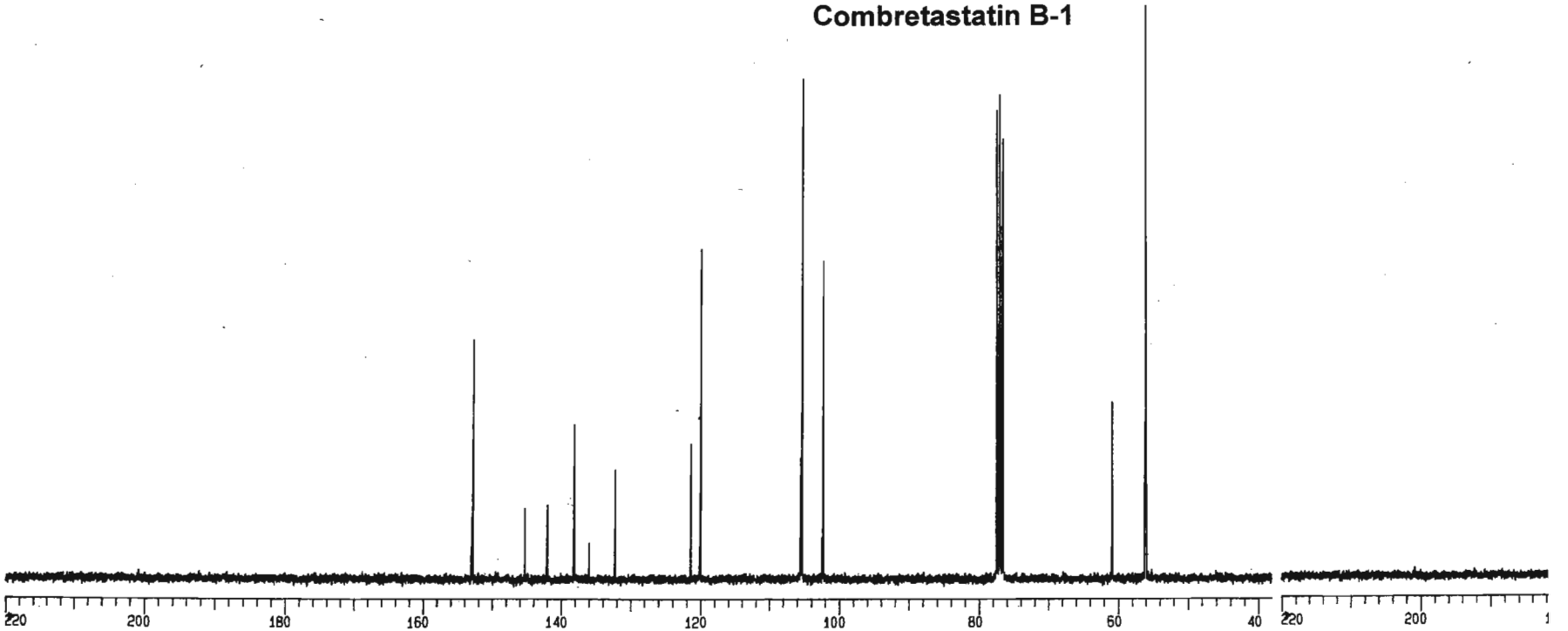


3 Acetate H-1 NMR

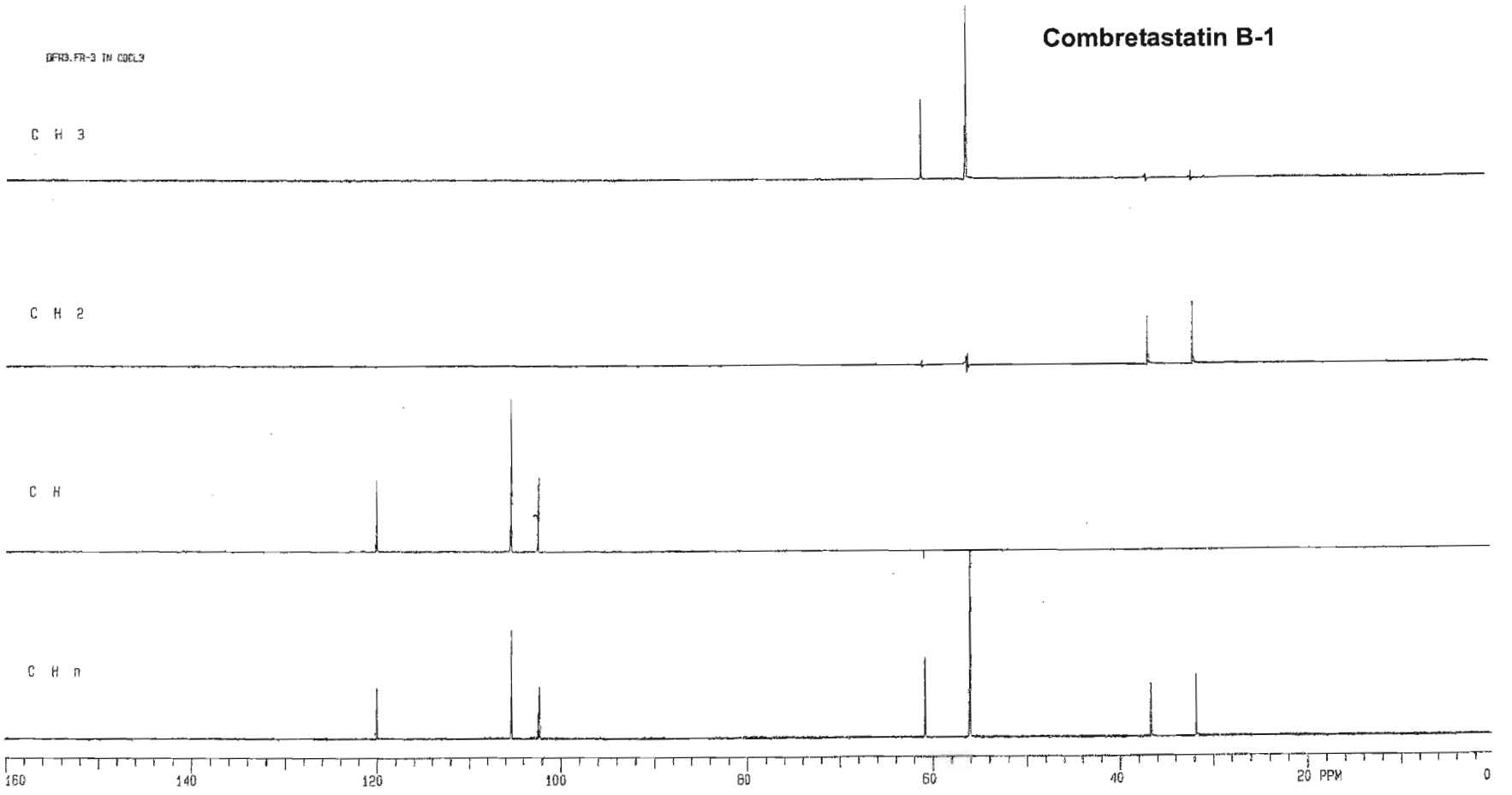

Combretastatin B-1
TRANSMITTANCE (%)
100
80
60
40
20
0
4000 cm-1
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600



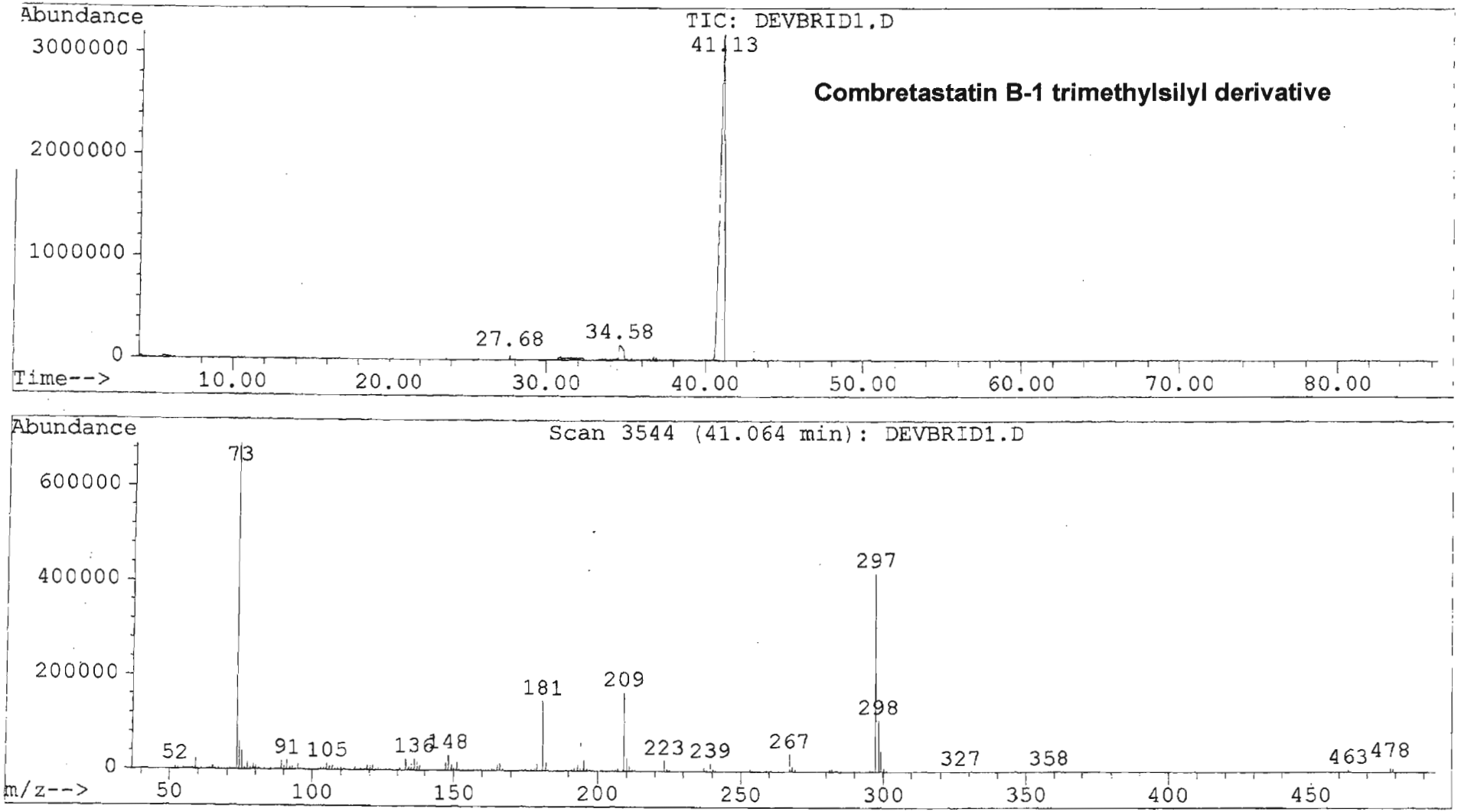
4 H-1 NMR



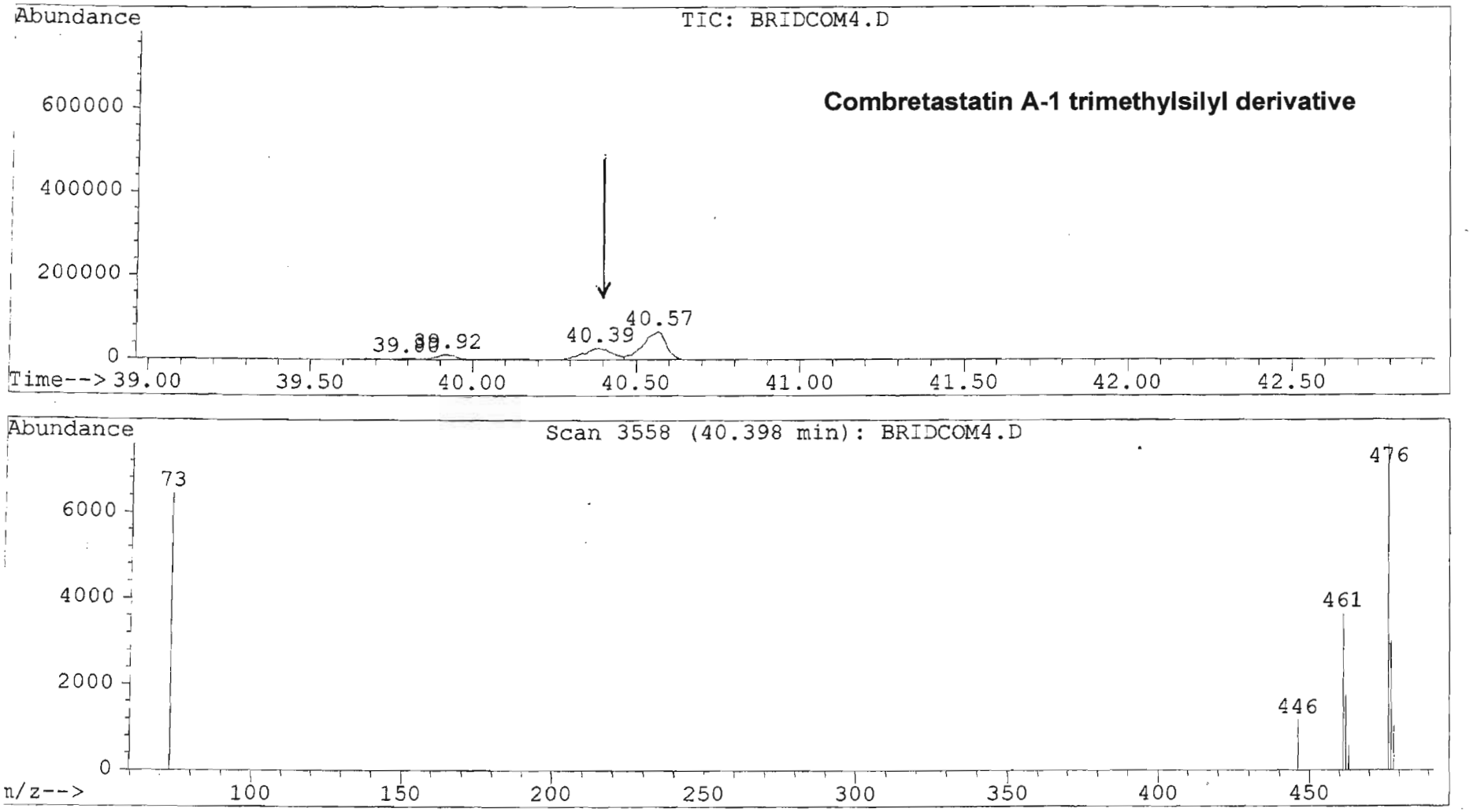
4 C-13 NMR


Combretastatin B-1
DFR3.FR-3 IN CDCL3
C H 3
C H 2
C H
C H n
160
140
120
100
80
60
40
20 PPM
0

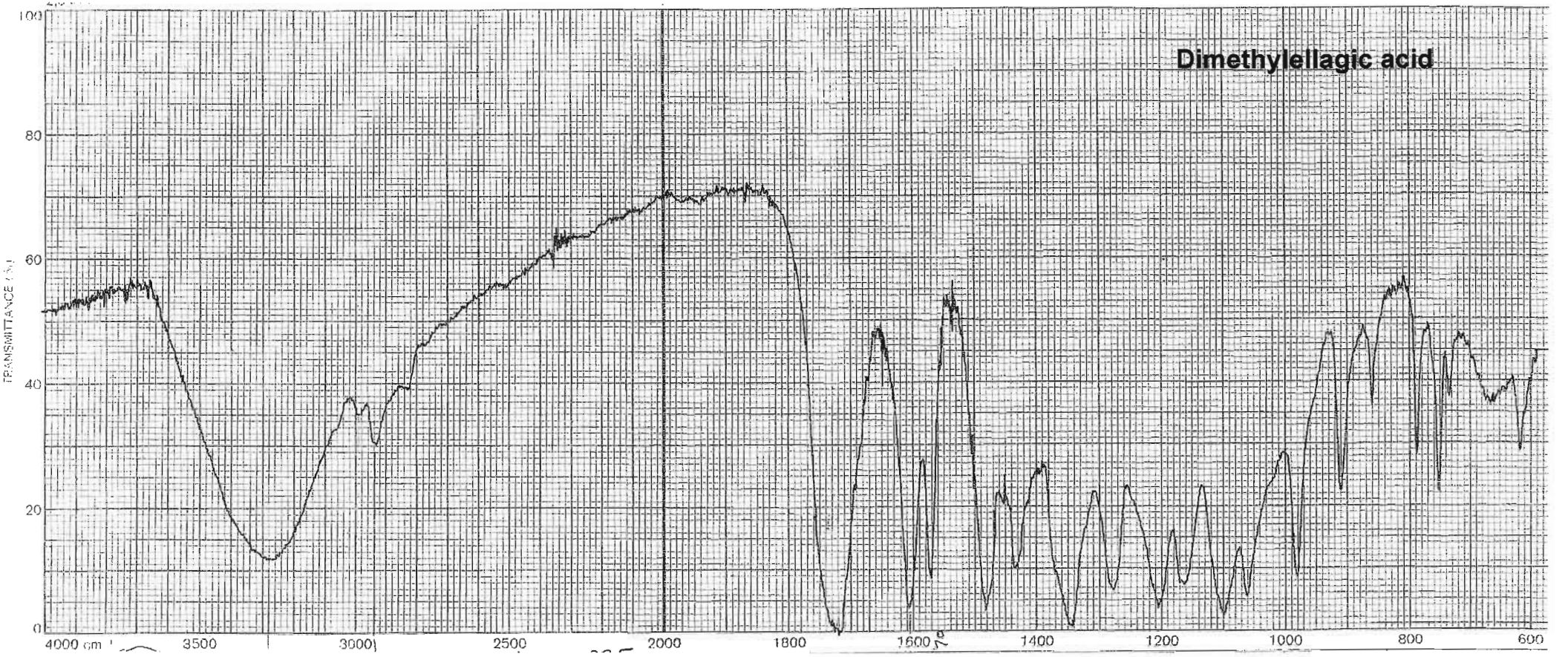
4 DEPT NMR



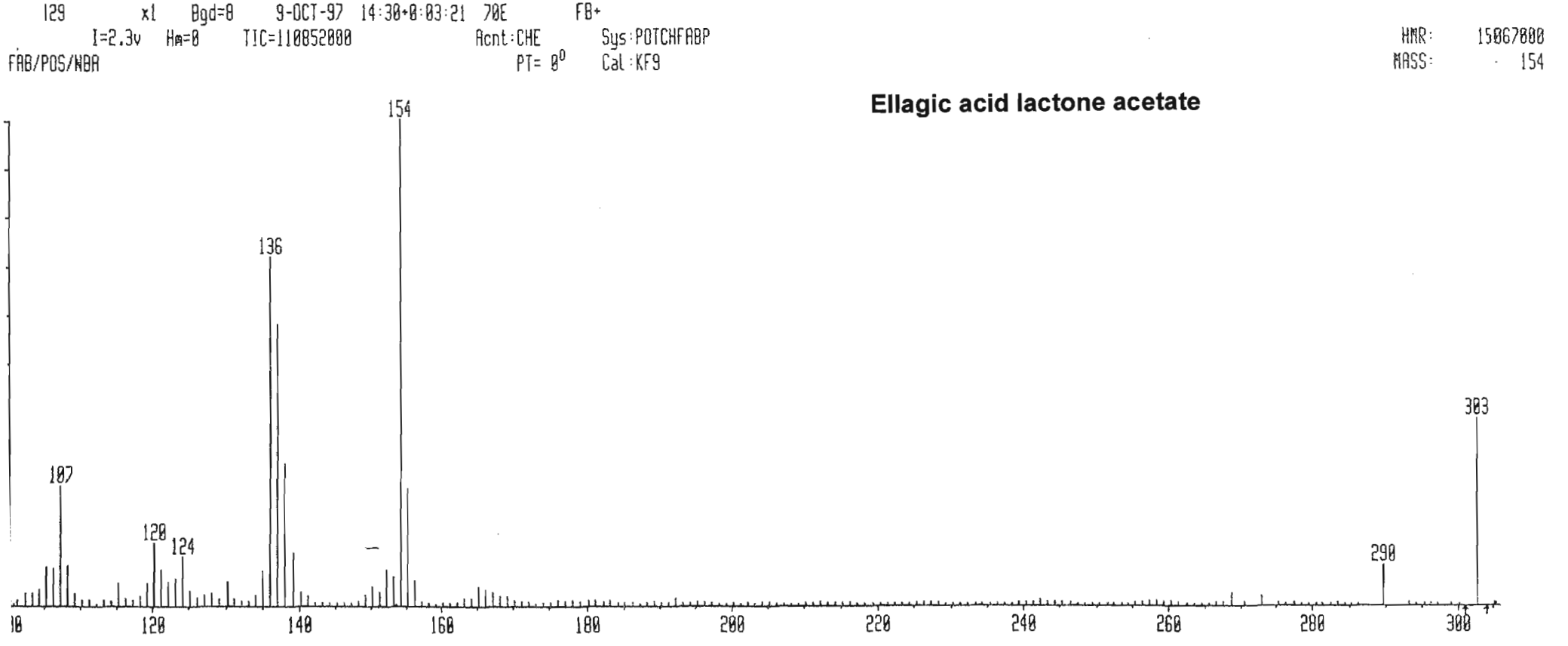
4 trimethylsilyl derivative GC-MS



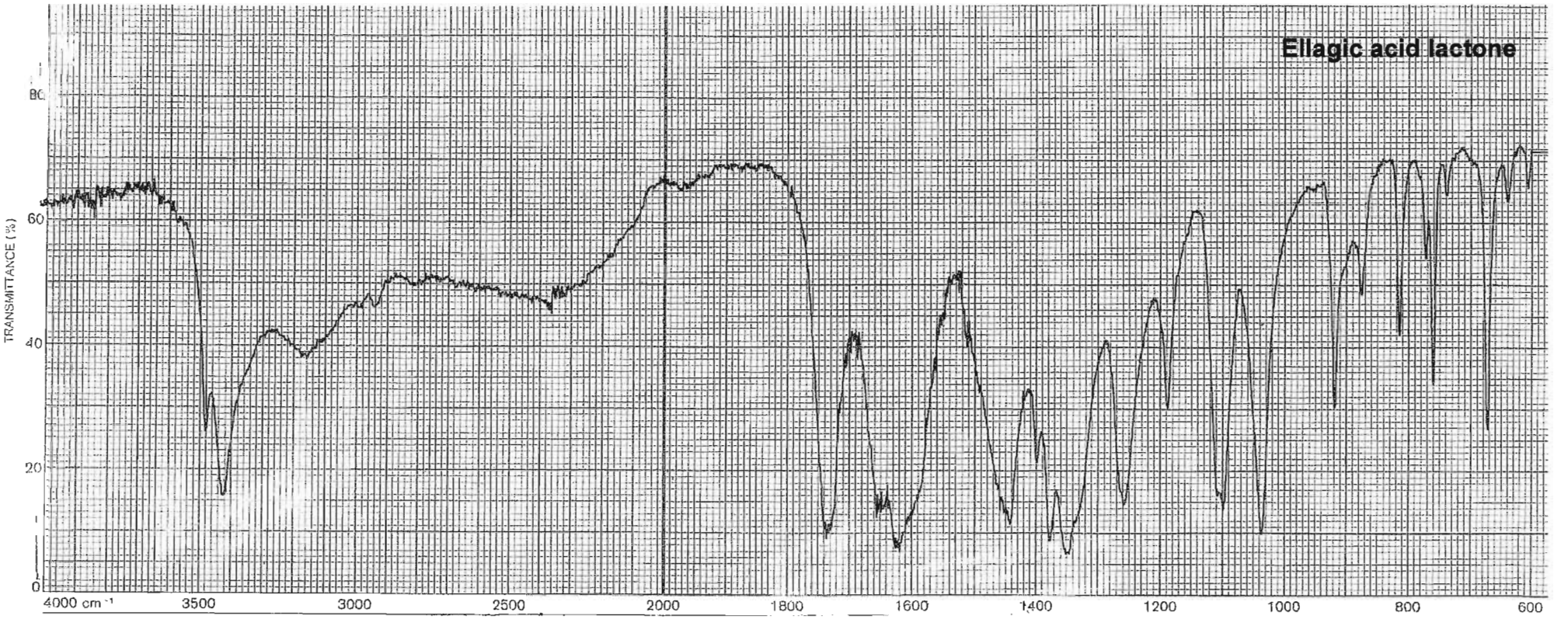
5 trimethylsilyl derivative GC-MS


Dimethylellagic acid
100
80
60
40
20
0
4000 cm
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600

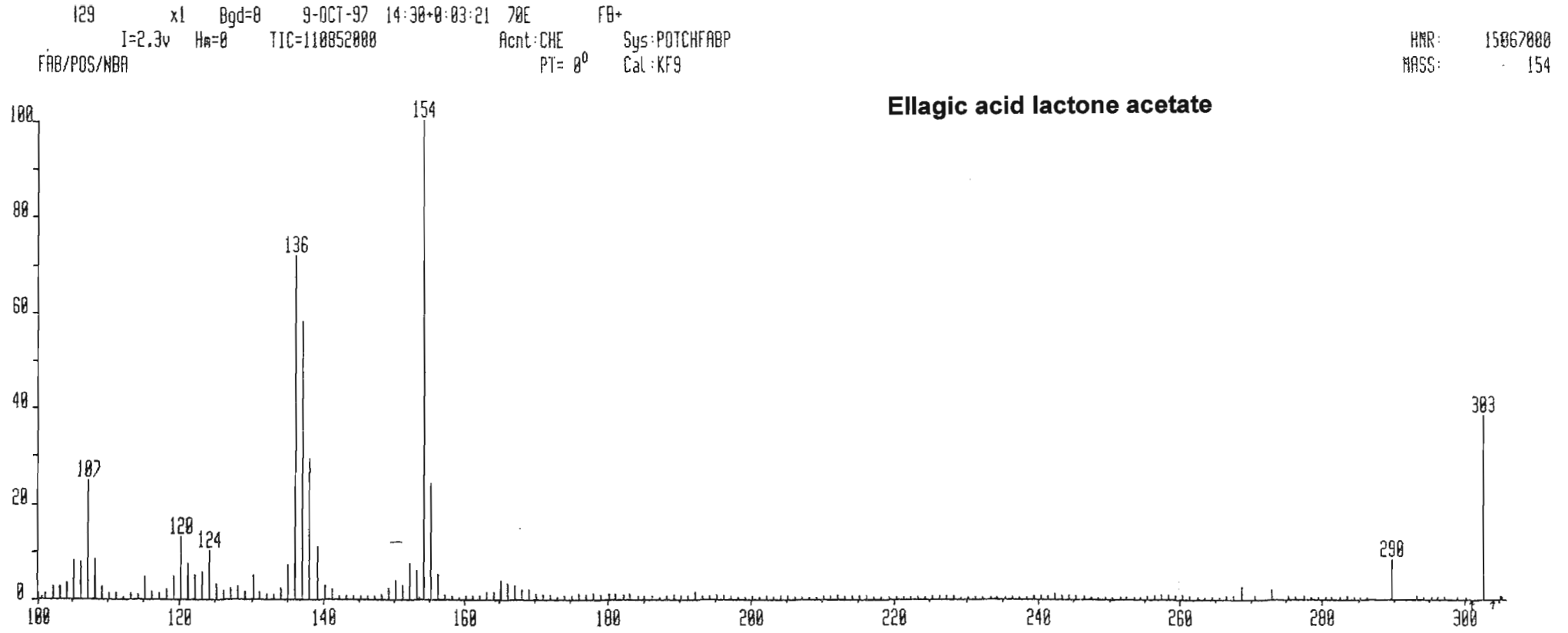
6 IR



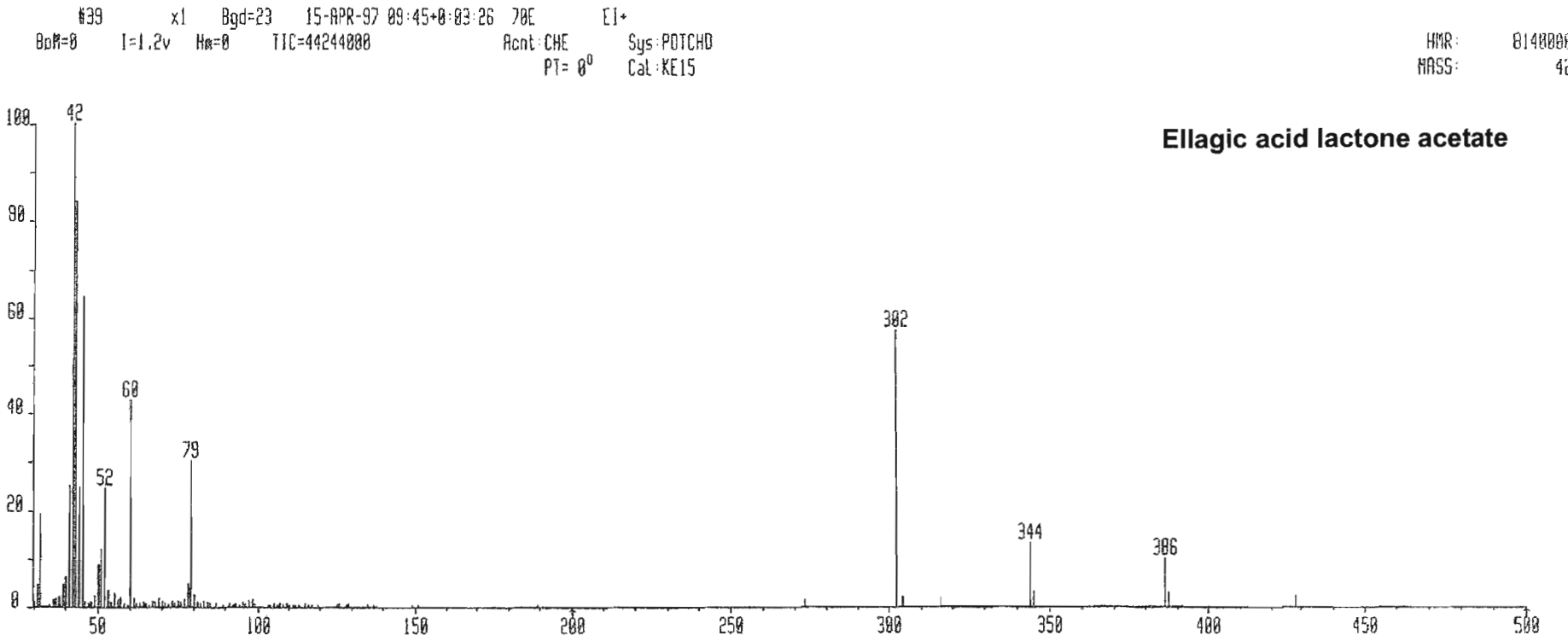
7a Acetate FAB-MS


Ellagic acid lactone
TRANSMITTANCE (%)
80
60
40
20
0
4000 cm⁻¹
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600

7a IR

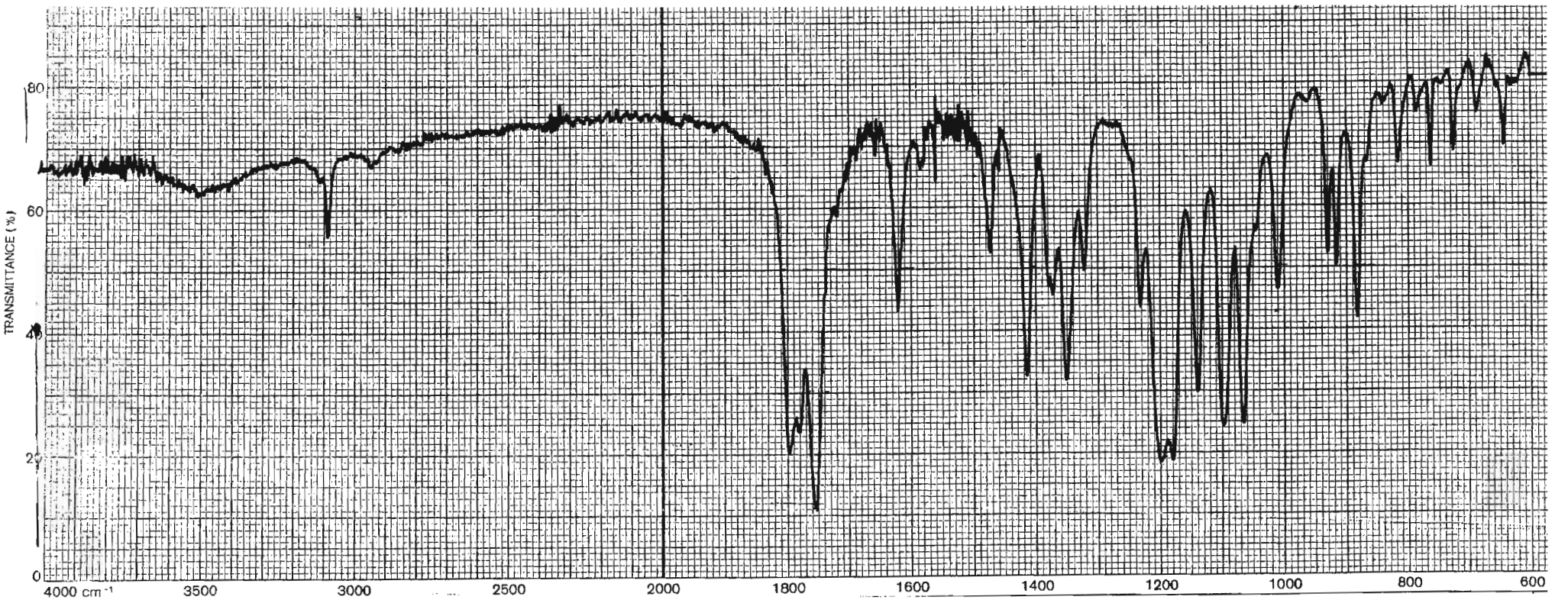


7a Acetate FAB-MS

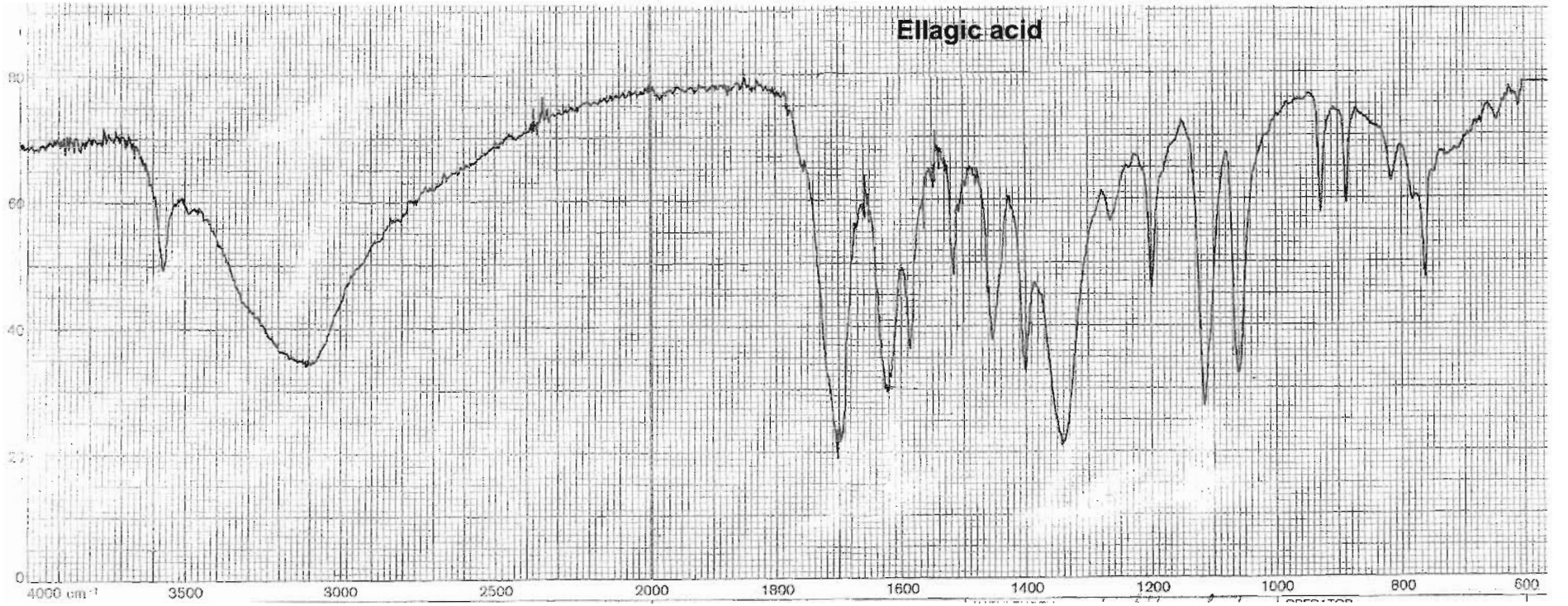


7a Acetate EI-MS

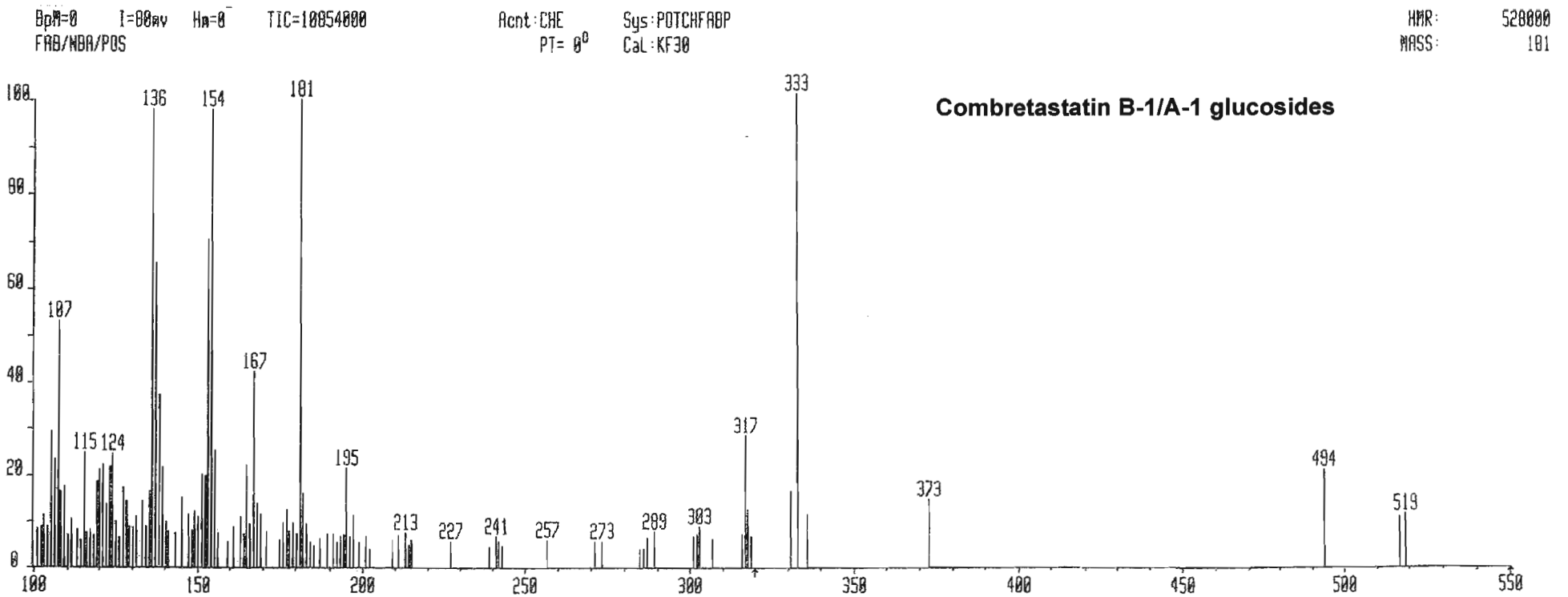
Ellagic acid lactone acetate



7a Acetate IR

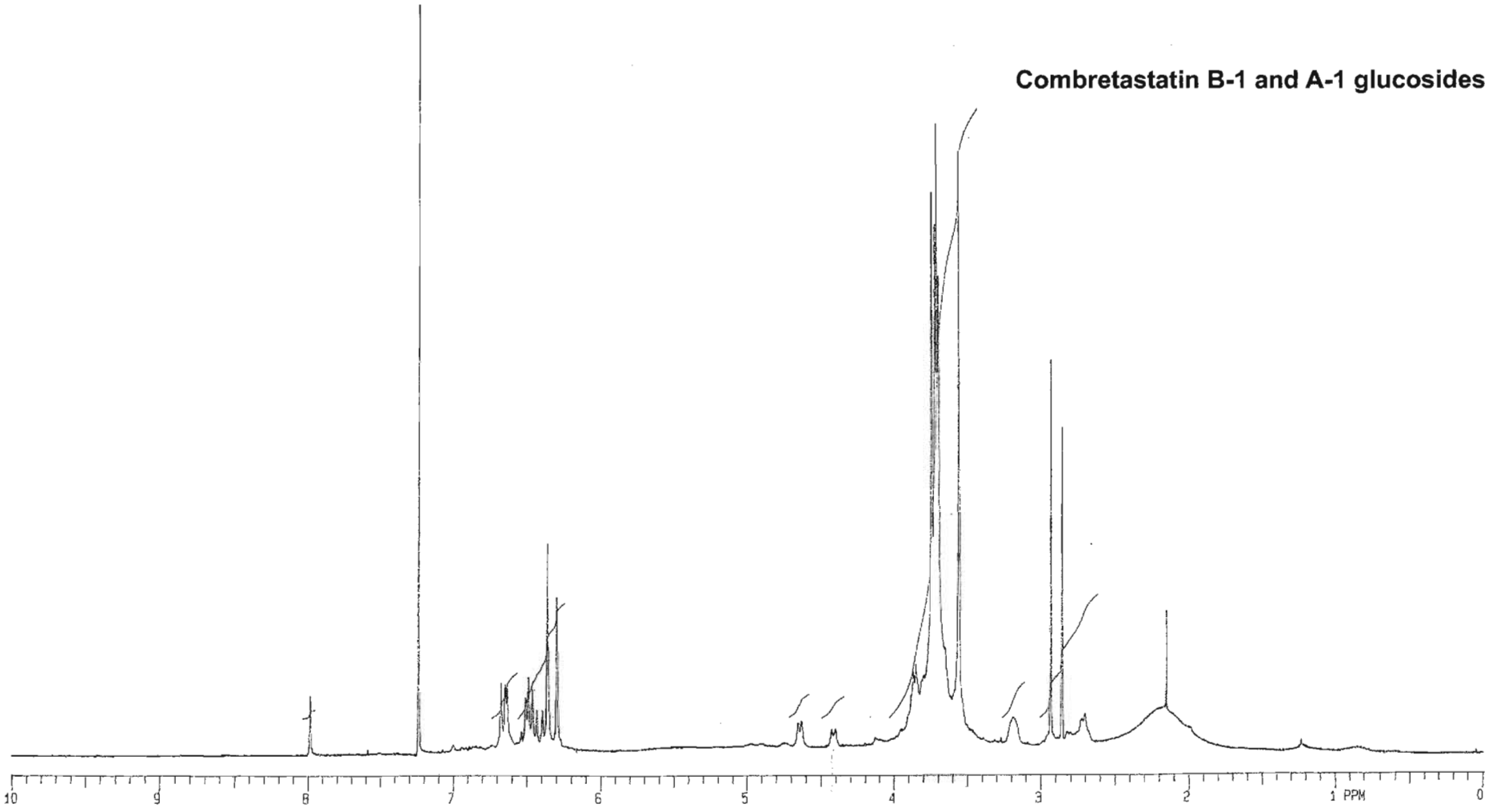

Ellagic acid
80
60
40
20
0
4000 cm⁻¹
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600

7b IR


BpM=0 I=88mv Hm=0 TIC=10854888
FAB/NBA/POS
Acnt:CHE
PT= 0°
Sys:POTCHFABP
CaL:KF30
HMR: 528888
MASS: 181
Combretastatin B-1/A-1 glucosides
100
80
60
40
20
0
100
150
200
250
300
350
400
450
500
550
107
115
124
136
154
167
181
195
213
227
241
257
273
289
303
317
333
373
494
519

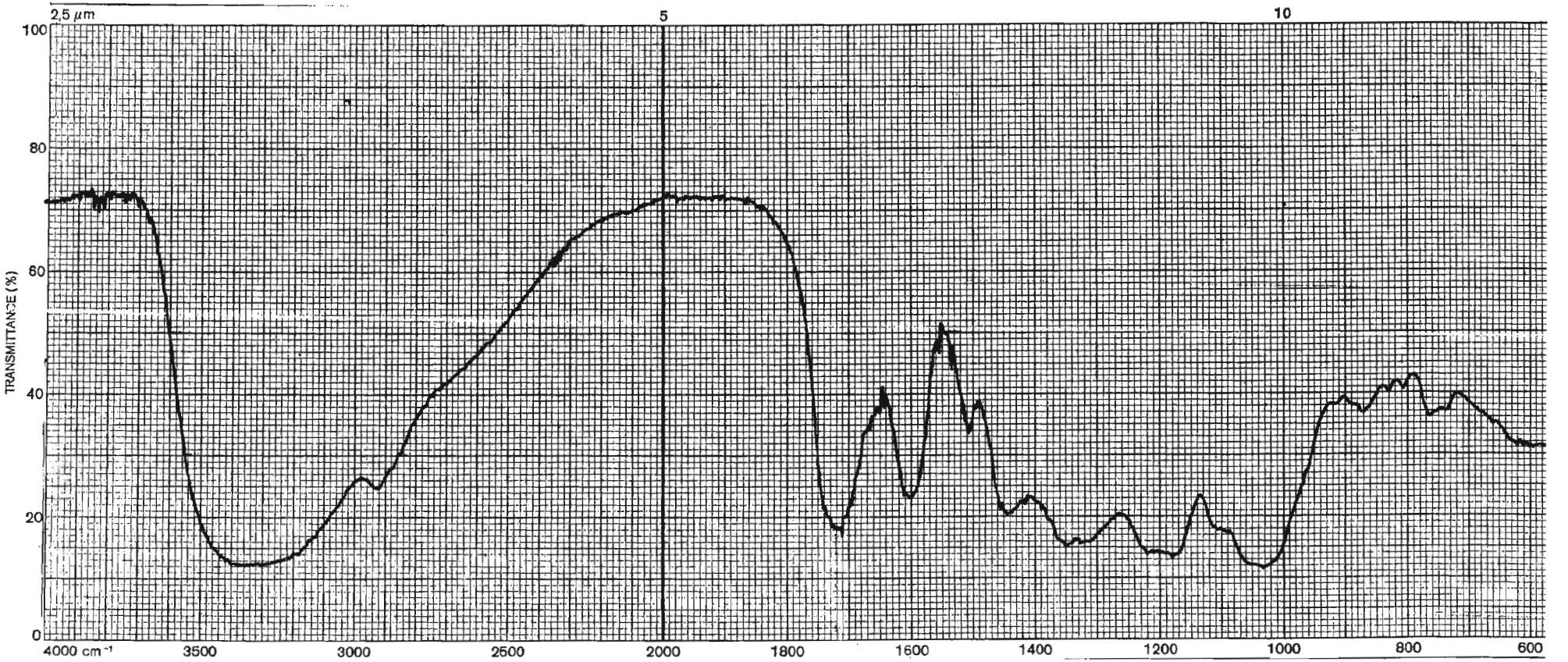
8+9 FAB-MS

Combretastatin B-1 and A-1 glucosides

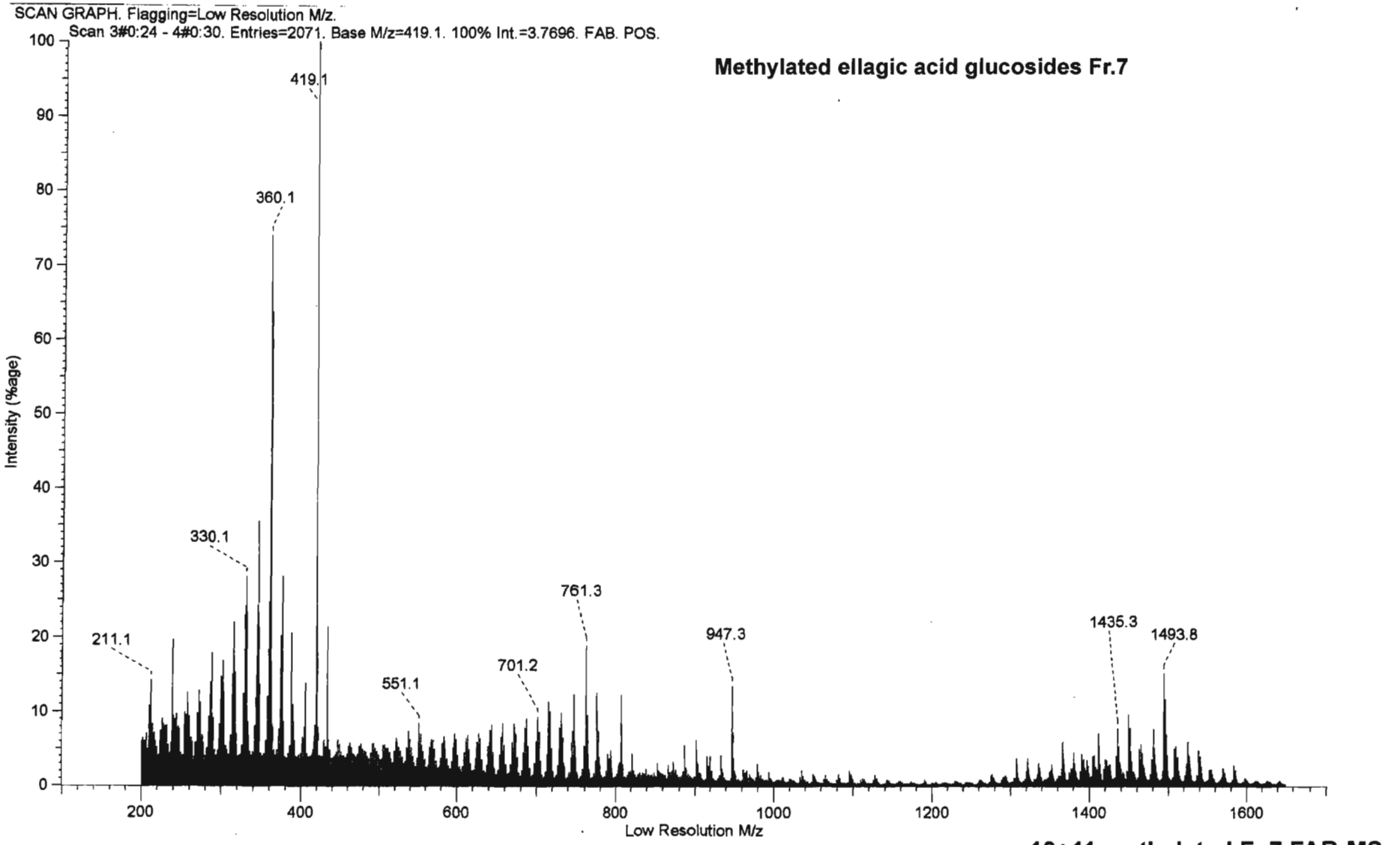


8+9 H-1 NMR

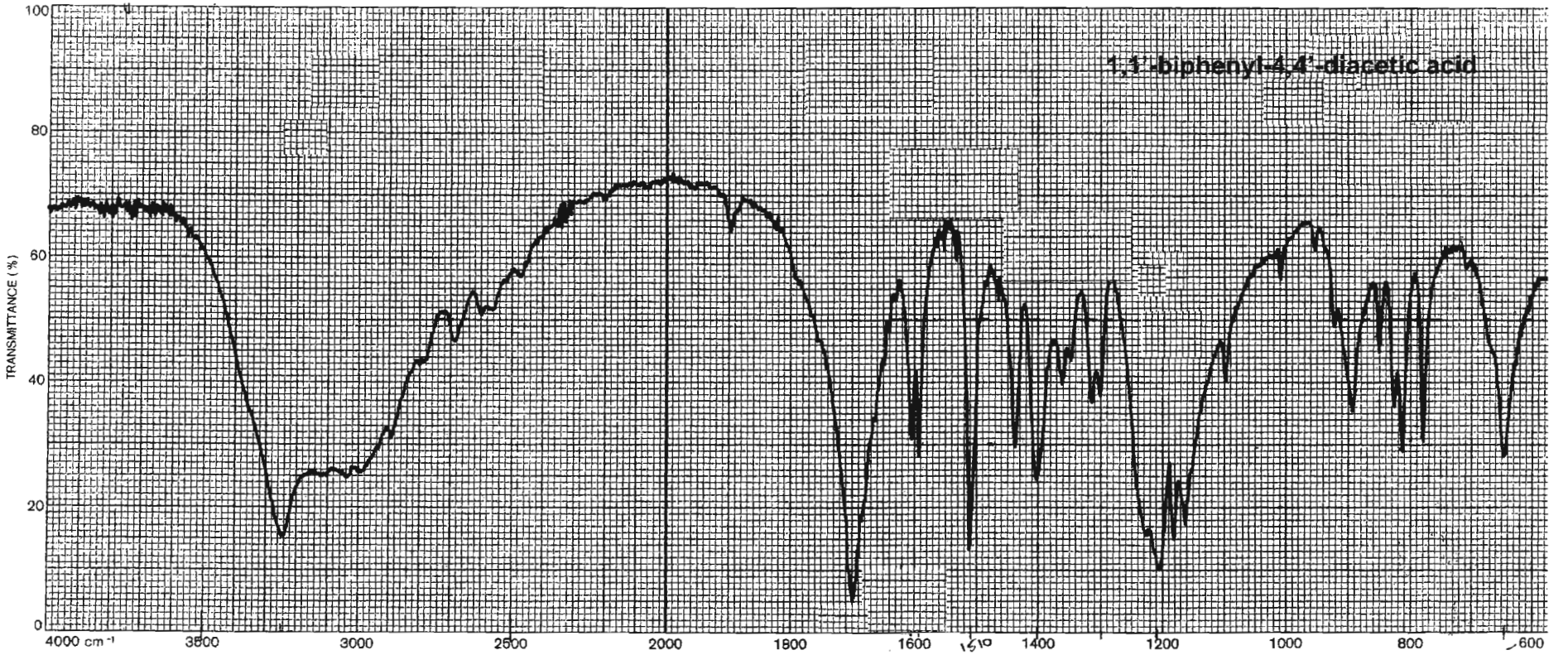
Ellagic acid glucosides 10 + 11



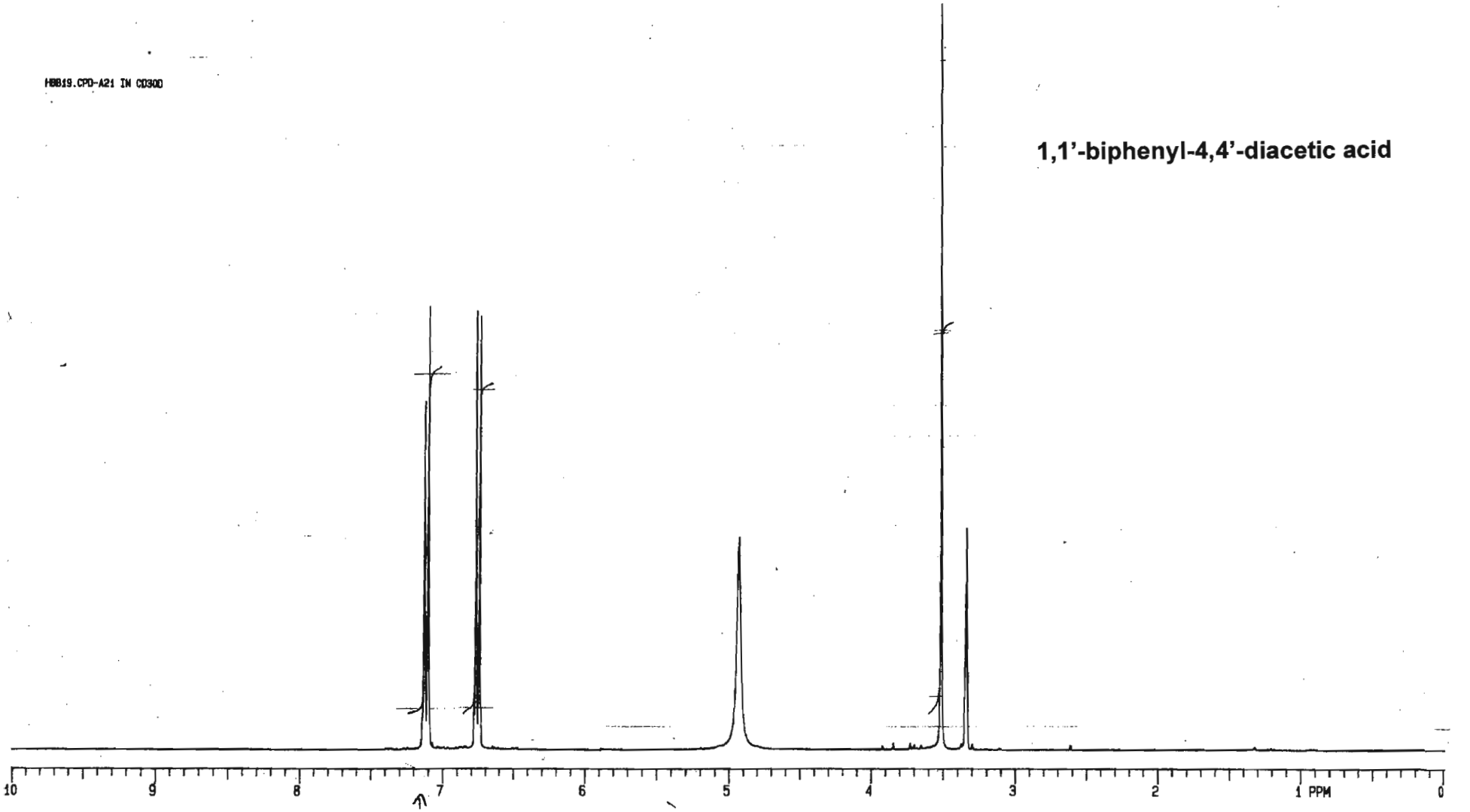
10 + 11 IR



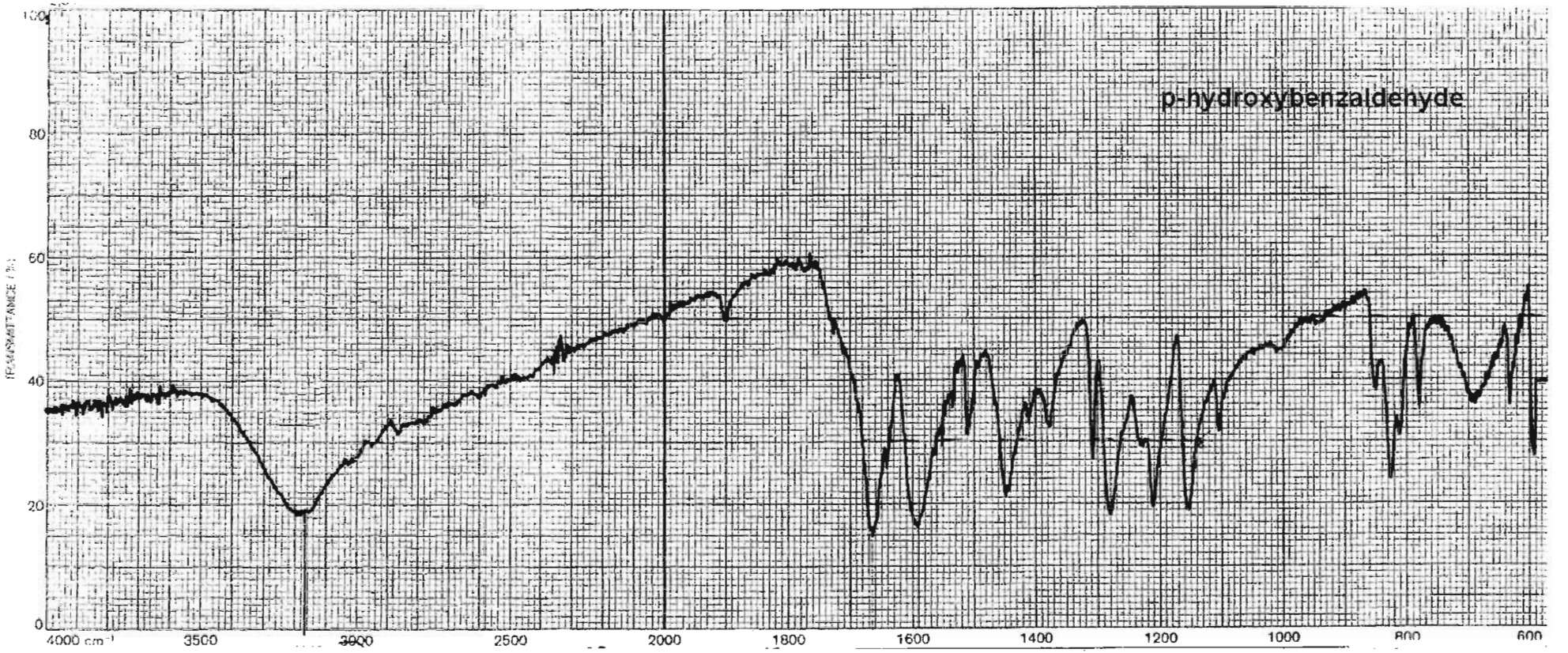
10+11 methylated Fr.7 FAB-MS

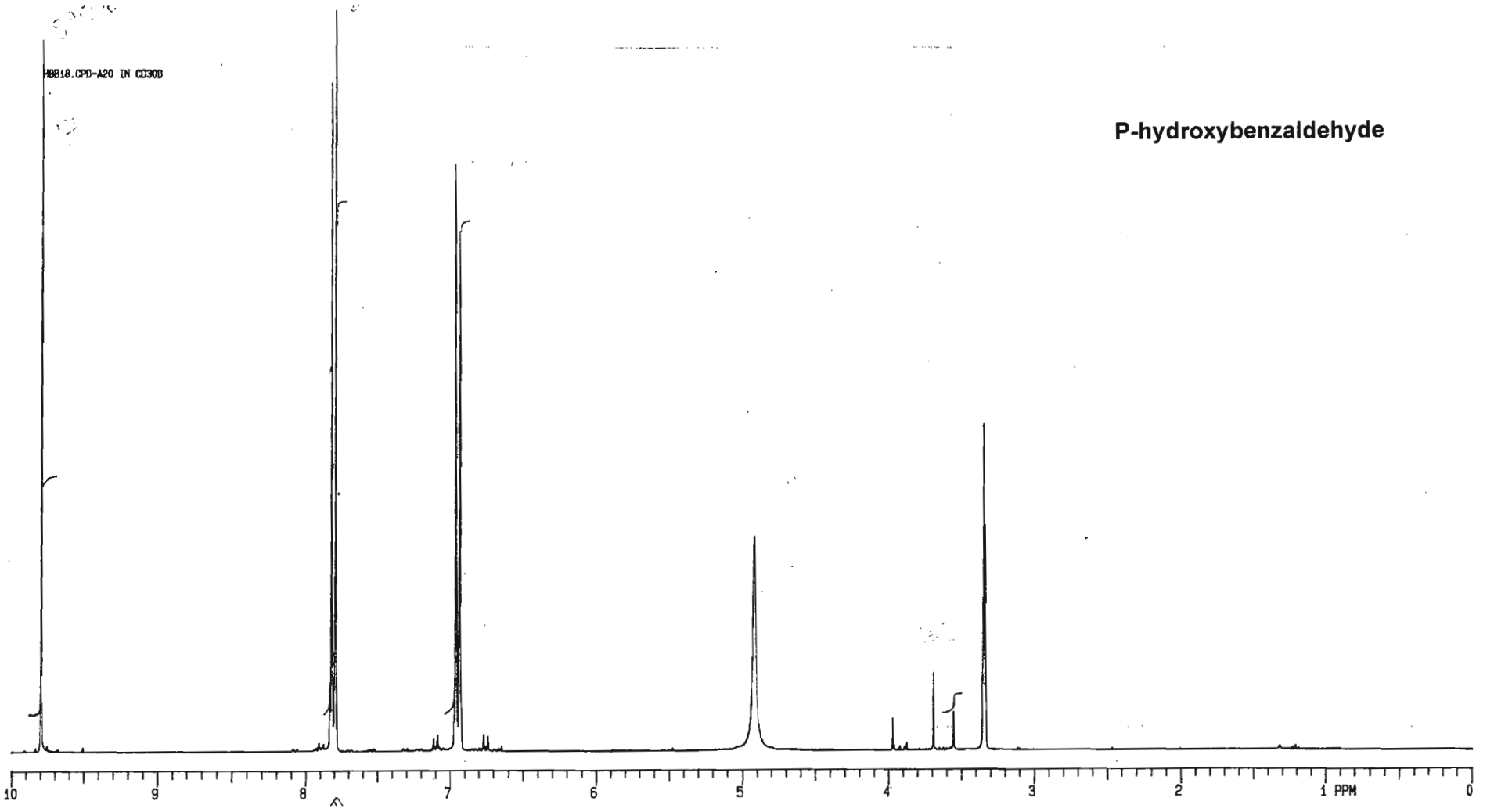

1,1'-biphenyl-4,4'-diacetic acid
TRANSMITTANCE (%)
100
80
60
40
20
0
4000 cm⁻¹
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600

14 IR

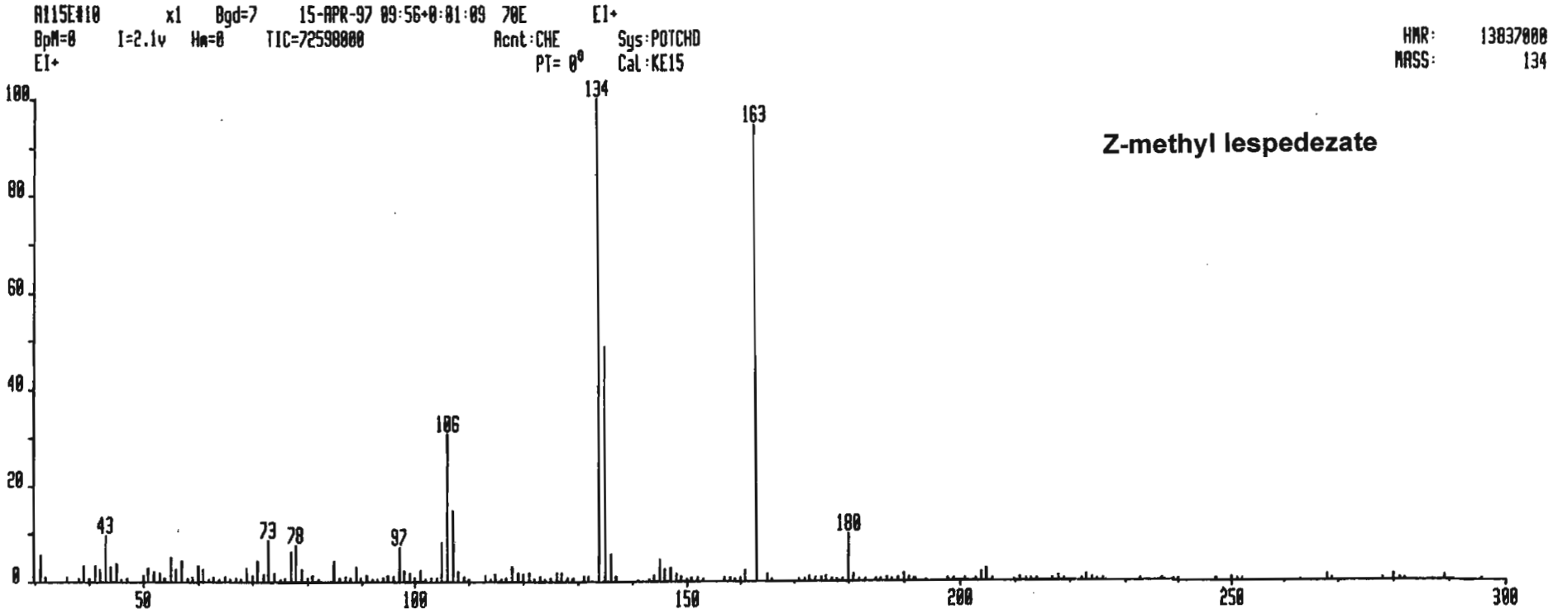

HBB19.CPD-A21 IN CD3OD
1,1'-biphenyl-4,4'-diacetic acid
10
9
8
7
6
5
4
3
2
1 PPM
0

14 H-1 NMR

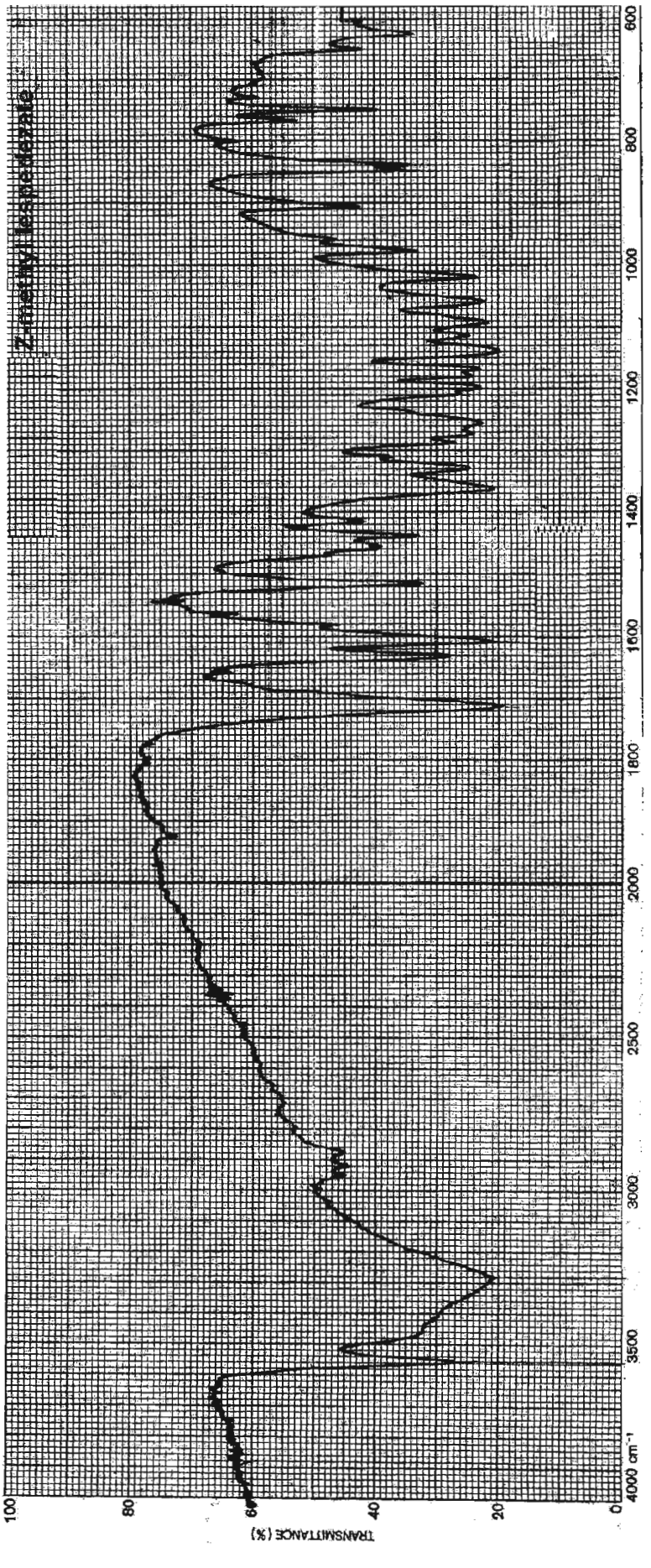

p-hydroxybenzaldehyde
100
80
60
40
20
0
4000 cm⁻¹
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600

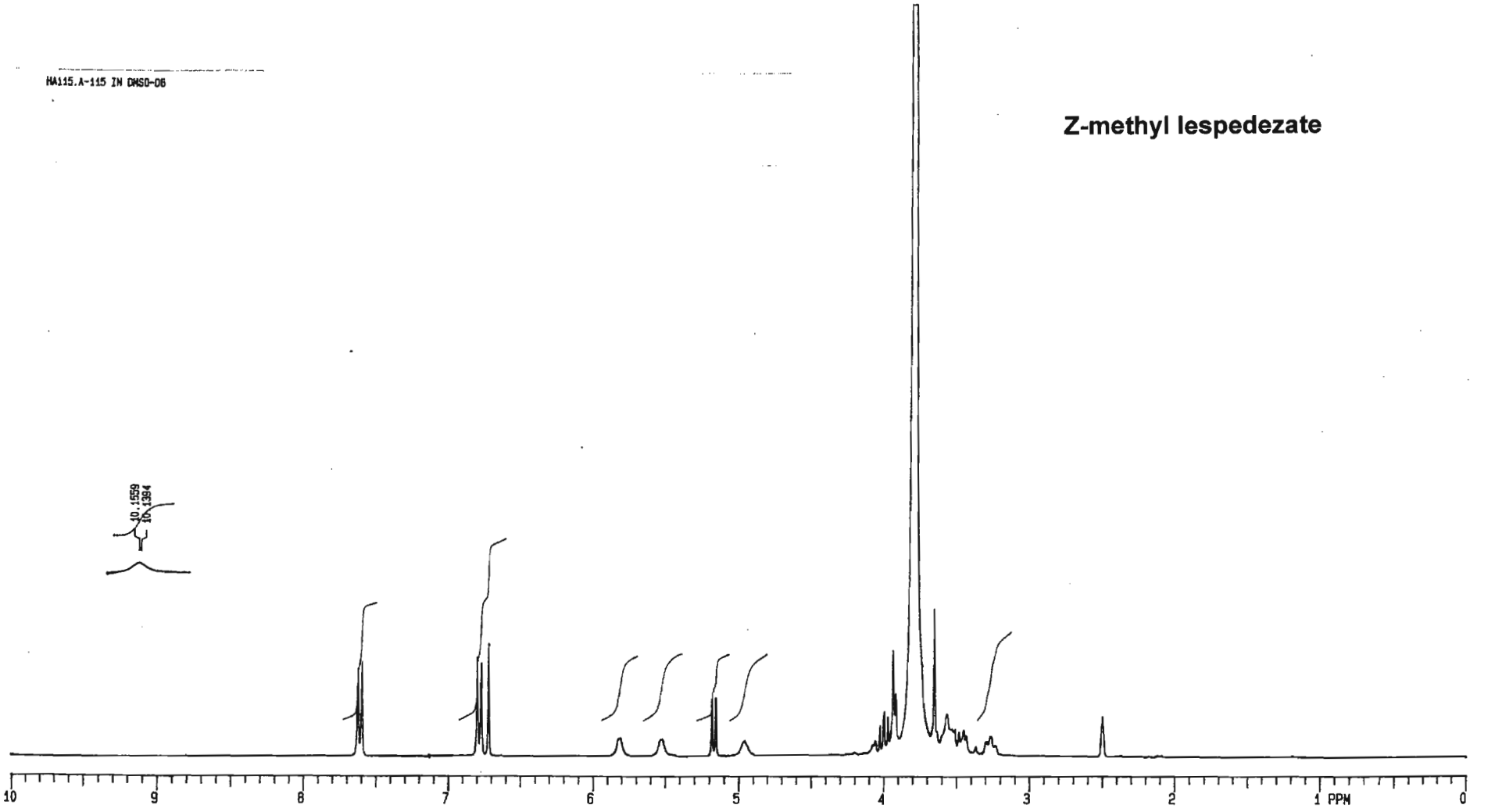

HBB18.CPD-A20 IN CD3OD
P-hydroxybenzaldehyde
10
9
8
7
6
5
4
3
2
1 PPM
0

15 H-1 NMR


A115E#10 x1 Bgd=7 15-APR-97 09:56+0:01:09 70E EI+
BpM=0 I=2.1v Hm=0 TIC=72598000 Acnt:CHE Sys:POTCHD
EI+ PT= 0° Cal:KE15
HMR: 13837000
MASS: 134
Z-methyl lespedezate
134
163
106
43
73
78
97
180
100
80
60
40
20
0
50
100
150
200
250
300

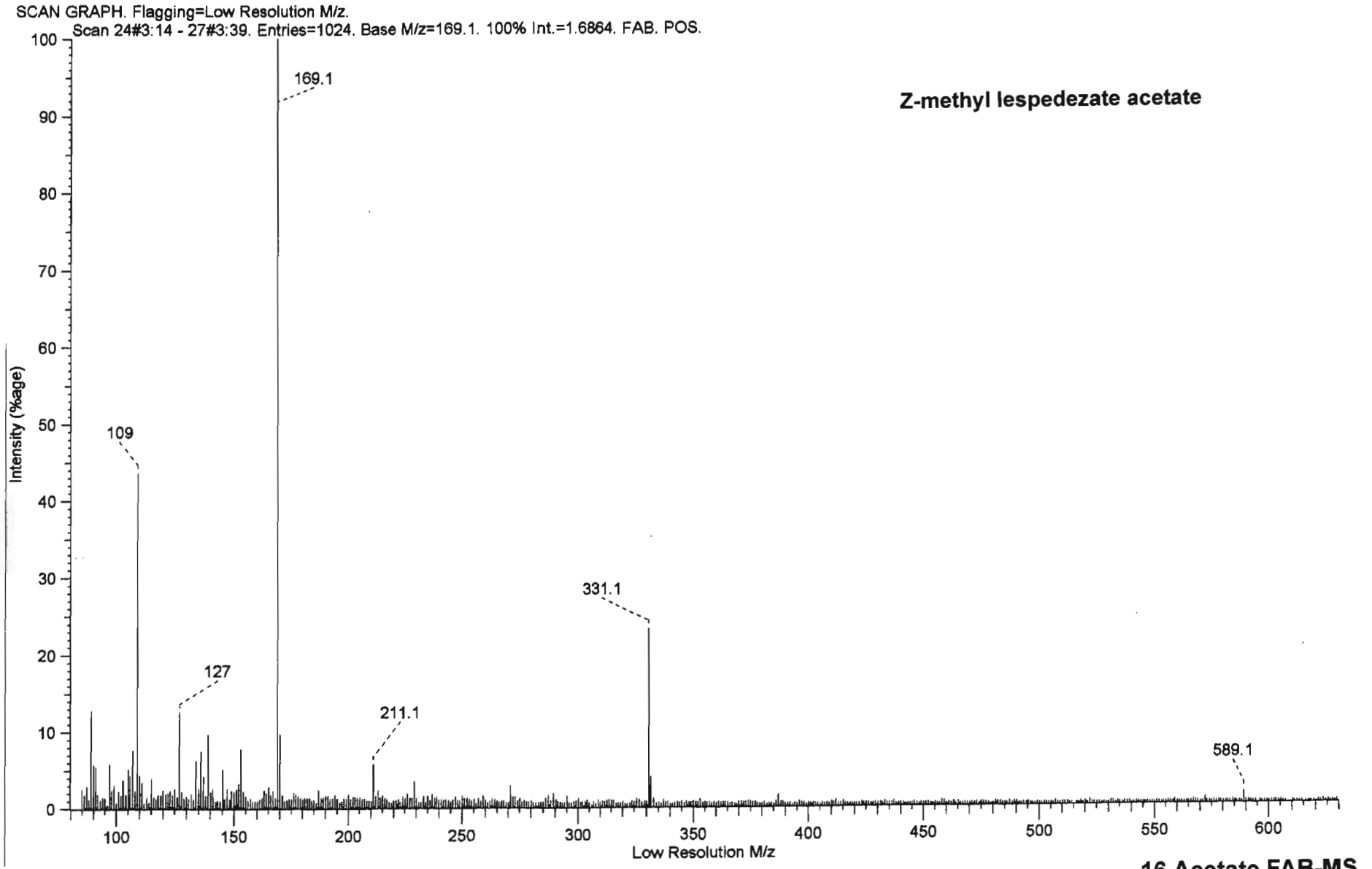
16 EI-MS


Zinostatin stimalamer
TRANSMITTANCE (%)
100
80
60
40
20
0
4000 cm⁻¹
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600

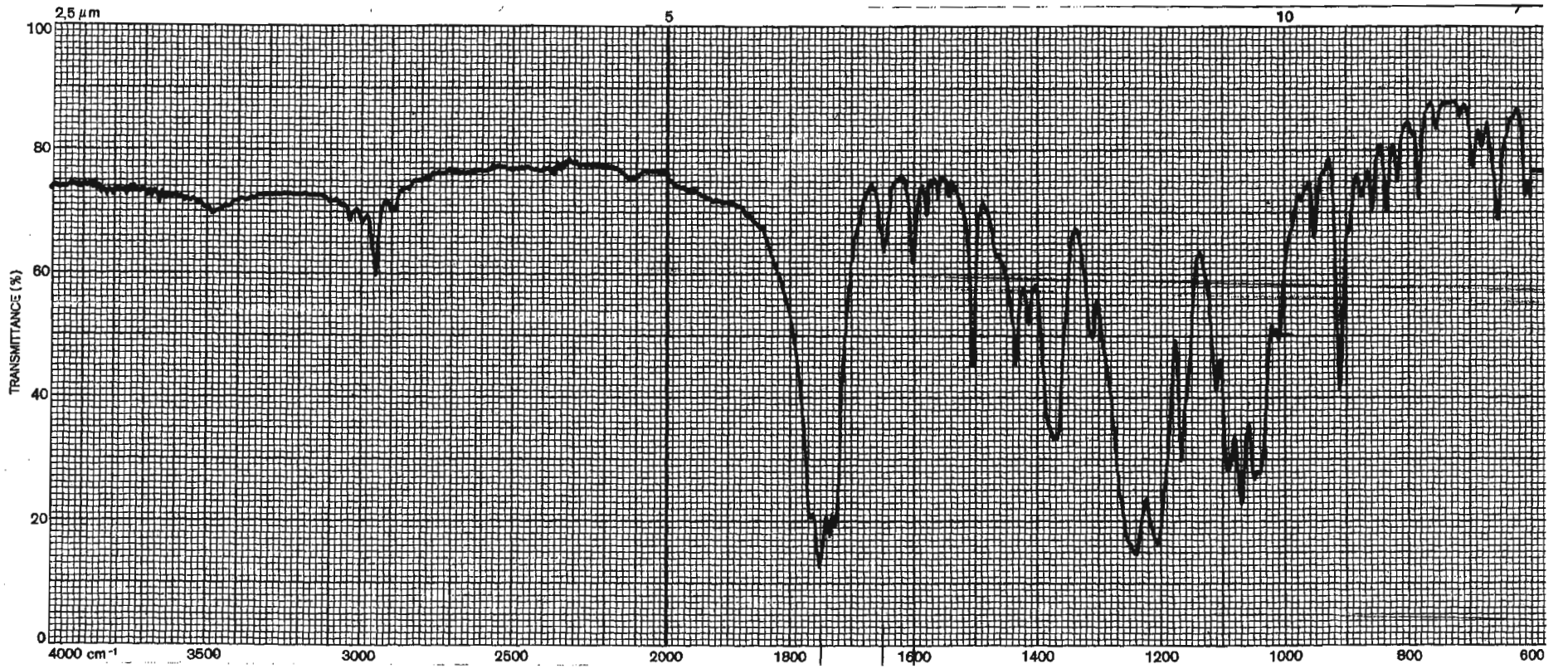


Z-methyl lespedezate

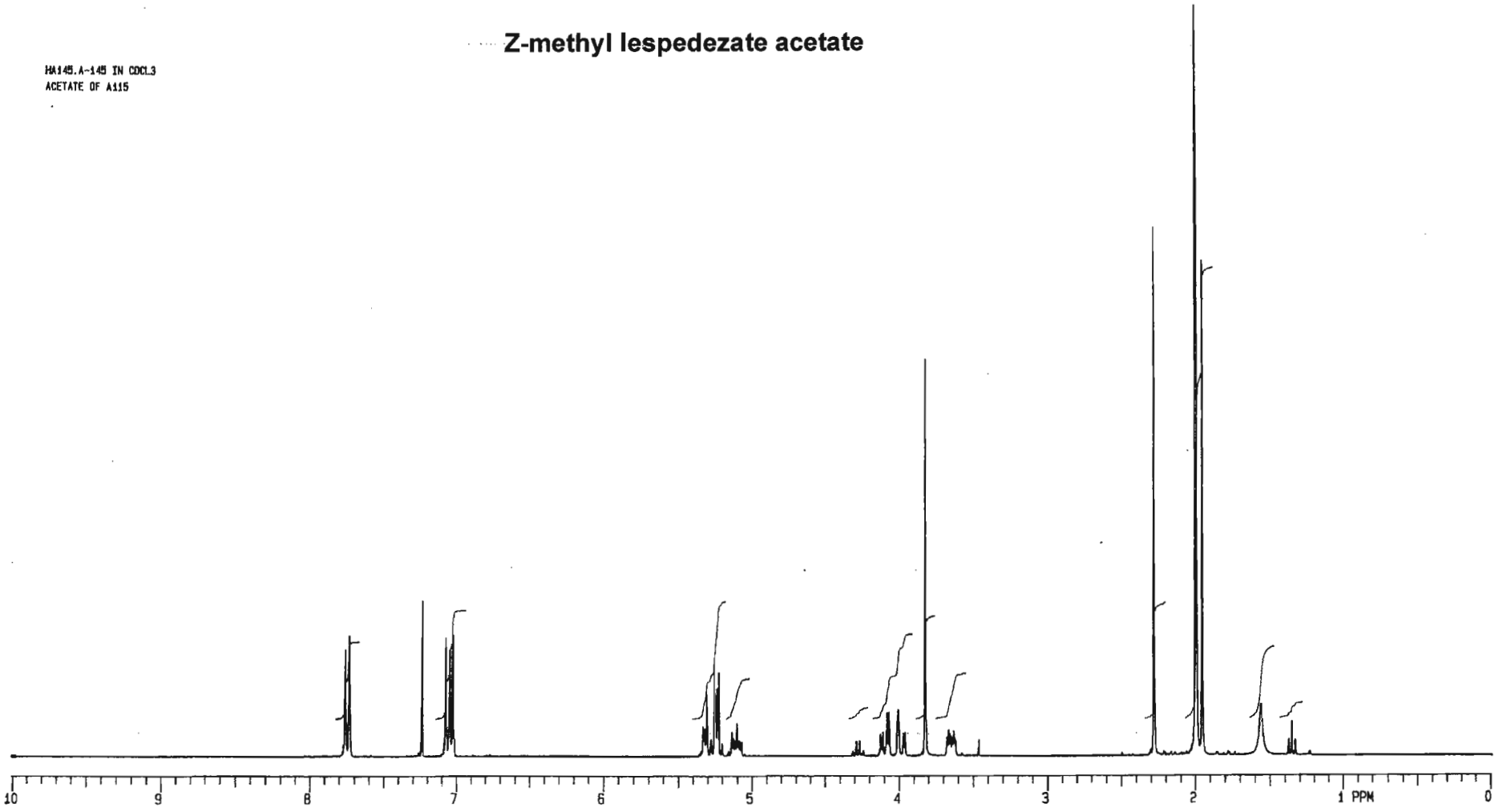
16 H-1 NMR


SCAN GRAPH. Flagging=Low Resolution M/z.
Scan 24#3:14 - 27#3:39. Entries=1024. Base M/z=169.1. 100% Int.=1.6864. FAB. POS.
Intensity (%age)
Low Resolution M/z
169.1
109
127
211.1
331.1
589.1
Z-methyl lespedezate acetate
16 Acetate FAB-MS

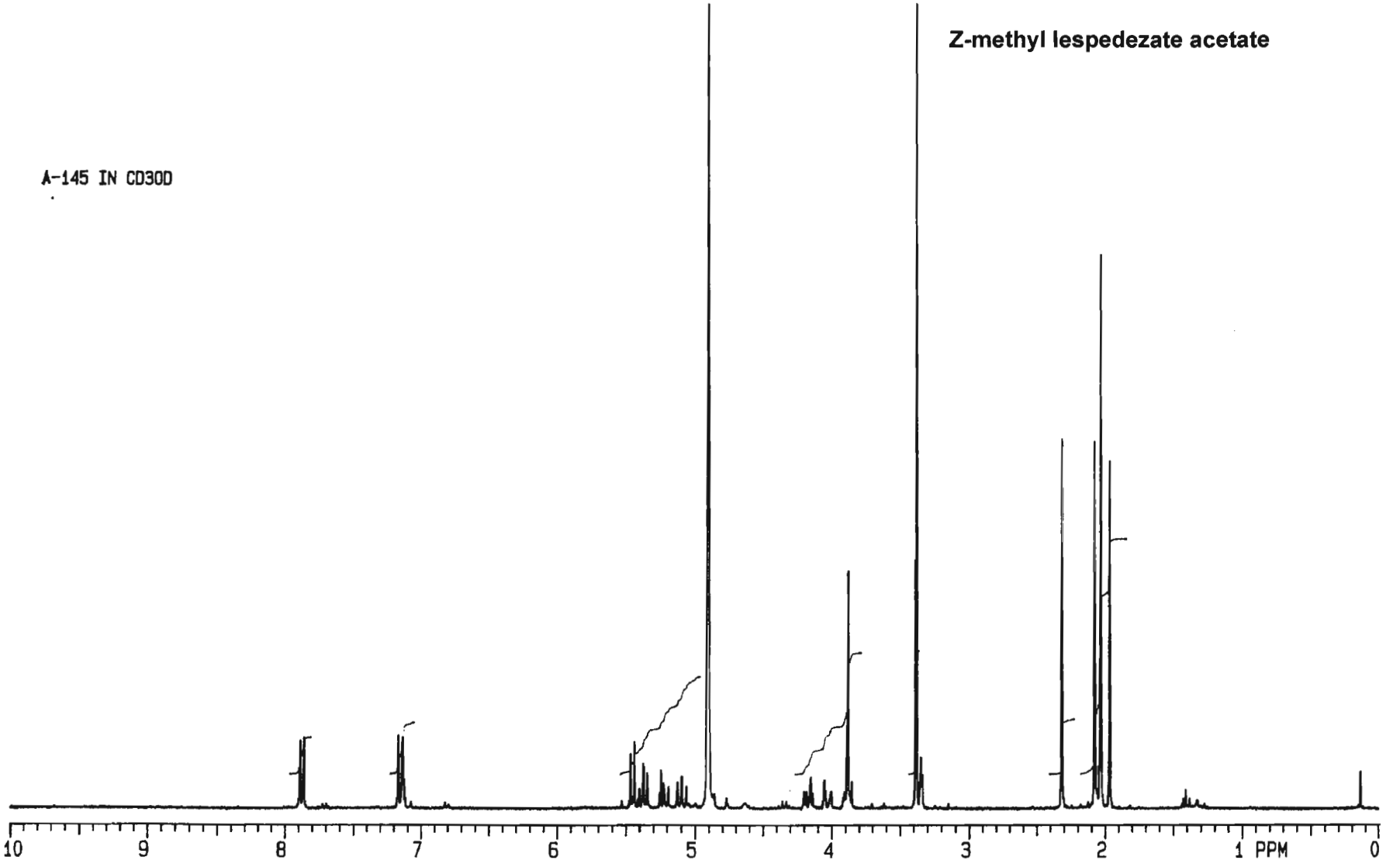
Z-methyl lespedezate acetate



Z-methyl lespedezate acetate

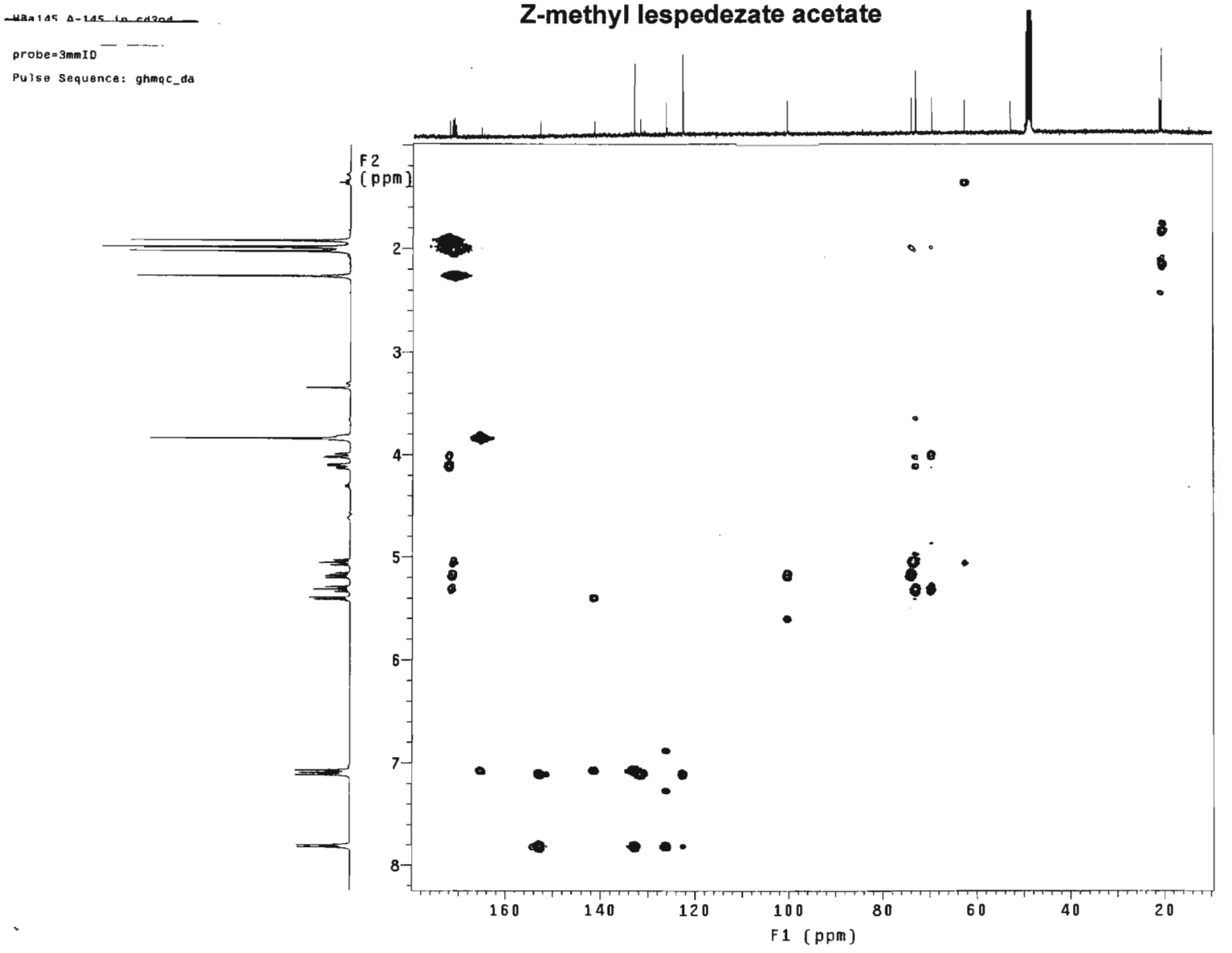


16 Acetate H-1 NMR in CDCl3



16 H-1 NMR in CD_3OD

Z-methyl lespedezate acetate

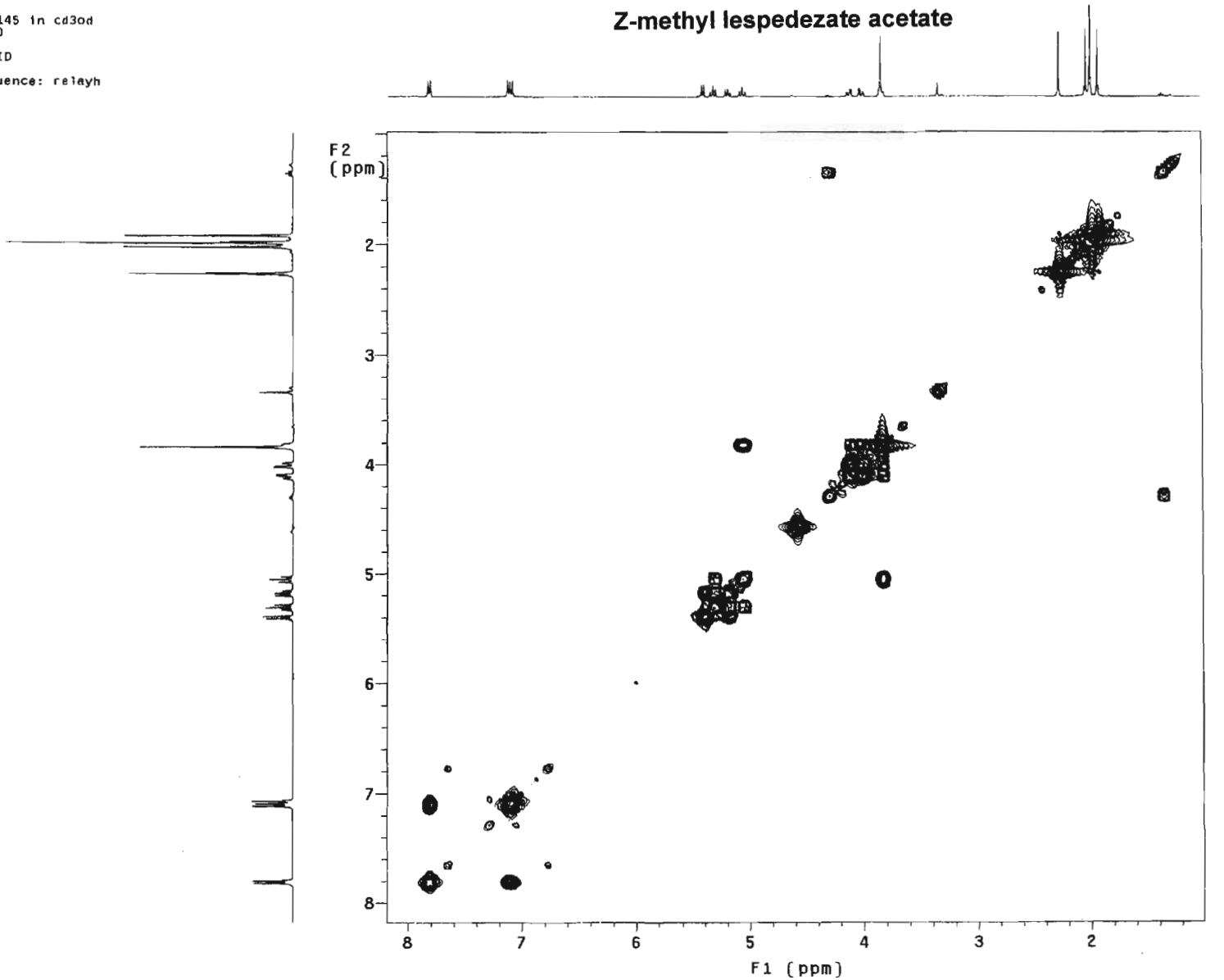


16 Acetate HMBC

cya145.A-145 in cd3od
1H Cosy-90
temp=45
probe=3mmID

Pulse Sequence: relayh

Z-methyl lespedezate acetate



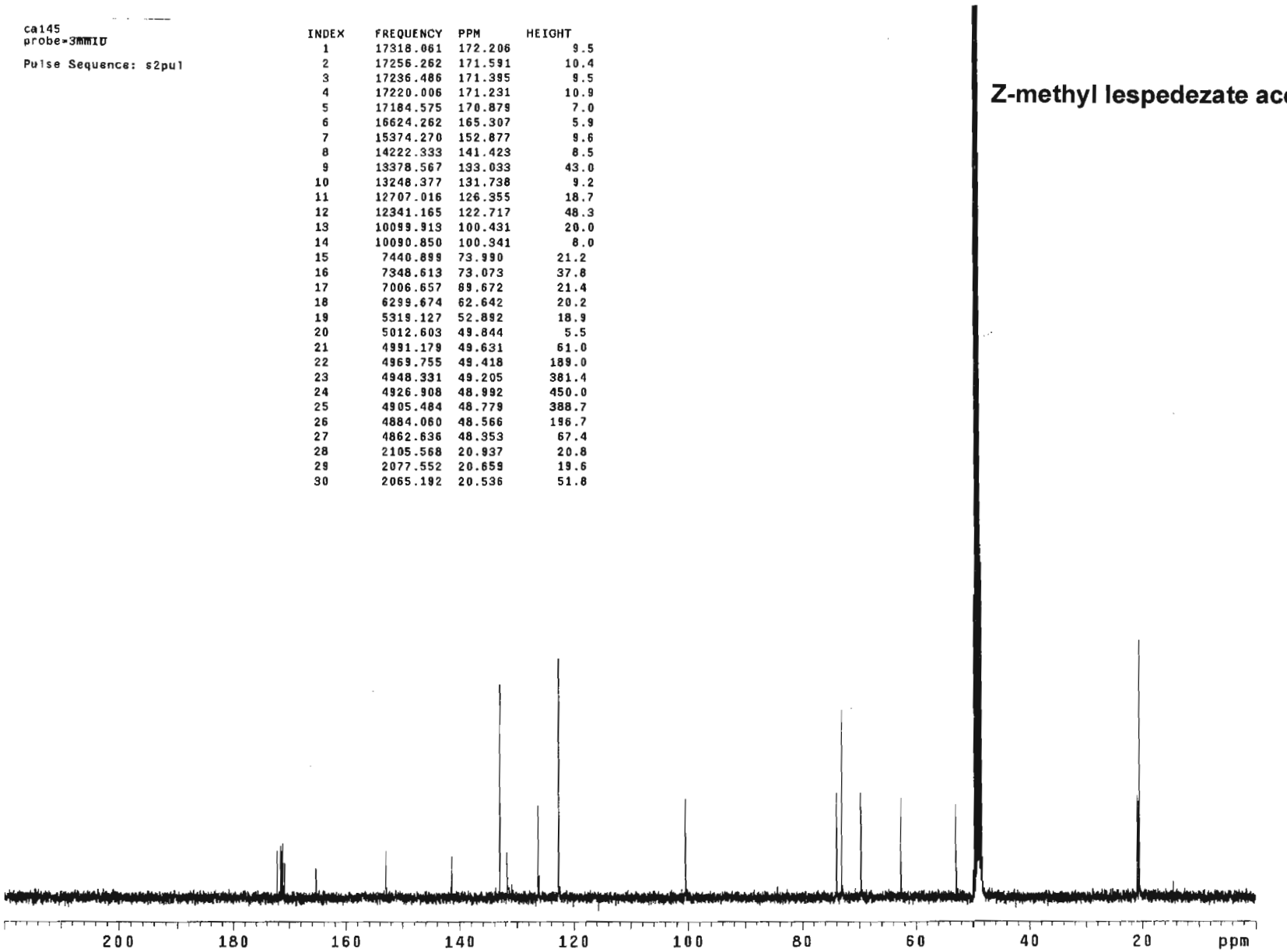
16 Acetate COSY

ca145
probe=3mmID

Pulse Sequence: s2pul

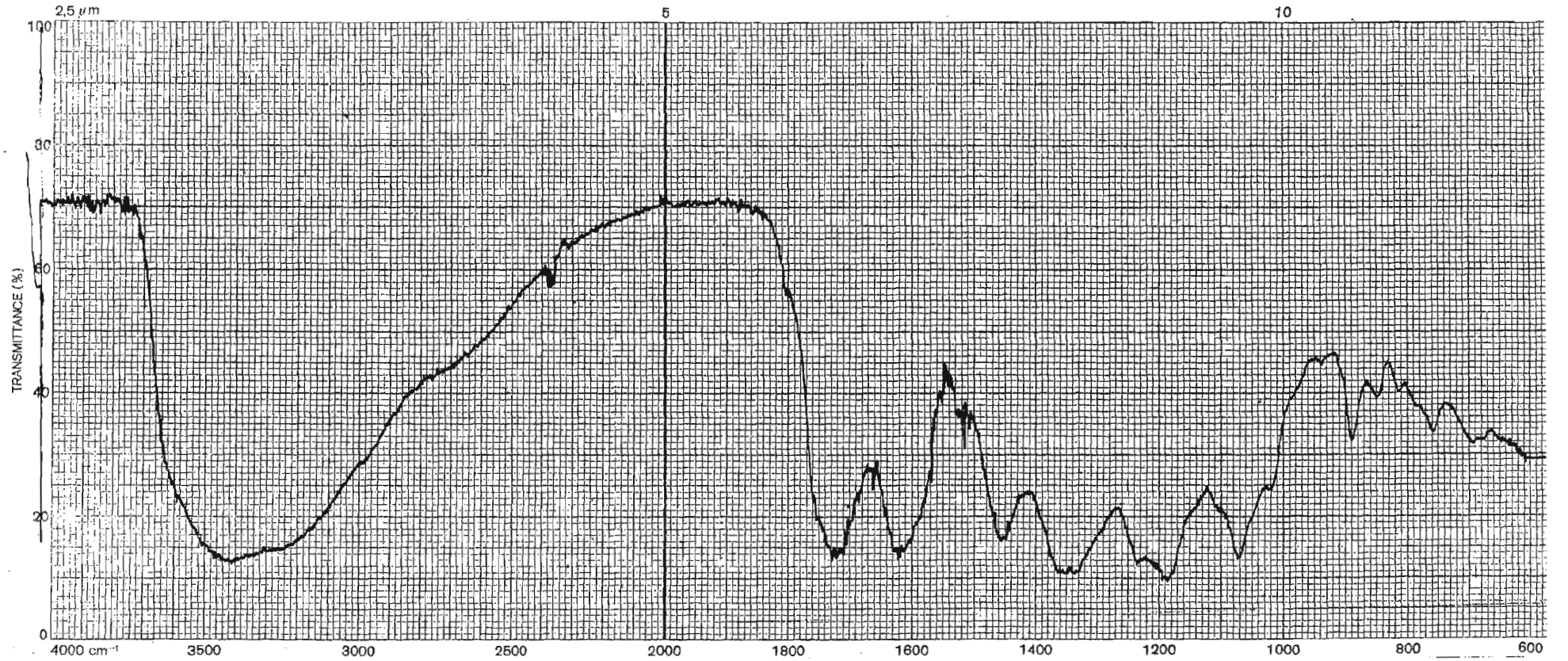
INDEX	FREQUENCY	PPM	HEIGHT
1	17318.061	172.206	9.5
2	17256.262	171.591	10.4
3	17236.486	171.395	9.5
4	17220.006	171.231	10.9
5	17184.575	170.879	7.0
6	16624.262	165.307	5.9
7	15374.270	152.877	9.6
8	14222.333	141.423	8.5
9	13378.567	133.033	43.0
10	13248.377	131.738	9.2
11	12707.016	126.355	18.7
12	12341.165	122.717	48.3
13	10099.913	100.431	20.0
14	10090.850	100.341	8.0
15	7440.899	73.990	21.2
16	7348.613	73.073	37.8
17	7006.657	69.672	21.4
18	6299.674	62.642	20.2
19	5319.127	52.892	18.9
20	5012.603	49.844	5.5
21	4991.179	49.631	61.0
22	4969.755	49.418	189.0
23	4948.331	49.205	381.4
24	4926.908	48.992	450.0
25	4905.484	48.779	388.7
26	4884.060	48.566	196.7
27	4862.636	48.353	67.4
28	2105.568	20.937	20.8
29	2077.552	20.659	19.6
30	2065.192	20.536	51.8

Z-methyl lespedezate acetate

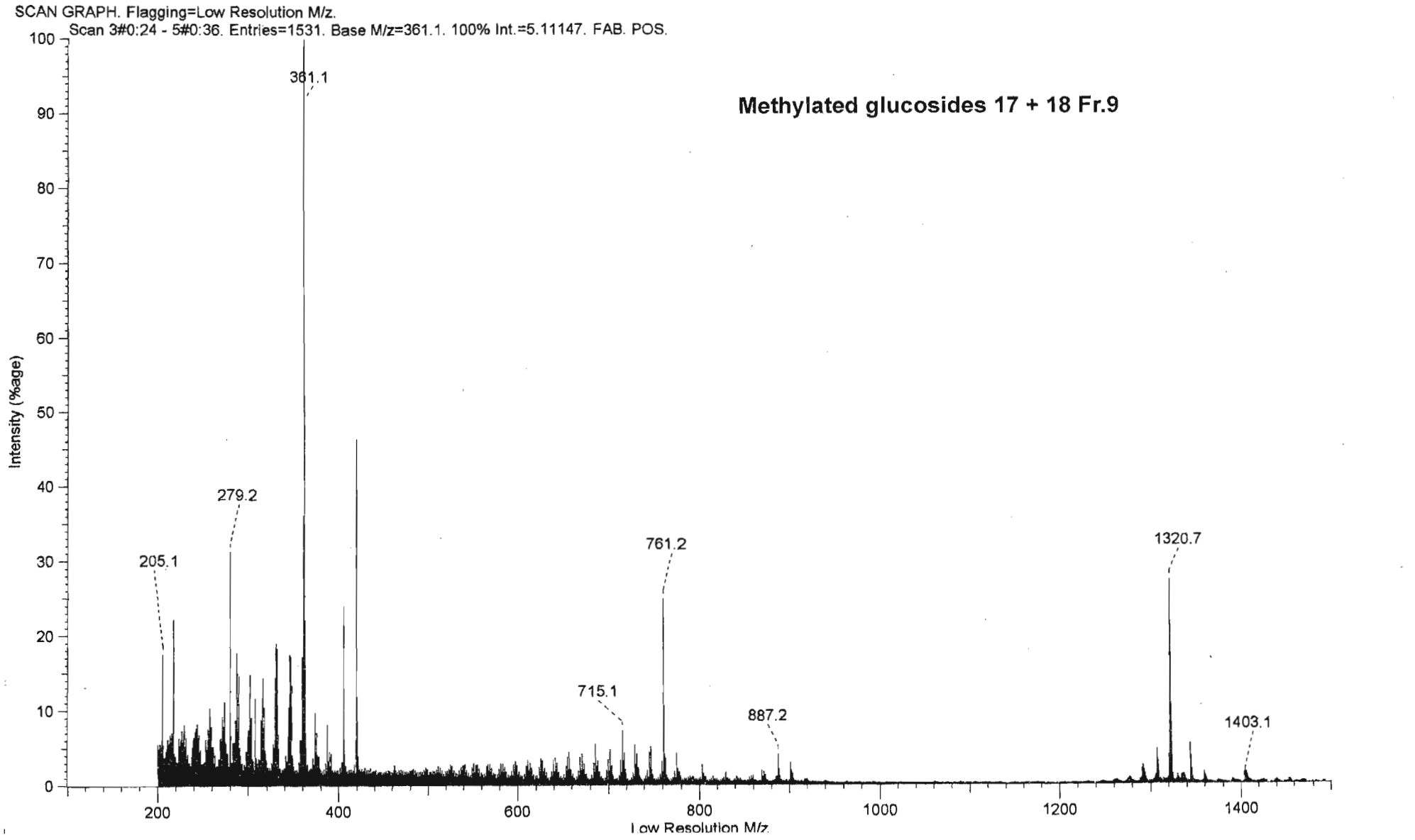


16 Acetate C-13 NMR

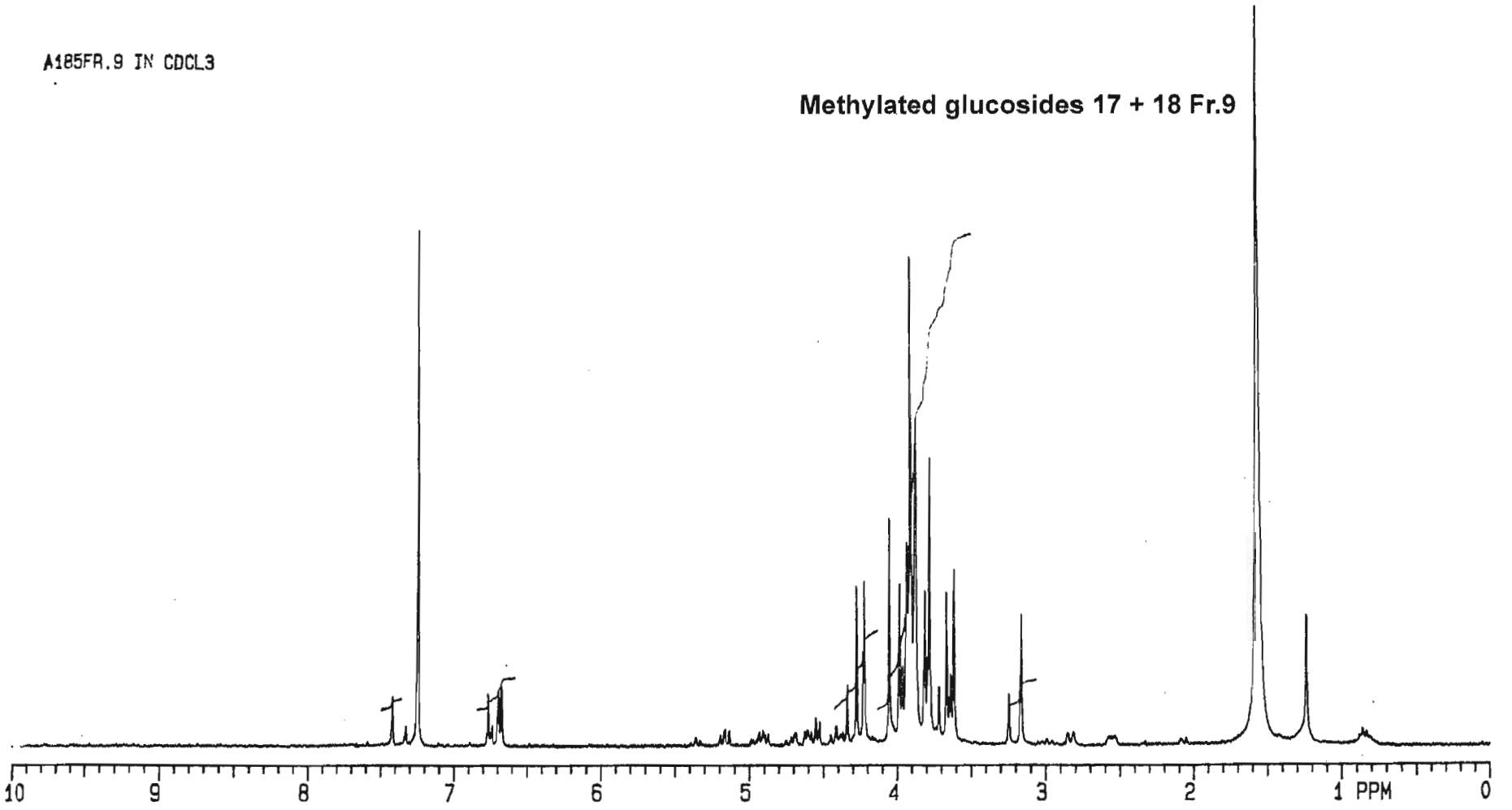
Glucosides 17 + 18



17 + 18 IR



Methylated 17 + 18 Fr.9 FAB-MS

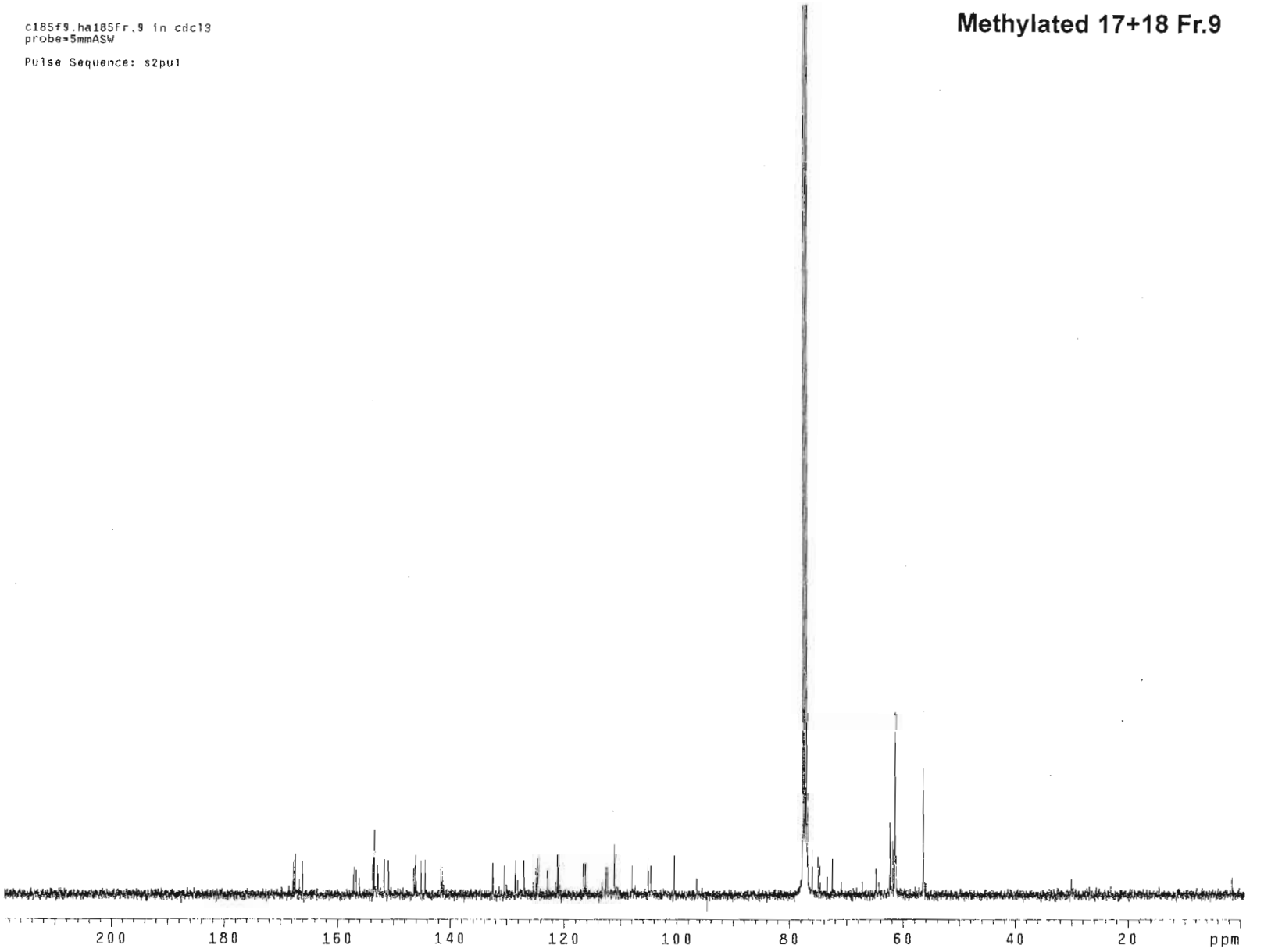


Methylated 17 + 18 Fr.9 H-1 NMR

c185f9.ha185fr.9 in cdcl3
probe=5mmASW

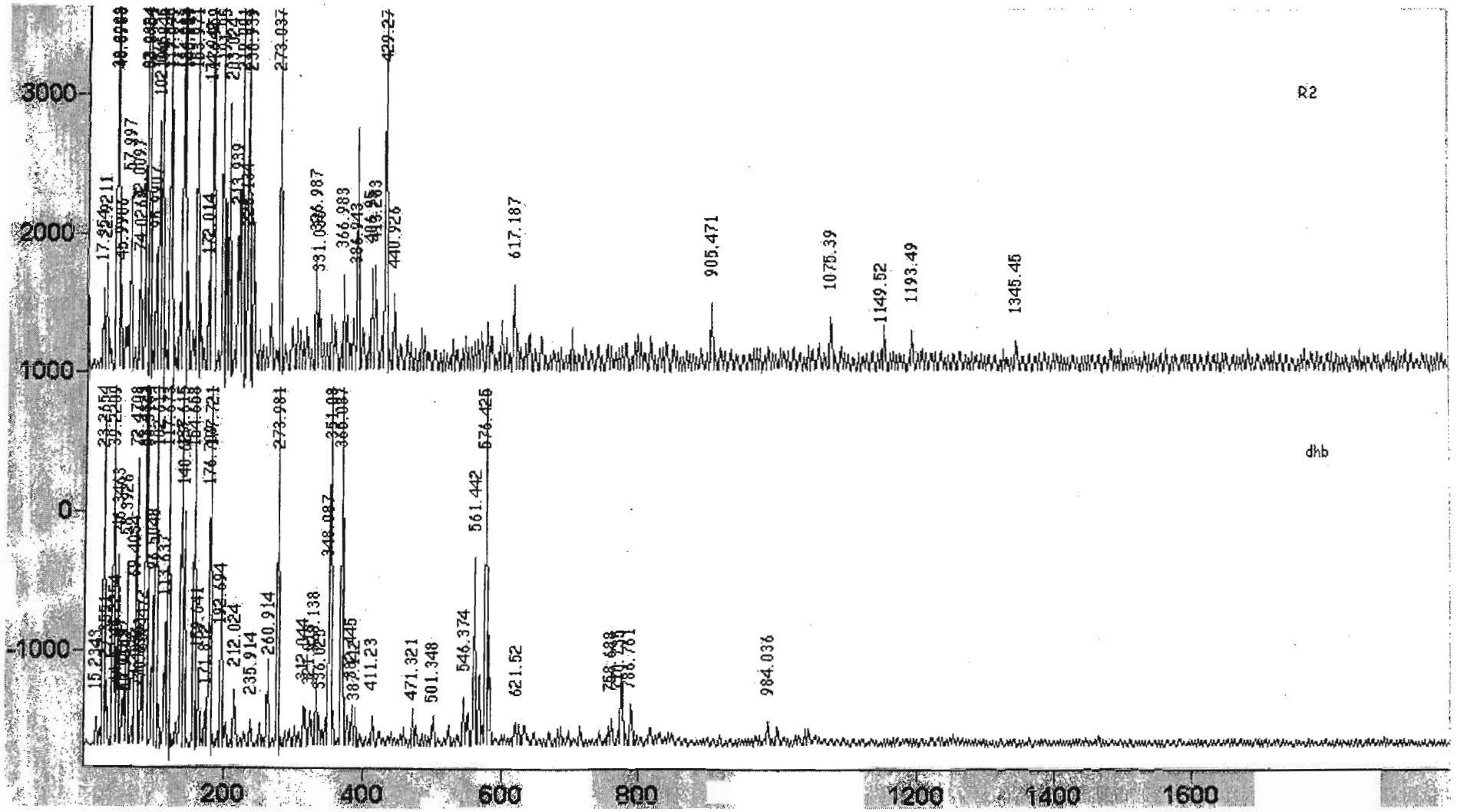
Pulse Sequence: s2pul

Methylated 17+18 Fr.9

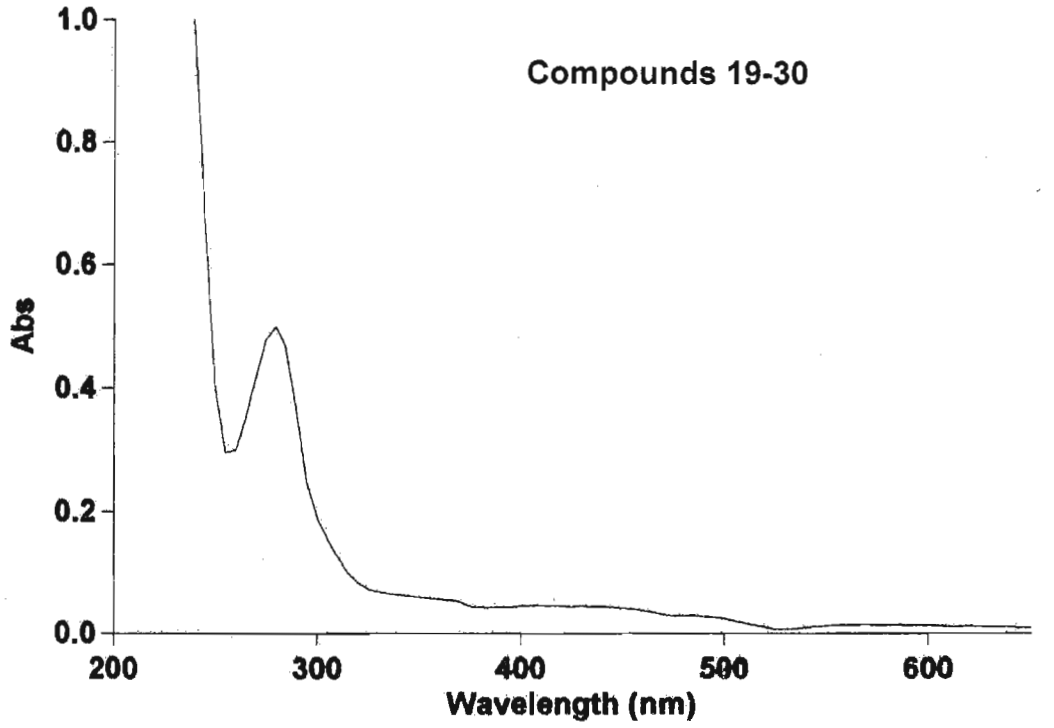


Methylated 17+18 Fr.9 C-13

Compounds 19-30 in methanol extract



19-30 MALDI-TOF MS



Scan Analysis Report

Report Time : Mon 20 Mar 03:05:56 PM 2000
Batch:
Software version: 02.00(25)
Operator: Bridget

Sample Name: sample4 dil. 1:100

Collection Time	3/20/00 3:06:16 PM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.0100
Range	649.9nm to 199.9nm

Wavelength (nm)	Abs
280.0	0.4999

19-30 UV

0.70 cm/min Attenuation = 247 Zero Offset = 34%
0.000 min End Time = 30.002 min Min / Tick = 1.00
-20
-10
0
10
20
30
0.022
1.667
1.845
1.952
2.324
2.217
2.334
2.418
2.628
2.797
3.116
3.550
3.884
4.420
5.133
5.854
6.481
6.978
8.565
9.399
10.115
10.873
11.296
12.179
12.698
13.597
14.153
14.685
15.491
16.994
19.621
20.292
20.838
21.282
22.133
24.006
25.330
27.206
28.263
<WI=2.0
<WI=4.0
<WI=8.0
<WI=16.0
Compounds 19-30 HPLC (1)
19-30 HPLC (1)

Compounds 19-30 Alpha Labs. HPLC

```
Injection Date   : 12/6/2002 11:00:56 AM          Seq. Line :   4
Sample Name      : sample #5458                      Vial :   4
Acq. Operator    : ebl                               Inj :   1
                                                 Inj Volume : 5 µl

Acq. Method      : C:\HPCHEM\5\METHODS\SBCAT.M
Last changed     : 12/6/2002 8:57:43 AM by ebl
Analysis Method  : C:\HPCHEM\5\METHODS\SBCAT.M
Last changed     : 12/6/2002 2:37:18 PM by ebl
                   (modified after loading)
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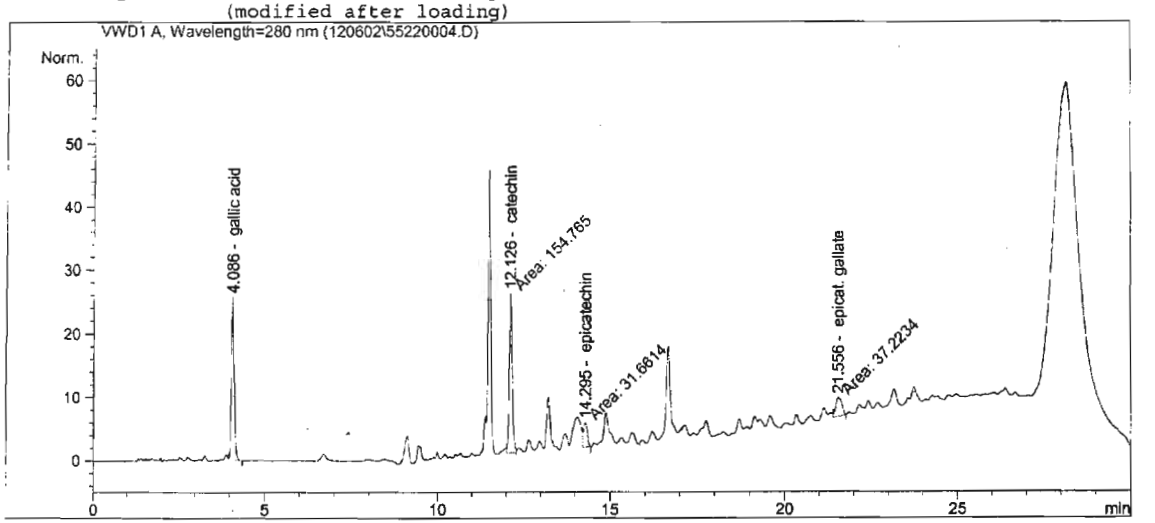


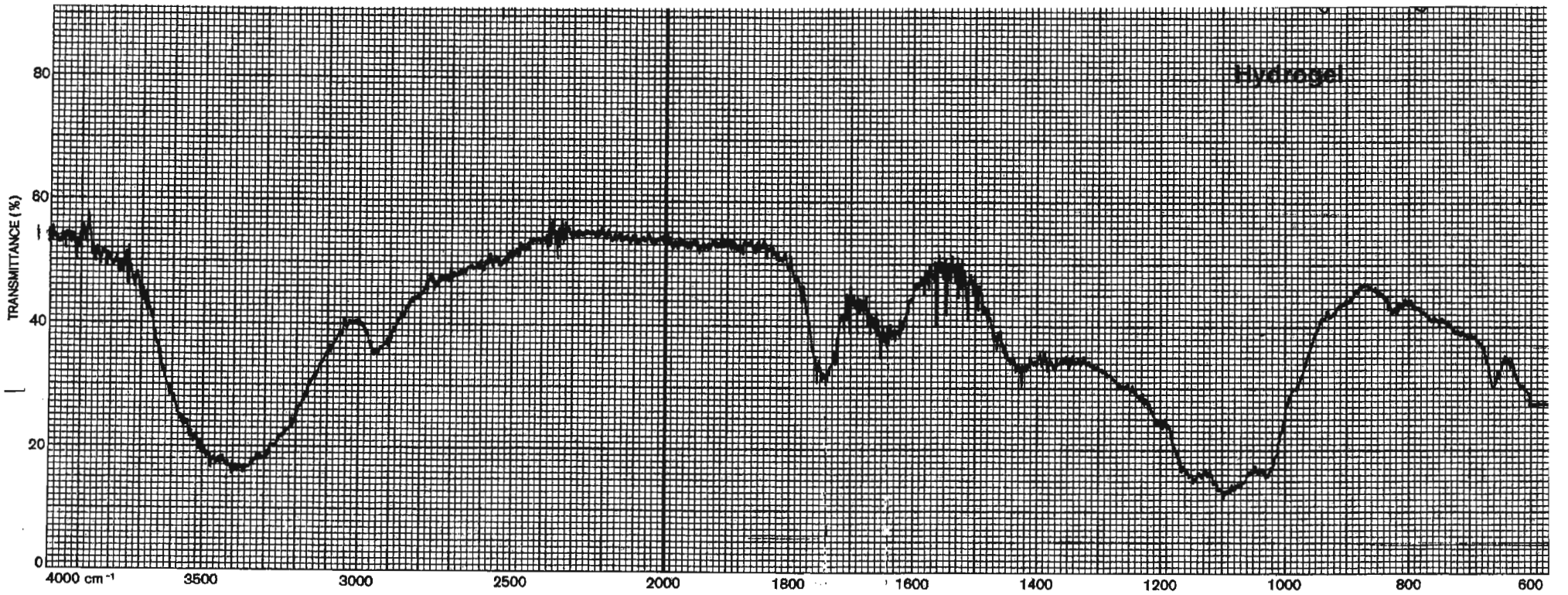

```
=====================================================================
                        ESTD Percent Report
=====================================================================

Sorted By             :      Signal
Calib. Data Modified  :      12/6/2002 2:37:14 PM
Multiplier            :      1.0000
Dilution              :      1.0000
Sample Amount         :      6.27800  [mg/ml]

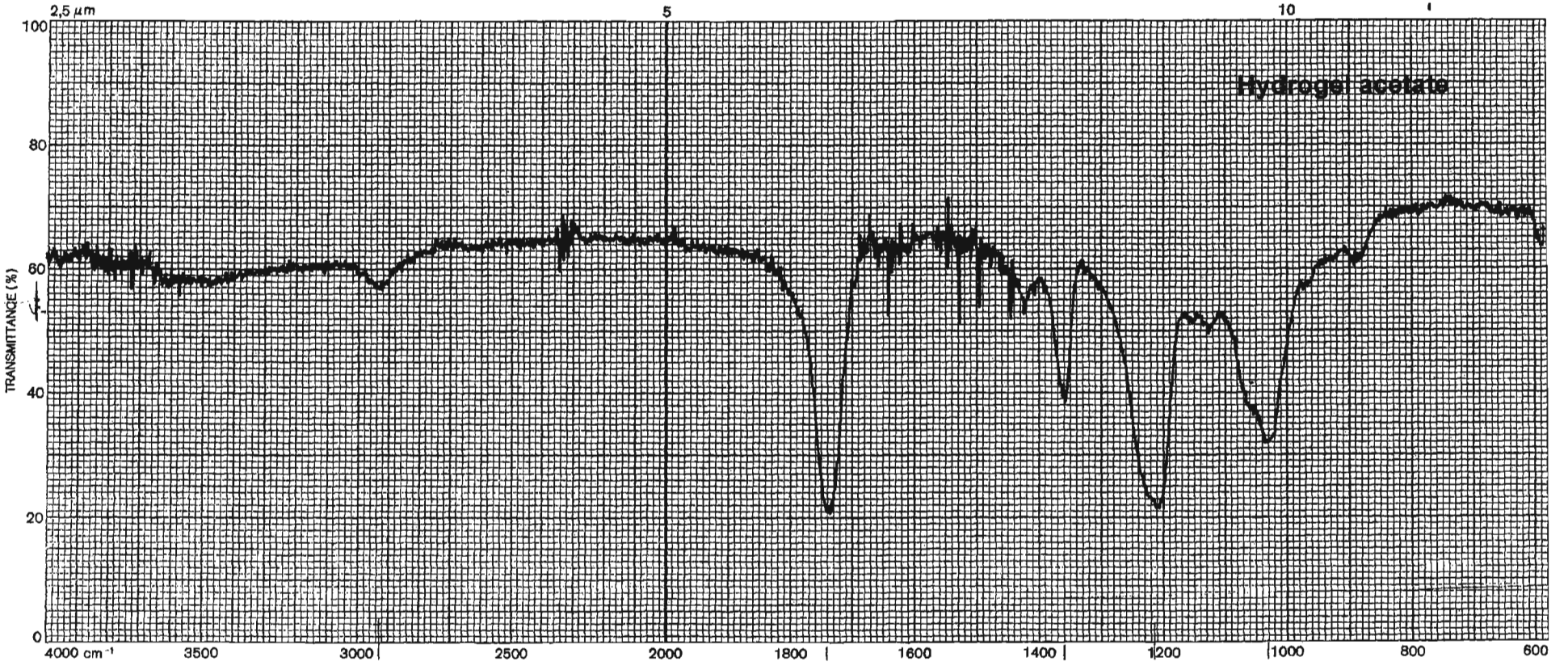
Signal 1: VWD1 A, Wavelength=280 nm

RetTime  Type      Area      Amt/Area     Amount    Grp   Name
 [min]          mAU   *s                    %
-------|------|----------|----------|----------|--|------------------
 4.086 VP       141.42526 8.78152e-5   0.197822     gallic acid
10.975            -          -          -          EGC
12.126 MF       154.76515 3.45806e-4   0.852480     catechin
14.057            -          -          -          caffeine
14.295 FM        31.66141 3.34212e-4   0.168551     epicatechin
16.213            -          -          -          EGC gallate
17.454            -          -          -          GC gallate
21.556 MF        37.22336 1.43001e-4 8.47880e-2    epicat. gallate

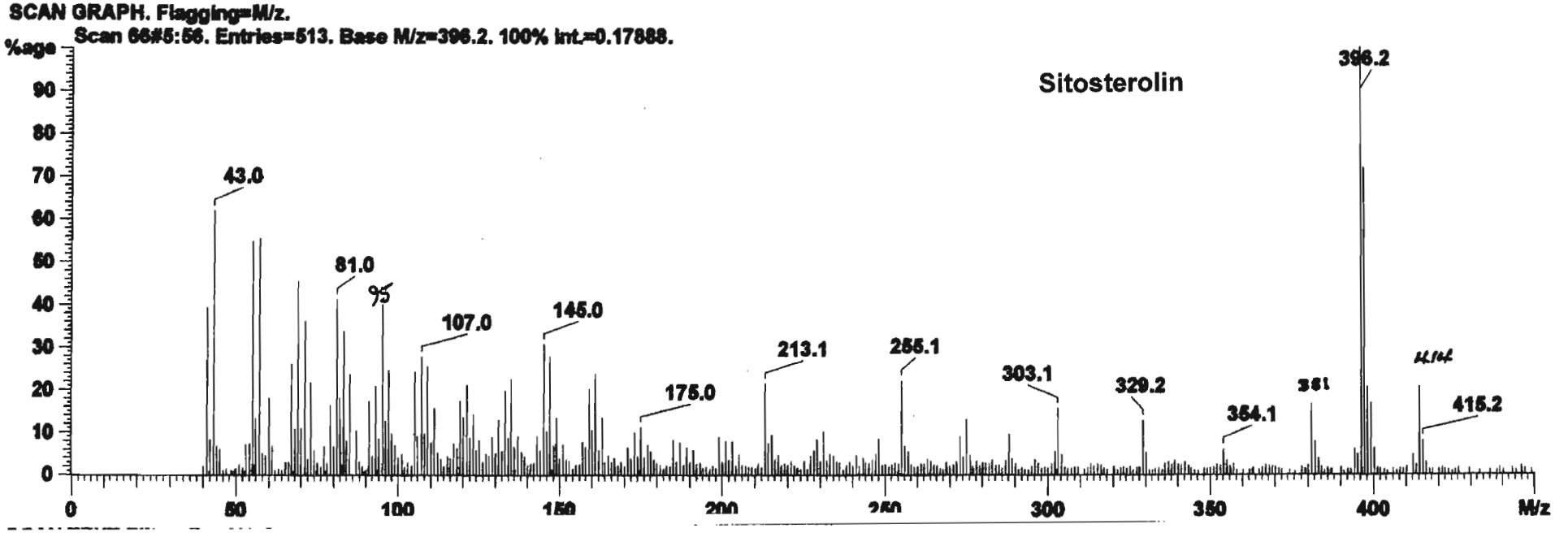
Totals :                                1.303642
```


Hydrogel
TRANSMITTANCE (%)
80
60
40
20
0
4000 cm⁻¹
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600

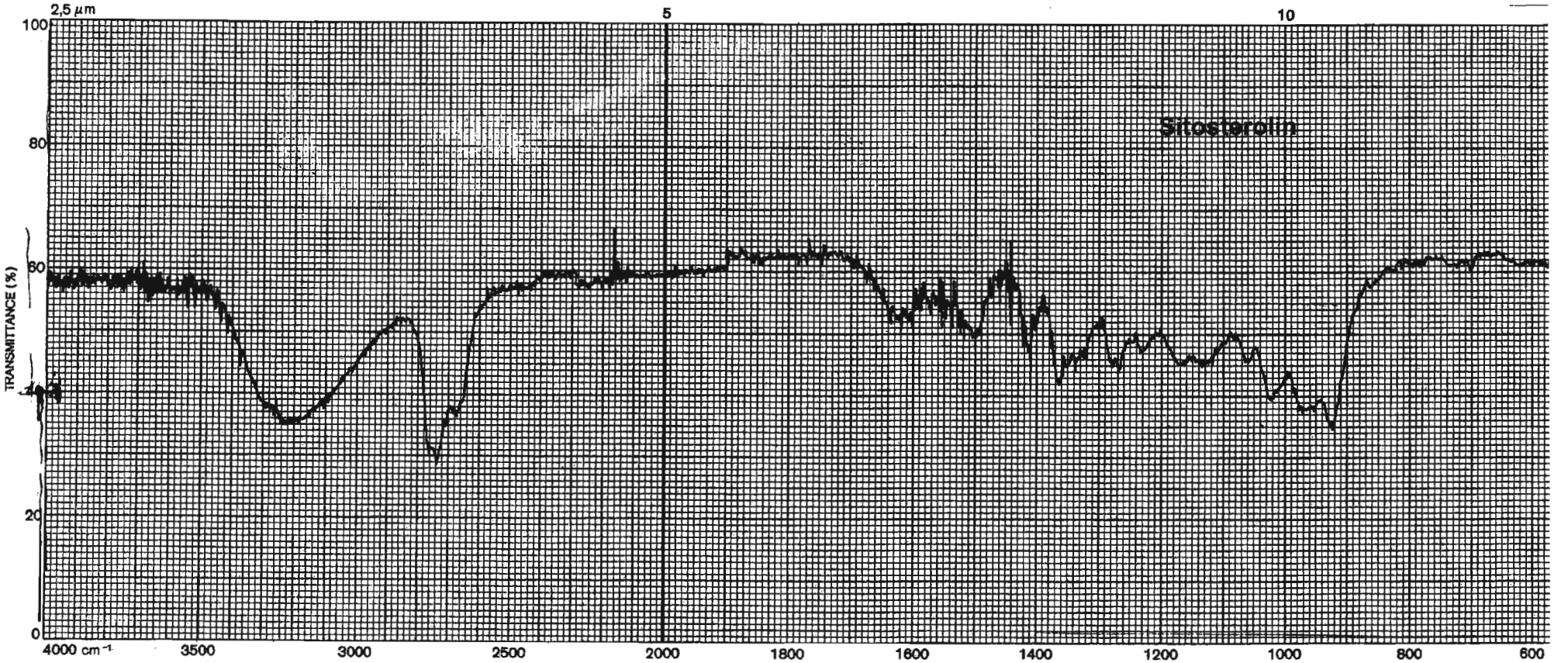
31 IR



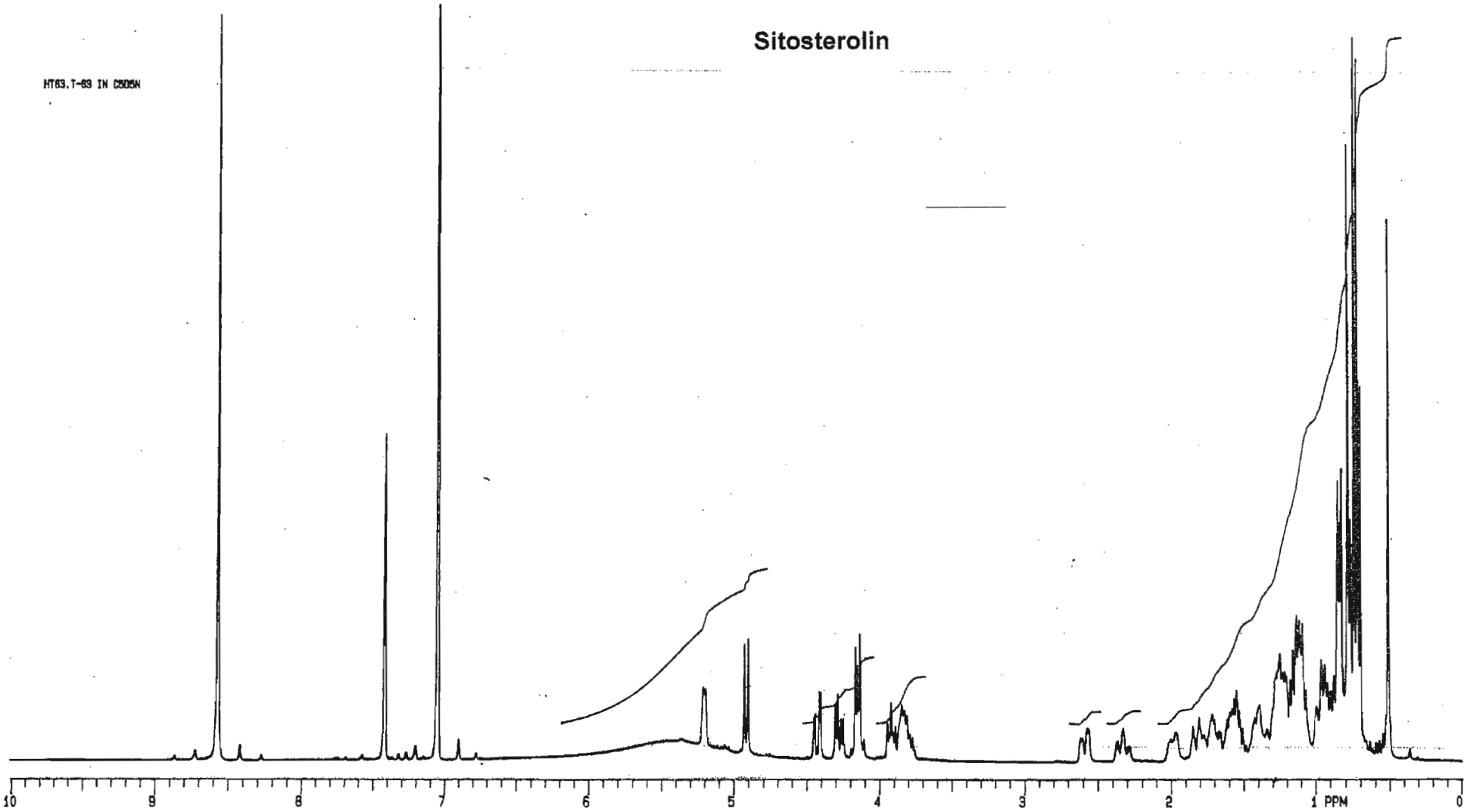
31 Acetate IR


SCAN GRAPH. Flagging=M/z.
Scan 66#5:56. Entries=513. Base M/z=396.2. 100% int.=0.17888.
%age
Sitosterolin
396.2
43.0
81.0
95
107.0
145.0
175.0
213.1
255.1
303.1
329.2
354.1
381
415.2
M/z

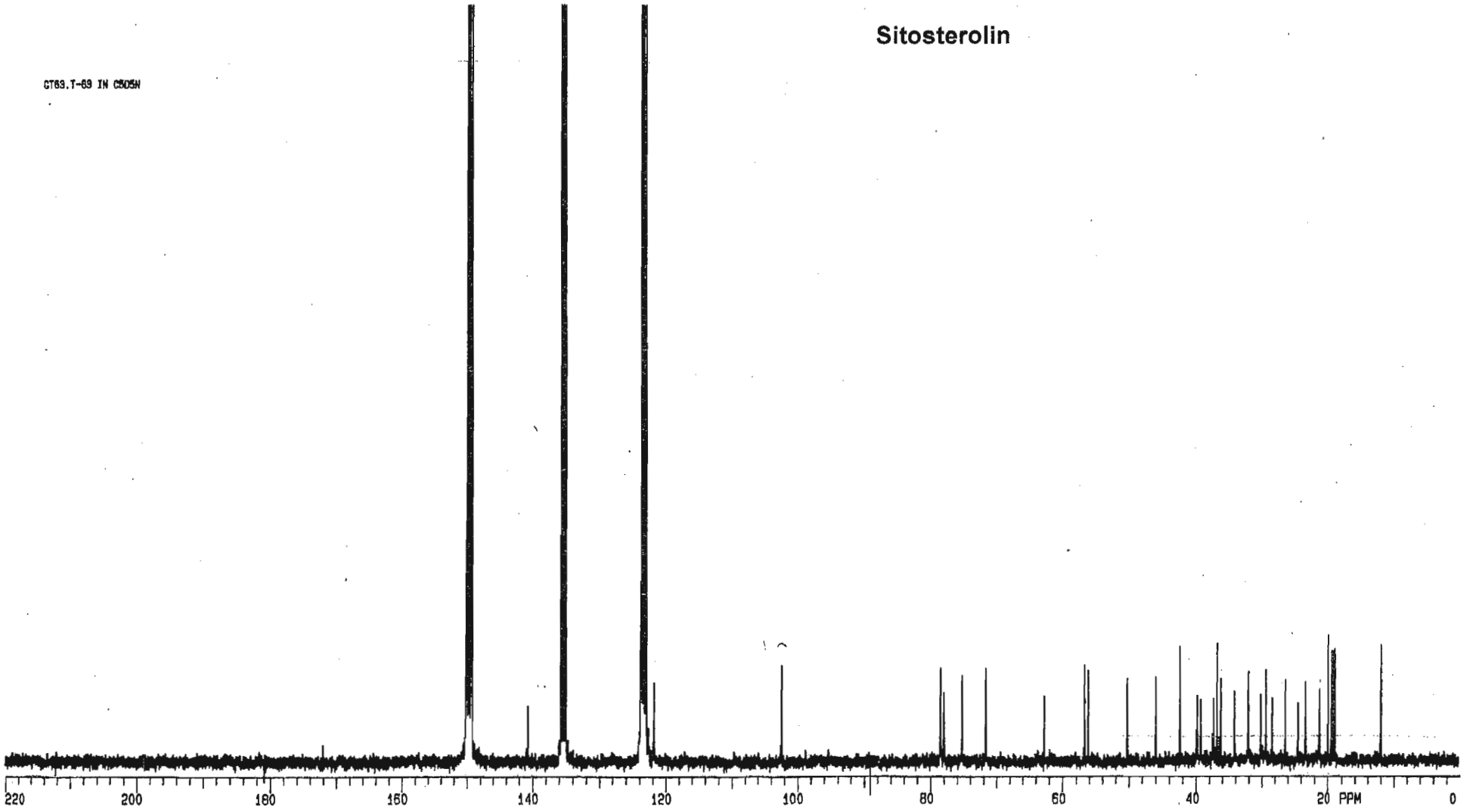
33 NC-MS


2,5 µm
5
10
Sitosterolin
TRANSMITTANCE (%)
100
80
60
40
20
0
4000 cm⁻¹
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600

33 IR

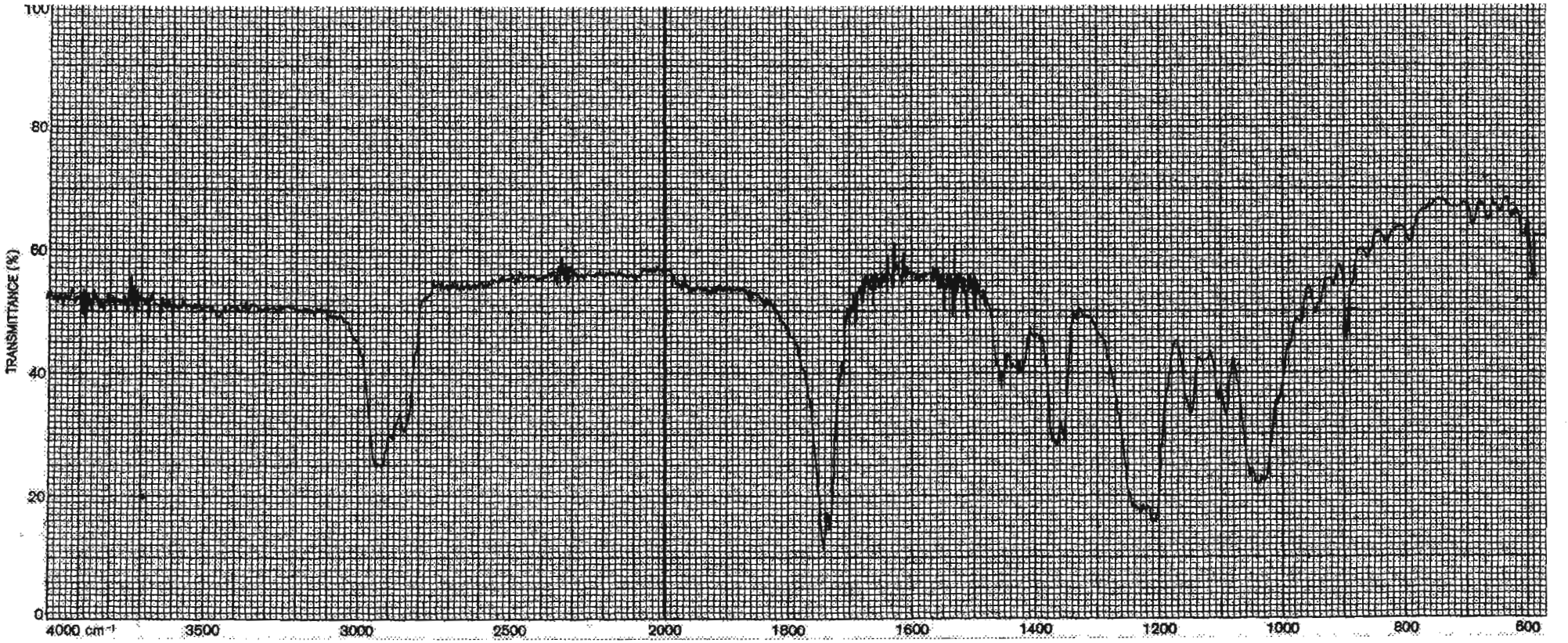


33 H-1 NMR

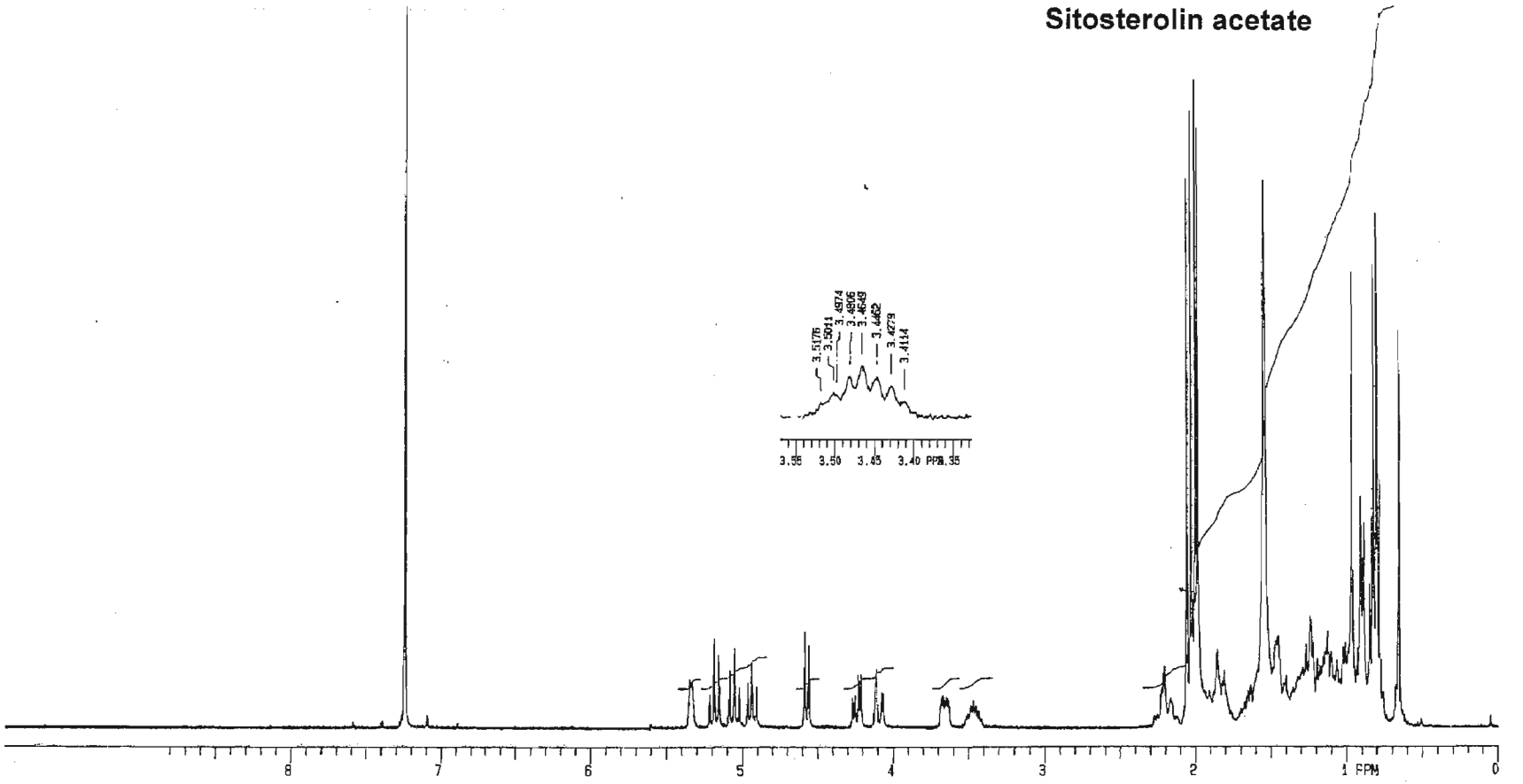

Sitosterolin
CT63.T-69 IN C5D5N
220
200
180
160
140
120
100
80
60
40
20 PPM
0

33 C-13 NMR

Sitosterolin acetate

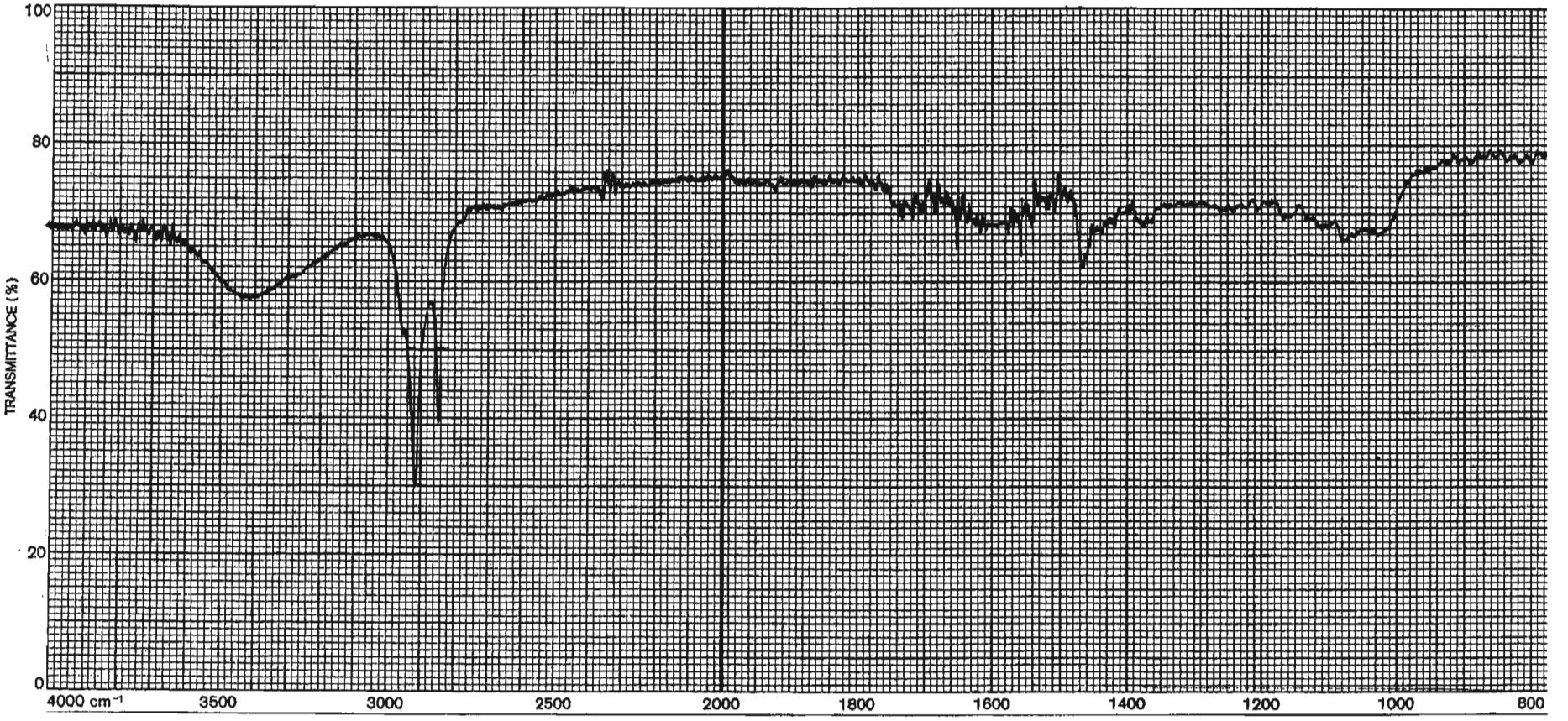


33 Acetate IR

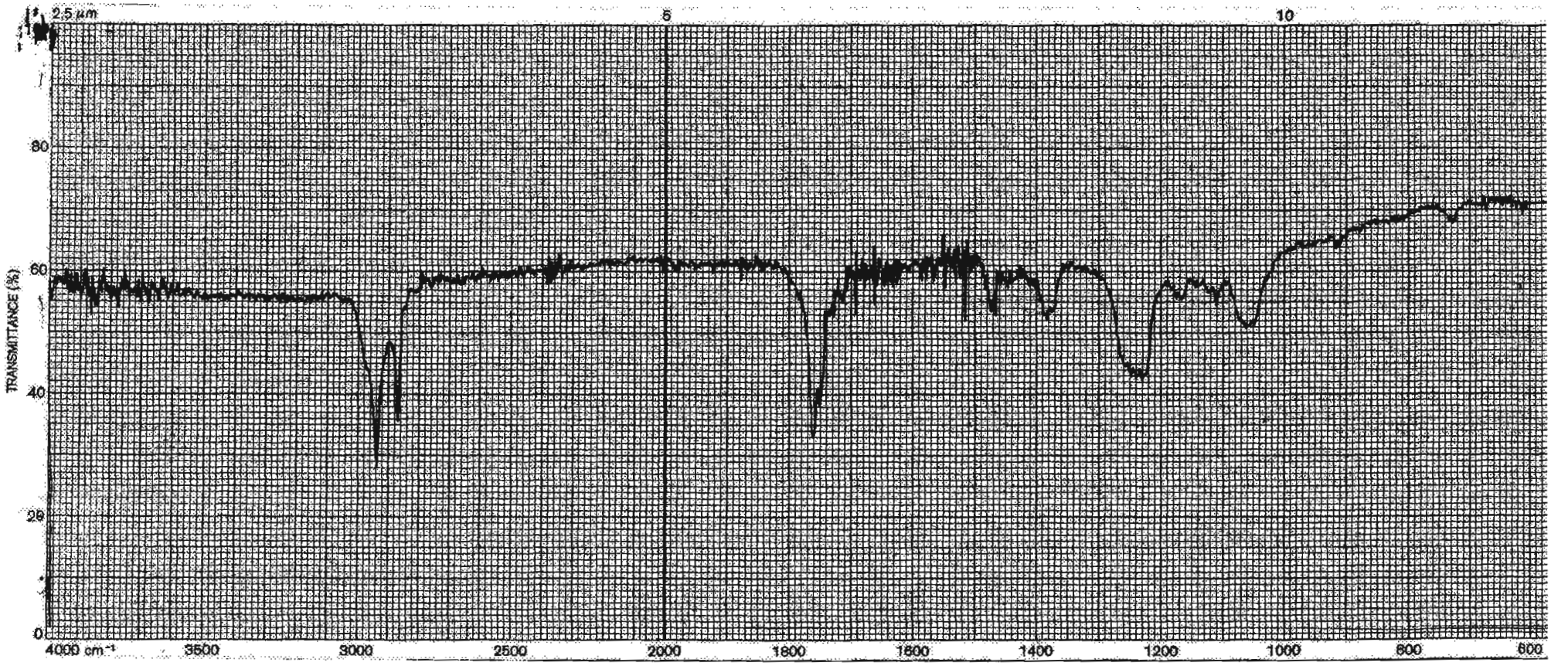


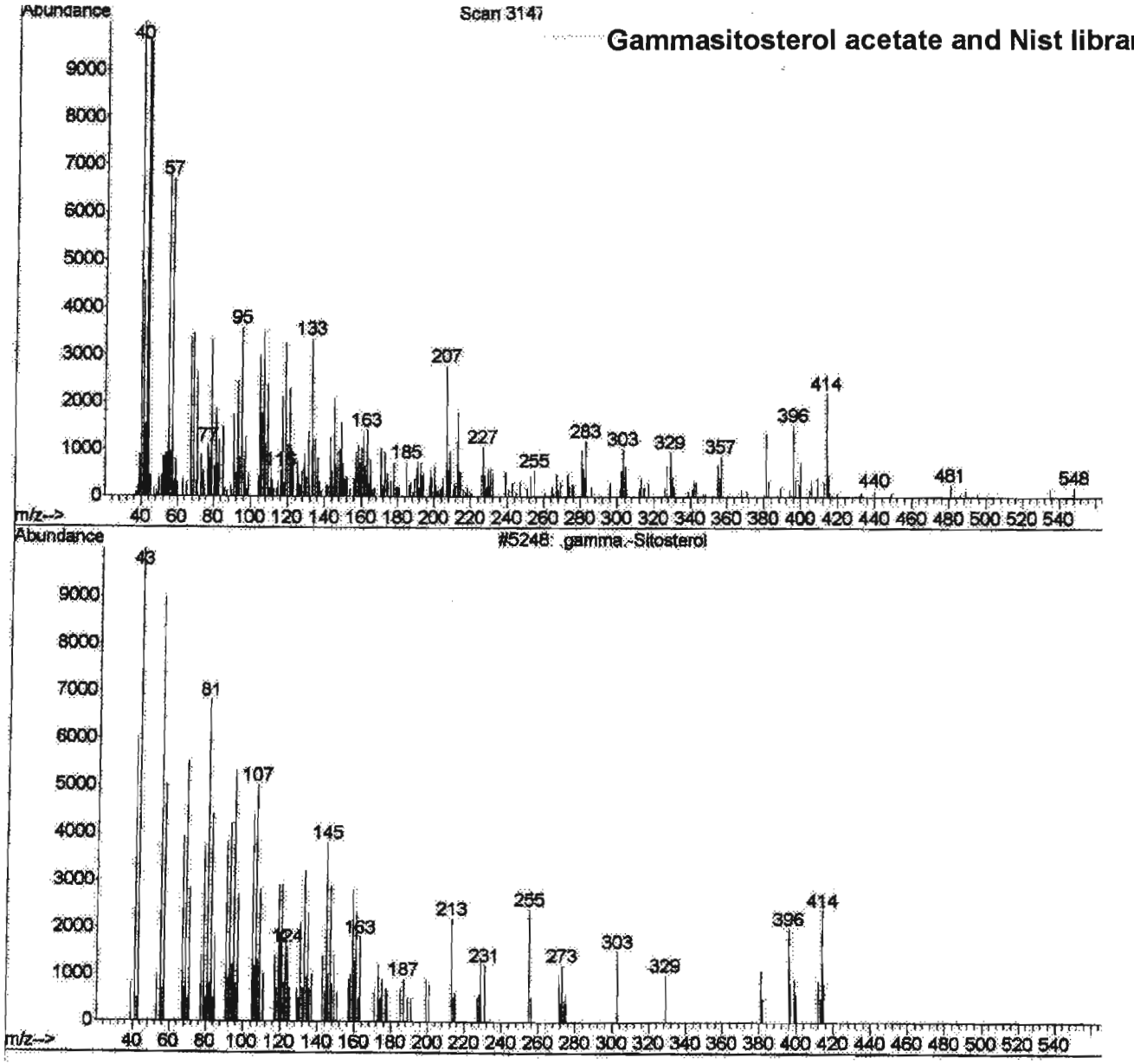
33 Acetate H-1 NMR

Gamma sitosterol

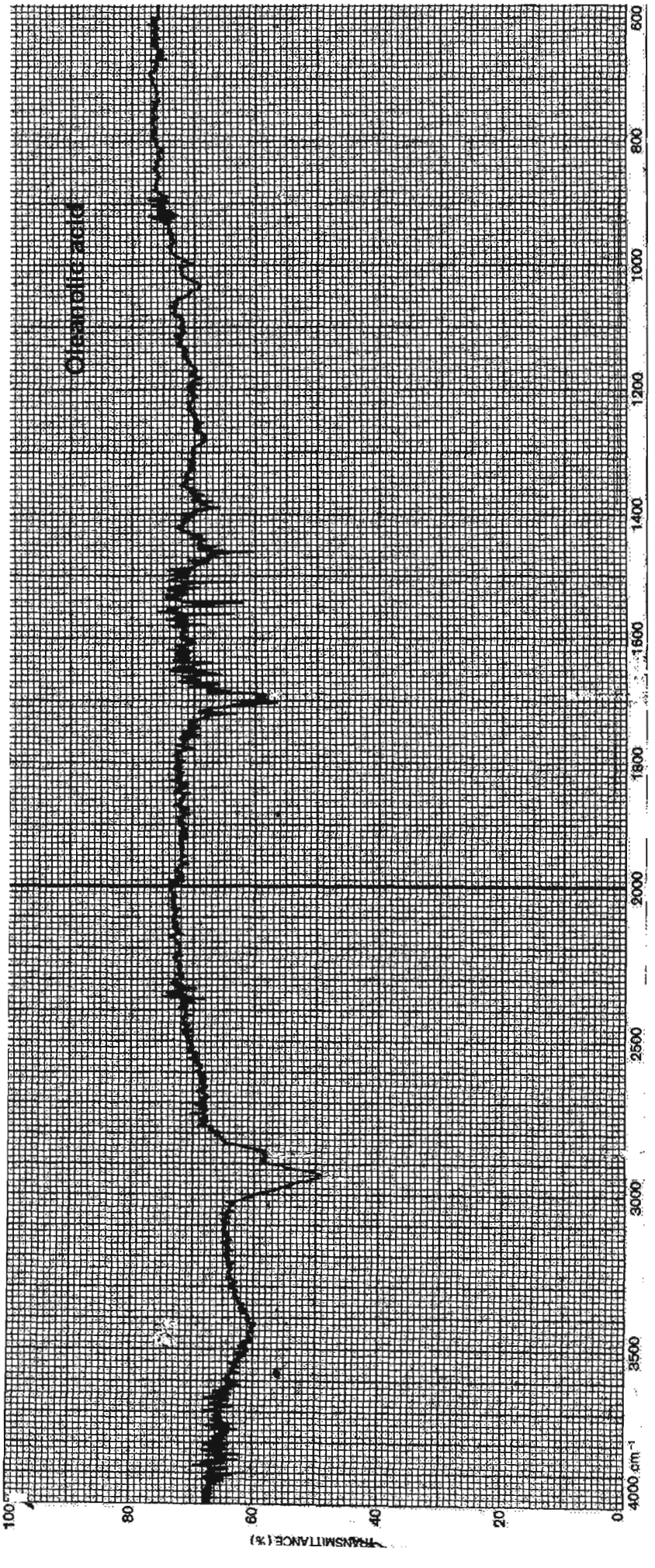


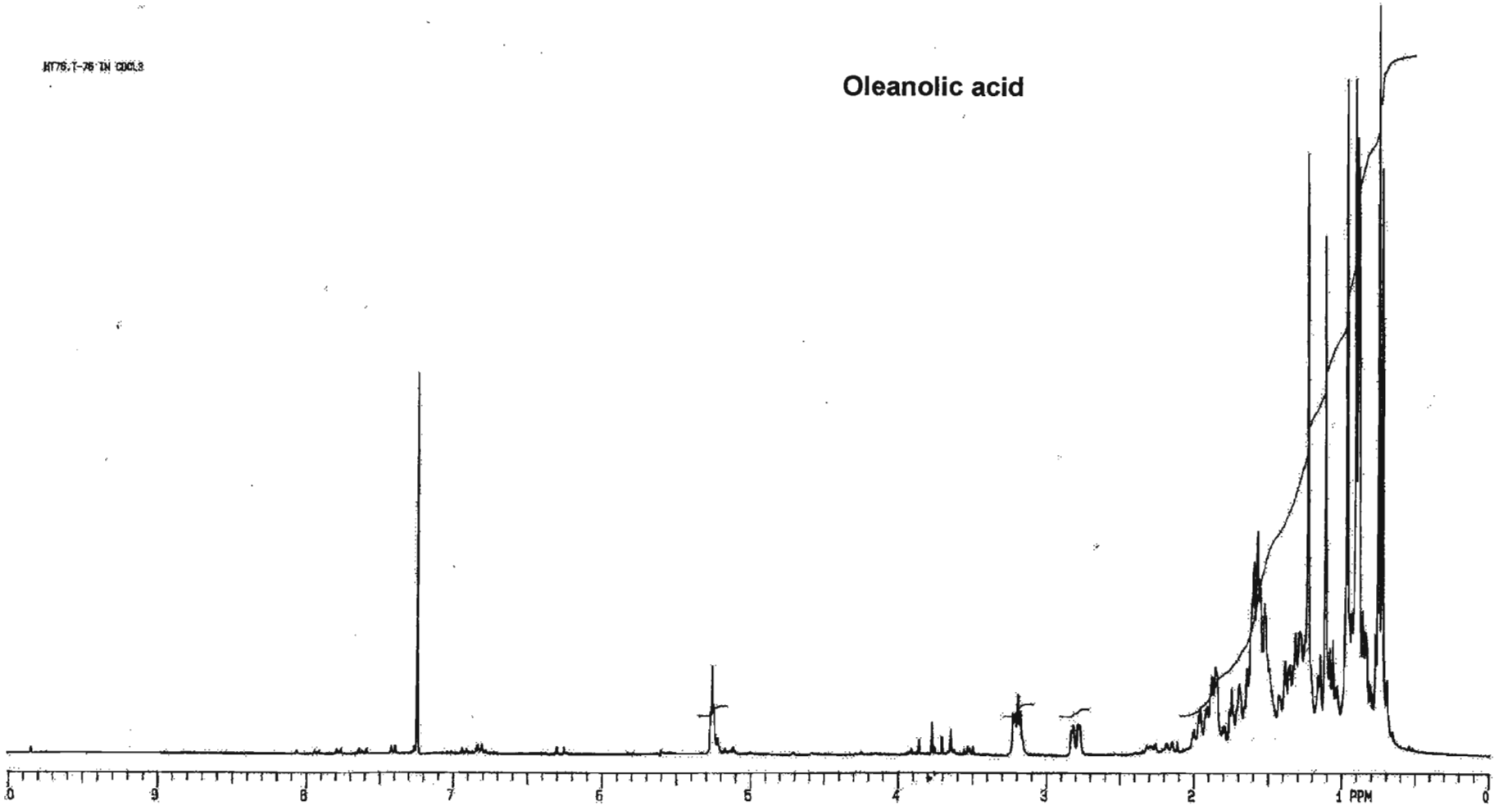
Gamma sitosterol acetate





Gammasitosterol acetate and Nist library match


Oleanolic acid
TRANSMITTANCE (%)
100
80
60
40
20
0
4000 cm⁻¹
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600



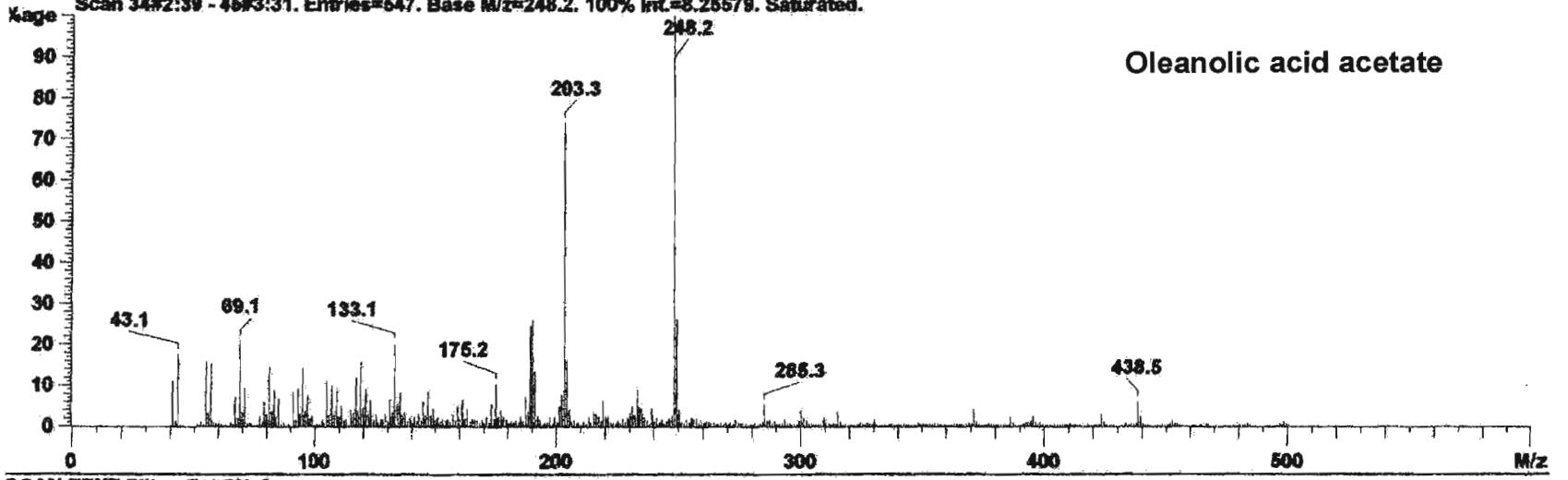
35 H-1 NMR

File Title	: T-96	
Operator	: Dr Philip Boshoff/NMSC	248·1800
Instrument	: VG70-250SEQ-MS2	248·1776
Notes	: B.BROOKES	≡ $C_{16}H_{24}O_2$ Best fit.

438·3475
438·3498 ≡ $C_{30}H_{46}O_2$

SCAN GRAPH. Flagging=M/z.

Scan 34#2:39 - 45#3:31. Entries=647. Base M/z=248.2. 100% Int.=8.25579. Saturated.



Oleanolic acid acetate

SCAN TEXT.Filter=[Int:3%.].

Scan 34#2:39 - 45#3:31. Sorted on M/z (ascending). Entries=76. Base M/z=248.2. 100% Int.=8.25579. Saturated.

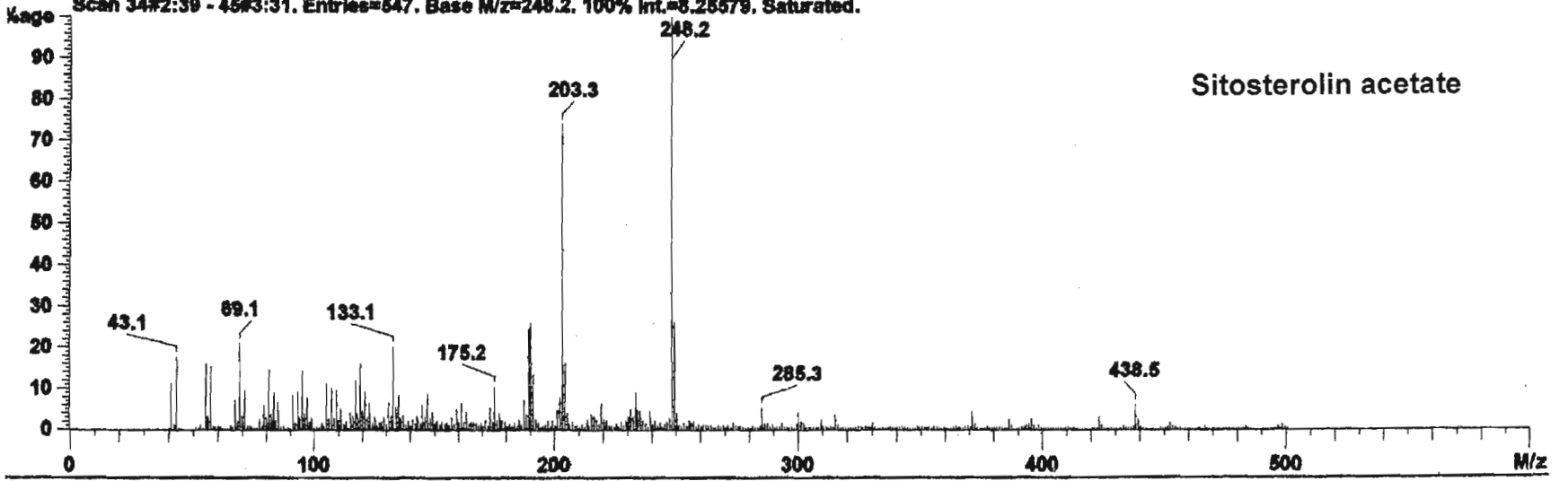
---M/z--	--%age--	---M/z--	--%age--	---M/z--	--%age--	---M/z--	--%age--
41.1	10.95	107.1	9.97	146.2	3.08	216.2	3.15
43.1	17.59	109.2	9.50	147.2	8.65	219.2	6.31
55.1	15.83	111.1	4.96	149.2	4.26	230.2	3.17
56.1	3.22	115.1	3.98	159.2	4.95	231.2	4.78
57.1	15.17	116.1	3.21	161.2	6.32	233.2	8.95
67.1	7.04	117.1	11.84	163.2	4.19	234.2	4.64
69.1	20.92	118.1	3.77	173.2	5.22	235.2	4.57
70.1	3.24	119.1	15.78	175.2	10.37	239.3	4.38
71.1	9.42	120.1	4.44	177.2	4.04	248.2	s 100.00
79.1	5.74	121.1	9.07	187.2	7.39	249.3	26.06
81.1	14.47	123.1	6.24	188.2	3.67	250.3	3.92
82.1	3.64	125.2	3.06	189.2	24.57	285.3	5.23
83.1	8.78	131.1	6.49	190.2	26.04	300.3	4.09
85.1	6.73	132.1	3.43	191.2	13.49	315.3	3.39
91.1	8.33	133.1	20.19	201.2	4.75	371.3	4.18
93.1	9.03	134.2	5.55	202.2	7.62	438.5	5.96
95.1	14.18	135.2	8.14	203.3	73.99		
96.1	3.59	137.2	3.55	204.3	16.24		
97.1	7.43	143.1	3.21	205.3	4.07		
105.1	11.15	145.2	5.94	215.2	3.66		

File Title	: T-96	
Operator	: Dr Philip Boshoff/NMSC	248.1800
Instrument	: VG70-250SEQ-MS2	248.1776
Notes	: B.BROOKES	≡ $C_{16}H_{24}O_2$ Best fit.

438.3475
438.3498 ≡ $C_{30}H_{46}O_2$

SCAN GRAPH. Flagging=M/z.

Scan 34#2:39 - 45#3:31. Entries=547. Base M/z=248.2. 100% Int.=8.25579. Saturated.



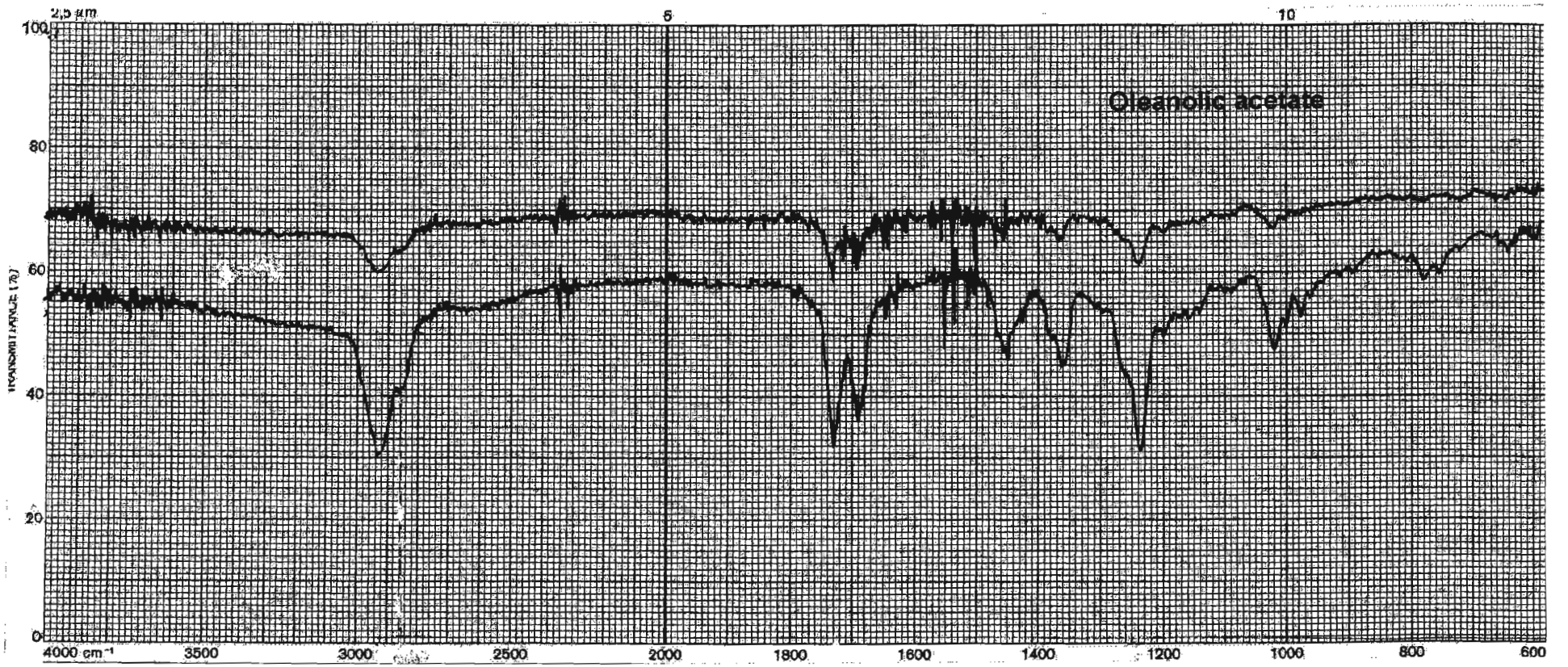
Sitosterolin acetate

SCAN TEXT.Filter=[Int:3%.].

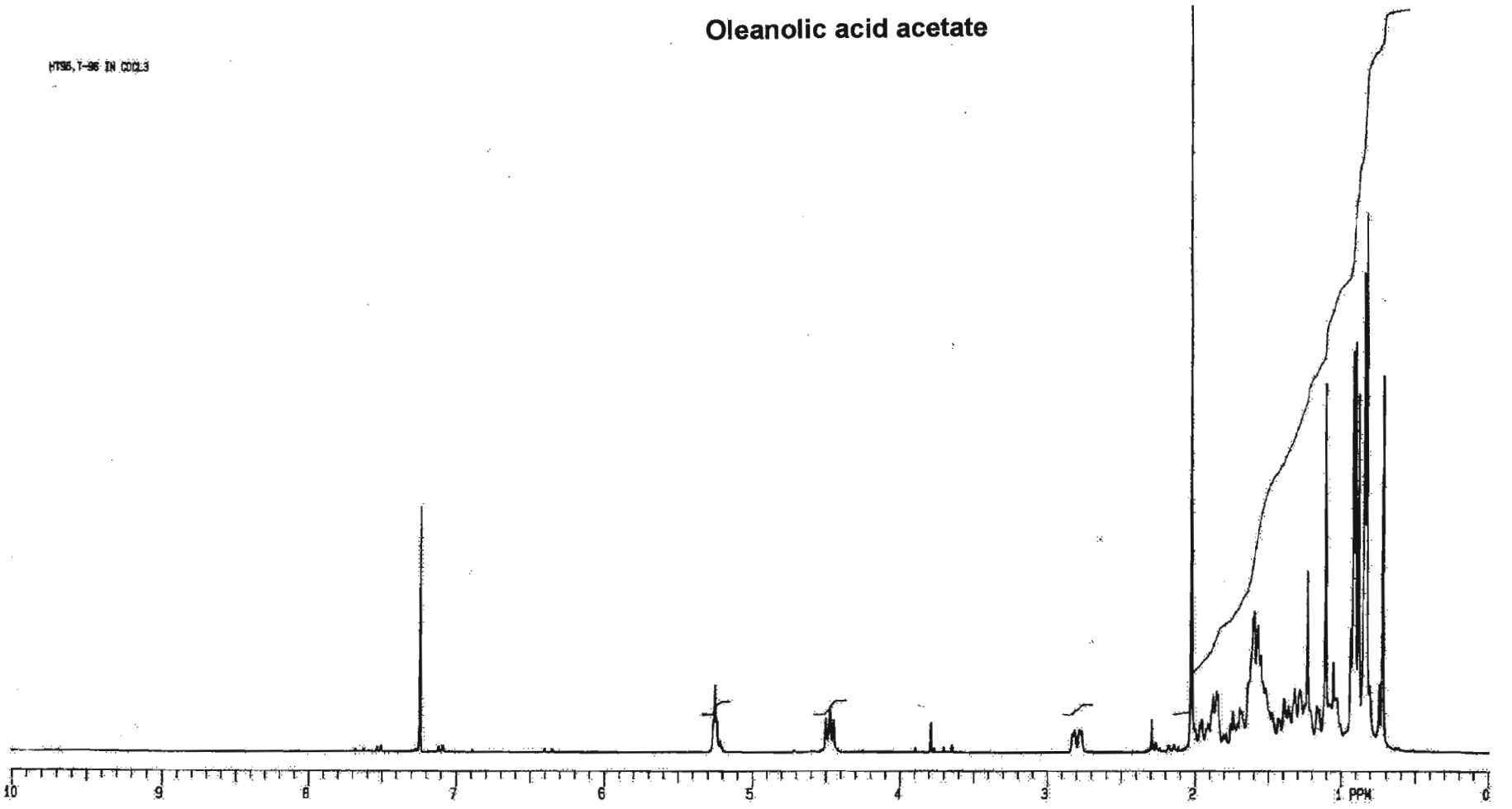
Scan 34#2:39 - 45#3:31. Sorted on M/z (ascending). Entries=76. Base M/z=248.2. 100% Int.=8.25579. Saturated.

---M/z--	--%age--	---M/z--	--%age--	---M/z--	--%age--	---M/z--	--%age--
41.1	10.95	107.1	9.97	146.2	3.08	216.2	3.15
43.1	17.59	109.2	9.50	147.2	8.65	219.2	6.31
55.1	15.83	111.1	4.96	149.2	4.26	230.2	3.17
56.1	3.22	115.1	3.98	159.2	4.95	231.2	4.78
57.1	15.17	116.1	3.21	161.2	6.32	233.2	8.95
67.1	7.04	117.1	11.84	163.2	4.19	234.2	4.64
69.1	20.92	118.1	3.77	173.2	5.22	235.2	4.57
70.1	3.24	119.1	15.78	175.2	10.37	239.3	4.38
71.1	9.42	120.1	4.44	177.2	4.04	248.2	s 100.00
79.1	5.74	121.1	9.07	187.2	7.39	249.3	26.06
81.1	14.47	123.1	6.24	188.2	3.67	250.3	3.92
82.1	3.64	125.2	3.06	189.2	24.57	285.3	5.23
83.1	8.78	131.1	6.49	190.2	26.04	300.3	4.09
85.1	6.73	132.1	3.43	191.2	13.49	315.3	3.39
91.1	8.33	133.1	20.19	201.2	4.75	371.3	4.18
93.1	9.03	134.2	5.55	202.2	7.62	438.5	5.96
95.1	14.18	135.2	8.14	203.3	73.99		
96.1	3.59	137.2	3.55	204.3	16.24		
97.1	7.43	143.1	3.21	205.3	4.07		
105.1	11.15	145.2	5.94	215.2	3.66		

35 Acetate EI-MS



35 Acetate IR



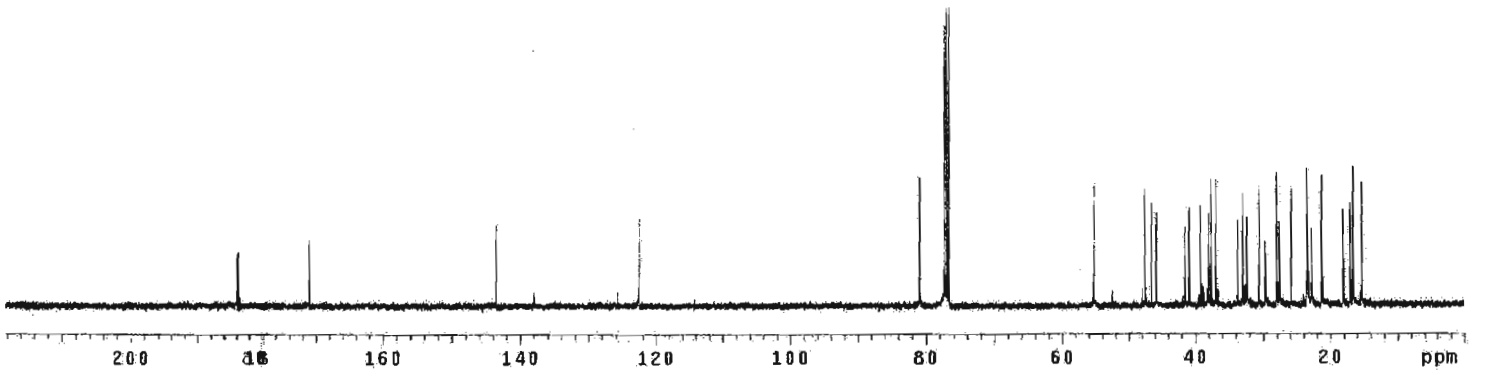
35 Acetate H-1 NMR

probe=[redacted]

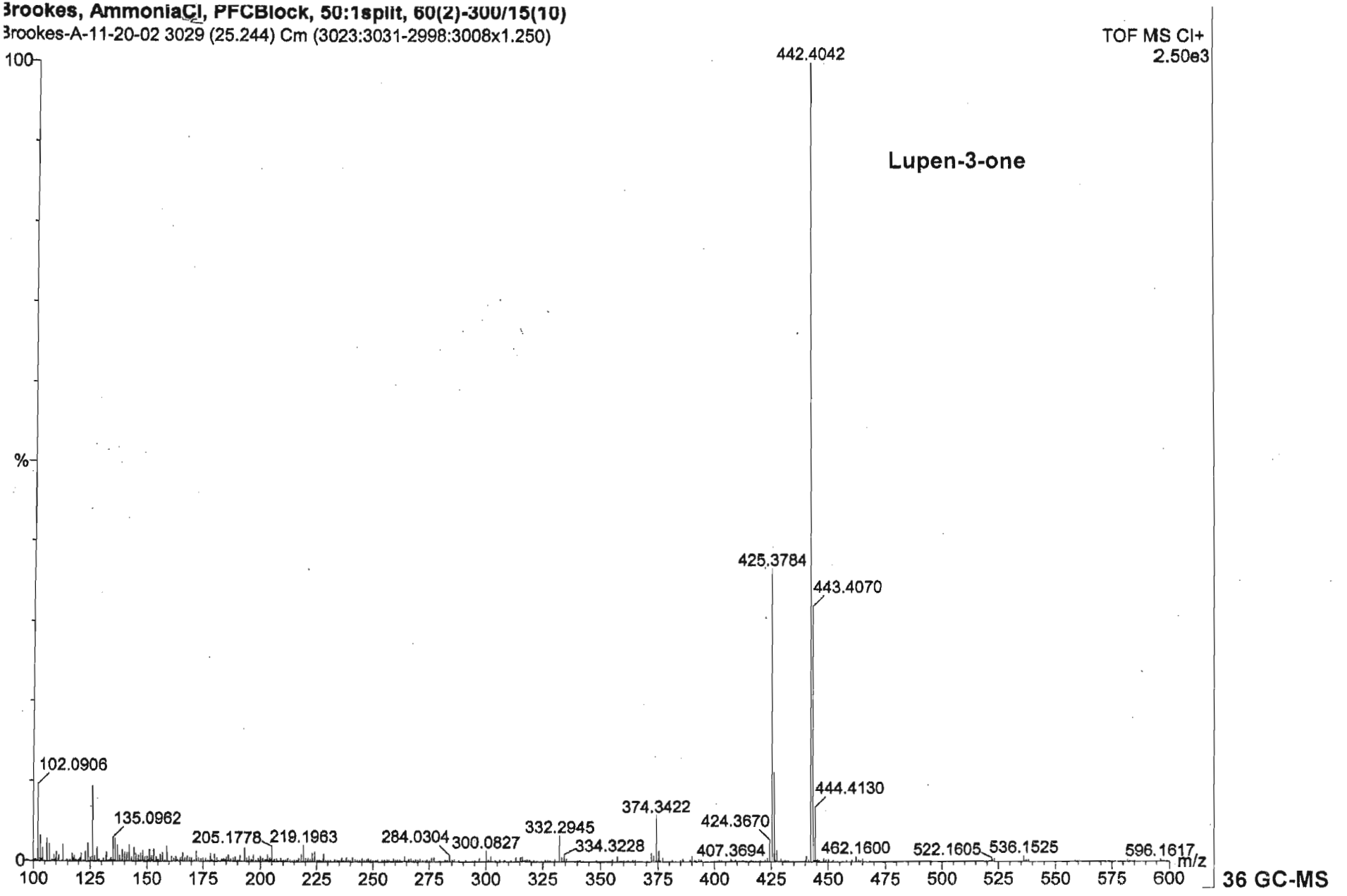
Pulse Sequence: s2pul

INDEX	FREQUENCY	PPM	HEIGHT	
1	18493.288	183.893	9.0	
2	17202.920	171.062	11.0	
3	14436.435	143.573	13.6	
4	12321.607	122.524	14.8	
5	8136.564	80.908	21.6	
6	7775.657	77.320	47.0	} $CHCl_3$
7	7743.521	77.000	50.0	
8	7711.388	76.680	50.0	
9	5556.853	55.254	20.3	
10	4777.983	47.511	19.7	
11	4677.456	46.512	17.3	
12	4604.945	45.791	15.6	
13	4174.823	41.514	13.2	
14	4112.200	40.891	16.5	
15	3945.754	39.236	16.8	
16	3823.803	38.023	15.5	
17	3787.548	37.663	21.4	
18	3715.861	36.950	21.0	
19	3393.681	33.748	14.3	
20	3321.994	33.033	18.9	
21	3288.786	32.484	15.0	
22	3258.547	32.402	14.9	
23	3081.389	30.641	19.6	
24	2984.882	29.682	10.8	
25	2816.888	28.011	22.3	
26	2778.161	27.625	14.1	
27	2601.627	25.872	19.6	
28	2368.636	23.553	23.1	
29	2362.046	23.488	17.4	
30	2348.862	23.357	15.4	
31	2295.351	22.840	12.9	
32	2142.041	21.300	21.9	
33	1823.981	18.137	16.2	
34	1720.982	17.113	17.3	
35	1672.367	16.630	23.4	
36	1544.648	15.360	20.7	

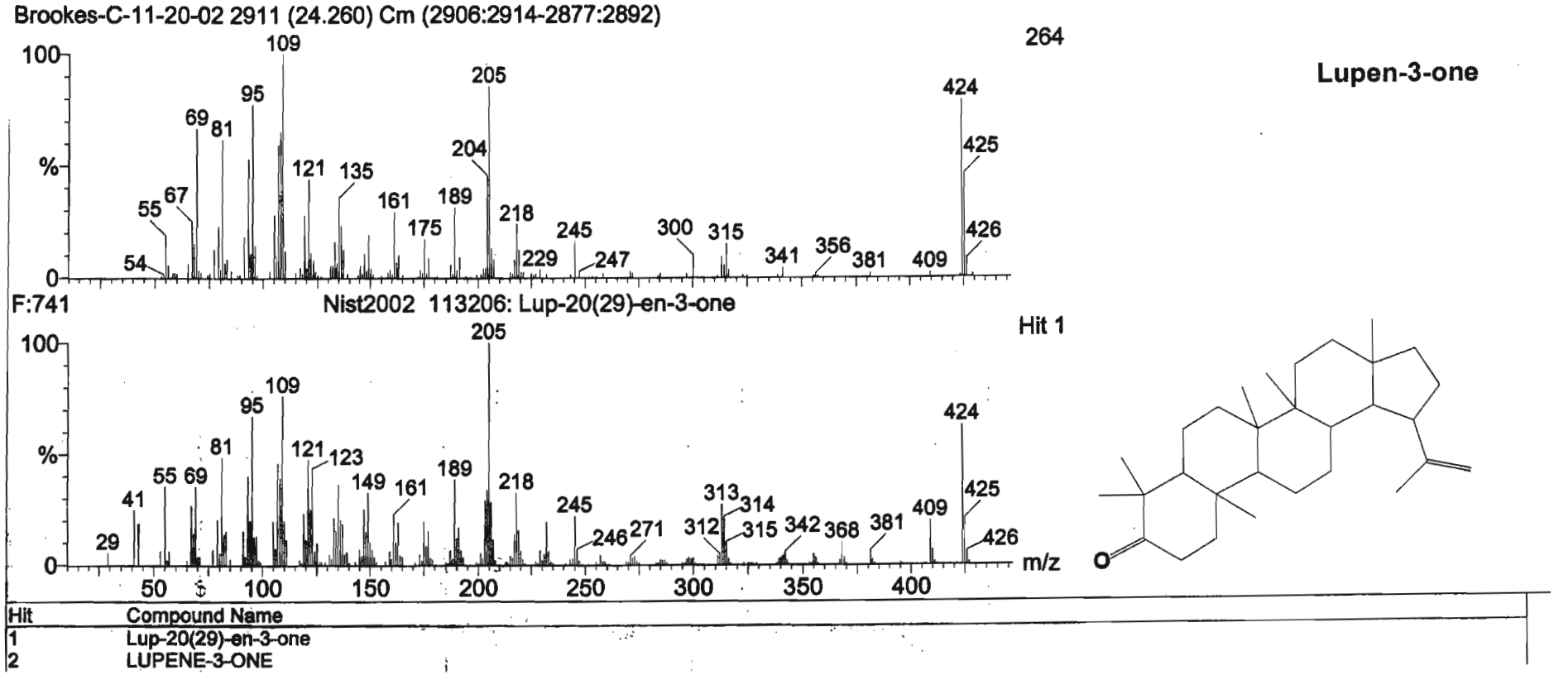
Oleanolic acid acetate



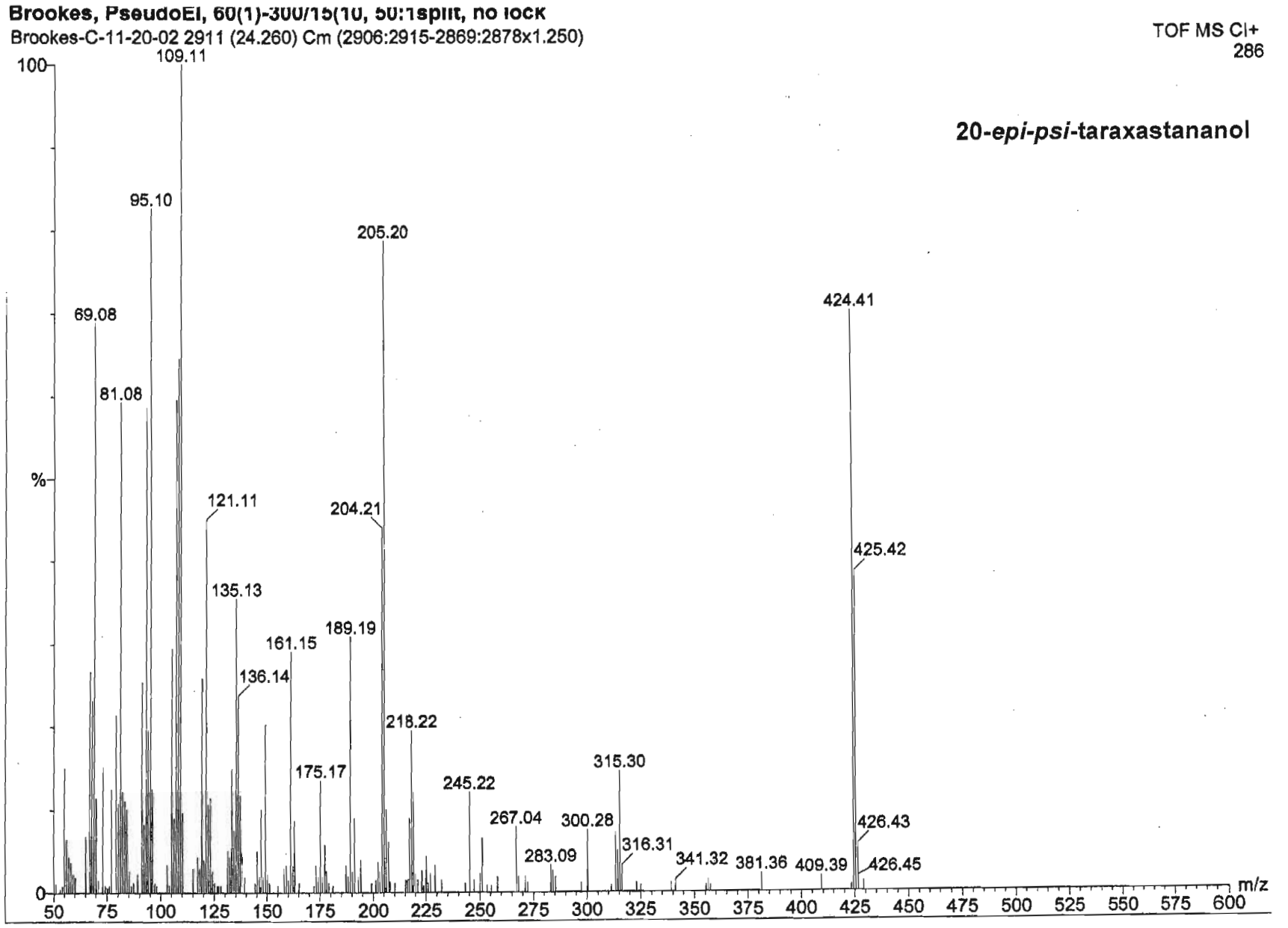
35 Acetate C-13 NMR


Brookes, AmmoniaCI, PFCBlock, 50:1split, 60(2)-300/15(10)
Brookes-A-11-20-02 3029 (25.244) Cm (3023:3031-2998:3008x1.250)
TOF MS CI+
2.50e3
Lupen-3-one
100
%
0
442.4042
425.3784
443.4070
102.0906
135.0962
205.1778
219.1963
284.0304
300.0827
332.2945
334.3228
374.3422
407.3694
424.3670
444.4130
462.1600
522.1605
536.1525
596.1617
m/z
100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550 575 600

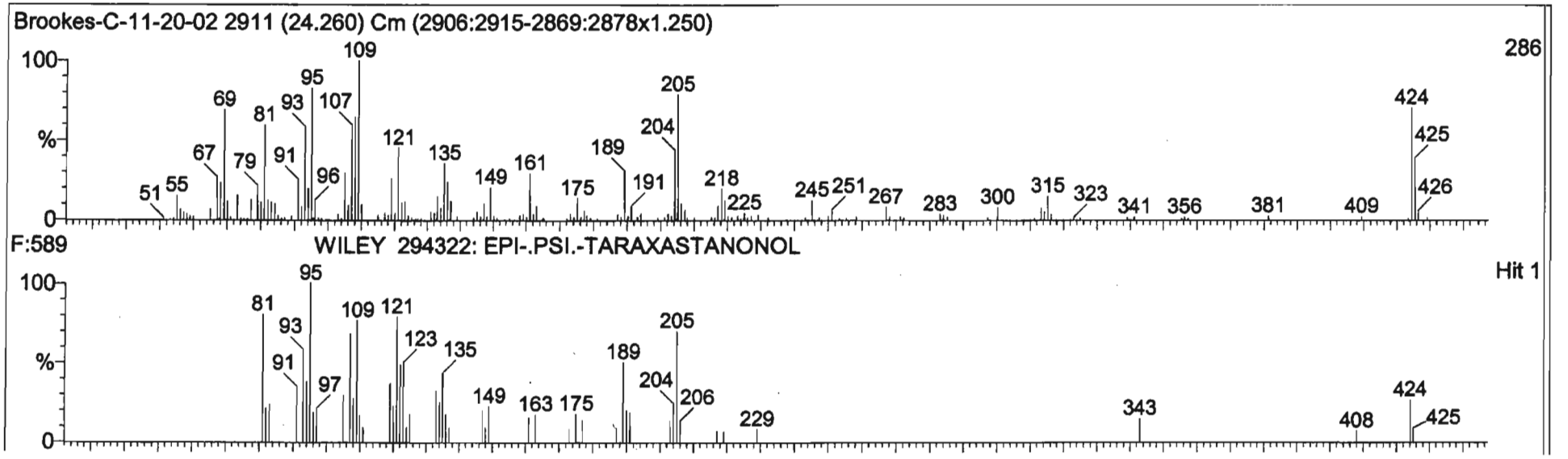
36 GC-MS



36 NIST Library match


Brookes, PseudoEI, 60(1)-300/15(10, 50:1split, no lock
Brookes-C-11-20-02 2911 (24.260) Cm (2906:2915-2869:2878x1.250)
TOF MS CI+
286
20-epi-psi-taraxastananol
100
%
0
109.11
95.10
205.20
69.08
424.41
81.08
121.11
204.21
425.42
135.13
189.19
161.15
136.14
218.22
315.30
175.17
245.22
267.04
300.28
426.43
316.31
283.09
341.32
381.36
409.39
426.45
m/z
50 75 100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550 575 600

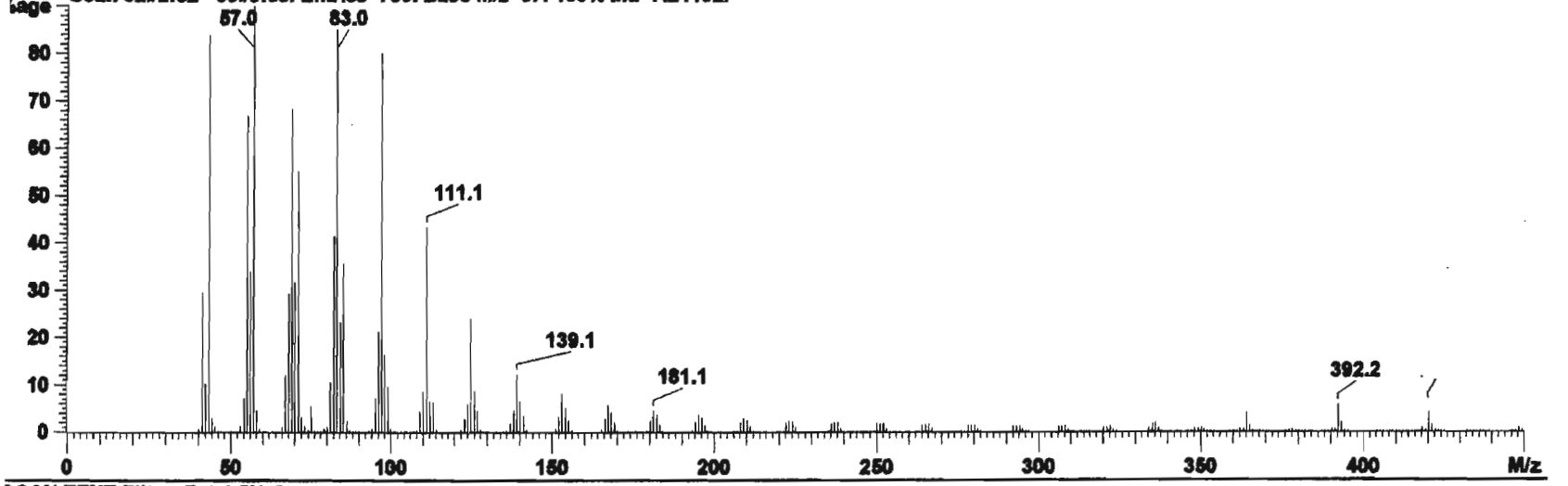
***Epi-psi*-taraxastananol and Wiley library match**



File Name : c:\maspec2\data\[redacted]ms2
Operator : Dr Philip Boshoff/NCMS
Instrument : VG70-250SEQmsii

Triacontanol

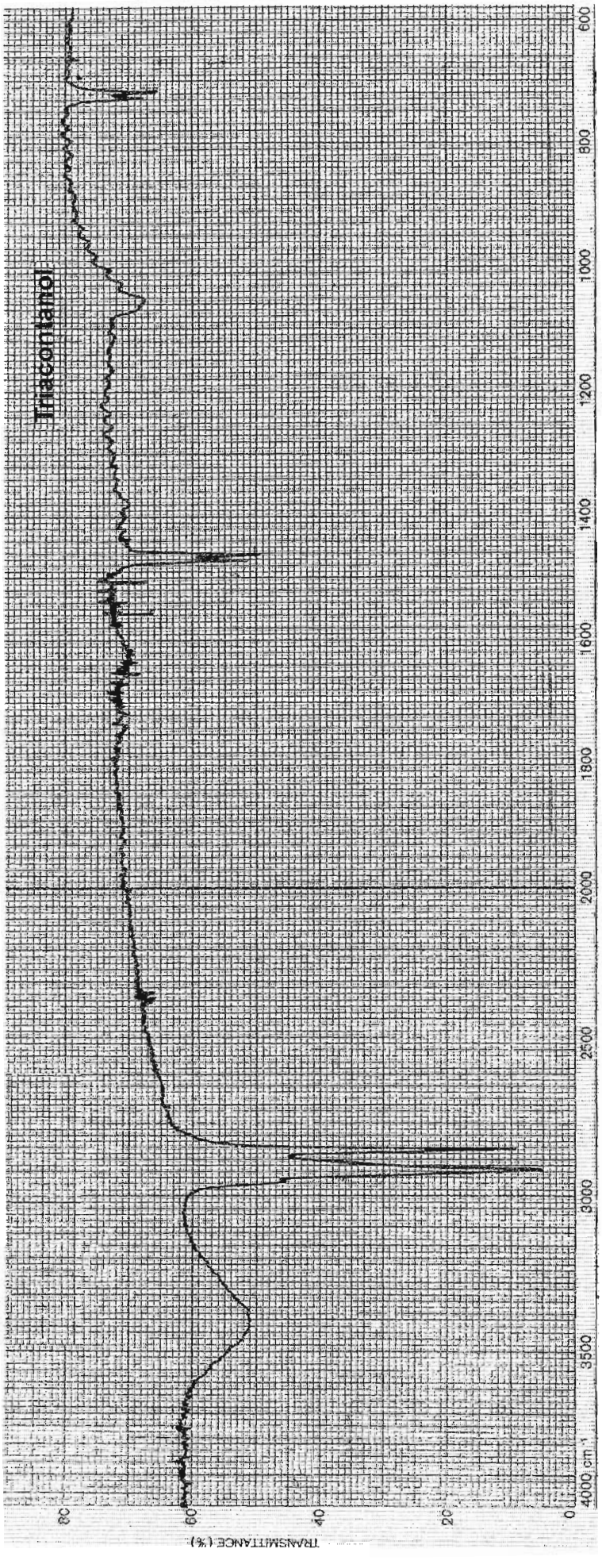
SCAN GRAPH. Flagging=M/z.
Scan 32#2:52 - 39#3:30. Entries=739. Base M/z=57. 100% Int.=7.24192.

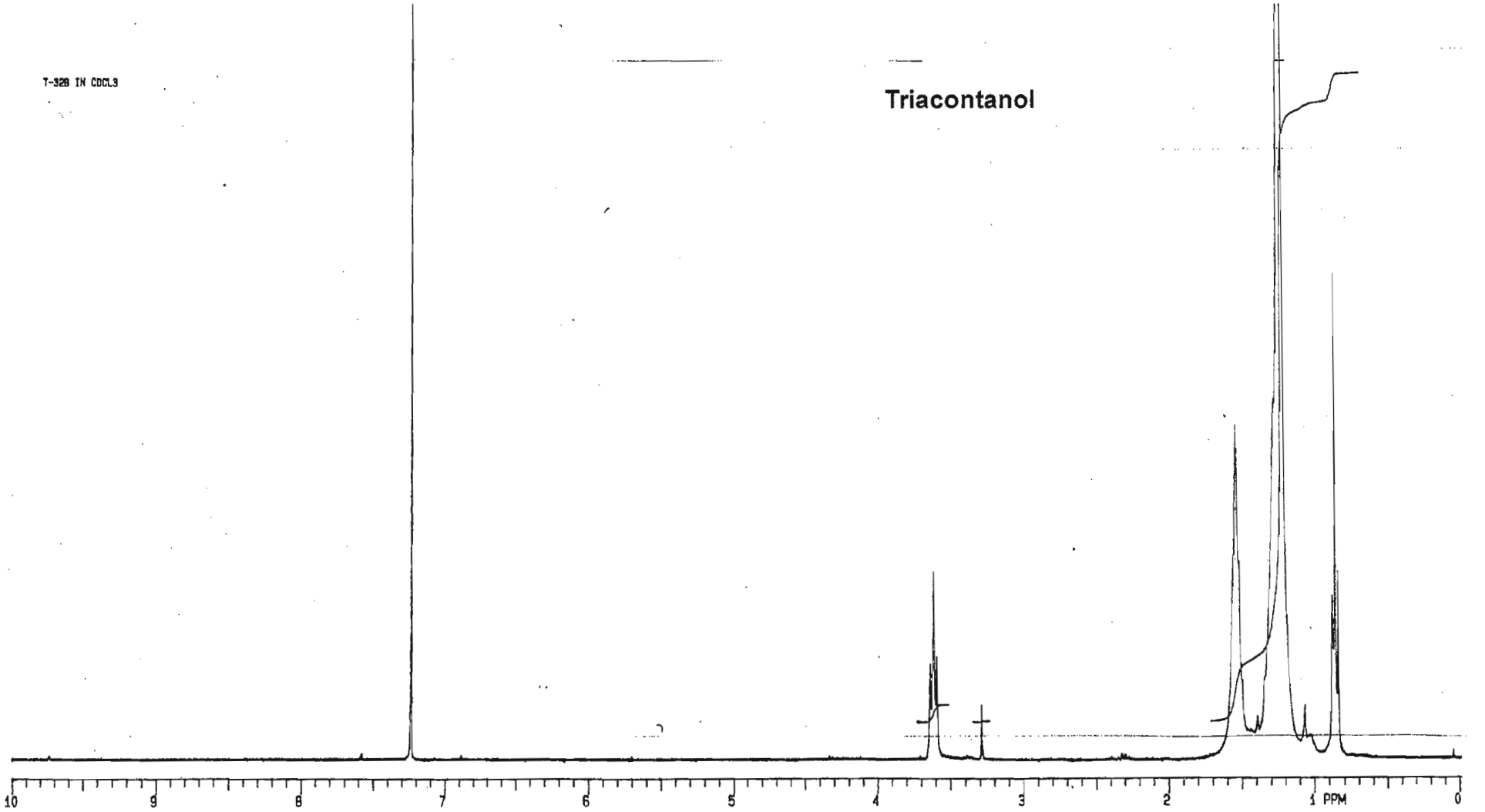


SCAN TEXT.Filter=[Int:1.5%.].
Scan 32#2:52 - 39#3:30. Sorted on M/z (ascending). Entries=75. Base M/z=57. 100% Int.=7.24192.

---M/z--	--%age--	---M/z--	--%age--	---M/z--	--%age--	---M/z--	--%age--
41.0	29.38	86.1	2.39	152.0	3.14	236.1	1.68
42.0	10.14	95.1	7.09	153.0	7.96	237.1	1.92
43.0	83.76	96.0	21.22	154.1	5.03	238.1	1.93
44.0	2.81	97.0	79.97	155.0	2.32	250.1	1.58
54.0	7.03	98.0	16.19	166.1	2.62	251.1	1.61
55.0	66.70	99.1	9.55	167.1	5.72	252.1	1.66
56.0	33.82	109.0	4.34	168.1	4.08	266.1	1.51
57.0	100.00	110.0	8.45	169.1	2.03	336.2	1.86
58.0	4.46	111.1	43.36	180.1	2.21	364.2	3.99
67.0	11.83	112.1	6.38	181.1	4.42	392.2	5.58
68.0	29.10	113.1	6.36	182.1	3.48	393.2	1.88
69.0	68.03	123.0	2.81	183.1	1.65	420.3	3.96
70.0	31.52	124.0	5.79	194.1	2.06		
71.0	54.86	125.0	23.83	195.1	3.48		
72.0	3.07	126.0	8.52	196.1	2.89		
75.0	5.34	127.0	4.47	208.1	1.90		
81.0	10.35	137.0	1.83	209.1	2.75		
82.0	41.39	138.1	4.47	210.1	2.45		
83.0	85.01	139.1	12.06	222.1	1.83		
84.1	23.17	140.1	6.40	223.1	2.23		
85.1	35.51	141.1	3.35	224.1	2.13		

38 CI-MS


Triacontanol
TRANSMITTANCE (%)
100
80
60
40
20
0
4000 cm-1
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600

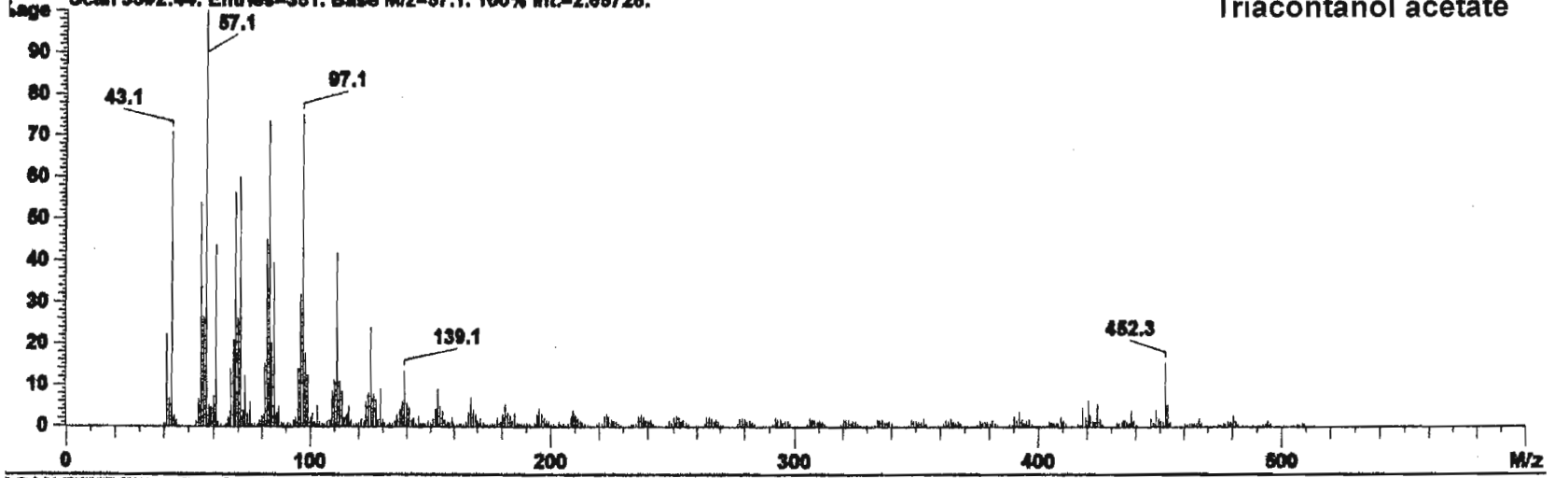


38 H-1 NMR

File Title : [redacted]
Operator : Dr Philip Boshoff/NMSC
Instrument : VG70-250SEQ-MS2
Notes : B.BROOKES

SCAN GRAPH. Flagging=M/z.
Scan 35#2:44. Entries=351. Base M/z=57.1. 100% Int.=2.65728.

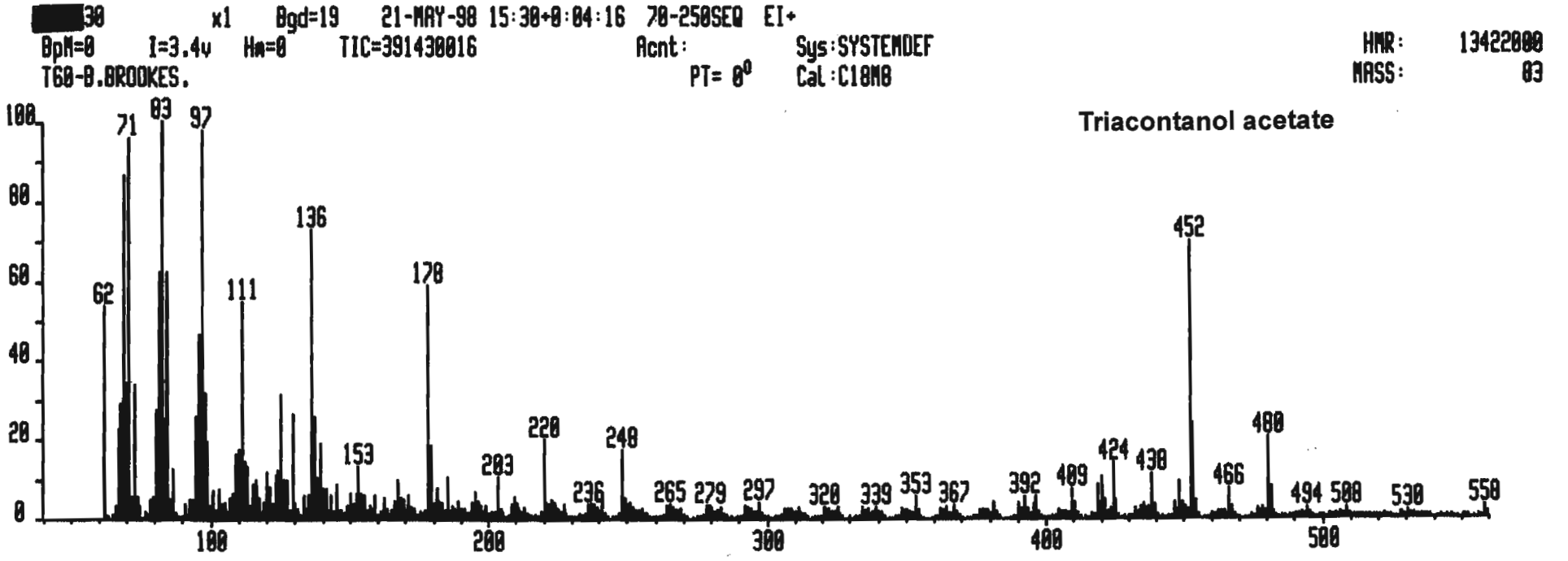
Triacontanol acetate



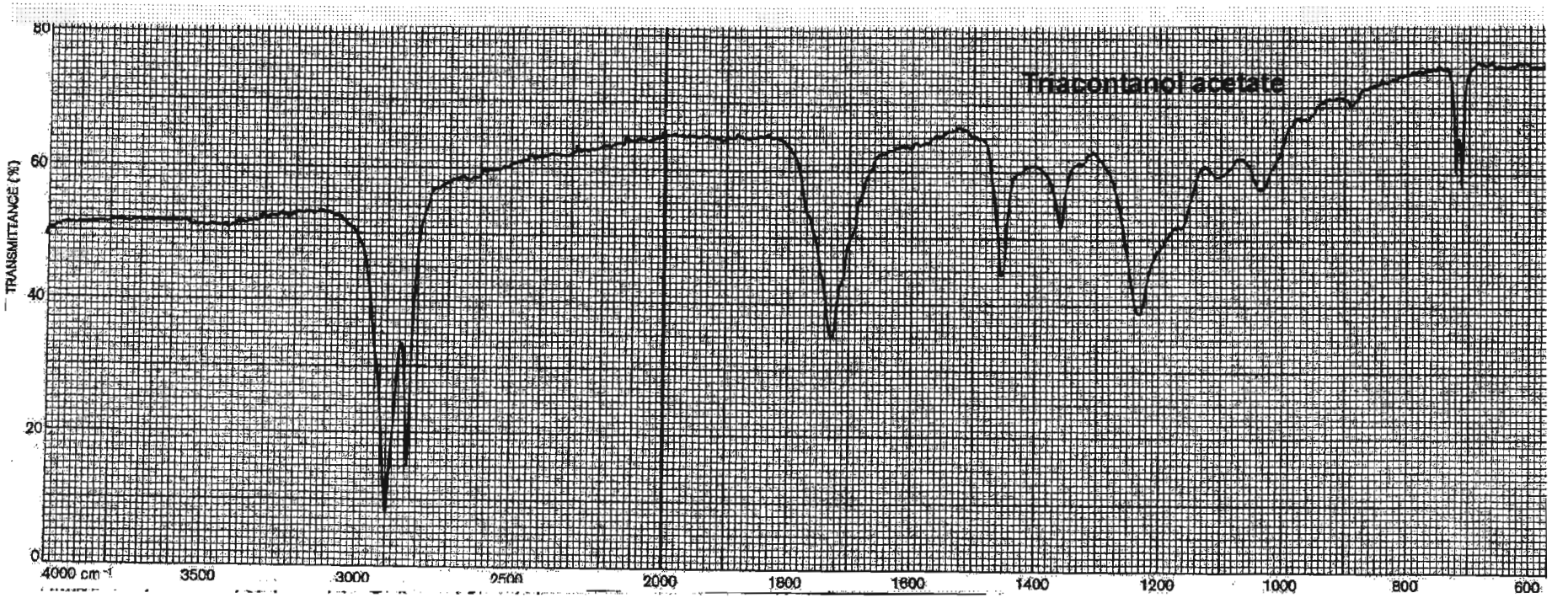
SCAN TEXT.Filter=[Int:5%.].
Scan 35#2:44. Sorted on M/z (ascending). Entries=48. Base M/z=57.1. 100% Int.=2.65728.

---M/z--	--%age--	---M/z--	--%age--	---M/z--	--%age--
41.1	22.13	84.1	20.23	140.2	5.67
42.1	6.73	85.1	39.40	153.1	8.97
43.1	70.69	95.1	13.89	167.2	6.87
54.0	6.50	96.1	31.72	181.2	5.26
55.0	53.61	97.1	75.05	420.4	6.08
56.0	26.45	98.1	17.74	424.3	5.38
57.1	100.00	99.1	12.52	452.3	15.41
58.1	5.12	109.1	8.42	453.3	5.30
60.0	7.23	110.1	11.12		
61.0	43.67	111.1	41.79		
67.0	13.73	112.1	11.01		
68.1	21.07	113.1	8.41		
69.0	56.14	123.1	5.88		
70.1	25.89	124.1	7.88		
71.1	59.80	125.1	24.01		
73.0	12.08	126.1	7.54		
75.0	5.71	127.1	6.31		
81.1	15.19	129.1	8.76		
82.1	45.01	138.1	6.05		
83.1	73.39	139.1	13.32		

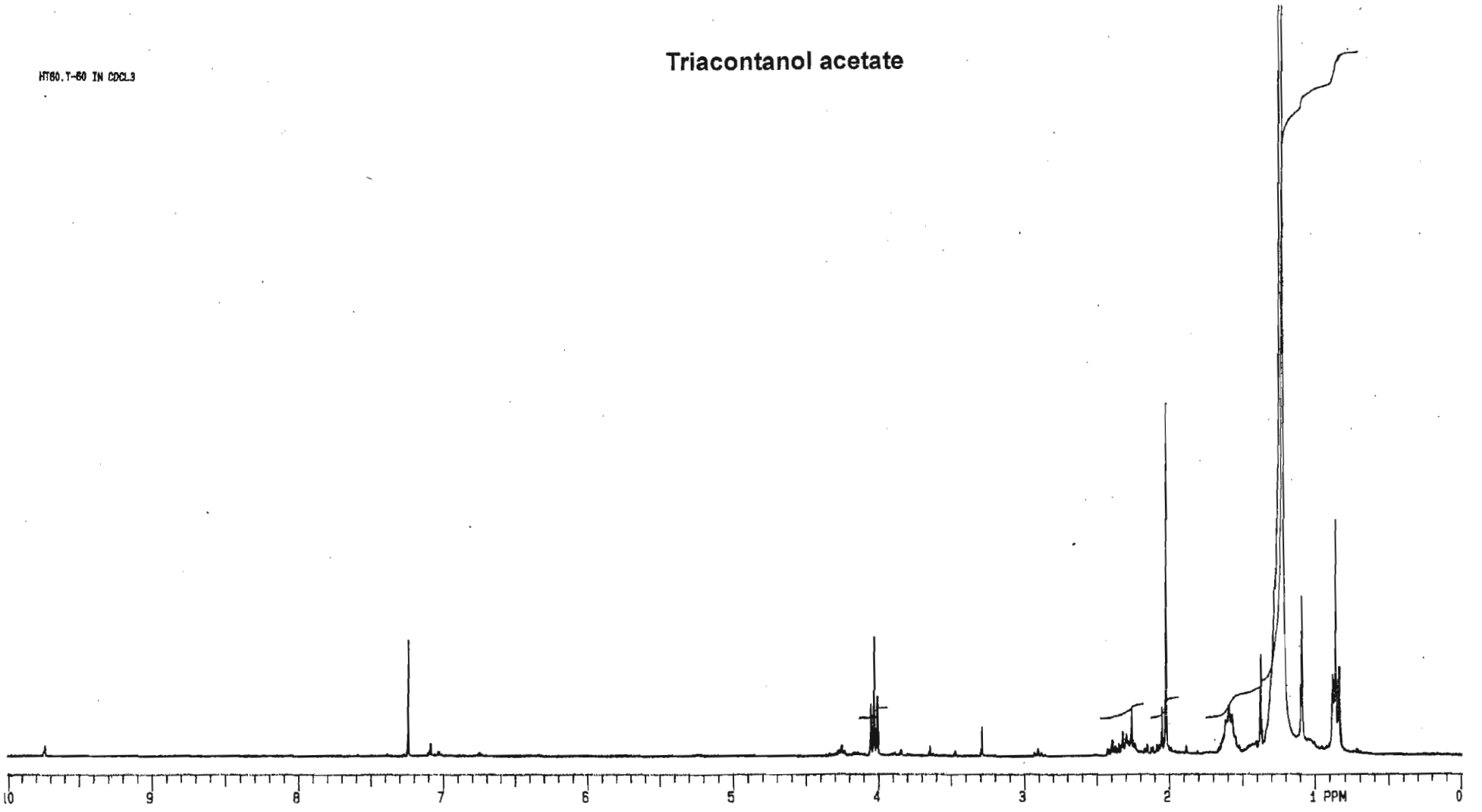
38 Acetate CI-MS



38 Acetate EI-MS



38 Acetate IR



38 Acetate H-1 NMR

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