A PHYTOCHEMICAL STUDY OF *ILEX* AND *BETULA* SPECIES

By

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ABSTRACT

A phytochemical study of the outer bark of native common holly (*Ilex aquifolium*), led to the isolation and identification of nine novel fatty acid esters of the pentacyclic triterpene α-amyrin. These compounds were the oleate, linoleate, heptadectrienoate, decanoate, myristate, pentadecanoate, palmitate, heptadecanoate and stearate esters of the triterpene.

Studies on the outer bark of native birch (*Betula pubescens*) led to the isolation and identification of the pentacyclic triterpenes betulin and lupeol. Oxidation of betulin led to the formation of betulonic acid, which in turn was reduced and acetylated to yield betulinic monoacetate. Both of these derivatives are naturally occurring pentacyclic triterpenoids.

The barks of two non-native or exotic species of birch (*Betula ermanii* and *Betula papyrifera*) were also investigated and this study resulted in the isolation of two further pentacyclic triterpenes; oleanolic acid and oleanolic monoacetate.

The molecular structures of these compounds and their synthetic derivatives were elucidated using the following modern chemical and spectroscopic methods and techniques; ^{13}C & ^{1}H NMR, FT-IR and GC/MS.

In addition, an extensive literature review for the medicinal and commercial applications of the triterpenoids was undertaken.

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TO ROSE.....

INTRODUCTION

Aims and Objectives of this Research

This research work was undertaken to investigate native and exotic species of birch, as well as native holly, for their viability as possible sources of novel triterpenoid compounds. The economic importance of this chemical family, in both the field of ethnobotany and that of ethnopharmacology prompted and motivated this research. The work also provided the author with an opportunity to explore and gain proficiency with modern chemical and spectroscopic techniques and methods, commonly employed in the isolation and elucidation of large organic molecules from natural product sources.

Methods of Investigation

Plant material was extracted in classical fashion with a range of organic solvents of increasing polarity. Compounds of interest were identified from these extracts by means of TLC. Isolation and purification of these compounds was undertaken by way of recrystallisation from organic solvent mixtures and / or flash chromatography. Synthetic derivatives of the isolated molecules were prepared, in order that functionality and stereochemistry could be investigated. Analysis, and in some instances separation, of these derivatives and the original compounds from which they were prepared, was achieved by means of GC, GC / MS, EIMS, FT-IR, ^{13}C, ^{13}C DEPT and ^{1}H NMR spectroscopies. The results of, and conclusions drawn, from all this work are reported in the following chapters and pages, in accordance with the principles and conventions established in the current phytochemical literature.

Results and Conclusions

These studies resulted in the isolation, purification and identification of nine novel fatty acid esters of the pentacyclic triterpene α-amyrin, from the bark of the native common holly (*Ilex aquifolium*). From the outer bark of native birch (*Betula pubescens*) were isolated and identified the pentacyclic triterpenes betulin and lupeol. Due to its contemporary, pharmacological significance in the treatment of skin diseases, such as

the cancer; malignant melanoma, a derivative of betulinic acid was prepared from the isolated and purified betulin. This derivative was synthesised by oxidising the isolated betulin to yield betulonic acid and in turn reducing and acetylating this product to give betulinic monoacetate. Finally, phytochemical investigation of the barks of the two exotic species of birch (*Betula ermanii* and *Betula papyrifera*) resulted in the isolation and identification of two further pentacyclic triterpenes, namely; oleanolic acid and one of this compound's naturally occurring derivatives, oleanolic monoacetate.

The conclusions which can be drawn from this *corpus* of work is, that the species of plant life studied, are valuable sources of pentacyclic triterpenes and their saponins (noted, but not elucidated). This fact, when viewed economically, may prove very beneficial in the fields of ethnobotany and ethnopharmacology.

THE ROLE OF TRITERPENOIDS IN ETHNOBOTANY AND ETHNOPHARMACOLOGY

In most developing countries where coverage by health services is limited or non-existant, it is to the traditional practitioner or to folk medicine that the majority of the population will turn when unwell. The treatment they receive is based on the use of medicinal plants. Early in this century the greater part of medical therapy in industrialised countries depended on medicinal plants, but with the growth of the pharmacuetical industry, their use fell out of favour as they were largely replaced by synthetic compounds.

However the last fifteen years has seen a renewed and growing interest in drugs of plant origin in the developed world. This is reflected in the number of symposia held on medicinal aspects of natural products, as well as in the publication of a number of new journals devoted to phytochemical analysis and ethnopharmacology. At the same time the trade in herbal products continues to expand rapidly, with an estimated value of $1 300 million being spent annually in this regard, in the USA alone. The impetus for this turn-about has come largely from the World Health Organisation, as a result of its 'Health for all by the year 2000 programme'.

In 1987 the World Health Assembly, reaffirming earlier resolutions and reommendations and encouraged by the success of the Chinese in incorporating traditional medicine into modern practice, recommended member states to encourage the development and practice of their traditional systems of medicine. Member states were urged to ;

1. optimise the use of traditional systems of medicine in primary health care,
2. initiate programmes for the identification, evaluation, preparation, cultivation and conservation of medicinal plants used in traditional medicine,
3. support research into traditional methods of treating ailments, promoting family

health, nutrition and well-being.

Consequently, a number of countries initiated the suggested programmes. While the future need for plants as sources of pharmacologically active substances is questioned by some, new and important plant based drugs are constantly being introduced. The economic significance of these naturally occurring pharmaceuticals is quite considerable (Principe, 1989).

Historically, the early botanists concentrated on the economic potential of plants used by aboriginal societies while the early anthropologists considered how different perceptions of the natural world could influence subsistence decisions. In India and other parts of Asia many projects are, today, directed at documenting knowledge of traditional medicinal plants. In Africa traditional agricultural knowledge is increasingly being incorporated into rural development. While in Australia, traditional methods of vegetation management are receiving considerable attention from both environmentalists and ecologists.

A significant proprtion of current ethnobotanical research is conducted on the American continent. Up to 41% of all the studies carried out there are due, in no small part, to that continent's wealth of biological and cultural diversity and its rich archaeological record. Despite American dominance in this field, European ethnobotanists continue to make invaluable contributions to it, particularly in the disciplines of ethnopharmacology and palynology.

Today, the ethnobotanical approach is a well recognised tool, used by the pharmaceutical industry in the search for new pharmaceuticals. Many research projects in the fields of ethnobotany and ethnopharmacology are interdisciplinary in nature, involving scientists from the disciplines of anthropology, botany, medicine,

pharmacology and chemistry. Pharmaceuticals provide the connection between chemistry and biology and do so in two respects;

1. a drug is a chemical manipulator designed to set right the biochemical malfunctions which cause the symptoms of a particular disease state, and
2. drugs can also be used as tools for the study of biochemical balances and processes .....for example, if a drug molecule alters a normal biochemical process, this alteration may be reflected in some biological event, subsequently occurring (Fisher and Christie, 1982).

Table 1. Naturally occurring pharmaceuticals and their therapeutic uses

Pharmaceutical	Natural Source	Therapeutic Use
Ephedrine	*Ephedra vulgaris*	Asthma Treatment
Quinine	Cinchona	Anti-malarial
Digitalis	*Digitalis lanata*	Pulmonary Oedema
Reserpine	*Rauwolfia vomitoria*	Anti-Hypertensive
Morphine	*Papaver somniferum*	Analagesic
Emetine	*Cephaelis ipecacuanha*	Amoebic Dysentery Treatment
Cocaine	*Erythroxylon coca*	Local Anaesthetic
Atropine	*Atropa belladonna*	Parkinson's Disease
Warfarin	Sweet Clover	Anti-Coagulant
Penicillin	*Penicillium chrysogenum*	Antibiotic
Streptomycin	*Streptomyces griseus*	Antibiotic
Colchicine	*Colchicum autumnale*	Gout Treatment

G.T. Prance of the Royal Botanic Gardens, Kew has made the point that many drugs currently available in our pharmacies have come to us, either from folk use or use by indigenous cultures. **Table 1** above, illustrates some of the well-established pharmaceuticals that have been isolated from natural sources.

Prance argues however that these naturally occurring drugs are often used in the context of modern medicine for purposes different from those of the native cultures. Therefore folk medicine, both historically and traditionally, is a very useful indicator of the presence of biologically active substances within a plant (Prance, 1994).

North American science, which deeply influences medical and academic Latin American institutions, disregarded the information about the use of medicinal plants by indigenous cultures during the early years of the WHO's campaign. Thus, it was 20 years later that North American pharmaceutical companies came to the search for new plant drugs. At this stage Asian and European companies were already marketing new herbal remedies.

Scientists from some US universities in the interim period resuscitated some once popular ideas about the importance of the study of the use of plants amongst Latin Amerindian groups. They created academic institutions for participation in research in this field in some Latin American countries with the collaboration of local scientists. The concept of the 'ethnosciences' was promoted, including ethnobotany, ethnopharmacology and ethnomedicine. The avalanche of medicoanthropological and ethnobotanical studies on medicinal plants in Latin America performed during the first decade (1973-1983) of this revival occurred, in the majority of cases, without support from the international pharmaceutical industry.

Plant sampling for drug discovery can follow different approaches, including collection of plants at random, collections guided by chemotaxonomy and collections based on ethnopharmacological (ethnomedical) data. The ethnomedical approach involves selecting for investigation plants identified from traditional and popular knowledge as having active compounds. Practice is demonstrating that the Western 'soft herbal remedies' prepared as closely as possible to the traditional form used for centuries are

preferred by the modern urban consumer. An African or Latin American healer will regard these products as the correct and intelligent approach to the use of medicinal plants.

International agencies have recognised that the 1980's saw a significant increase in the use of herbal remedies around the world. Many countries are now seeking assistance in identifying safe and effective herbal remedies for use in national health-care systems. The term 'herbal remedy' now appears more frequently in legal and medical language. Governments are facing problems in classifying and regulating herbal remedies that do not fit into the common legislative framework for pharmaceutical drugs or foods. New scientific evidence supports a future increase in the use of such herbal remedies. A new pharmacology of natural products is being developed all around the world (Lozoya, 1994).

However, a major cause of strain between traditional and modern systems of medicine is the theoretical incompatability of western and purely traditional medical systems. Chinese, African or Indian traditional medicines are national constructions, originating from basic conceptions of the universe and philosophies relating to man. These traditional or indigenous systems of medicine strove to treat man holistically rather than just his isolated diseased anatomical parts, and to think of him in terms of his emotional sphere and physical environment.

With such a difference in basic philosophy, any attempt to compare the performances of the two systems should be done with caution. In considering effective integration of the two systems of medicine, the prevailing socio-economic circumstances as well as the cultural pattern and the wishes of society must be considered. The integration of these two systems of medicine, in whatever form, will involve a public pronouncement by government based on policy decisions, modification of professional attitudes, and

public enlightenment campaigns to guide and shape public sentiment towards change in the existing pattern. There will undoubtedly be opposition to integration of traditional medicine or even its co-recognition with modern medicine, and governments of developing countries should be aware of, and be in a position to deal with and if necessary legislate for this difficulty (Sofowora, 1982).

Historical Origins of Phytochemistry and Ethnobotany

Historically, botany and medicine came down the ages hand in hand until the seventeenth century. Then both arts became scientific and went their separate ways. No new herbals were compiled and botanical books ignored the medicinal properties of plants, while medical books contained no plant lore. Herbals were a combination of traditional plant lore and the medicinal properties of herbs in conjunction with their taxonomic classification. It was not until 1629 that Culpeper's popular herbal was discredited amongst the scientific community because of its reliance on astrology. As a result of and since the demise of the herbal, modern herbalists and scientists have identified the need to redocument in terms of modern scientific principles this *corpus* of forgotten knowledge.

The names of plants can hold interesting clues as to their medicinal value, because these names are often derived from their original use in medicine. The traditional use of the plant has often originated from some pecularity of it, based on the 'Doctrine of Signatures'. From about the 11th to the 18th centuries this dogma was the sole means of attributing medicinal value to certain plants. It involved the plant's shape, growth, colour, scent, taste or habitat. Thus, the worm-shaped embryo of *Chenopodium* (wormseed) suggested it to be of value as an anthelmintic, the yellow colour of saffron served to point out its value in liver disorders, the serpentine shape of *Rauwolfia* roots (snakeroot) indicated that they should be useful in treating snakebite, etc. The classical name of a plant often embodies a tradition that goes back to the medieval period and

Cox, an expert on Samoan ethnobotany and his co-worker Balick highlight an interesting point when they indicate that until the 1950's, almost all pharmaceutical research relied heavily on flowering plants and ferns as opposed to fungi and microscopic organisms as sources of medicines (Cox and Balick, 1994). Other areas of research that have increased interest in medicinal plants include Chinese *materia medica* and the examination of plants used for medicinal, narcotic and other purposes by indigenous peoples.

As a result of modern isolation and pharmacological testing procedures, new plant drugs usually find their way into medicine as purified substances rather than in the form of the older galenical preparations. Such usage of individual purified compounds, including synthetic drugs, is not without its limitations, and in recent years there has been a considerable revival in herbal, homoeopathic and Ayurvedic systems of medicine, all of which rely heavily on plant sources. Whilst pharmacognosy has been generally pursued for utilitarian ends and may thus be called an applied science it has played an important role in the development of the pure sciences, such as descriptive botany, plant taxonomy and phytochemistry (Trease and Evans, 1983a).

Natural Products and Drug Development

Cox observed that by the mid-1980's most pharmaceutical manufacturers had abandoned the ethnobotanical approach in their search for new drugs (Cox and Balick, 1994). However, this trend is now reverting to a realisation and an appreciation that plants used in traditional medicine can serve as sources of novel therapeutic agents. Cox and Balick had discovered that 86% of the plants used by Samoan healers display significant biological activity in a variety of assays.

Fieldwork involving the exploration of the medicinal uses of plants by indigenous peoples in remote parts of the world, in conjunction with the introduction of sophisti-

cated bioassays, has facilitated the discovery of bioactive molecules produced by medicinal plants. Indeed some of these molecules show promise as possible therapeutic agents suitable for use against a range of diseases, including AIDS and cancer.

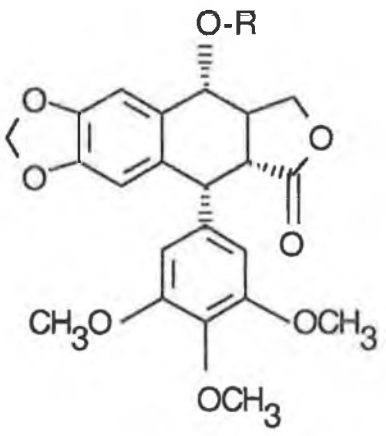
The ethnobotanical approach is an effective methodology that can be applied to choosing plants for pharmacological studies. It is estimated that of the 265 000 flowering species that grace the planet, less than 0.5% have been studied exhaustively for their chemical composition and medicinal value. The history of drug discovery indicates that the ethnobotanical approach is the most productive of the plant surveying methods used. Recent findings by Cox lend support to this latter contention (Cox and Balick, 1994).

However, Cox and Balick believe that ethnobotany despite its manifest successes, is unlikely to ever become a major driving force behind commercial drug discovery programmes. It is too rigorous and time consuming, requiring long-term field work in remote areas of the world. Many sponsors of drug research programmes perceive the ethnobotanical approach to be archaic, unscientific and unworthy of attention. Nevertheless, the approach's demonstrated ability to produce valuable leads for drug discovery suggests that for the immediate future, at any rate, it will occupy an expanding role in the development of new drugs.

Cox and Balick are also of the opinion that few compounds exhibiting activity in laboratory test conditions will in fact become new pharmaceuticals. Some will turn out to be identical to or less potent than existing agents. Others will prove too toxic for commericial use. In any event demonstrating activity in a bioassay is a necessary first step in the development of new drugs (Cox and Balick, 1994).

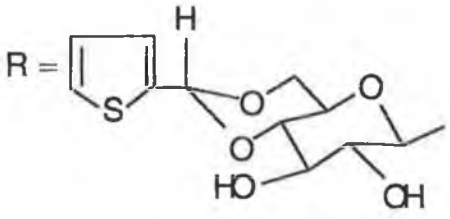
In the 1960's Cox and Balick drew attention to the fact that the standard screening assay methods for potential drugs involved injecting test material into rodents and, then waiting to see if the animal prospered, became ill, or showed some other behavioural or health change. This was a time-consuming, imprecise and costly process. Today bio-assays are faster and far more specific. Such modern assay techniques involve the use of human tissue cultures, while other assays assess the ability of an extract to influence the activity of a single enzyme involved in the biochemical interactions that underlie a particular disease.

Chemists using modern chromatographic and spectroscopic methods, isolate and elucidate the structure or structures of the molecule or molecules from extracts that display significant activity in such bioassays. If it transpires on the basis of comparative analysis that the isolated compound is a novel one, it may then be subjected to further investigation. In some instances a synthetic version will instead be examined where it proves viable. Where an active compound exhibits severe side-effects when administered in the pure isolated form, a synthetic derivative of it may be prepared in order to provide a less toxic alternative. This was the case with the lignan podophyllotoxin and its less toxic derivative teniposide. Teniposide, a semi-synthetic derivative is prepared from podophyllotoxin, isolated from *Podophylum pelatum.* The plant is traditionally used by N. American Indians to treat skin cancers and warts. Podophyllotoxin although of therapeutic value in the treatment of cancer, viral and protozoal infections, is also highly cytotoxic.

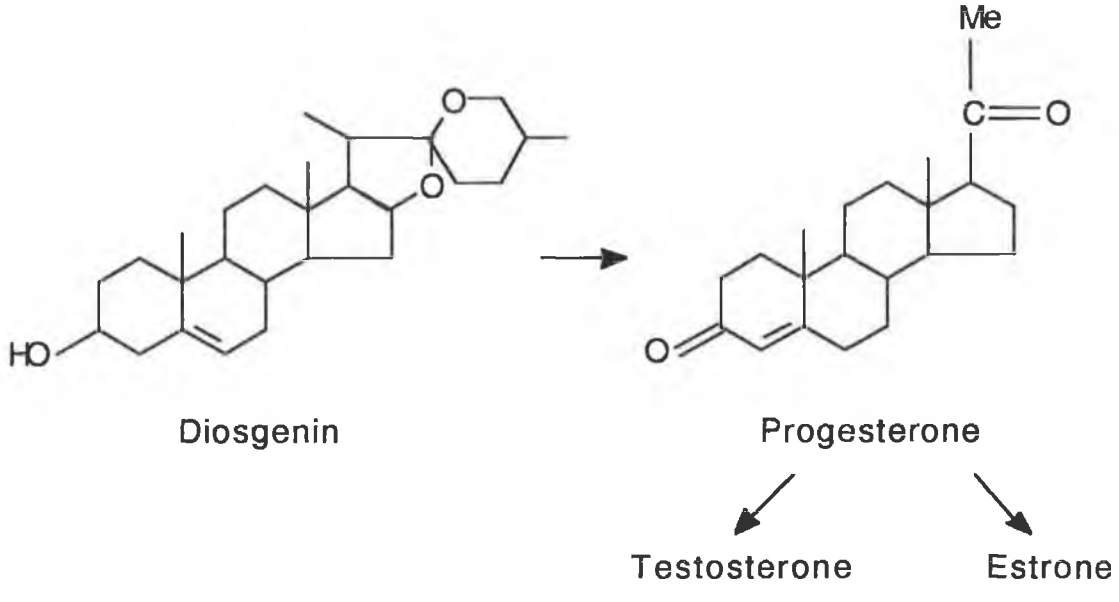


Podophyllotoxin; R = OH

Teniposide;



In yet other cases, the extracted substance will serve as raw material capable of being structurally altered and so producing the desired activity. This was the case in the celebrated Marker degradation, whereby the sapogenin (plant steroid) diosgenin (the biosynthetic precursor of which is cholesterol) was converted by a series of degradative steps to the steroidal hormones progesterone, testosterone and estrone. The diosgenin itself, was isolated from specimens of *Dioscorea* (yam) collected in Mexico and in particular, one known to the natives as 'barbasco' (*D. composita*).



The Marker degradation

The international pharmaceutical company Syntex Laboratories was established to commercially exploit this process, which it continues to do successfully today. The great irony in this success story however, lies in the fact that in 1975 the Mexican Government moved to protect their country's supplies of *D. composita* as a natural resource. This move was blocked by the International Pharmaceutical Industry and today Mexico has to import steroidal hormones because the large pharmaceutical companies would not accept its Government's conditions for exploitation of their country's natural resource (Lehmann *et al.*, 1973; Lozoya, 1994).

A distinct approach was taken by Shaman Pharmaceutical Inc. which has a colloborative drug-discovery and development relationship with Eli Lilly & Co. Shaman was established in the US in 1990 by a group of research scientists, who, employing the ethnobotanical approach collected tropical medicinal plant species and as a result of investigating these species they managed in a period of two years to introduce two anti-viral agents into human clinical trials, namely Provir ^{R} and Virend ^{R}. These agents are indicated as follows;

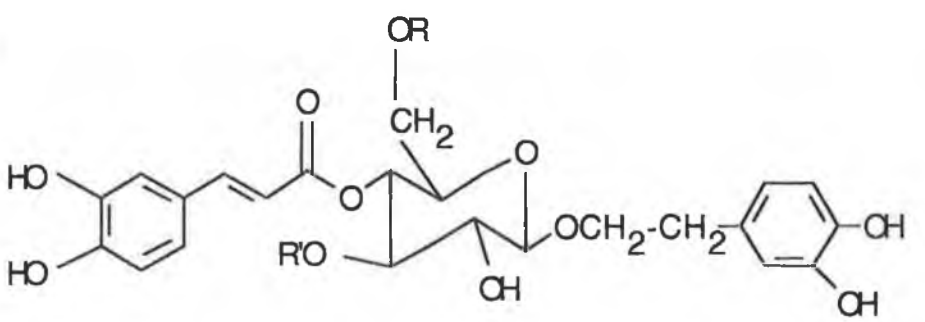
1. **Virend ^{R}:** a topical treatment for the herpes virus and
2. **Provir ^{R}:** an oral treatment for respiratory viral infections.

This company recognised the important contribution ethnobotany has to make to the identification and isolation of biologically active molecules and has made extensive use of botanical databases, language interpreters and the scientific knowledge of western-trained medical practitioners.

Echinacea (*E. augustfolia, E. pallida, E. purpurea* and other species) drugs were used medicinally by the N. American Indians against a wide variety of illnesses, including application to wounds and burns, swellings of the lymph glands (mumps), insect bites,

toothache, headache, stomach cramps, coughs, chills, measles and gonorrhoea. There are also frequent reports of the use of *Echinacea* as an antidote for rattlesnake bite and other types of poisoning. The roots were the most frequently used part of the plant and in addition, the Amerindians also used the juice or a paste of macerated fresh plant material for medicinal purposes. Pharmacologically, the mechanism of the plant's drug action could not be explained in analogy with direct acting drugs such as antibiotics. But in the last 16 years relevant immunological *in vitro* and *in vivo* assays have been developed and as a result of these it has been possible to show that *Echinacea* acts primarly via a stimulation of the unspecific immune system.

Moreover, it has been possible to assign the immunostimulatory activity of the plant's extracts to certain groups of constituents. The species contain a wealth of natural products which include; caffeic acid derivatives, flavonoids, essential oils, polyacetylenes, alkylamides, alkaloids and polysaccharides.



R = Glucose (1,6-)
R' = Rhamnose (1,3-)

Echinacoside

Stoll described the antibacterial action of a complex caffeic acid derivative (echinacoside) from the roots of *E. augustfolia* (Stoll, Renz and Brack, 1950). More recently, Bonadeo showed pharmacologically that a polysaccharide fraction from

E. purpurea promoted wound healing (Bonadeo, Bottazzi and Lavazza, 1971), while over fifteen years later Tubaro, Tragni and their co-workers demonstrated that a crude polysaccharide mixture from *E. augustifolia* roots displayed anti-inflammatory activity (Tubaro *et al.*, 1987). An alkylamide fraction from *E. purpurea* roots was shown by Wagner to be a possible anti-inflammatory principle (Wagner *et al.*, 1989).

In 1967 the isolation of four polyacetylene compounds with bacteriostatic and fungistatic activity were reported by Schulte (Schulte *et al.*, 1967). These compounds were extracted from the roots of *E. purpurea* and *E. augustfolia.* That same year Jacobson reported that the alkylamide, echinacein (dodeca-(2E,6Z,8E,10E)-tetraenoic acid isobutylamide) or α-sansho oil from *E. augustfolia* showed insecticidal activity against *Musca domestica* (housefly) (Jacobson, 1967).

In 1972 Voaden and Jacobson reported that a pentane extract from *E. pallida* roots and a pentane soluble essential oil of *E. augustfolia* root displayed inhibitory activity towards Walker carcinoma 256 and P-388 lymphocyte leukaemia. These workers subsequently isolated (Z)-1,8-pentadecadiene, which displayed weak oncolytic activity. Then in 1978 Wacker and Hilbig reported that *E. purpurea* extracts possessed 'interferon like' activity against influenza, herpes and vesicular stomatitis viruses. In the same year May and Willuhn reported a marked inhibition of herpes, influenza and poliovirus by aqueous extracts of *E. purpurea* (Bauer and Wagner, 1991).

Those who truly value and appreciate the worth of the ethnobotanical approach are in a constant race against time. Plant knowledge is sadly disappearing faster than the forests themselves, generations of accumulated medical wisdom has died with the indigenous practitioners of the ancient craft and has tragically gone unrecorded. Ethnobotanists however, have the capability of capturing much of the remaining wisdom but only if the research is funded and conducted in the short term.

There is a growing interest in, and a demand for, the numerous systems of alternative or complementary medicines that utilise plants and their extracts by the public at large. In economic terms alternative medicine is the second largest growth industry next to microelectronics and some $1.4 billion are spent *per annum* on it in Europe alone. The reasons for the increased use of herbal medicines are numerous but amongst them is a clear dissatisfaction with orthodox proprietary pharmaceuticals.

There can be no doubt that herbal remedies are economically successful. For example, plants are sources of potent medicinal agents such as digoxin, morphine and tubucurarine. In fact they continue to act as the sole source of these pharmaceuticals. Many herbal remedy ingredients are not in this potent category and, although they have been used previously in medical practice, their use has declined in orthodox medicine in both Ireland and the U.K. Critics may argue that this is because the plants had no activity and that they have therefore, gradually been superseded by truly effective agents. Whilst this may be true in some instances, many other plants possess demonstrable pharmacological effects in laboratory animals, even though it may have proved difficult or completely impossible to isolate chemically characterised, active principles from them.

That situation is slowly being remedied however, by the comparatively recent use of spectacularly successful modern experimental techniques which have been developed for the preparative purification and characterisation of natural products. In particular HPLC, GC, GC/MS and Counter Current Chromatography have proved invaluable. These instrumental techniques have led to the isolation of the active principles of plants such as feverfew, with its sesquiterpene lactones, ginseng with its saponins, ginkgo which contains diterpenoid gingkolides and valerian which contains iridoid glycosides and valerates.

As discussed, many secondary metabolites exhibit interesting biological / therapeutic activities which are useful to humans but because of the degree of chiralty which many of these compounds exhibit their synthesis has proved difficult and uneconomic. For this reason it is often commercially, as well as scientifically more sensible to utilise biological production techniques, such as plant cell culturing to produce and harvest such complex molecules.

Such is the current interest in pharmacognosy today, that a new international journal, *Phytotherapy Research* was launched in 1987 with the aim of bringing together the wealth of biological, therapeutic and analytical information on pure natural products, plant extracts and associated pharmaceutical preparations (Phillipson, 1988).

Terpenoid History

The terpenes which form the subject-matter of this dissertation possess a history which spans the centuries of civilization. They have been the subject of chemical study from the early beginnings of chemistry. The essential oils which consist of a mixture of monoterpenes, particularly oil of turpentine, were known to the ancient Egyptians whilst they also received mention in Dioscorides' (Greek physician and botanist of the 1st century AD) "De Materia Medica". The astringent and toxic properties of sesquiterpenoid and diterpenoid bitter principles, mainly lactones, figure in many folk medicines. Camphor was first introduced to Europe from the East by the Arabs and is recorded in several eleventh-century manuscripts. The process of obtaining oils by fat extraction, the so-called process of 'enfleurage' was known by the Middle Ages.

Arnald de Villanova, who died in 1311, described distilled oils from rosemary and sage. His "oleum mirabile" was an alcoholic solution of oil of turpentine and rosemary. The development of the art of distillation belongs to the sixteenth century and is recorded in books written by Brunschwig and Lonicer. There is evidence from

the writings of Walter Ryff of Strasburg in 1550 and of Quercetanus in 1607 that a vestigial essential oil industry existed in France producing oil of lavender and oil of juniper. Eau de Cologne appears to date from the early eighteenth century. Analyses of oil of turpentine were recorded in 1818 by J.J. Houton de la Billardiere who showed that the carbon-hydrogen ratio was five to eight. In fact much of the early work done on the chemistry of the terpenoids was made possible by the rich supplies of material obtainable from the oils and resins of pine, cedar, juniper and redwood (Hanson, 1972a).

Terpene chemistry has provided an immense fund of structural problems whose solutions have, in many instances, provided the stimulus for theories that are now fundamental to organic chemistry. The Wagner-Meerwein rearrangement of α-pinene, the various descriptions of the non-classical carbocation and the theories of conformational analysis have firm foundations in terpene chemistry. The same applies to the Woodward-Hoffman rules of cycloaddition reactions. The juxtaposition of diverse functionality on a terpenoid framework permits the chemist to study the interaction between functional groups within molecules. These interactions are often reflected in the spectroscopic properties of terpenoid substances.

Terpenoid Distribution

Terpenoids are widely dispersed throughout nature, being found in fungi, liver-worts, lichens, algae, ferns, insects and bacteria (both photosynthetic and non-photosynthetic) as well as in all parts of the higher plants. In the plant, terpenes are stored in ducts, lactifers or specialised epidermal cells or are adsorbed onto a lipophilic matrix such as ligin or the cuticle. In most instances the storage cells are dead and so are immune to the toxic effects of many terpenes (Wink, 1990). The majority of plant terpenoids are lipophilic substances, however some of the polyhydroxy steroids and glycosides are lipophobic. Over 15 000 naturally occuring terpenoids have been isolated and character-

ised, while dozens more are reported weekly. In excess of 4 000 naturally occuring triterpenoids have been isolated so far and more than forty skeletal types have been identified. In the case of the monoterpenes at least 38 different skeletal types have been recognised while over 200 sesquiterpenoid skeletal types have been reported.

The largest class of triterpenoids is the oleanane group and most oleananes, as well as many other triterpenes, exist in nature as hydrophilic saponins. Saponins are high-molecular weight glycosides, consisting of one or more sugar units linked to a triterpenoidal or steroidal aglycone. The aglycone is also termed the genin or sapogenin. Monodesmosidic saponins possess a single sugar chain, usually attached at position C-3 of the aglycone. Bidesmosidic saponins possess two sugar chains most often linked at position C-3 of the aglycone via an ether linkage and at position C-28 of the aglycone via an ester linkage. The glycone moiety of these compounds are generally oligosaccharides, linear or branched, attached to a hydroxyl or carboxyl group or both. Tridesmosidic saponins sometimes occur in nature, but on the whole are extremely rare. Triterpenoid saponins possess the property of forming stable froth when shaken with water (Mahato *et al.*, 1988).

In the early days of phytochemistry saponin containing extracts were hydrolysed in HCl because separation techniques then in existance for glycosides were very limited in scope. This fact led to the structures of the intact saponins remaining undetermined. Structural elucidation was confined to the aglycone portion of the molecule. Today, using modern chromatographic techniques, in particular 'counter-current chromatography' many of these biologically important hydrophilic-compounds have been isolated and have had their chemical and physiological properties fully investigated.

Terpenoid Biosynthesis

Clasically, terpenoids have been identified through recognition of an 'isoprene' pattern in their carbon skeletons. It is the number of these significant C_5, isoprene units in a compound that has given rise to a simple primary classification system. The organisation of the isoprenyl carbon skeleton within each primary class then gives rise to the various secondary classes. Thus the terpenes have been classified primarily on their carbon number (C_n), as shown in **Table 2**;

Table 2. Classes of Terpenoids

Name	C_n
Hemiterpenoid	C_5
Monoterpenoid	C_{10}
Sesquiterpenoid	C_{15}
Diterpenoid	C_{20}
Sesterterpenoid	C_{25}
Triterpenoid	C_{30}
Carotenoid	C_{40}

and then on their carbon skeleton (Devon and Scott, 1972). In 1887 Wallach proposed his 'Isoprene Rule', which stated that all monoterpenoids were hypothetically constructed by linked isoprene (2-methylbutadiene) units. By 1894 Wallach's rule had been modified to take account of newer structures which had at that time been recently determined, thus giving rise to the 'Modified Isoprene Rule'. This rule stated that the isoprene units were linked 'head-to-tail' in the terpenoid compounds. However, the problem with this interpretation was that it appeared not to be always obeyed. In fact it transpired that structural interrelationships based on it posed baffling problems. This difficulty was further compounded by virtue of the fact that the Wagner-Meerwein rearrangement, which occurs in terpenoids at low pHs, went unrecognised at that time,

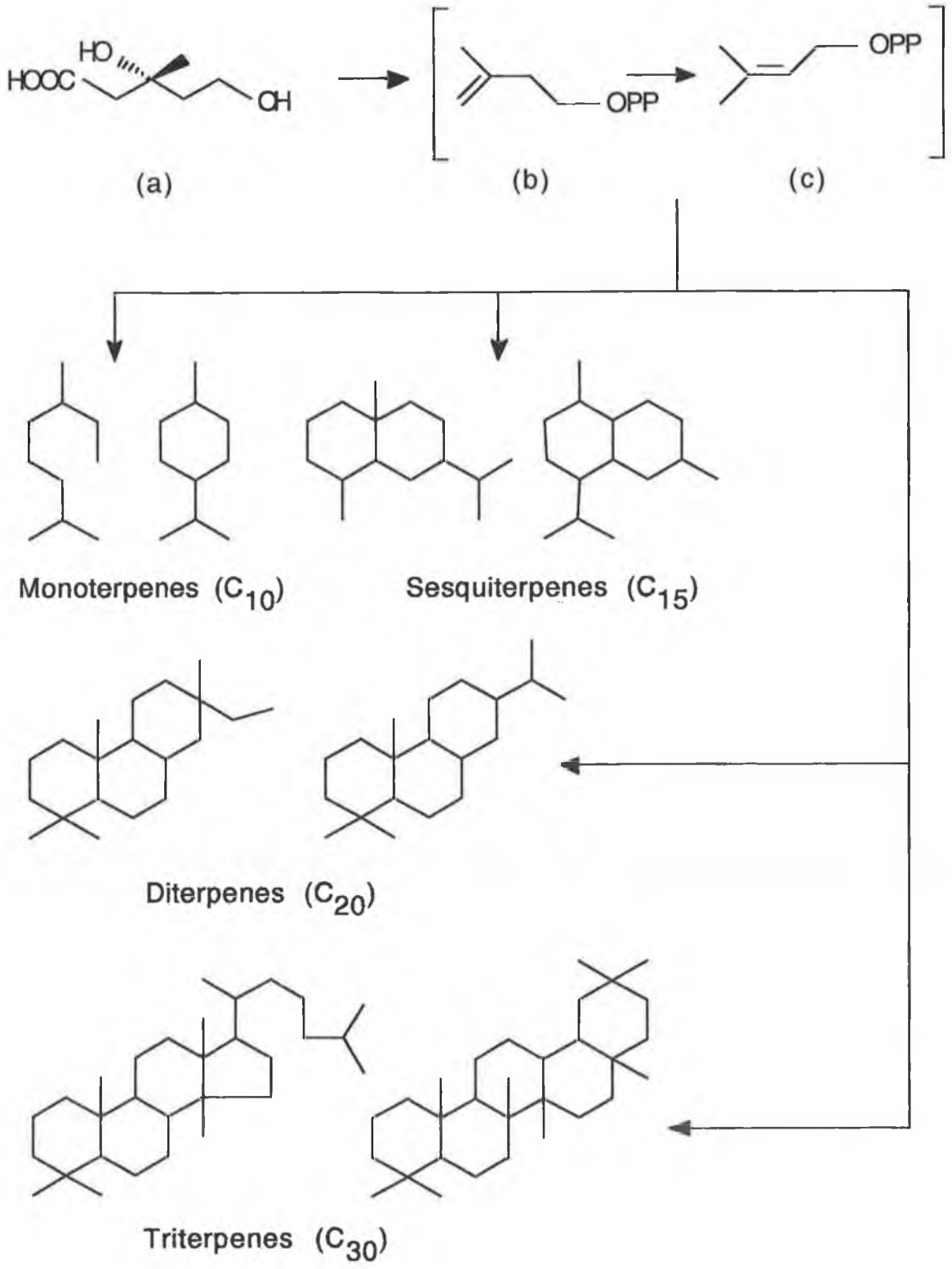
and the reality of the situation was that many of the then proposed structures were in fact incorrect.

As a result of the ever-increasing number of skeletal types that arose within each class of terpenoid, Ruzicka, Arigoni and Eschenmoser managed in the 1950's to rationalise these structures in terms of their 'Biogenetic Isoprene Rule'. This rule stated that each member of a terpenoid group or subgroup was derived from a single parent compound that was unique to that particular group, and that the parents were related in a single homologous fashion, or put another way that each class of terpenoid is formed from an acyclic precursor which is cyclised and further elaborated according to a limited number of well-defined stereo-electronic principles. As with other 'intuitive' hypotheses, including those of Winterstein and Robinson, the 'Biogenetic Isoprene Rule' was merely an attempt on the part of organic chemists to throw some light on the biosynthetic processes that lead to the *in vivo* formation of the terpenoids.

These early theories were based upon comparisons of the molecular architecture of natural compounds and intelligent 'dissections' of their structures into 'building blocks' of biologically acceptable nature. It was never claimed, however, that they were anything more than indications of general trends along which nature probably works. Nevertheless, it is the triumph of the 'Biogenetic Isoprene Rule' as a hypothesis that it successfully accommodates in constitutional and configurational detail the great diversity of terpenoid structures found in nature (Connolly and Overton, 1972).

Although highly speculative for their time and, naturally, received with great scepticism by the early biochemists, the biogenetic hypotheses had and still have an enormous impact on structural organic chemistry. They could be applied with such success in very different areas of natural product chemistry that there was little doubt that many of them, did indeed contain the fundamental principles of biosynthesis.

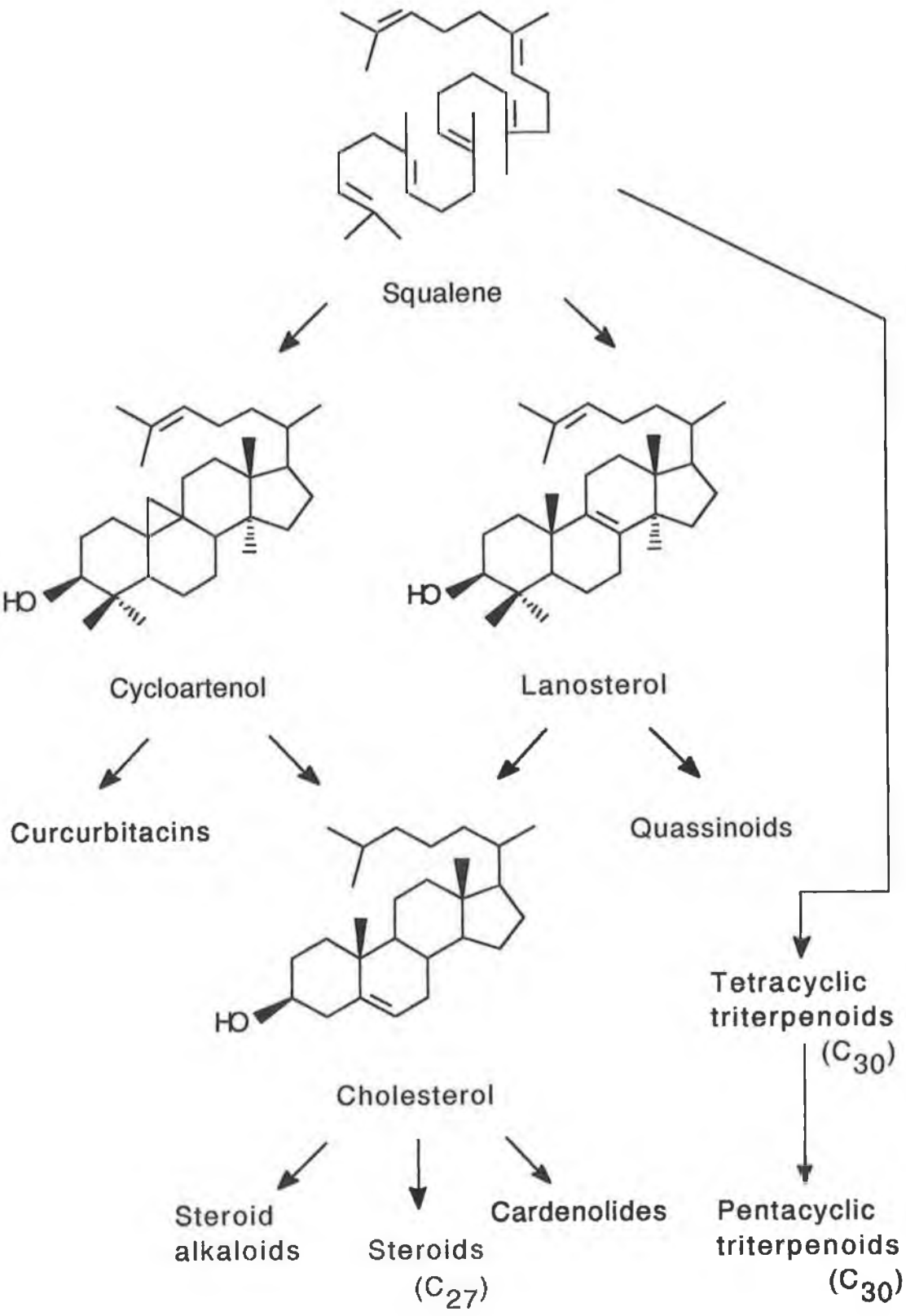
Since that time some of these hypotheses have been put on a much firmer basis by biosynthetic studies using radioactive tracers (Erdtman, 1968). For example Ruzicka went on to propose as shown in **Fig. 1**, that mevalonic acid (a) derived from acetic acid, was the common precursor to all the terpenoids and that by means of enzyme-catalysed reactions, this acid was first converted to isopentenyl pyrophosphate (b), then to 3,3-dimethylallyl pyrophosphate (c), and subsequently to the various terpenoid compounds. The 'Biogenetic Isoprene Rule' was experimentally verified by the use of [^{14}C]-mevalonic acid and [^{14}C]-acetate, and the sequences shown in **Fig's. 2** and **3** below, were established (Natori, 1974).



Biosynthesis of various terpenoid skeletal types

(OPP = Pyrophosphate)

Fig. 1



Biosynthesis of triterpenes and steroids

Fig. 2

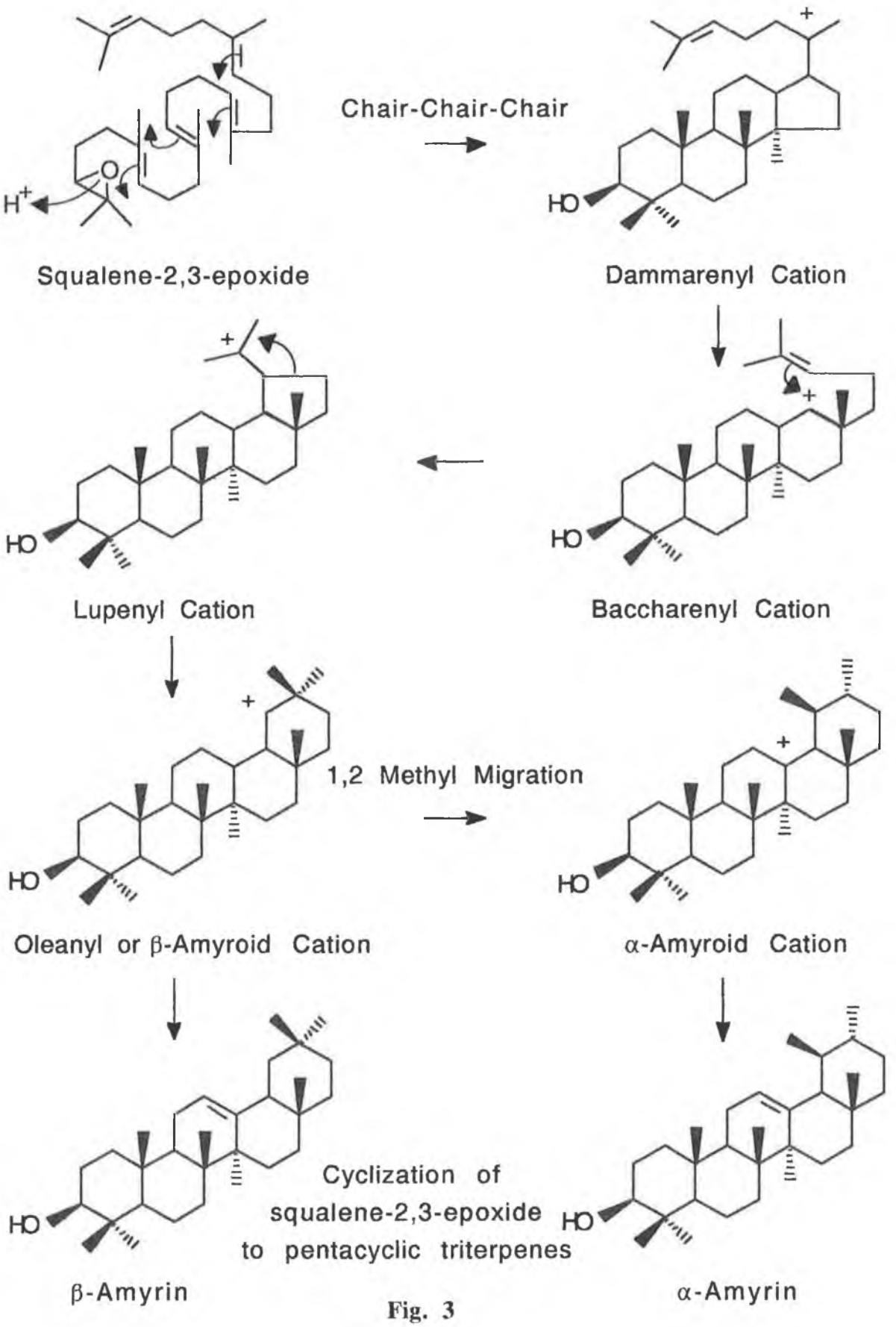

Chair-Chair-Chair
Squalene-2,3-epoxide
Dammarenyl Cation
Lupenyl Cation
Baccharenyl Cation
1,2 Methyl Migration
Oleanyl or β-Amyroid Cation
α-Amyroid Cation
Cyclization of
squalene-2,3-epoxide
to pentacyclic triterpenes
β-Amyrin
α-Amyrin

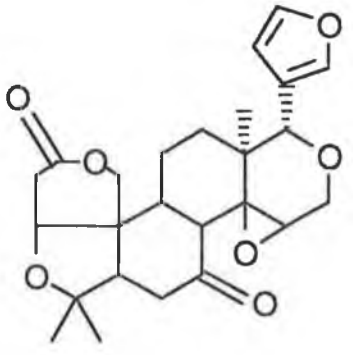
Fig. 3

Therefore, the triterpenoids are all derived from the common precursor squalene ($C_{30}H_{50}$) or more specifically squalene-2,3-epoxide. The all-*trans* polyene squalene, was first discovered in whale-liver oil, it was again detected in shark oil in the 1920's, and was further detected in small quantities in other mammals. However, despite this early work its presence in plants remained speculative until tracer and enzymic studies confirmed it to be an obligatory intermediate in the biosynthesis of triterpenoid molecules.

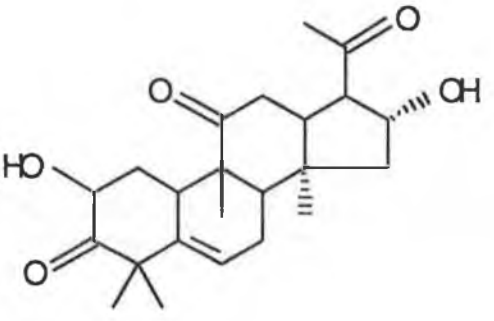
According to the biogenetic isoprene rule, the cyclization of all-*trans* squalene proceeds via a well-defined sequence of prechair and preboat conformations. It was proposed that the transformation takes place according to the rules of antiperiplanar cationic 1,2-addition, 1,2-rearrangement and 1,2-elimination, the entire process being a concerted one (Mahato and Sen, 1997). However, in 1982 van Tamelen proposed that a single transition state for a complex polycyclization is less likely, and that the cyclization proceeds through a series of discrete conformationally rigid, partially cyclized carbocationic intermediates. Several monocyclic and bicyclic triterpenes, which are thought to be partially cyclized intermediates, have been isolated from nature. The isolation of these triterpenoids supports the above postulate of van Tamelen (van Tamelen, 1982).

The majority of triterpenes possess the conventional skeleta arising from the various cyclizations of squalene-2,3-epoxide. This in turn gives rise to various fused polycylic products. The isoprene units that form squalene are arranged symmetrically about the middle of the molecule. At this point there is a head-to-head linkage; in the rest of the chain the units are joined by the usual head-to-tail linkage (Beyer and Walter, 1996). As a result of the total number of possible rearrangements that can occur during biosynthesis there are more triterpenes produced in plants than any other group of terpenoids.

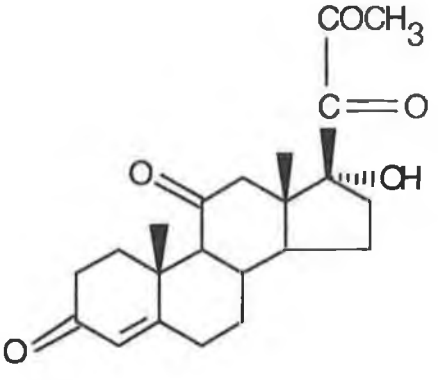
During the later stages of biosynthesis small carbon fragments may be removed from squalene-2,3-epoxide to produce molecules with less than thirty carbon atoms, these are termed *nor*-triterpenes. Some *nor*-triterpenoids are formed by loss of the methyl at C-4 and C-14 of the corresponding triterpenoid. In the case of the *nor*-triterpene limonin, truncation of the side-chain by 4 carbons followed by furan ring formation also occurs (Hanson, 1972b; Mann *et al.*, 1994a). Hexa*nor*-curcurbitacin D and the adrenocortical hormone cortisone are other examples of *nor*-triterpenoids. Saponins with *nor*-triterpenoid aglycones are present in *Guaiacum officinale* and certain other plants such as *Celmisia petriei* and *Isertia haenkeana*. The aglycone in the *Guaiacum* saponins is 30-norolean-12,20(29)-dien-28-oic acid, a C_{29} triterpene (Hostettmann and Marston, 1995a).



Limonin

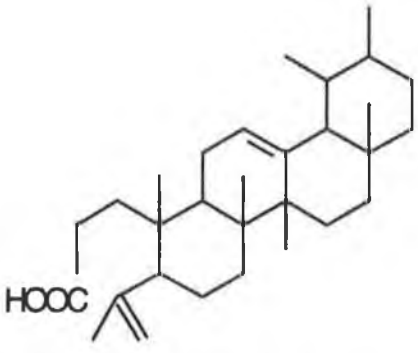


Hexa*nor*-curcurbitacin D

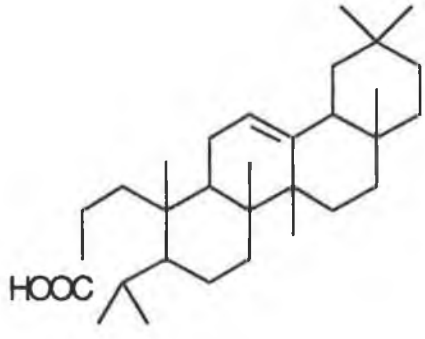


Cortisone

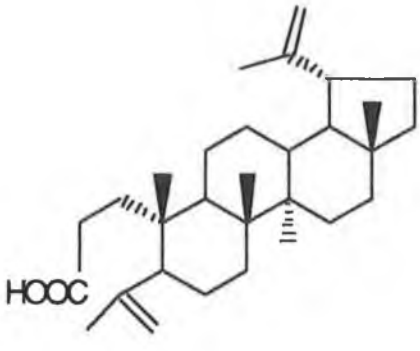
Seco -triterpenes are also known, for example the 3,4-*seco*-3-acids such as roburic acid from *Cynips mayri* and nyctanthic acid from *Nyctanthes arbor tristis* are probably produced by simple fission of the A-ring from the intact precursors α- and β-amyrin (Baas, 1985). The *seco*-triterpenes possess open rings, this usually occurs in ring A or they may possess constituents other than carbon in their rings, for example oxygen or nitrogen, which in respect of the former will give rise to lactones, as in the case of buxaheptalactone (Hanson, 1972b). Interestingly, limonin mentioned in relation to the *nor*-triterpenoids is also an example of a *seco*-triterpene. Canaric acid is a lupane-type *seco*-triterpene acid from the gum produced by the bark of the tree *Dacryodes edulis.* It is also found in the oleoresin of *Canarium zeylanicum.*



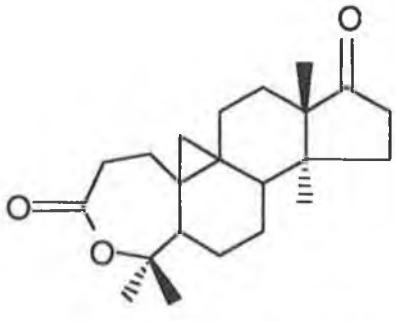
Roburic Acid



Nyctanthic Acid



Canaric Acid



Buxaheptalactone

Very little biosynthetic data is available on *seco*-A-formation of non-degraded triter-

penes in plants, although a number of formal pathways been suggested to explain their formation. Ring-cleavage mechanisms involving a leaving group at C-3 or on a C-4 methyl have been suggested, as has an enzymatically controlled Baeyer-Villiger type reaction with the 3-ketone as intermediate. This type of conversion may be the usual route to 3,4-*seco*-triterpenes since in plants 3-ketones are often found together with the corresponding *seco*-derivatives (Baas, 1985).

It is also speculated that insects have a part to play in the enzymatically controlled formation of *seco*-triterpenes, since such compounds have not been detected in the wax of uninfected tissue of *Quercus robur* (common oak), but are present in the surface wax of their galls. The triterpene metabolism of the tree is thought to have been altered by the gall-making insect (Baas, 1985). Regular reviews on the isolation of new triterpenoids and biosynthetic pathways appear in the scientific literature (Hill, 1993).

Terpenoids in Medicine

Some plants contain large quantities of triterpenes in their latex and resins and the physiological function of these compounds is generally believed to be a chemical defence against pathogens and herbivores. It is expected, therefore, that triterpenes should act against related pathogens that cause human and animal diseases. However, with the exception of the steroids, application of triterpenes as successful therapeutic agents is limited thus far. Perhaps this fact may primarily be ascribed to the hydrophobic nature of most triterpenes. The recent development in techniques of drug solubilization are likely to overcome this problem and widespread reports in recent years on useful biological activities of triterpenes indicate their varied potential (Mahato and Sen, 1997).

The majority of natural products used medically are either terpenoids (which include the steroids) or alkaloids. The mono, sesqui and diterpenes exhibit greater than 26 differ-

ent pharmacological activities alone, ranging from anaesthetic to vitamin, and including analgesic, antibiotic, anticancer, anti-inflammatory, hypotensive and sedative pharmacological properties. The monoterpenes, better known as the essential oils, are much utilised in modern forms of alternative medicine, as well as being used as flavourings and perfumes. The application of terpeneoids as pharmaceutical agents is next considered under the following 'therapeutic class' headings;

1. Antiviral Agents,
2. Anticancer / Antitumor,
3. Anti-Inflammatory / Anti-ulcerative,
4. Cardiovascular Agents,
5. General *Panacea*,
6. Hypolipidaemic,
7. Anti-migraine,
8. Antihypertensive Agents.

Antiviral agents

In 1978 it was reported that synthetic antiviral compounds presented significant drawbacks, such as narrow spectrums of activity, limited therapeutic usefulness and variable degrees of toxicity (Van Den Berghe *et al.*, 1978). In fact only seven pharmaceuticals are licensed for use as antivirals in the US and, to date, these antiviral drugs have enjoyed limited success. On the other hand, the prevalence of virally induced diseases is of growing concern; therefore, the development of new and better antiviral compounds is most desirable.

The threat of the AIDS virus since the 1980's and the fact that many infections are, in any event viral in nature, prompted a search for new antiviral drugs from botanical sources. Antivirals, depending on their mode of action, fall into one of two categories;

direct or indirect acting. The former agents aim for direct inhibition of viral replication in the infected host, whereas the indirect agents aim at the stimulation of the host's defence mechanisms.

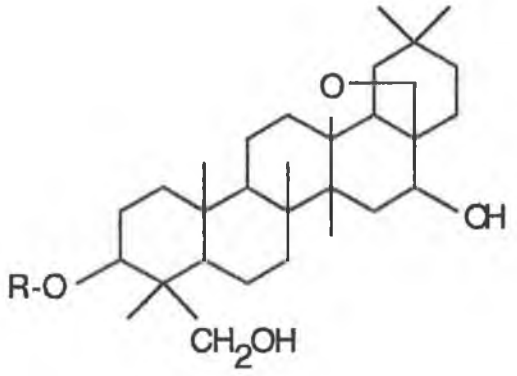
Infections of indigenous peoples tend to be viral in nature, in fact they are very often found to be viral-gastrointestinal disorders. Rotavirus for example, is responsible for at least 50% of infections that lead to acute diarrhoea. It is not surprising then, that the *materia medica* of indigenous cultures would include a large number of antiviral compounds. Furthermore, it has been demonstrated that viruses respond to plant extracts in a manner distinct from their response to synthetic antivirals (Van Den Berghe *et al.*, 1978). Thus, it was predicted that the ethnobotanical approach would lead to the isolation and characterisation of new antiviral agents.

During the past 25 years approximately 4 000 plants, randomly selected from around the world have been screened for antiviral properties. It was found that roughly 10% of them showed activity against one or more viruses. Studies of the antiviral effects of many plant extracts and natural products clearly show that they provide a useful source of new antiviral compounds. In the search for plant-derived antiviral agents the screening of a relatively low number of randomly selected plants has afforded a remarkably high number of active leads in comparison with the screening of synthetic compounds. In comparing different approaches to plant collecting, it was reported in 1991 that folk-based collections give a five times higher rate (circa 25%) of active leads, whereas random collections offer fewer leads but more novel compounds (Elisabetsky and Posey, 1994).

Shaman Pharmaceuticals, who discovered the previously mentioned antiviral agents Provir ^{R} and Virend ^{R}, found that in virtually all cases where a plant had been used traditionally to combat viral infections both *in vivo* and *in vitro* laboratory testing prov-

ed positive in showing the presence of antiviral agents. Of the biologically active extracts identified only a handful have been further investigated or have had their active principle(s) isolated. In instances where isolation and determination of the antiviral agents have taken place, a variety of structural types have been identified, namely; the alkaloids, flavanoids and tannins as well as the terpenoids and their glycosides.

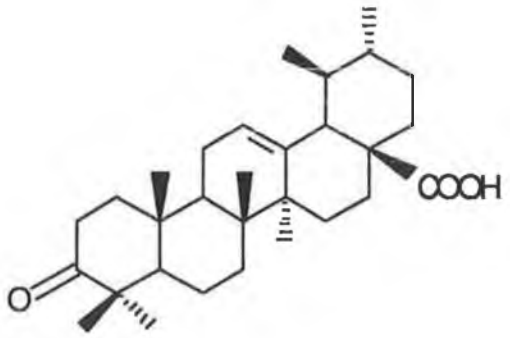
A series of saponins containing acylated β-amyrin aglycones were tested against influenza A virus and many were found to be active (Rao *et al.*, 1974). An extract of *Anagallis arvensis* is reported to show antiviral activity against herpes simplex type 1 and the polio viruses. This led to the isolation of two novel triterpene saponins (1) and (2), the first of these protoprimulagenin A glycosides (1) was found to inhibit both the cytopathic effect and the replication of herpes simplex type 1 and polio type 2 viruses.



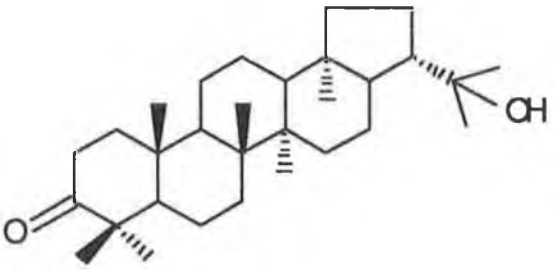
(1) R = glu-glu-xyl
|
ara

(2) R = glu-glu-glu-xyl
|
ara

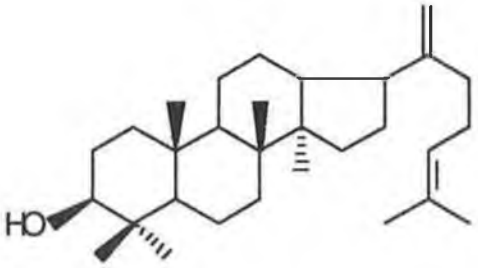
During the late 1980's, nine triterpenes were isolated from dammar resin and their antiviral activities against herpes simplex types 1 and 2 were reported. These antiviral compounds include the following triterpenes; ursonic acid, hydroxyhopanone, dammaradienol, hydroxyoleanonic lactone and shoreic acid (Poehland *et al.*, 1987).



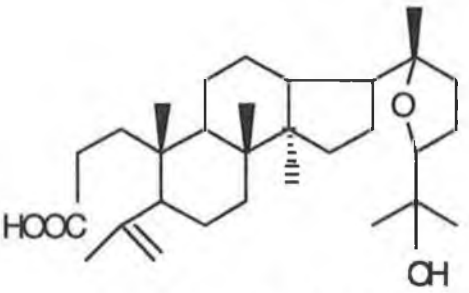
Ursonic Acid



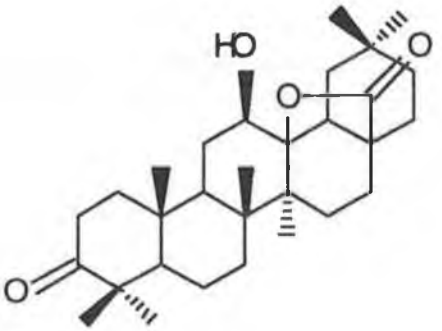
Hydroxyhopanone



Dammaradienol

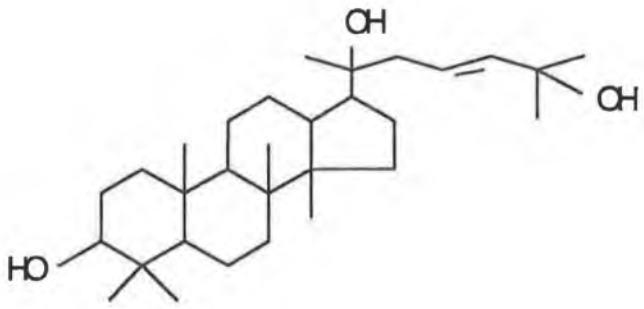


Shoreic Acid



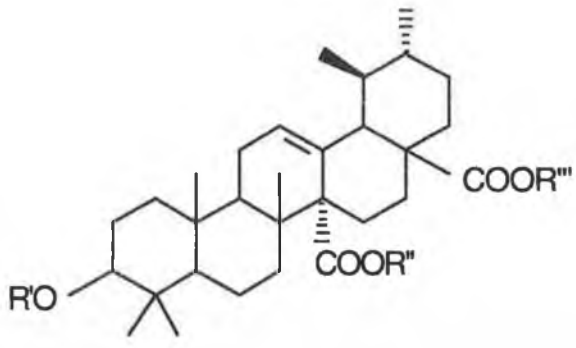
Hydroxyoleanonic Lactone

Isofouquierol was shown in 1987, by Poehland, to be a major antiherpetic constituent of *Gierocarpus intricatus* (Poehland *et al.*, 1987)



Isofouquierol

In 1989 the isolation of several quinovic acid glycosides from *Uncaria tomentosa* and *Guettarda platypoda* were reported, these compounds showed an inhibitory effect against vesicular stomatitis virus (Aquino *et al.*, 1989).



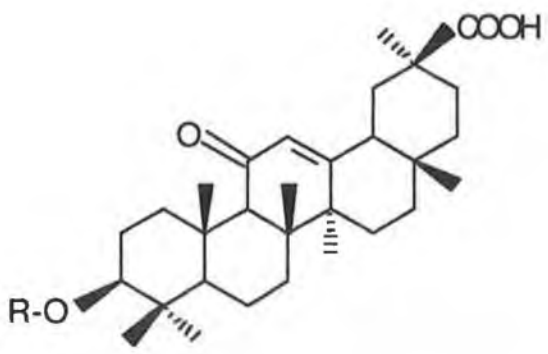
R', R'' & R''' = H or sugar unit/s

Quinovic acid glycosides

Liquorice root (*Glycyrrhiza glabra*) extract or as it is more commonly known by its old Galenical name; *Liquiritiae radix*, is an oriental medicine used in the treatment of chronic liver disease (Hino *et al.*, 1981; Kumada *et al.*, 1983; Kiso *et al.*, 1984). *Liquiritiae radix* comes from the yellow-coloured varieties of *G. glabra*, a widely distributed shrub having several commercial uses, including the preparation of teas, extracts and liquorice. The root is reputed as a condiment and a flavouring for foods while large quantities are used in the tobacco industry as a curing agent. Apart from

their use as sweetners and taste modifiers pure compounds and extracts from *G. glabra* are useful expectorants and demulcients. Liquorice sweets stimulate saliva production and the need to swallow, thus the cough reflex is reduced.

The pharmacological actions of liquorice root have been well documented (Fujisawa *et al.*, 1980; Hikino, 1985; Ren and Wang, 1988; Ngo *et al.*, 1992) The best known constituent of the extract is the triterpene saponin glycyrrhizin. The root contains up to 14% of this sweet-tasting compound. In the form of its potassium and calcium salts; the aglycone, 18β-glycyrrhetinic acid lacks a sweet taste. It has been reported that glycyrrhizin and glycyrrhetinic acid were effective anti-inflammatory agents (Yamasaki *et al.*, 1967; Fukahori *et al.*, 1990).



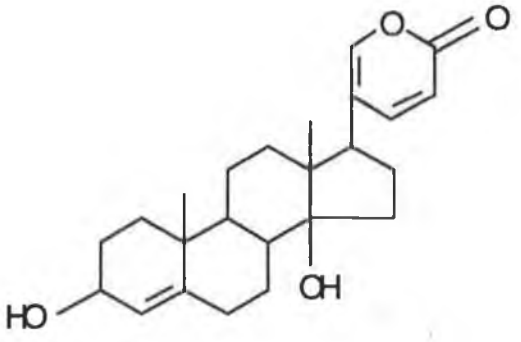
Glycyrrhizin; R = β,β'-glucuronido-glucuronide

Glycyrrhetinic acid; R = H

Both glycyrrhizin and the glycyrrhiza extract were found to be anti-mutagenic of several (Ngo *et al.*, 1992). Glycyrrhizin and its acid were found to inhibit the growth of melanoma cells in mice, while the herbals report the extract to possess wound-healing properties. In Japan preparations containing the compound have been widely used for chronic hepatitis and liver cirrhosis (Suzuki *et al.*, 1983; Hikino, 1985). A recent discovery in relation to glycyrrhizin and its aglycone, glycyrrhetinic acid is that they possess antiviral activity. Among a wide spectrum of biological activities, glycyrrhizin

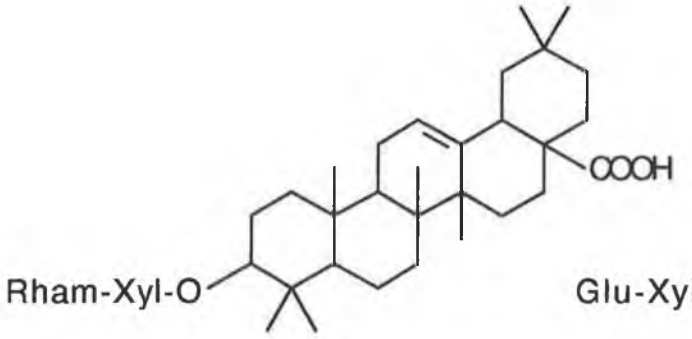
is effective in treating chronic viral hepatitis (Fujisawa *et al.*, 1980). Both compounds inhibit the *in-vitro* growth and cytopathogenicity of several DNA and RNA viruses, including herpes simplex virus type 1, Newcastle disease virus, vesicular stomatitis virus, vaccinia (Pompei *et al.*, 1979; Pompei, 1981) and varicella-zoster virus (Baba and Shigeta, 1987). Other antiviral related biological effects of glycyrrhizin include the activation of natural killer cells and the induction of interferons (Djeu *et al.*, 1979; Abe *et al.*, 1982). An intravenously admistered solution containing glycyrrhizin, cysteine and glycine (Stronger Neo-Minophagen C) has been used in Japan as an antihepatitis drug. This pharmaceutical preparation was developed for clinical use in 1989 following reports that glycyrrhizin inhibited replication of the human immunodeficiency virus type 1. Two years later it was reported that the 11-deoxo derivative of glycyrrhizin is equipotent with it, against HIV-1 induced cytopathogenicity at low concentrations (Hikino and Kiso, 1988; Pisha and Pezzuto, 1994; Hostettmann and Marston, 1995b). The acid is also both antipyretic and antimicrobial.

Bufadienolides or bufotoxins are naturally occuring steroid molecules incorporating a six-membered lactone ring. They occur in frogs, toads and the squill family of plants. Such compounds are no doubt responsible for the inclusion of toads' organs in esoteric recipes in folk medicine and black magic (Mann *et al.*, 1994b).

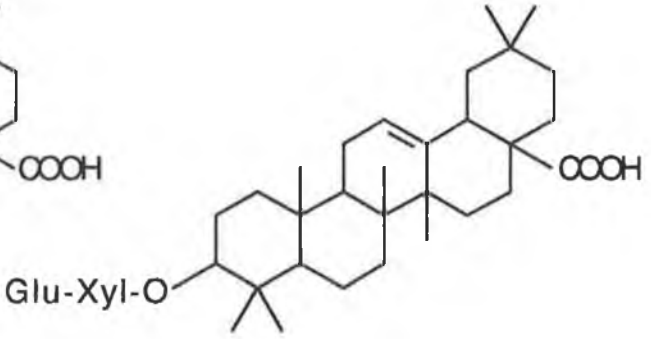


Scillarenin

Scillarenin is a plant bufadienolide (steroid) which inhibits replication in certain viruses, in particular rhinoviruses and poliovirus, *in vitro*. However, *in vivo* usage of this compound proves too toxic. Two triterpene saponins; giganteaside D and flaccidin B from *Anemone flaccida* exhibit inhibitory effects on the avian myeloblastosis virus reverse transcriptase enzyme (Che, 1991).

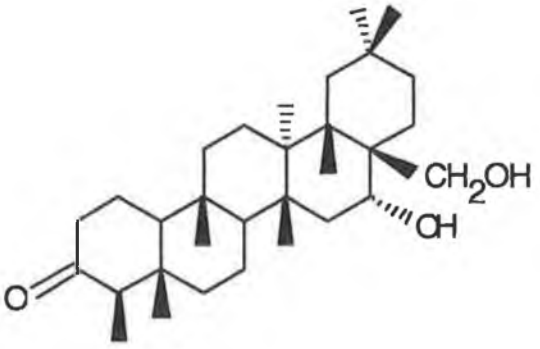


Giganteaside D



Flaccidin B

The plant *Celastrus hindsii*, possesses a triterpene, celasdin-B with anti-AIDS properties; it is found to exhibit 'anti-HIV replication activity' in H9 lymphocyte cells (Kuo and Yang Kuo, 1997).



Celasdin-B

Anticancer / antitumour agents

Cancer may be defined as any malignant tumour arising from the abnormal and uncontrolled division of cells that then invade and destroy the surrounding tissues.

Cancer cells begin to replicate and grow without reference to the needs of the organism to which they belong and this can occur in almost any organ. It is one of the characteristics of the growth of malignant cells that the normal cells that turn to malignancy tend to lose their differentiation (i.e. the process by which unspecialised cells or tissues become specialised for particular functions and hence bring variation to an organism) and the faster they grow and divide the more primitive they become. Consequently, tumours are classified as being well, moderately, or poorly differentiated.

Metastasis (the spread of cancer cells to unaffected areas) may occur via the blood stream or the lymphatic channels or across body cavities such as the pleural and peritoneal spaces. This spreading phenomenon results in the setting up of secondary tumours at sites distant from the original tumour. Each individual primary tumour has its own pattern of local behaviour and metastasis. The symptoms attributable to such growths may be those related to the presence of a large mass of cells in an unaccustomed place, for example, a mammary lump; or to those resulting from a change in the function of an organ, for instance liver failure; or to an obstruction, such as a growth in the oesophagus or colon. There are many causitive factors in relation to cancers, they include; genetic inheritance, ionising radiation, chemical and environmental factors. Treatment of cancer is dependant upon the form involved, the site of the primary tumour and the extent of metastasis.

The histological diagnosis of cancer rests on two sources of evidence. The first is the macroscopic evidence associated with the term tumour (i.e. a swelling in the body) - a discernible alteration in body structure or function. The second is the microscopic: an alteration in cell structure and tissue architecture. Historically, the fatal outcome of cancer was recognised as early as 300 BC and it was differentiated from benign growths such as carbuncles and ulcers and treated, where possible, by excision or else

by arsenical and similar ointments with a strong corrosive action. Over the last sixty-five years, literally hundreds if not thousands of compounds (carcinogens), among them the polynuclear aromatic hydrocarbons and dyestuff intermediates have been shown to induce carcinomas. Even virusses have been reported to cause cancer in laboratory animals, in fact the first virus-induced cancer, chicken leukosis was described by Ellerman in 1908 (Harris, 1962a). Since then many such cancer inducing virusses have been described.

It has long been the hope of all those who treat cancer patients that the chemists, biochemists, and pharmacologists would discover a drug that, like a guided missile, would target all cancer cells in the body and destroy them without harming any normal cells in the same body. In the past the cytotoxic agents used in cancer chemotherapy, which included compounds such as the nitrogen mustards, folic acid or the purine antagonists were unspecific for malignant cells and thus targeted all 'fastly' replicating cells, such as germ cells and cells associated with the production of hair and nails, and proceeded to actively destroy them. Coupled with this problem was the fact that cancer cells possessed the ability to develop resistance to these cytotoxic agents.

Modern cytotoxic drugs can be classified into four main groups, namely; alkylating agents, cytotoxic antibiotics, antimetabolites and vinca alkaloids. They are all designed to terminate cell division in cancer cells, however, normal cells and particularly those with a rapid rate of mitosis (non-germ, cellular division), can also be affected. Toxic effects normally observed include severe nausea and vomiting, alopecia and bone marrow suppression. Myelosuppression necessitates checking blood counts prior to each stage of treatment, with dosage modification or delayed treatment being employed, accordingly.

The sex hormones and antagonists are another class of pharmaceuticals used in the

battle against cancer. They are mainly used for the treatment of prostatic and breast cancer. Hormonal treatment of advanced prostatic cancer is based on the concept that hormone-dependent neoplasms will regress when deprived of hormone stimulation. In prostatic carcinoma, which in general is androgen-dependent, oestrogens are often effective but their use is limited by cerebro- and cardiovascular side effects (Letoha, 1996).

The margin of efficacy over toxicity is a major problem associated with finding appropriate anticancer remedies from plants and other sources. More often than not the ED_{50} is dangerously close to the LD_{50} and in pharmacological terms this translates to mean that the dosage required to elicit a desirable biological response from the agent is perilously close to that capable of eliciting a fatal or medically undesirable response. This scientific fact reiterates in modern medical terms the old principle of drug administration expounded by the Greek physician Paracelsus, many centuries ago, when he stated that it is merely the dosage that differentiates a medicine from a poison. Paul Ehrlich of Salvarsan ('the magic bullet') fame certainly believed that a drug with specificity for malignant cells could be found, and perhaps now with natural product treatments such as taxol, his belief is at last being realised (Harris, 1962b).

Studies at the US National Cancer Institute have shown that most potent anticancer agents are frequently isolated from plants which are toxic to animals, for example, those used as arrow and fish poisons or as vermifuges. However, these compounds are not generally well tolerated by large mammals, including humans. The long and legendary history of the yew (*Taxus* spp.) as a poison and the more recent discovery of the antitumour activity of some of its constituents have made the genus *Taxus* one of the most intensely investigated, from the chemical point of view. The yew tree contains secondary metabolites that are unique in their structure and outstanding in their pharmacological properties. Phytochemical investigations on the yew in recent years

have grown exponentially, primarily as a result of the commericial exploitation of this plant as a source of antitumour agents.

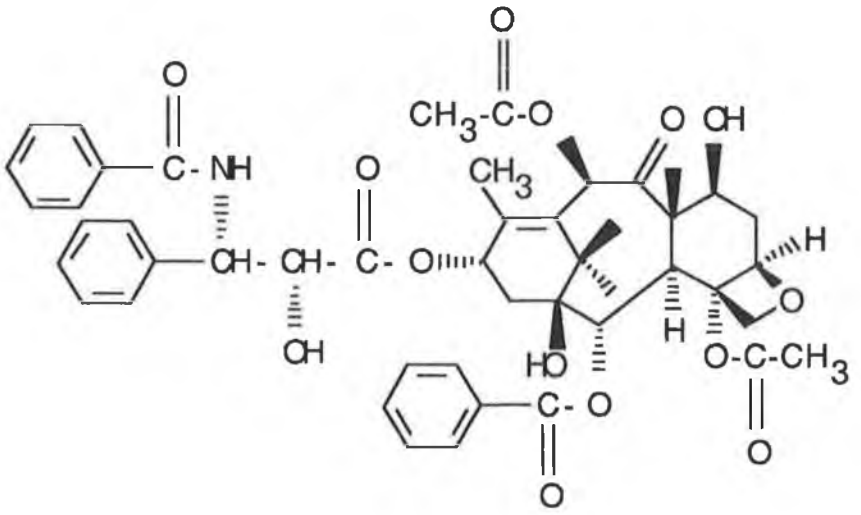
Paclitaxel (Taxol ^{R}) is isolated from the wood and bark of several species of yew; *Taxus brevifolia* Nutt. (Pacific Yew), *Taxus baccata* Barron var. *barroni* (European Yew) and *Taxus cuspidata* Sieb. and Zucc. *Taxus brevifolia* is a slow-growing tree which is only found in localized coastal areas of Calfornia, Washington and Oregon in the USA Taxol ^{R}, a heavily substituted diterpene, is extremely active against a number of forms of cancer. This is most interesting, as many diterpenes, particularly the phorbols which are present in the latex of many spurges (*Euphorbia* spp.) are tumor promoters. Taxol ^{R} occurs in the leaves / needles of all *Taxus* species, together with key precursors that can be readily converted to active paclitaxel analogues. One of these analogues 10-deacetyl-baccatin is the starting material for the synthesis of docetaxel (Taxotere ^{R}) which is currently undergoing advanced clinical development (Cragg, *et at.*, 1994)

Taxol ^{R} displays very good activity against the B16 melanoma and MX-1 mammary xenograft systems. It shows moderate activity against the L1210, P388 and P1534 leukemia systems, the CX-1 colon and LX-1 lung xenografts. It is effective by means of a mechanism very different from other cancer agents currently available and in use, that act on DNA, RNA or protein synthesis. Mechanistically it inhibits mitosis, acting as a mitotic spindle poison (Blasko and Cordell, 1988). Taxol ^{R}'s structure was elucidated in the early 1970's and the compound is currently considered by many oncologists to be one of the best cancer chemotherapy agents discovered in many years. There had been a lull of ten years between the compound's discovery and the work leading to its development as a pharmaceutical. The drug's efficacy in the treatment of refractory ovarian cancer is very well established and the bark of *T. brevifolia* is the only currently approved source of Taxol ^{R} used in the treatment of this form of cancer.

The compound has also shown promise in the treatment of breast and lung cancers. Interestingly, paclitaxel or Taxol ^{R} also shows impressive antimalarial activity, being highly effective against chloroquinine and pyrimethamine-resistant parasites.

Unfortunately, the procedure for the extraction of the compound is difficult, low yielding, expensive and results in the destruction of the tree. In an attempt to save the tree from extinction, other renewable sources of the chemical such as the twiggs, needles and leaves are under investigation. Efforts are also being made to cultivate yew on a large scale. Researchers world-wide are working hard to produce Taxol ^{R} by other methods such as tissue and fungal culture, as well as trying to improve on its industrial synthesis. It is somewhat ironic however, that an important new innovative drug like paclitaxel did not come from a remote rainforest or the depths of the ocean, but instead from a tree that is an important element of urban parks and gardens (Appendino, 1995).

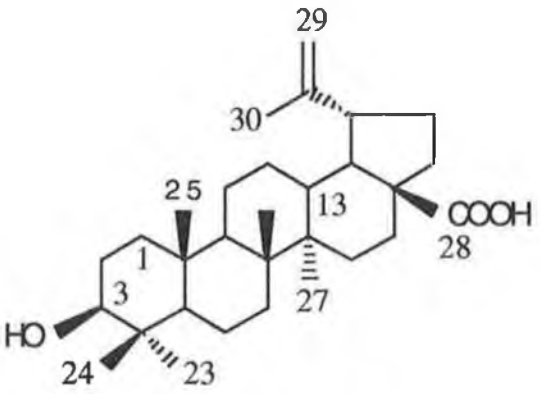
Structurally, Taxol is a very complex, heavily substituted diterpene molecule, having eleven assymmetric centres and thus a possible 2 048 diastereomers. Its total synthesis posed a formidable challenge, even to the most outstanding organic chemists. But in any event such a synthesis was achieved in 1994 by Holton and Nicolaou and in 1995 by Danishefsky (Phillipson, 1990a; Flores, 1992; Holton *et al.*, 1994; Wall and Wani, 1994; Masters *et al.*, 1995). Whether these synthesis procedures will prove to be commercially viable processes or not, remains to be seen.



Taxol

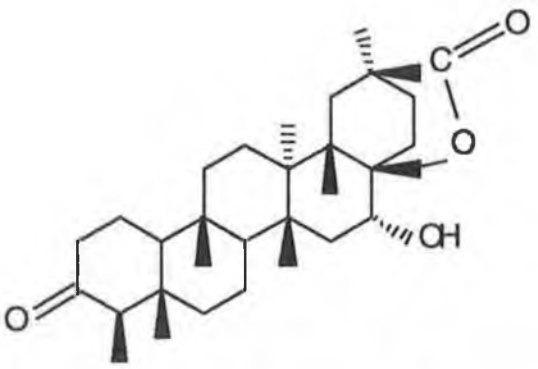
Another terpene showing considerable promise in the treatment of melanomas is betulinic acid. This compound which can be isolated from Birch bark destroys melanoma cells, while leaving other cell types unharmed. The compound's pharmacological activity was realised during a massive screening programme funded by the US National Cancer Institute, which tested 2 500 plant extracts for toxicity to laboratory cultures of human cancer cells.

The research work undertaken by Pezzuto at the University of Illinois indicates that the triterpene triggers apoptosis (programmed cell death) in cultured melanoma cells but has no affect on any other type of cells tested. The compound also shrank human melanomas induced in mice and the treatment produced no apparent side effects. Bristol-Myers Squibb, a leading pharmaceutical company has this triterpenoid acid under evaluation and if it proves successful in clinical trials, it will provide the medical profession with a much needed drug to combat this form of cancer (*Anon.*, 1995). Large amounts of this compound are naturally occurring and thus readily available for medical research and application.



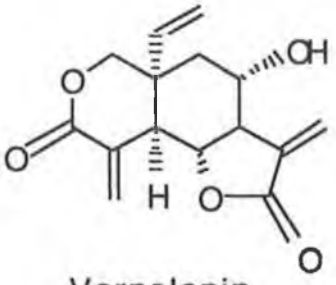
Betulinic acid

Four new pentacyclic triterpene molecules were recently isolated from *Celastrus hindsii* and one of them a cytotoxin, named maytenfolone-A, possesses antitumor properties.



Maytenfolone-A

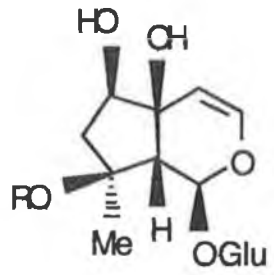
Biological evaluation of this compound has shown that it possesses demonstrable cytotoxicity against hepatoma (HEPA-2B) and nasopharynx carcinoma (KB) (Kuo and Yang Kuo, 1997). Vernolepin isolated from the *Vernonia* species also possesses antitumour activity.



Vernolepin

Anti-inflammatory and anti-ulcerative agents

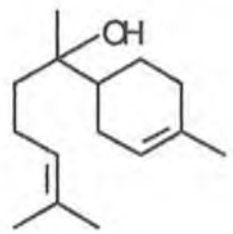
The herb 'devil's claw' (*Harpagophytum procumbens*) has a wide reputation for the treatment of rheumatoid disease. One of its iridoids, harpagoside, possesses anti-inflammatory activity. Iridoids are monoterpenoid cyclopentanoid lactone congeners that occur in plants both in the free state and the glycosidic form. Many of these iridoid compounds are toxic to animals and insects. The first examples of this compound-class were isolated from the sub-tropical *Iridomyrmex* genus of ants. In fact the class takes its name from the simplest compound in it, iridodial, which was first isolated from the common meat ant *Iridomyrmex detectus* by Cavill in 1956 (Bate-Smith and Swain, 1966).



Harpagoside

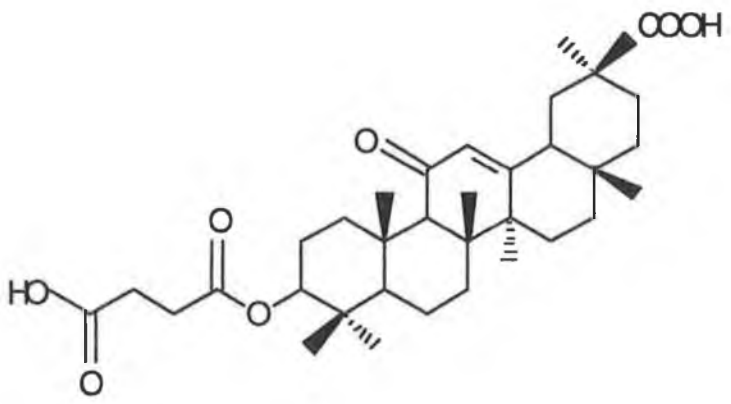
R = trans-cinnamoyl

The sesquiterpene bisabolol, from *Chamomilla recutita,* also possesses anti-inflammatory properties.



Bisabolol

The active ingredients of *Commiphora mukul* extracts are reported to be triterpenes and have been used in Ayurvedic (traditional Indian) medicine for the treatment of arthritis and gout (uric acid retention). One of these compounds, the pentacyclic triterpene carbenoxollone, was discovered to possess anti-inflammatory and anti-ulcerative activity in 1968 (Taylor and Kennewell, 1981). Carbenoxolone sodium, the water-soluble disodium salt of the compound, was the market leader in the treatment of gastric ulcers during the 1960's.



Carbenoxolone

More complex diterpenes, the gingkolides are found in *Gingko biloba.* Some of them are capable of inhibiting the binding of 'platelet aggregation factor' to platelets and leucocytes and so possess potential as drugs for the treatment of inflammation, allergies and asthma.

Glycyrrhizin isolated from the root of *Glycyrrhiza glabra* (the liquorice plant), and previously discussed in the context of its antiviral properties, also possesses important pharmacological activity in relation to the gastro-intestinal tract. A phytochemical investigation of the roots and rhizomes of *G. glabra* showed that the active factor responsible for curing peptic ulcers was in fact, glycyrrhizin. Gibson in 1978 reported

that while the action of the compound might in fact be due to its anti-inflammatory activity, other factors such as the inhibition of gastric secretion, promotion of mucus formation, activation of gastric membrane protective factors and activation of natural body protection mechanisms are also important.

On the other hand, contemporary medical research has shown that the bacterium *Heliobacter pylori* colonizes the human stomach causing an inflammation termed type B gastritis. This micro-organism is strongly associated with peptic ulcer formation and it is also linked with the occurence of gastric cancer (Macpherson, 1995). Infections due to this bacterium can persist for years unless treated and it is quite conceivable that glycyrrhizin's mode of action is in fact antibacterial in nature. As a result of its anti-inflammatory properties in conjunction with those of glycyrrhetinic acid, glycyrrhizin has been exploited in the preparation of skin cosmetics and in the treatment of dermatoses and pruritis. Interestingly, the compound is used in Egypt as a substitute for the anti-inflammatory steroid, cortisone.

Cardiovascular agents

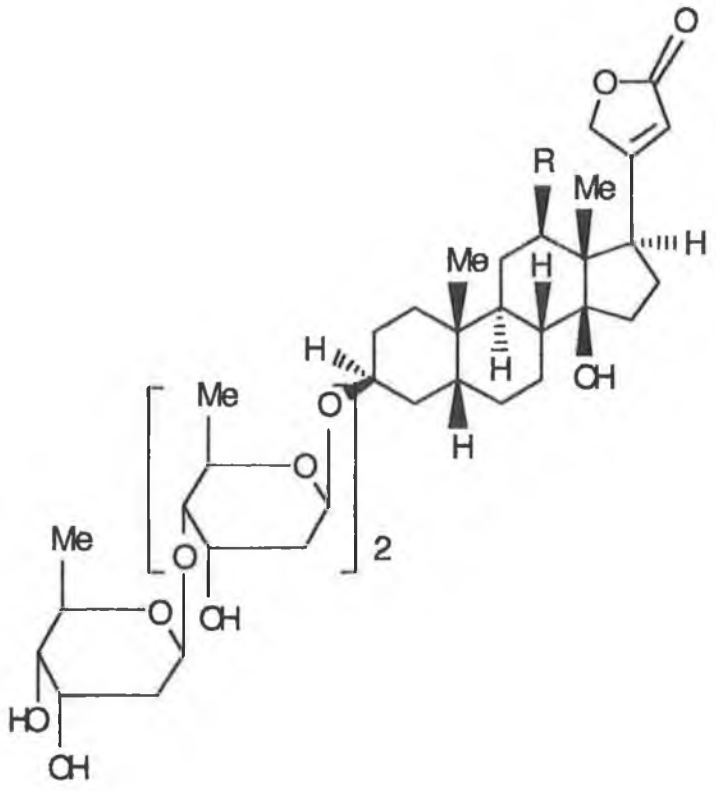
Foxglove was well known to the ancient Egyptians, while later on the Romans employed it as a diuretic, heart tonic, emetic, and rat poison. *Digitalis* was being written about in the year 1250 by Welsh physicians, who appear to have been administering it externally. It was a traditional English folk remedy for dropsy and Parkinson recommended its application in 1640. In fact its use by an old woman in Shropshire and a carpenter in Oxford led the English physician Withering to undertake his scientific study of it from 1775 to 1785 (Robbers *et al.*, 1996c). But despite his advocacy regarding its use, its application in the treatment of dropsy lapsed within the following century. By contrast with its neglect in dropsy, its effect on the heart beat gained emphasis. Bouillaud lauded it in 1835 as 'le veritable opium du coeur' and administered it by blistering the precordium and covering the area with powdered digit-

alis.

By 1873 Roberts had discovered that digitalis relieved the pulmonary symptoms of mitral disease. It was the work of Mackenzie, a general medical practitioner in Lancashire that gave digitalis its established position in the treatment of heart failure, especially in the treatment of that form of arrhythmia shown to be atrial fibrilation. He recognised what Withering had not .....that the relief of dropsy was directly due to the action of the heart. A cardiac glycoside was first isolated in 1875 when digitoxin was prepared from *D. purpurea* .

Digitalis is therefore, a classic example of the ethnobotanical approach to drug discovery. It is today, a much used traditional medicine of great importance. In recent decades, in addition to *Digitalis* many other valuable medicines have made the transition from the realm of traditional medicines to the registries of approved pharmaceuticals of even the most scientifically and legally fastidious nations (Schmidt, 1990; Hollman, 1992).

Plants in general are an important source of other cardiovascular drugs. In many instances the naturally occurring drug can be used without chemical modification as is the case with digoxin. The spirostanol saponins, to which digoxin and digitoxin belong are a family of terpenoids which have given rise to an industry of their own as a result of their extensive use as oral contraceptives, sex hormones and anti-inflammatory corticosteroids. For example digoxin, which is commercially isolated from *Digitalis lanata* and *Digitalis purpurea* (Foxglove) as previously discussed, is commonly prescribed in the treatment of congestive heart failure. Because digoxin is preferred clinically to digitoxin, it has resulted in the latter becoming a waste product. However, digitoxin is not discarded, instead it has been stockpiled and is used as a target molecule for conversion to digoxin.



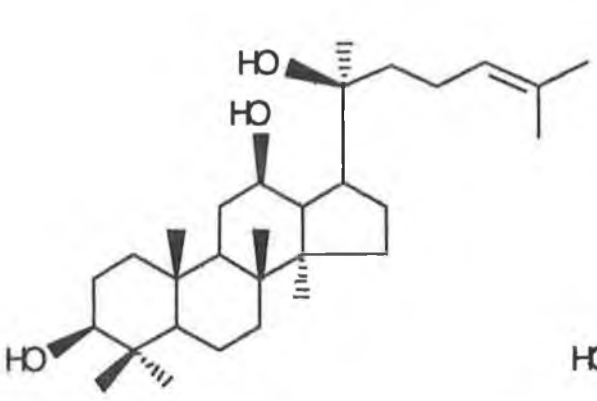
R = OH, Digoxin

R = H, Digitoxin

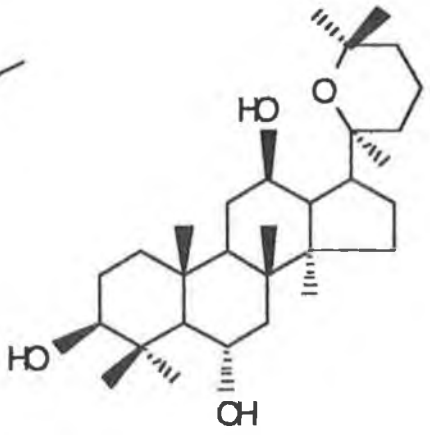
General *panacea*

Ginseng prepared from the roots of *Panax ginseng* has been used as a drug in oriental countries for more than 5 000 years. Traditional Chinese medicine recommends ginseng for increasing mental efficiency, recovering physical balance, stimulating metabolic function, treating anaemia, diabetes, gastritis and sexual impotence, as well as many other conditions arising from the onset of old age. The drug is also official in the former USSR, and in the West, too, and it has in recent years become an extremely popular remedy. *P. ginseng* roots have been thoroughly studied by modern methods of chemical analysis and, while many compounds have been isolated, the medicinal activity of the plant appears to reside in a number of saponins termed ginsenosides by Japanese workers and panaxosides by Russian workers. The saponins belong to the dammarane family of triterpenoids, protopanaxadiol and panaxatriol being examples of

two of their aglycones (Trease and Evans, 1983c; Hill, 1993b).



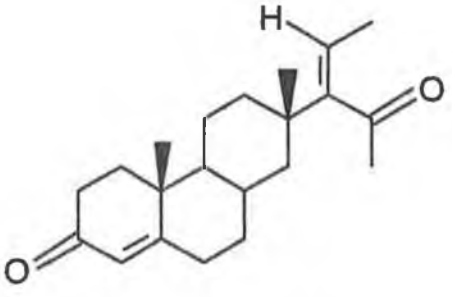
Protopanaxadiol



Panaxatriol

Hypolipidaemic agent

Z-guggulsterone, one of the triterpenoids from *Commiphora mukul*, is used in India as a hypolipidaemic. Its mode of action is attributable to its ability to inhibit cholesterol biosynthesis and to prevent platelet aggregation (Phillipson, 1990b). Other triterpene constituents from this plant have been used in Ayurvedic medicine for the treatment of arthritis and gout.



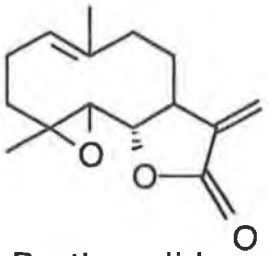
Z-Guggulsterone

Anti-migraine agent

Migraine affects approximately 15% of the adult population. It is characterised by a

severe headache, nausea and vomiting. Occasionally, the condition is preceded by neurological symptoms, especially visual disturbances such as blurred vision, blind spots and sensitivity to light. Feverfew (*Tanacetum parthenium*), which takes its name from its tonic and fever-dispelling properties, is used in folk medicine for the treatment of fever, headache and arthritis. The leaves of this plant were infused as a tea or ingested whole in a sandwich. Dr. Stewart Johnson, a pharmacologist at King's College, London carried out a survey amongst the users of the plant and found that while it was capable of producing unpleasant side effects, it successfully relieved the migraine symptoms in 43% of its users. In recent years, following extensive studies carried out by Johnson, which involved extraction of the plant and isolation of its active ingredients, it has been demonstrated to be prophylactic in the treatment of migraine.

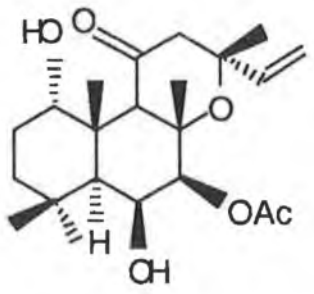
The mode of action of the active compound(s) is believed to be vasodilatory. The reason for this belief lies in the aetiology of the disease, which proceeds as follows; at the beginning of a 'migraine attack' the blood vessels in the head and brain constrict, and during the course of the headache they dilate. It is believed that the constriction is induced by endogenous substances from neuron endings or blood platelets, probably prostaglandins. The feverfew compound(s) isolated by Johnson may decrease the activity of blood vessels in response to these endogenous substances (*Anon.*, 1983). One of the plant's sesquiterpene constituents, parthenolide, is implicated as an active ingredient.



Parthenolide

Antihypertensive agent

A diterpene named coleonol from *Coleus forskohlii* is cardiotonic and antihypertensive, it has also been involved in clinical trials for the treatment of glaucoma.



Coleonol

Terpenoids in Chemical Ecology

'Chemical ecology' may be defined as that part of the science of ecology that considers the various chemical interactions that occur between plants and other plants, plants and animals, plants and micro-organisms, plants and insects, etc., and in more general terms it may be regarded as the study of the chemical interactions of plants within their ecosystem. In recent times, difficult ecological problems have had great scientific and social impact, due to a heightened environmental awareness amongst people, globally. These ecological problems relate to air, water and soil pollution and have had a major influence on the development of the relatively new discipline of chemical ecology. As individuals grow more reluctant to contaminate the environment with synthetic substances of broad and lasting effect, the impact of 'ecological awareness' on industry, agriculture and forestry is assuming a greater significance (Sondheimer and Simeone, 1970).

Plant terpenoids have effectively dominated the subject of chemical ecology and terpenoid molecules have been implicated in almost every possible interaction between plant and animal, plant and plant or plant and micro-organism. They act as insect anti-

feedants, defence agents, pheromones and allelochemicals. Some are highly toxic to the animal system, while others possess the ability to interfere hormonally with insect metamorphosis, animal growth and reproduction.

The majority of higher plants synthesise secondary metabolites and this fact may be regarded as one of their characteristic features, the major functions of which, are;

1. defence against herbivores and insects,
2. defence against micro-organisms and
3. metabolic or ecological functions such as UV-protection, attraction of insects and animals for pollination purposes.

The diverse functions of the terpenoids also include regulation of plant growth, accessory pigment activity in photosynthesis and attractant activity for the purposes of pollination. The terpenoids also provide effective defence against herbivory, and are by far the largest single class of natural products found in all living organisms. Another abundant source of terpenoids, many of which are toxic, are the marine algae in which they seem to play a protective role in that they prevent overgrazing of seaweeds by fish and molluscs. The marine terpenoids also play an important part in chemotaxonomy, as do their terrestrial counterparts. The ecological roles played by various natural products are considered next, under the following headings;

1. Plant-plant interactions,
2. Plant-insect,
3. Plant-mollusc,
4. Plant-animal and
5. Plant-fungi interactions.

Plant-plant interactions

Plants do not grow haphazardly; they are arranged in very definite associations and communities. The associations are mostly expressed as differences in life forms such as trees, shrubs and herbs; the communities are based on differences in species. Such groupings of species can be highly differentiated. It is difficult to envisage this differentiation in terms of physical factors such as light, temperature or humidity alone. For example if different degrees of shading were involved, a whole succession of communities should be found around a lone tree in a field, which is not the case. Therefore, we are more or less forced to assume that the fine differentiations in plant occurence must have an organic chemical basis, and research during recent decades partially substantiates this conclusion (Went, 1970a). Some instances of the chemical effects of plants are accepted as sufficiently established when two points are demonstrated:

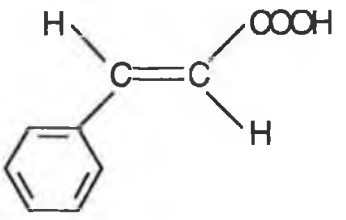
(1) that an effective inhibitory chemical is being produced and occurs at a potentially effective concentration in the soil, and
(2) that the inhibition is not an effect of plant competition for light, water and nutrients, nor one due to animal activities.

In cases where the effects are proven to be chemical, the phenomenon is referred to scientifically as allelopathy and the chemicals responsible for them are termed allelopathic substances. Allelopathic substances are released from a higher plant (directly or by way of decay processes) and they inhibit the germination, growth or occurrence of other plants in the vicinity of the plant releasing the substances. For example the terpenes; cineole and camphor are allelopathic compounds found emanating from the low shrubland plants that form the soft chaparral found in Southern California. Such volatiles, as a result of photochemical transformation, can give rise to blue hazes or smogs. These substances may be traced from the leaves by heat volatilis-

ation into the atmosphere surrounding the shrubs and to the soil where they are adsorbed onto soil particles. Experimentation has shown that terpenes such as camphor and cineole have a marked inhibitory effect on germination and seedling growth and on the life-cycles of certain soil bacteria (Whittaker, 1970).

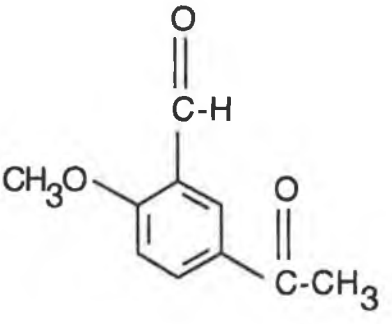
In 1966 Muller claimed that bare areas around aromatic shrubs like *Salvia mellifera* were caused by their own volatile emanations. In fact the quantities of volatile organic materials formed by plants is truly enormous. As mentioned volatilized terpenes can be activated photochemically, and this probably occurs through the production of free radicals. Thus polymerization of the terpenes can occur in the atmosphere leading to the formation of the blue hazes previously spoken of, which in turn result in a change in the radiation environment of the plants (Went, 1970b).

There are numerous examples of instances where plants excrete substances which cause inhibition of growth of other plants in their neighbourhood. For example *Parthenium argentatum* or guayule is a Mexican composite containing rubber, which in its natural habitat is very evenly spaced in the desert with considerable distances between adjacent shrubs. Laboratory experimentation performed in the 1940's by Bonner and Galston confirmed the existence of a substance produced by the plants roots, which inhibits the growth of other flora, including the young *Parthenium* plants themselves. Chemical isolation of the most active of the root secretions yielded *trans*-cinnamic acid as the main inhibitor. Thus, there is good scientific evidence to suggest that by secreting this acid the shrubs inhibit the growth of other plants in their environment, at least as far as their roots can reach.



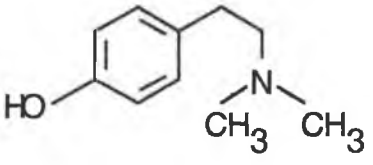
trans-Cinnamic Acid

In a similar manner *Encelia farinosa* inhibits the germination of other plants in its immediate surroundings due in part to the production of 3-acetyl-6-methoxybenzaldehyde, which was isolated from its leaves (Gray and Bonner, 1948).

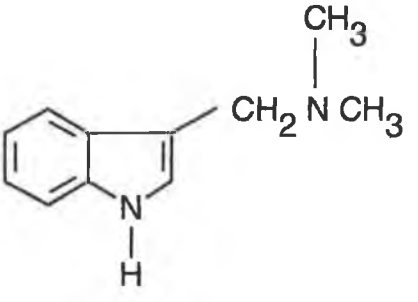


3-Acetyl-6-Methoxybenzaldehyde

Hordeum sativum (barley) is a so-called 'smother crop' since it inhibits the growth of weeds in fields, especially if it has been sown thickly. In 1966 it was reported that the plant's roots secrete inhibitors which were tentatively identified as hordenine and gramine, two alkaloids (Went, 1970c).



Hordenine



Gramine

The interesting aspect of this study is that wild barley, *Hordeum spontaneum*, which is closely related to *H. sativum* grows in the wild state in almost pure stands, indicating that even there, it inhibits the growth of other plants. There are many more examples of plant species inhibiting other plants growing close to them in their habitat and it is generally believed that this phenomenon is attributable to inhibitory substances excreted by the roots or given off by aerial parts of the plant. The inhibitors are found primarily in shrubs and plants from dry climates and while these compounds inhibit the growth of a number of other plants, the strongest inhibition they produced is in fact against their own seedlings.

All plants, whether algae, liverworts or higher plants produce measurable quantities of terpenoids. Went indicates that while no beneficial physiological or metabolic role has been ascribed to the majority of these compounds, their ubiquity makes it unacceptable to regard them as mere waste products or metabolic errors (Went, 1970b).

Plant-insect interactions

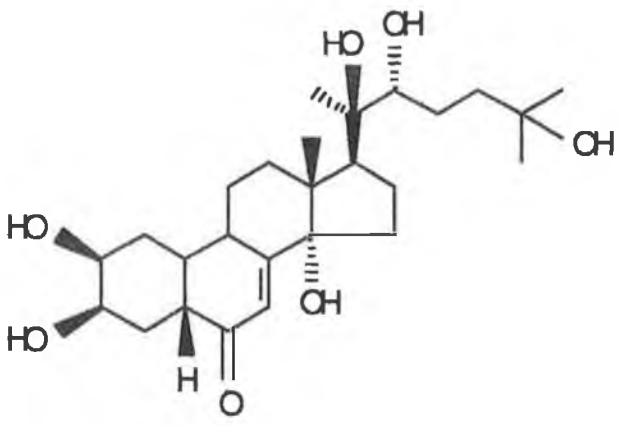
In 1987 Harmatha reported a spirastan-type saponin in the flowers of *Allium porrum* (the leek) (Harmatha *et al.*, 1987). The compound protects the plant from being fed upon by the leek moth larva. Saponins have also been shown to protect young holly leaves from insect attack. The oligophagus southern red mite feeds on *Ilex opaca* (American holly) leaves, only after they have expanded and their structural defences have become fully developed.

The young leaves, although rich in nutrients, are generally avoided by the mite and this appears to be due to the high saponin levels found in them. Such leaves are also avoided by insects like the 'fall webworm' and the 'eastern tent caterpillar'. However, this situation changes rapidily as the leaves mature, and the saponin concentrations drop to a relatively low level. These low levels are then maintained throughout the rest of the

plant's life-cycle.

Terpenoid insecticides

An interesting discovery made in the field of terpenoid phytochemistry in the 1960's was the identification of the ecdysteroid insect moulting hormone 20-hydroxyecdysone. This compound is present in very low concentrations in insects and is responsible for causing moulting or the shedding of the insect's outer cuticle.

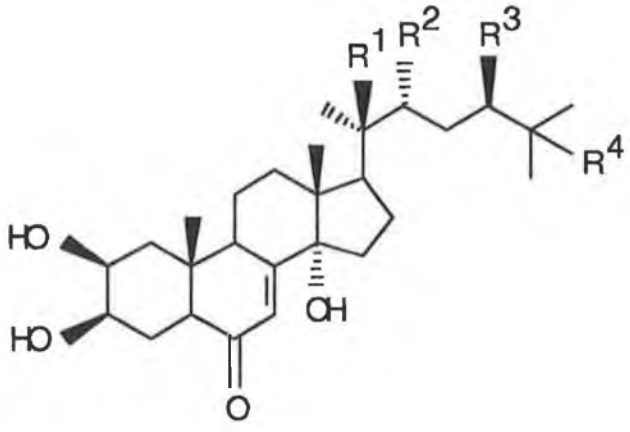


20-Hydroxyecdysone

Butenandt and Karlson in 1954, reported the isolation of 25 mg of pure ecdysone from 500 kg of silkworm (*Bombyx mori)* pupae (Butenandt and Karlson, 1954). Shortly after ecdysone's structure had been determined from the silkworm extract it was discovered in plant tissues; in *Podocarpus elatus* (the Australian conifer) and, in *Polypodium vulgare* (the fern) in high concentrations. There is also evidence that such phytoecdysteroids play an antifeedancy role in plants in order to protect them from the ravages of phytophagous insects. A total of 102 phytoecdysteroids are now known and their natural sources have been identified (Camps, 1991).

These componds include those isolated from the various species of yew (*Taxus*). As well as the widespread sterols; β-sitosterol, stigmasterol and campesterol, the yew tree

also contains ecdysones and as a result the tree is rarely attacked by insect pests. The insect moulting activity of yew extracts was recognised in the mid-sixties, and several ecdysteroids were isolated from the needles of various *Taxus* species among those compounds isolated were; β-ecdysone (a), ponasterone A (b) and makisterone A (c). The concentrations of these phytoecdysones in yew needles is relatively high. It has been calculated that 25 g of dried needles of European yew (*Taxus baccata* L.) contains the same amount of β-ecdysone obtained from the extraction of half a ton of silkworms (Appendino, 1995).



(a) $R^1 = R^2 = R^4 = OH$; $R^3 = H$

(b) $R^1 = R^2 = OH$; $R^3 = R^4 = H$

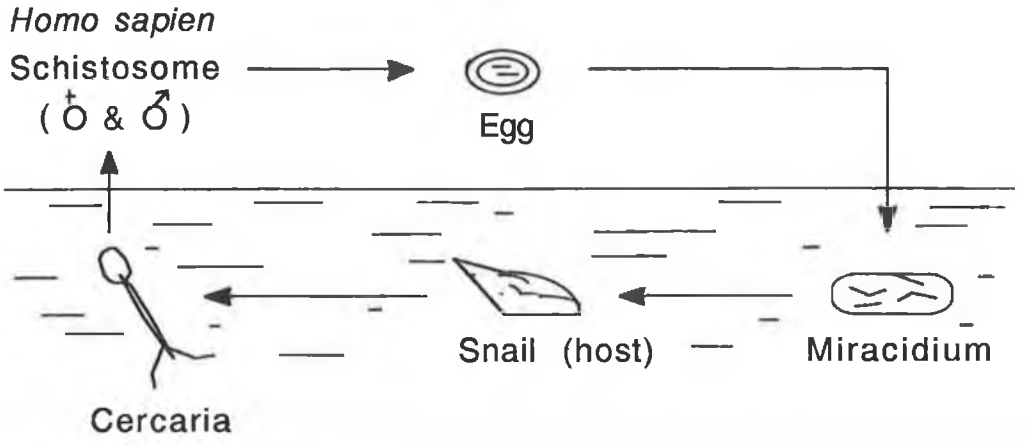
(c) $R^1 = R^2 = R^4 = OH$; $R^3 = Me$

Plant-mollusc interactions

Second only to malaria, schistosomiasis (bilharzia) is the most serious parasitic disease affecting the peoples of tropical and subtropical regions of the world. The World Health Organisation estimates that over 200 million cases of it occur worldwide, with half the deaths in Egypt being indirectly attributable to this disease. The disease is caused by small parasitic flatworms termed trematodes, flukes or schistosomes which live in the intestinal blood vessels of many mammals including man. They cause liver

cirrhosis, bladder cancer, dysentery, anaemia and diarrhoea.

The life-cycle of these schitosomes (see **Fig. 4**) is very similar to that of the common liver-fluke (*Fasciola hepatica*), found in Ireland. It involves an aquatic snail as an intermediate host. Incidence of infection is increasing as more dams and irregation systems are constructed in tropical and sub-tropical regions of the world, since these hydrological constructions inadvertently provide ideal breeding sites for the snail vectors that transmit the disease.



Life cycle of *Schistosoma* species

Fig. 4

Fluke eggs from the faeces and urine of infected persons enter water courses and produce miracidia, which in turn infect carrier-snails of the genus *Biomphalaria.* These then multiply into thousands of cercariae. The cercariae on leaving the host snail can penetrate the skin of humans, who come into contact with the water courses infected by the parasite. Once inside the skin they develop into mature trematodes or flukes. These trematodes are termed schistosomes, and belong to one of three main species; *Schistosoma haematobium*, *Schistosoma mansoni* and *Schistosoma japonicum*, which then mate and produce eggs. The eggs in turn are excreted in the faeces or urine of the

infected human and so the cycle continues. By destroying the intermediate snail host the life-cycle of the parasite is interrupted and so human infection is prevented.

There is a pressing need for some selective and efficient molluscicides for controlling these snails. Synthetic molluscicides such as sodium pentachlorophenate, dinitro-o-cyclohexyl phenol and copper sulphate have been used to control the snails but with uneven success. Furthermore, the costs of most of these synthetic agents is economically prohibitive for most 3rd World countries.

Terpenoid molluscicides

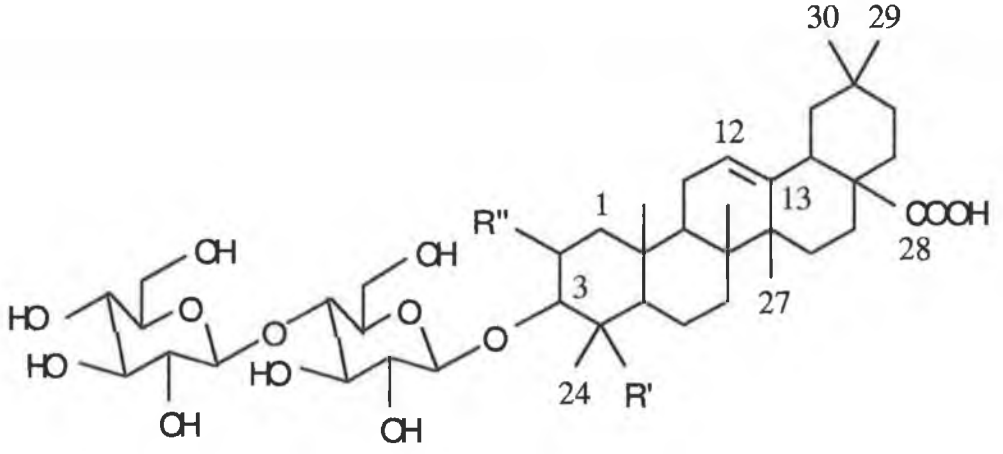
Phytolacca dodecandra (endod or soapberry) a member of the pokeweed family is native to Ethopia and other parts of Africa, its small berries when dried, powdered and placed in water produce a foaming detergent solution which is used in place of soap by indigenous peoples. Plant materials containing saponins (Latin *sapo*, soap) have long been used in many parts of the world for their detergent properties, in addition, saponins are toxic to fish, causing paralysis of the gills. They are also capable of haemolysing erythrocytes.

In Europe the root of *Saponaria offinicalis* was used, while in S. America the bark of *Quillaia saponaria* was extracted for this purpose. Lemma first observed the molluscidal properties of *P. dodecandra*; he noted that in places along streams and rivers where people washed clothes with the aid of the powdered berries, there appeared to be comparatively more dead snails than in adjacent areas, whether upstream or downstream of the wash-sites (Lemma, 1965). It was subsequently shown that a preparation of the plant possessed molluscicidal properties.

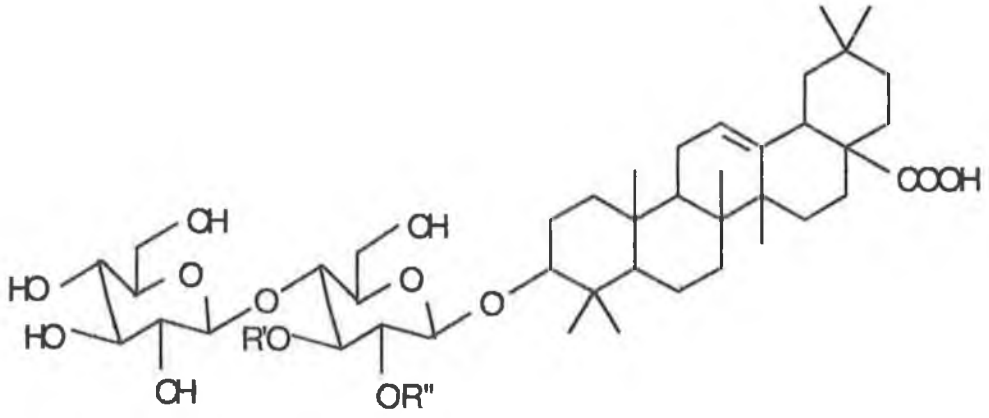
Since then investigations of the berries of this plant for novel molluscicides has been the subject of numerous scientific articles, books, international workshops and several

patents. As possibly the most studied and certainly the most promising plant molluscicide, Lemma reported that soapberry kills schistosomiasis-transmitting snails at low concentrations. It has been shown that this potency remains stable over a wide range of pH values, temperature changes and varying conditions of UV irradiation. Importantly, the molluscicidal activity of crude aqueous extracts of *P. dodecandra* berries approaches that of available synthetic molluscicides.

After much investigation, the monodesmosidic triterpene saponins of the plant were shown to be responsible for these effects. Dried soapberry fruits contain upto 25% saponins and the structures of some of those with molluscicidal activity have been determined and are shown below. The first structure is a general representation of a molluscicide molecule, it consists of a Δ^{12} oleanene series triterpene with a disaccharide sugar moiety attached at the C-3 position by means of an ether-linkage. The sugar moiety renders the normally hydrophilic triterpene water soluble.



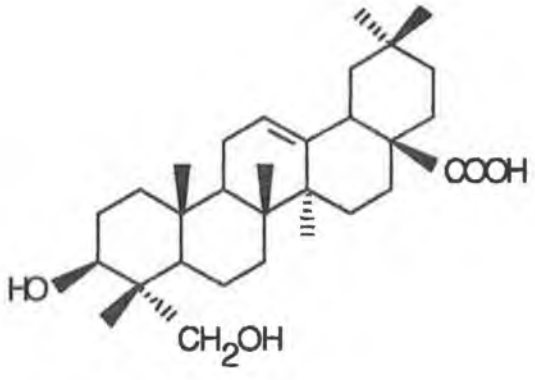
In general terms, active (molluscicidal) saponins possess an aglycone of the oleanane series with a sugar chain attached at position C-3 and a free carboxylic acid group at C-28. The carboxylic acid group is imperative for molluscicidal activity.



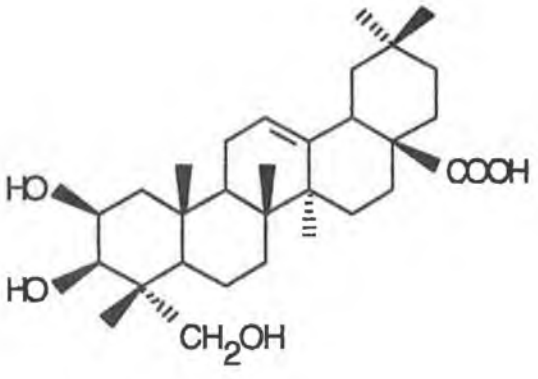
Oleanoglycotoxin-A; R' = H, R" = Glc

Lemmatoxin; R' = Gal, R" = H

Other molluscicidal saponins isolated from *P. dodecandra* possess as their aglycones hedragenin and bayogenin.



Hederagenin



Bayogenin

The chemistry of *P. dodecandra* was extensively studied during the 1980's by Hostettmann and Marston (Hostettmann and Marston, 1985; Hostettmann and Marston, 1986; Marston and Hostettmenn, 1991). They introduced rapid and selective screening techniques for plant extracts. The dried berries were extracted using the classical method with solvents of increasing polarity as follows; petroleum spirit, $CHCl_3$,

MeOH and H_2O. The extracts were then subjected to 'rotation locular countercurrent chromatography', in the search for novel compounds. The net result of all this work was an improvement of the extraction procedure of *P. dodecandra*. Surprisingly, none of the extracts thus obtained showed any molluscicidal activity, whereas direct water extraction yielded a very potent solution.

Six bidesmosidic saponins were isolated from the MeOH extract. None showed any molluscicidal activity against the carrier-snails. However, the corresponding prosapogenins obtained by cleavage of the glucose unit esterified at position C-28 exhibited strong activity. Direct water extraction of the plant afforded the monodesmosidic saponins and only traces of the bidesmosidic saponins were detectable. Hence, the bidesmosidic saponins are easily hydrolysed during the aqueous extraction procedure. The hydrolysis process which is very probably occurring, involves the more labile ester-linked glycosides being cleaved, while the more stable ether-linked molecules remain intact.

These findings were interesting because they suggested that the addition of an alkali (normally performed with weak solutions of KOH (Domon and Hostettmann, 1984)) during the course of the H_2O extraction procedure could improve the degree to which hydrolysis of the sugar unit esterified at position C-28 of the triterpene aglycone occurs. Thus the overall net result would be an increase in the yield of active monodesmosidic saponins available to perform the molluscicidal function.

Saponins also possess haemolytic properties, and when injected into the blood stream, are highly toxic. However, when ingested orally saponins are comparatively harmless. Toxicological studies have demonstrated that the great advantage of soapberry is that its acute oral toxicity to mammals, birds and plants is very low. Thus, there is very little risk to flora and fauna resulting from the use of the plant in molluscicidal concentrations

in the field. On the other hand, soapberry extracts are lethal to fish, as are all the other currently available molluscicides whether of synthetic or natural origins.

Additional studies on *P. dodecandra* have revealed that its monodesmosidic saponins show remarkable spermicidal effects, which indicates that the compound may possess pharmacological potential also. Furthermore BuOH extracts of the plant are toxic to mosquitoes at very low concentrations indicating possible usage in the control of the vector responsible for the transmission of the world's most prevalent tropical disease, malaria. The concentration required for such insecticidal activity is less than that needed to kill snails and fish.

Since the discovery of the molluscicidal properties of soapberry in the 1960's and 1970's, naturally occurring molluscicides have recieved considerable attention and a number of active compounds have been isolated from other plant sources such as; *Talinum tenuissimum*, *Swartzia madagascariensis* and *Sesbania sesban*. Identification of the active constituents of plants such as these, is pharmacologically essential, for the purposes of toxicity, stability and dosage studies. This identification work is also important for structure-activity investigations.

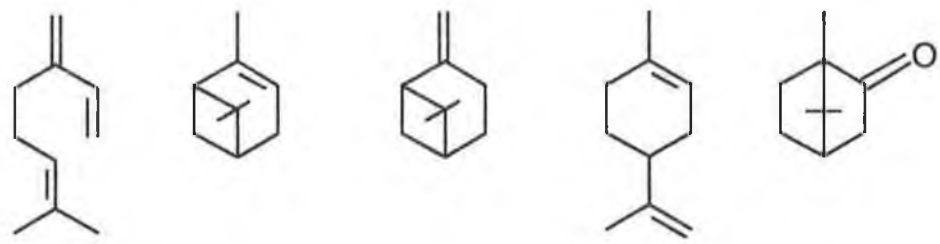
S. madagascariensis mentioned above, is a common tree found in many parts of Africa, in fact the tree is particularly abundant in East Africa where it flowers after the short rainy season. Since the 1930's reports indicate that its fruits possess molluscicidal activity. Its seed pods grow upto 30 cm in length and dry out between July and August. The plant is well known to the native population for its medicinal uses, as well as its use as a fish poison. Borel and Hostettmann reported the isolation and structural elucidation of saponins responsible for molluscicidal activity from an aqueous extract of the dried fruits of this plant (Borel and Hostettmann, 1987). These saponins were shown to be glucuronides of oleanolic acid and gypsogenin. All the saponins carried a

rhamnopyranosyl unit at position C-3 of the glucuronic acid moiety. One of the compounds a bidesmosidic saponin, carried an additional sugar moiety at position C-28 of the oleanolic acid aglycone. Therefore, unlike the others, this latter compound exhibited no molluscicidal activity.

Tekle showed that the dried, powdered leaves of the tree *S. sesban* were toxic to snails (Tekle, 1977). These leaves may be harvested at anytime of year without endangering the plant's existance. The tree is very fast growing and is widely distributed throughout Ethiopia and many other African countries. Following the reports on the toxicity of *S. sesban* leaves to snails, a phytochemical investigation revealed the presence of four oleanolic acid glycosides (Dorsaz *et al.*, 1988). The molluscicidal activity of the leaf extract was ascribed to the monodesmosidic saponin 3-O-[α-L-rhamnopyranosyl-(1->3)-β-D glucuronopyranosyl] oleanolic acid, which was previously isolated from *S. madagascariensis* (Borel and Hostettmann, 1987). Dorsaz reported the isolation of six glucuronides of oleanolic acid from *S. sesban* (Dorsaz *et al.*, 1988).

In general the molluscicidal activities of saponins vary with the nature of the saccharide chains, the sequencing of these sugars, the inter-glycosidic linkages involved and the substitution patterns of the aglycone. As water is the medium in which the plant molluscicide has to perform its snail-killing role, the water-solubility of this class of compound is an important feature in their favour.

Terpenoid antifeedants



Myrcene α-Pinene β-Pinene Limonene Camphor

(a) Pine Oil

This constitutes a mixture of monoterpenes including, α- and β-pinene, limonene, myrcene and several monoterpene alcohols. It is an effective feeding repellant to voles and snowshoe hares (*Lepus americanus*), since these mammals do not become habituated to the pine-odour, according to Bell and Harestad, who reported their findings in 1987 (Bell and Harestad, 1987).

(b) Camphor

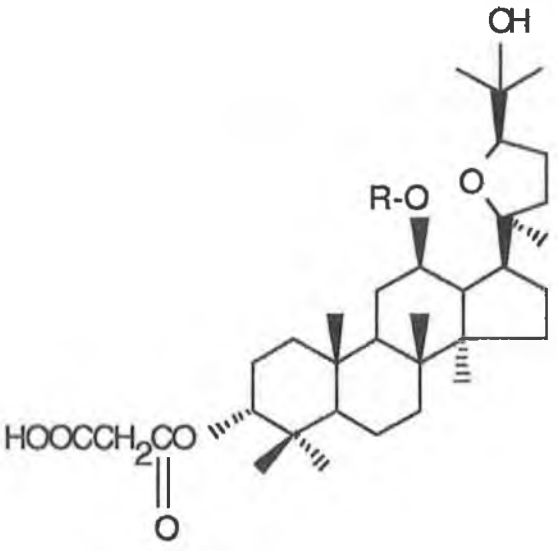
Detailed studies undertaken by Sinclair on the resistance of *Picea glauca* (white spruce) to browsing snowshoe hares, were reported in 1988 (Sinclair *et al.*, 1988). From these studies it is apparent that the monoterpene camphor is a specific antifeedant to this species of hare. Interestingly, camphor is only produced in sufficient quanities to protect the juvenile growth from herbivory, since the mature foliage is out of reach of the animal. This is evidenced by the fact that the camphor content of the juvenile twigs and leaves of the plant is four times that of the mature specimens.

(c) Papyriferic acid

In 1984, Reichardt reported on studies undertaken in relation to the defensive role

played by papyriferic acid in birch (*Betula papyrifera* subsp. *humilis* = *resinifera*), against winter-browsing by snowshoe hares (Reichardt *et al.*, 1984). The triterpenoid is present in enormous concentrations (up to 30% of the dry weight) in the winter-dormant juvenile twigs, thus serving to protect the tree only when it is at its most vulnerable to attack. Feeding experiments have shown that the triterpene is highly distasteful to the hares. The compound was fed to them in oatmeal at 2% of dry weight during these experiments (Harbone, 1993a).

In older trees the high concentrations of the triterpene drop off, because much of the winter-dormant tissue is out of reach of the herbivores. There is also further evidence to suggest that papyriferic acid is a feeding deterrent to other mammals such as moose and certain species of rodent. Feeding deterrency may be linked with mammalian toxicity, since papyriferic acid kills laboratory mice when administered at a relatively low doses.



Papyriferic acid; R= Ac

3-O-malonylbetulafolientriol oxide 1; R= H

Following the discovery that papyriferic acid played an important role in defending

Alaskan paper birch from browsing by vertebrate herbivores, Reichardt initiated a phytochemical investigation of other birches found in boreal regions (Reichardt *et al.*, 1987). In particular, attention was drawn to Alaskan populations of dwarf birch (*Betula nana* subsp. *exilis*) which like the paper birch had resinous deposits on the exterior of the bark of its current year twigs. Interestingly, these twigs also proved rather unpalatable to snowshoe hares.

A novel triterpene, structurally similar to papyriferic acid termed; 3-O-malonylbetulafolientriol oxide 1 was isolated. Although present in this species at rather high concentrations (*ca* 1% by weight), there is no scientific evidence as yet, for its contribution towards the unpalatability of the species to vertebrate herbivores. On the other hand, it has been argued that the snowshoe hare's use of dwarf birch in British Columbia is in fact governed by nitrogen concentrations (Reichardt *et al.*, 1987).

Plant-fungi interactions

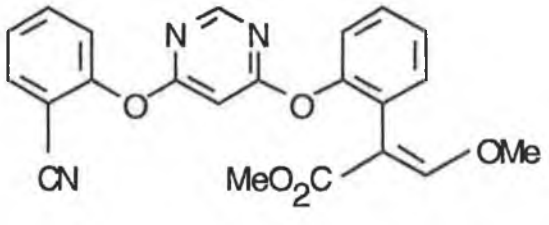
A major breakthrough in the study of chemically based, disease resistance mechanisms in higher plants came in the 1940's with the enunciation of the 'Phytoalexin Theory' by Muller and Borger, who were also responsible for coining the term 'phytoalexin' (Muller and Borger, 1940). This theory proposed that certain chemical substances were produced by plants *de novo*, at the time of infection and they came into action 'as compounds warding off (*alexos*) disease-causing organisms from the plant (*phytos*)' (hence the term phytoalexin). The theory was developed from experiments carried out by Muller and Borger on resistance factors in potato (*Solanum tuberosum*) to the fungal blight organism, *Phytophthora infestans*. In 1958, Muller redefined phytoalexins as low molecular weight antibiotics and extended the definition to include other plant-pathogen interactions.

However, Muller and Borger were unsuccessful in demonstrating the existence of a

discrete chemical substance in potato with the properties of a phytoalexin. It remained for Perrin and Bottomley some twenty years after the theory was postulated to be the first to isolate and identify a phytoalexin, namely the substance pisatin from infected pea (*Pisum sativum*) tissues (Perrin and Bottomley, 1962). Since then, a large number of phytoalexins have been described from a wide range of plants (Brooks and Watson, 1985), and phytoalexin induction is now widely accepted as a major protective device used by higher plants (Harbone, 1993b). The most widely used working definition of the term phytoalexin, today, is; 'Phytoalexins are low molecular weight, antimicrobial compounds that are both synthesised by and accumulated in plant cells after exposure to micro-organisms' (Paxton, 1981).

Phytoalexins / fungicides

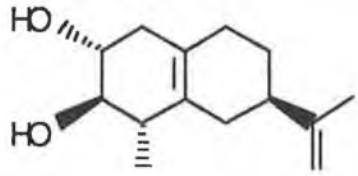
Recently, a natural product isolated from the edible woodland mushroom, *Oudemansiella mucida*, which grows on decaying wood, has been commerically exploited as a product named 'Amistar' by Zeneca, a world-leading pharmaceutical company. The new product is a novel fungicide belonging to a large family of natural products which incorporate the strobilurins, oudemansins and myxothiazols, all of which are derivatives of β-methoxyacrylic acid. The fungicide's active principle; azoxystrobin, unlike alternative commercial fungicides is effective at low rates of application and yet, is highly active against a broad spectrum of fungal diseases that attack food crops.



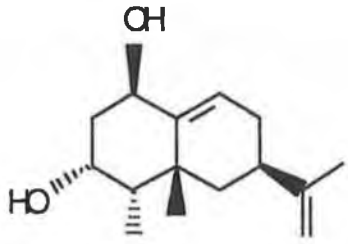
Azoxystrobin

It is intriguing if not indeed paradoxical from an ecological view point that fungi are able to biosynthesise fungicides, and moreover, it is not clear how they themselves survive the effects of these compounds. It seems likely however, that the ability to do so gives them an advantage as they compete for nutrients in their habitats. Azoxystrobin works by penetrating the cell wall of the fungus and attacking the mithochondria within the cell. The mitochondria are the organelles responsible for cellular respiration.

Thus, the compound effectively disrupts Kreb's Cycle (cellular respiration) in the mithochondria and so induce necrosis or cell death. This novel biochemical mode of action means that the compound is effective against fungi that have developed resistance to other fungicides. Most importantly, the new fungicide has a low acute oral toxicity. Commerically, the developers of this natural product expect to corner up to 50% of the global fungicide market from the date of launch of their new product (*Anon.*, 1997; Clough and Godfrey, 1995).



Rishitin

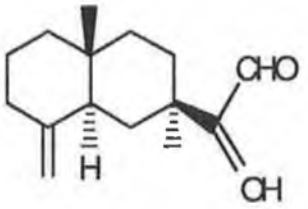


Capsidiol

Some plants, which do not normally synthesize fungicides, possess the latent ability to do so, when invaded by fungi. The phytoalexins previously mentioned are some examples of the type of compounds produced in response to such invasions. They are synthesised *de novo* in response to the fungal attack and are of several structural types. Research has indicated them to be terpenoid in nature, and the best studied group of

these terpenoid phytoalexins are the sesquiterpene alcohols, which include rishitin and capsidiol, shown above.

These compounds are produced extensively in red peppers, tobacco, potato and other species of Solanacea. The ability of plant cells to rapidily switch into phytoalexin production and temporarily switch off other pathways of terpenoid production is quite remarkable. 7-hydroxycostal is an example of a sesquiterpene, antifungal phytoalexin produced by the sweet potato (*Ipomoea batata*).



7-Hydroxycostal

RESULTS AND DISCUSSION

Introduction

The genus *Ilex* consists of over 400 species, which grow as trees or shrubs. They have alternate simple leaves, single or clustered small flowers and red, black or yellow berries. *Ilex aquifolium* which was one of the plant species investigated in this thesis is known by many common names, including Hulver bush, Holm, Holy tree, Common Holly and European Holly. *I. aquifolium* grows very slowly when planted among trees which are not more rapid in growth than itself. It possesses glossy leaves and clusters of scarlet berries. The common names of some of the better known species in the *Ilex* genus are; Paraguay tea, Jesuits' tea, Chimarrao, Yerba Mate and Mate (*Ilex paraguensis*), Black Alder Winterberry and Fever Bush (*Ilex verticillata*) (Grieve, 1992b). While *Ilex cornuta* , *Ilex opaca* and *Ilex crenata* are known as Chinese Holly, American Holly and Japanese Holly, respectively.

Ilex requires a reasonably wet, equable climate and while they enjoy a world-wide distribution, except for arid and arctic regions, their major centres of distribution are Central and South America, Asia and Europe. *I. aquifolium*, in particular, is native to most of the central and southern parts of the European continent. The *Ilex* genus is well documented in the fossil records, there being abundant fossil specimens relating to it from every geographic region from about 65 million years ago and a few from as far distant as 125 million years ago (Alkardis, 1987).

Ethnopharmacological uses of *Ilex* spp.

Ilex pubescens is a traditional Chinese medicinal plant used mainly for the treatment of coronary heart diseases. Phenolics and phenyl propanoids isolated from this plant have been shown to possess antipyretic, anti-inflammatory, analgesic, cardiovascular and circulatory activities. Flavonoid glycosides isolated from the bark are effective in the

treatment of hypertension, hyperlipidemia and hepatitis. *Ilex cornuta*, another traditional Chinese medicinal plant, had uses in the treatment of dizziness and hypertension. The leaves of this plant have been reported to contain triterpenoid glycosides with antithrombotic and anticholesteraemic action (Alikaridis, 1987).

Traditionally, *I. aquifolium* leaves have been used in the treatment of intermittent fevers because of their antipyretic properties, according to Grieve (Grieve, 1992c). They have also found use in the treatment of rheumatisms and are reported to possess astringent, diuretic and expectorant properties. Lewis and Elvin-Lewis indicate that *I. opaca* leaves possess pharmacological properties as a diuretic, tonic, purgative and cardiac stimulant (Lewis and Elvin-Lewis, 1997). A number of plants contain Cardioactive glycosides, and some of them have been employed for many years as cardiac stimulants and diuretics. Several are more potent than digitalis, but they are less reliable because their dosage cannot be properly controlled. Although most of these drugs (convallaria or lily-of-the valley root, apocynum or dog bane, adonis or pheasant's eye, olleander, strophanthus, squill and black hellebore) were recognised officially for years and were considered efficacious, they have been superseded by the digitalis glycosides. A few are currently under reinvestigation (Robbers *et al.*, 1996a). More recently, it was reported that a dried powder emulsion resulting from the leaves and the berries of *I. opaca* and *I. aquifolium* possesses the medicinal potency of digitalis.

In Amazonian Peru and Ecuador leaf decoctions of the rainforest holly *Ilex guayusa* which possess high caffeine concentrations are used as a morning stimulant. After daily ingestion, ritualistic vomiting by male Achuar Indians, better known as Jivaros, reduces excessive caffeine intake, so that blood levels of caffeine and biotransformed dimethylxanthines do not cause 'central nervous system' and other effects. Emesis is learned and apparently is not due to the presence of emetic compounds (Lewis *et al.*, 1991).

The beverage Mate or Paraguay tea is prepared virtually exclusively from the dried and cured leaves of *I. paraguensis* but may also contain other species of *Ilex*, small trees or shrubs indigenous to the region where Argentina, Paraguay and Brazil meet. The relatively high concentrations of caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine) in the leaves of *I. paraguensis* could be responsible for its traditional depurative, stimulant and diuretic actions. In addition to these compounds, the leaves of *I. paraguensis* contain the triterpenes α-amyrin, β-amyrin and ursolic acid as well as many other compounds (Duke, 1992; Alikaridis, 1987).

The records show that this beverage was known to the South American Indians prior to the invasion of the Spaniards, although, there is some academic debate surrounding this point. In any event, the plant was cultivated and promoted as a refreshing drink amongst the native Indians by Jesuit missionaries. It has been reported that Mate constitutes the primary source of methylxanthines in the diet of some groups in S. America (Alikaridis, 1987). Furthermore, it is said to be rich in vitamins and is used widely throughout S. America, while its consumption in Europe and the U.S.A. is increasing.

Mate has been shown to contain polyphenols, chlorogenic acids, caffeine and caffeic acid and is used in large doses as a laxative or purgative; it also has diaphoretic and diuretic properties (Robbers *et al.*, 1996b; Trease and Evans, 1983b; Gugliucci and Stahl, 1995). An interesting case-control study carried out at the Institute de Oncologia, Montevideo, Uruguay between the years 1988 and 1994 indicated that the risk of lung cancer in males is associated with Mate drinking. The study showed that there is a 1.6-fold increase in risk of incidence, for heavy drinkers compared to light drinkers of this beverage (Destefani *et al.*, 1996).

A similar caffeine-containing product is Cassina from the leaves of *Ilex cassine.* The mixed leaves of *Ilex cassine*, *Ilex vomitoria* and *Ilex dahoon* were used by the native peoples of N. America to prepare hot drinks (Yaupon or black drink) which possessed the ability to cause perspiration and vomiting and as a result, this beverage was used as part of ceremonial 'cleansing' rituals practiced by these tribes people (Alikaridis, 1987). *Ilex verticillata* is reputed to possess febrifuge properties. The following table lists the ethnopharmacological uses attributed to some of the other *Ilex* spp.;

Species	Medicinal Use
Ilex asprolla	Boils, abscesses, bruising, febrifuge, laryngitis, tonsillitis, wounds, decoagulant, etc.
Ilex guayusa	Amenorrhea, diuretic, stimulant, narcotic, emetic, diaphoretic, expectorant, etc.
Ilex vomitoria	Depurative, aperient, astringent, laxative, tonic, vermifuge, etc.
Ilex medica	Diuretic and stomachic.
Ilex pedunculosa	Haemorrides, dermal ailments, tonic etc.
Ilex verticillata	Antiseptic, astrintgent, laxative, vermifuge, etc.

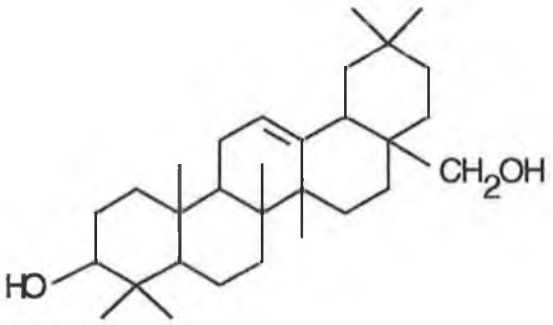
Non-medicinal uses of *Ilex* spp.

The uses of this genus stretches back over many centuries. It was used in festive rituals to both pagan and Christian Gods. Today, hollies are commercially grown and are used for decorative purposes as a result of their pleasing appearances. European and American hollies provide white timbers that are widely used in joinery and fine carpentry work and are sometimes dyed to imitate ebony. In addition 'bird lime', a glue-like substance is produced from the bark of some European and Japanese species, and is used to trap birds for the 'pet market', furthermore the substance is exported to warmer climates where it is used to control the 'pest element' associated with insects

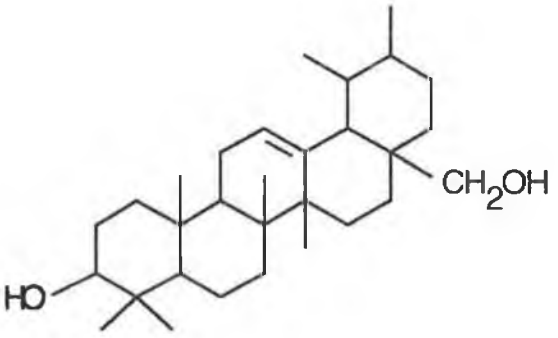
(Alikaridis, 1987). A more modern method of prepairing 'bird lime' involves boiling linseed oil until it becomes stringy to which the addition of a non-drying oil, such as castor oil or liquid paraffin is made (Mac Ewan, 1946). The occurrence of rubber in the bark of *Ilex* spp. has also been reported, while other reports have referred to the possibility of obtaining dyes from certain holly species (Alikaridis, 1987).

Chemical constituents of the *Ilex* genus

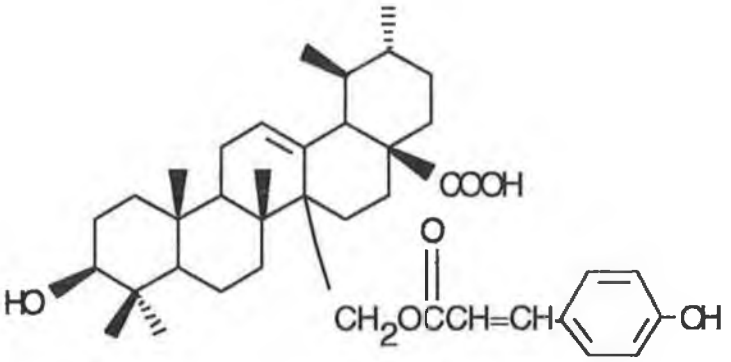
The genus *Ilex* contains many classes of chemical constituents such as; phenols and phenolic acids, phenyl propanoids, anthocyanins, flavonols and flavones, terpenoids, sterols, purine alkaloids, amino acids, fatty acids, miscellaneous nitrogen compounds, alkanes and alcohols, carbohydrates, vitamins and carotenoids (Mac Ewan, 1946). Personne in 1884 was the first to isolate a triterpene from *I. aquifolium*, which was named ilicic alcohol (Personne, 1884), and was later identified as α-amyrin in 1908 by Jungfleisch and Leroux (Jungfleisch and Leroux, 1908). Twelve years later, Nooyen reported the occurence of ursolic acid in *I. aquifolium* (Nooyen, 1920) and in 1930 Fischer and Linser confirmed its presence (Fischer and Linser, 1930). In 1978, Catalano isolated a large number of triterpenes from the leaves of this species, including; uvaol, ursolic acid, oleanolic acid, α- and β- amyrin, erythrodiol and baurenol (Catalano *et al.*, 1978). Thomas and Budzikiewicz in 1980 isolated α-amyrin, uvaol, ursolic acid, 27-*p*-coumaroxy ursolic acid and a bisnormonoterpene named Ilex lactone from the plant's fruits (Thomas and Budzikiewicz, 1980a,b).



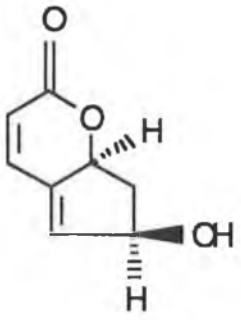
Erythrodiol



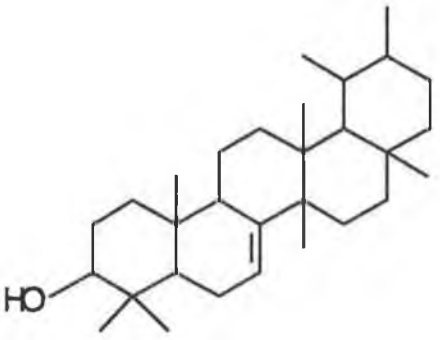
Uvaol



27-*p*-Coumaroxyursolic acid



Ilex lactone



Baurenol

Recently, Willems reported the presence of a toxin; a novel cyanogenic glucoside **Fig. 5**, in the ripe fruits, leaves and stem of *I. aquifolium*. This species is of course well

known for its toxic properties and children are regularly poisoned following ingestion of its fruits (Willems, 1988). *I. aquifolium*, one of the plant species investigated in this thesis, possesses α-amyrin derivatives in its bark but not free α-amyrin.

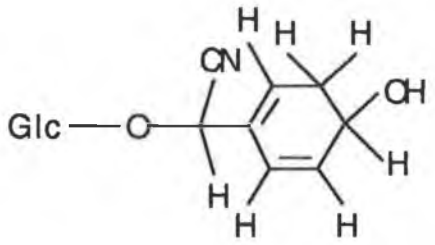
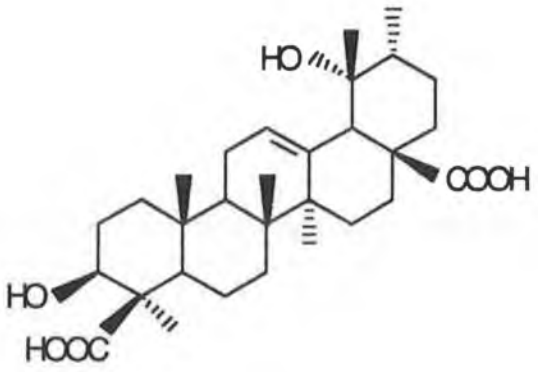


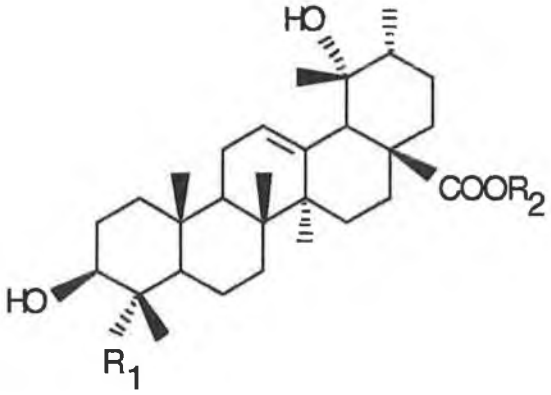
Fig. 5

The roots of *I. pubescens* contains the pentacyclic triterpene Ilexgenin A and its glycoside Ilexsaponin A1 (Hidaka *et al.*, 1987a,b).



Ilexgenin A

I. rotunda is reported to contain the triterpene rotundic acid **Fig. 6** in its seeds and winter fruits and the ester glycoside of this compound, peduncloside **Fig. 7** in its leaves and winter fruits. These fruits also contain rotundioic acid **Fig. 8** and rotungenic acid **Fig. 9** (Nakatani *et al.*, 1989).



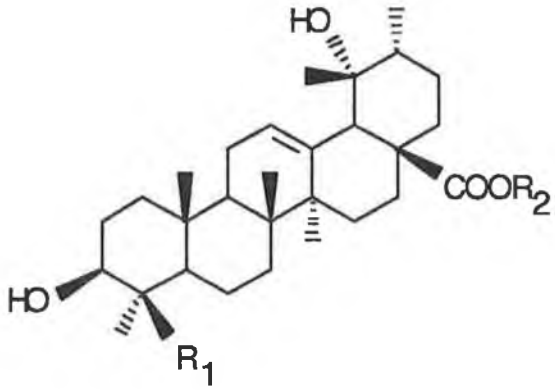


Fig.	R_1	R_2	Fig.	R_1	R_2
6	CH_2OH	H	**9**	CH_2OH	H
7	CH_2OH	Glu			
8	COOH	H			

The leaves of *I. latifolia* were shown to possess five triterpenoid saponins, latifolosides A-E, in addition to the triterpene ursolic acid, which also occurs in the fruits (Ouyang *et al.*, 1997; Alikaridis, 1987). The triterpene betulin, found in many plant families was isolated from the bark, fruit and leaves of *I. verticillata* (Duke, 1992). Phytochemical investigation of *I. cornuta* leaves by Gau resulted in the isolation and identification of the triterpenes lupeol and oleanolic acid (Gau *et al.*, 1983). The chemical constituents of the genus arranged by class, as well as the plants which contain them, are shown in the following tables;

Table 3
ANTHOCYANINS

Compound	*Ilex* species	Plant part	References
Cyanidin-3-xylosylglucoside	*I. amelanchier*	Fruit	(Santamour, 1973)
	I. aquifolium	Fruit	(Ishikura, 1971a,c)
	I. buergeri	Fruit	(Ishikura, 1975)

Table 3 contd.

Compound	*Ilex* species	Plant part	References
	I. cassine	Fruit	(Santamour, 1973)
	I. chinensis	Fruit	(Ishikura, 1971c; Santamour, 1973)
	I. coriacea	Fruit	(Santamour, 1973)
	I. crenata	Fruit	(Hayashi, 1942; Ishikura, 1971c; Santamour,1973; Ishikura and Sugahara, 1979)
	I.decidua	Fruit	(Santamour, 1973)
	I. geniculata	Fruit	(Ishikura, 1975; Ishikura and Sugahara, 1979)
	I. georgei	Fruit	(Santamour, 1973)
	I. glabra	Fruit	(Santamour, 1973)
	I. integra	Fruit	(Ishikura, 1971b,c)
	I. kiusiana	Fruit	(Ishikura, 1975)
	I. latifolia	Fruit	(Ishikura, 1971b,c)
	I. makropoda	Fruit	(Ishikura, 1975)
	I. mitis	Fruit	(Santamour, 1973)
	I. myrtifolia	Fruit	(Santamour, 1973)
	I. nipponica	Fruit	(Ishikura, 1975)
	I. opaca	Fruit	(Santamour,1973)
	I. paraguariensis	Fruit	(Santamour,1973)
	I. pedunculosa	Fruit	(Ishikura, 1971c; Santamour, 1973)
	I. rutunda	Fruit	(Ishikura, 1971c)
	I. serrata	Fruit	(Ishikura, 1971b,c)
	I. sugeroki	Fruit	(Ishikura, 1975; Santamour, 1973)
	I. vomitoria	Fruit	(Santamour, 1973)
Cyanidin-3-glucoside	*I. chinensis*	Fruit	(Ishikura, 1971c)
	I. coriaceae	Fruit	(Santamour, 1973)

Table 3 contd.

Compound	*Ilex* species	Plant part	References
	I. crenata	Fruit	(Ishikura, 1971c; Santamour, 1973; Ishikura and Sugahara, 1979)
	I. glabra	Fruit	(Santamour, 1973)
	I. micrococca	Fruit	(Ishikura, 1975)
	I. paraguariensis	Fruit	(Santamour, 1973)
	I. pedunculosa	Fruit	(Ishikura, 1971c; Santamour, 1973)
	I. rotunda	Fruit	(Ishikura, 1971c)
	I. sugeroki	Fruit	(Ishikura, 1975)
	I. latifolia	Spring leaves	(Yoshitama *et al.*, 1972)
	I. pedunculosa	Spring leaves	(Yoshitama *et al.*, 1972)
Cyanidin-3-sophoroside	*I. pubescens*	Fruit	(Santamour, 1973)
Cyanidin-3-rhamnoglucoside	*I. pedunculosa*	Spring leaves	(Yoshitama *et al.*, 1972)
Pelargonidin-3-xylosyl-glucoside	*I. aquifolium*	Furit	(Santamour, 1973; Ishikura, 1971a,c)
	I. buergeri	Fruit	(Ishikura, 1975)
	I. ciliospinose	Fruit	(Santamour, 1973)
	I. cornuta	Fruit	(Santamour, 1973)
	I. geniculata	Fruit	(Ishikura,1975; Ishikura and Sugahara, 1979)
	I. georgei	Fruit	(Santamour,1973)
	I. integra	Fruit	(Santamour, 1973; Ishikura, 1971b,c)
	I. kiusiana	Fruit	(Ishikura, 1975)
	I. latifolia	Fruit	(Santamour, 1973; Ishikura, 1971b,c)
	I. leucocladia	Fruit	(Santamour, 1973)

Table 3 contd.

Compound	*Ilex* species	Plant part	References
	I. nipponica	Fruit	(Ishikura, 1975)
	I. perado	Fruit	(Santamour, 1973)
	I. pernyl	Fruit	(Santamour, 1973)
	I. serrata	Fruit	(Santamour, 1973; Ishikura, 1971b,c)
	I. verticillata	Fruit	(Santamour, 1973)
Perlargonidin-3-glucoside	*I. aquifolium*	Fruit	(Santamour, 1973)
	I. corallina	Furit	(Santamour, 1973)
	I. cornuta	Fruit	(Santamour, 1973)
	I. leucocladia	Fruit	(Santamour, 1973)
	I. perado	Fruit	(Santamour, 1973)
	I. serrata	Fruit	(Santamour, 1973)
	I. verticillata	Fruit	(Santamour, 1973)

Table 4
TERPENOIDS

Compound	*Ilex* species	Plant part	References
α-Amyrin	*I.aquifolium*	Bark	(Personne, 1884; Jungfleisch and Leroux, 1908)
	I. aquifolium	Leaves	(Catalano *et al.*, 1978)
	I. aquifolium	Fruit	(Thomas and Budzikewiez, 1980b)
	I. crenata	Bark	(Yagishita, 1957b)
	I. goshiensis	Bark	(Yagishita and Nishimura, 1961)
	I. hanceana	Bark	(Yagishita, 1957b)

Table 4 contd.

Compound	*Ilex* species	Plant part	References
	I. integra	Bark	(Iseda *et al.*, 1954)
	I. latifolia	Bark	(Yagishita, 1957a)
	I. paraguariensis	Leaves	(Mendive,1940; Descartes, 1944)
Ursolic acid	*I. aquifolium*	Leaves	(Nooyen,1920; Fischer and Linser, 1930; Schindler and Herb, 1955; Catalano *et al.* 1978)
	I. aquifolium	Fruit	(Thomas and Budzikiewicz, 1980b)
	I. asprella	Leaves	(Arthur *et al.*, 1956)
	I. cinerea	Leaves	(Arthur *et al.*, 1956)
	I. crenata	Leaves	(Nooyen, 1920)
	I. dumosa	Leaves	(Heinzmann and Schenkel, 1995)
	I. hanceana	Leaves	(Arthur *et al.*, 1956)
	I. latifolia	Leaves	(Kariyone and Hashimoto, 1949, 1953; Kariyone *et al.*, 1953)
	I. latifolia	Fruit	(Koyama and Kato, 1954)
	I. memecylifolia	Leaves	(Arthur *et al.*, 1956)
	I. opaca	Fruit	(West *et al.*, 1977)
	I. paraguariensis	Leaves	(Nooyen, 1920; Hauschild 1935;

Table 4 contd.

Compound	*Ilex* species	Plant part	References
			Mendive,1940)
	I. perado	Leaves	(Nooyen, 1920)
	I. pubescens	Leaves	(Arthur *et al.*, 1956)
	I. rotunda	Leaves	(Arthur *et al.*, 1956)
	I. triflora	Leaves	(Arthur *et al.*, 1956)
β-Amyrin	*I. aquifolium*	Leaves	(Catalano *et al.*, 1978)
	I. crenata	Bark	(Yagishita, 1957b)
	I. hanceana	Bark	(Yagishita, 1957b)
	I. integra	Bark	(Iseda *et al.*, 1954)
	I. latifolia	Bark	(Yagishita, 1957a)
	I. latifolia	Leaves	(Yamada, 1966)
Oleanolic acid	*I. aquifolium*	Leaves	(Catalano *et al.*, 1978)
	I. *cornuta*	Leaves	(Gau *et al.*, 1983)
	I. *macrocarpa*	Leaves	(Arthur *et al.*, 1956)
	I. opaca	Leaves	(West *et al.*, 1977)
	I. pubescens	Leaves	(Arthur *et al.*, 1956)
Baurenol	*I. aquifolium*	Leaves	(Catalano *et al.*, 1978)
	I. crenata	Bark	(Yagishita, 1957b)
	I. integra	Bark	(Iseda *et al.*, 1954)
Neoilexonol	*I. buergeri*	Bark	(Yagishita and Nishimura, 1961)
	I. goshiensis	Bark	(Yagishita and Nishimura, 1961)
Lupeol	*I. cornuta*	Leaves	(Gau *et al.*, 1983)
	I. integra	Bark	(Iseda *et al.*, 1954)

Table 4 contd.

Compound	*Ilex* species	Plant part	References
	I. latifolia	Leaves	(Yamada, 1966)
Taraxerol	*I. latifolia*	Leaves	(Yamada, 1966)
Uvaol	*I. aquifolium*	Leaves	(Catalano *et al.*, 1978)
	I. aquifolium	Fruit	(Thomas and Budzikiewicz, 1980)
	I. latifolia	Leaves	(Yamada, 1966)
Erythrodiol	*I. aquifolium*	Leaves	(Catalano *et al.*, 1978)
3β-Hydroxylup-20(29)-en-30al	*I. cornuta*	Leaves	(Gau *et al.*, 1983)
27-*p*-Coumaroxyursolic acid	*I. aquifolium*	Fruit	(Thomas and Budzikiewicz, 1980 b; Budzikiewicz and Thomas, 1980)
Pedunculoside	*I. oldhami*	Leaves	(Hase *et al.*, 1973)
	I. pedunculosa	Leaves	(Hase *et al.*, 1973)
	I. rotunda	Leaves	(Hase *et al.*, 1973)
Latifoloside A	*I. latifolia*	Leaves	(Ochi *et al.*, 1975)
Ilexolide A	*I. pubescens*	Roots	(Zeng *et al.*,1982, 1984)
Ilexside I	*I. cornuta*	Leaves	(Nakanishi *et al.*, 1982; Otsuka Pharm Co., 1983)
Ilexside II	*I. cornuta*	Leaves	(Nakanishi *et al.*, 1982); Otsuka Pharm Co., 1983)
Hainanenside	*I. Hainanensis*	Leaves	(Min and Qin, 1984)
Abbeokutone glucoside	*I. sugeroki*	Leaves	(Ichikawa *et al.*, 1973)

Table 4 contd.

Compound	*Ilex* species	Plant part	References
Ilex lactone	*I. aquifolium*	Fruit	(Thomas and Budzikiewicz, 1980a,b)
Ziyu-glycoside I	*I. cornuta*	Leaves	(Wenjuan *et al.*, 1986)
Ziyu-glycoside II	*I. cornuta*	Leaves	(Wenjuan *et al.*, 1986)
Pomolic acid 3β-O-2-acetoxyarabino 28-O-glucoside	*I. cornuta*	Leaves	(Wenjuan *et al.*, 1986)
29-Hydroxyoleanolic acid 3β-O-arabino 28-O-glucoside	*I. cornuta*	Leaves	(Wenjuan *et al.*, 1986)
Ilexolic Acid A & B	*I. rotunda*	Leaves	(Amimoto *et al.*, 1993a,b,c)
Ilexrotunin	*I. rotunda*	Bark	(Wen, D.X. and Chen, Z.L., 1996)
Rotundanoic acid	*I. rotunda*	Bark	(Wen, D.X. and Chen, Z.L., 1996)
Ursolaldehyde	*I. aquifolium*	Leaves	(Vangenderen and Jaarsma, 1990)
Oleanolaldehyde	*I. aquifolium*	Leaves	(Vangenderen and Jaarsma, 1990)

Table 5
FATTY ACIDS

Compound	*Ilex* species	Plant part	References
Lauric acid	*I. crenata*	Seed	(Tahara and Sakuda, 1980)

Table 5 contd.
FATTY ACIDS

Compound	*Ilex* species	Plant part	References
	I. integra	Seed	(Tahara and Sakuda, 1980)
	I. latifolia	Seed	(Tahara and Sakuda, 1980)
	I. paraguariensis	Seed	(Cattaneo *et al.*, 1952)
	I. rotunda	Seed	(Tahara and Sakuda, 1980)
Myristic acid	*I. crenata*	Seed	(Tahara and Sakuda, 1980)
	I. integra	Seed	(Tahara and Sakuda, 1980)
	I. latifolia	Seed	(Kashimoto and Noda, 1958; Tahara and Sakuda, 1980)
	I. pedunculosa	Seed	(Tahara and Sakuda, 1980)
	I. rotunda	Seed	(Tahara and Sakuda, 1980)
Pentadecanoic acid	*I. aquifolium*	Leaves	(Catalano *et al.*, 1978)
	I. crenata	Seed	(Tahara and Sakuda, 1980)
	I. integra	Seed	(Tahara and Sakuda, 1980)
	I. latifolia	Seed	(Tahara and Sakuda, 1980)
	I. rotunda	Seed	(Tahara and Sakuda, 1980)
Palmitic acid	*I. aquifoliym*	Leaves	(Crombie, 1958; Catalano *et al.*,

Table 5 contd.
FATTY ACIDS

Compound	*Ilex* species	Plant part	References
			1978)
	I. crenata	Seed	(Tahara and Sakuda, 1980)
	I. integra	Seed	(Tahara and Sakuda, 1980)
	I. latifolia	Seed	(Kashimoto and Noda,1958; Tahara and Sakuda, 1980)
	I. paraguariensis	Seed	(Cattaneo *et al.*, 1952)
	I. pedunculosa	Seed	(Tahara and Sak uda, 1980)
	I. rotunda	Seed	(Tahara and Sakuda, 1980)
Heptadecanoic acid	*I. aquifolium*	Leaves	(Catalano *et al.*, 1978)
	I. crenata	Seed	(Tahara and Sakuda, 1980)
	I. integra	Seed	(Hirose *et al.*, 1971; Tahara and Sakuda, 1980)
	I. latifolia	Seed	(Tahara and Sakuda, 1980)
	I. rotunda	Seed	(Tahara and Sakuda, 1980)
Stearic acid	*I. aquifolium*	Leaves	(Crombie, 1958; Catalano *et al.*, 1978)
	I. crenata	Bark	(Yagishita, 1957b)
	I. crenata	Seed	(Tahara and Sakuda, 1980)

Table 5 contd.
FATTY ACIDS

Compound	*Ilex* species	Plant part	References
	I. intergra	Seed	(Tahara and Sakuda, 1980)
	I. latifolia	Bark	(Yagishita, 1957a)
	I. latifolia	Seed	(Kashimoto and Noda, 1958; Tahara and Sakuda, 1980)
	I. paraguariensis	Seed	(Cattaneo *et al.*, 1952)
	I. pedunculosa	Seed	(Tahara and Sakuda, 1980)
	I. rotunda	Seed	(Tahara and Sakuda, 1980)
Nonadecanoic acid	*I. integra*	Seed	(Hirose *et al.*, 1971)
Arachidic acid	*I. aquifolium*	Leaves	(Catalano *et al.*, 1978)
	I. crenata	Seed	(Tahara and Sakuda, 1980)
	I. integra	Seed	(Hirose *et al.*, 1971; Tahara and Sakuda, 1980)
	I. latifolia	Seed	(Kashimoto and Noda (1958); Tahara and Sakuda, 1980)
	I. paraguariensis	Seed	(Cattaneo *et al.*, 1952)
	I. pedunculosa	Seed	(Tahara and Sakuda, 1980)
	I. rotunda	Seed	(Tahara and Sakuda, 1980)

Table 5 contd.
FATTY ACIDS

Compound	*Ilex* species	Plant part	References
Behenic acid	*I. aquifolium*	Leaves	(Catalano *et al.*, 1978)
Lignoceric acid	*I. aquifolium*	Leaves	(Catalano *et al.*, 1978)
Cerotic acid	*I. latifolia*	Seed	(Kashimoto and Noda, 1958)
Pentadecenoic acid	*I. aquifolium*	Leaves	(Catalano *et al.*, 1978)
Palmitoleic acid	*I. aquifolium*	Leaves	(Crombie, 1958; Catalano *et al.*, 1978)
	I. crenata	Seed	(Tahara and Sakuda, 1980)
	I. integra	Seed	(Tahara and Sakuda, 1980)
	I. paraquariensis	Seed	(Cattaneo *et al.*, 1952)
	I. rotunda	Seed	(Tahara and Sakuda, 1980)
Oleic acid	*I. aquifolium*	Leaves	(Crombie (1958); Catalano *et al.*, 1978)
	I. crenata	Seed	(Tahara and Sakuda, 1980)
	I. integra	Seed	(Koyama and Toyama, 1957 a,b; Tahara and Sakuda, 1980)
	I. latifolia	Seed	(Kashimoto and Noda, 1958; Tahara and Sakuda, 1980)

Table 5 contd.
FATTY ACIDS

Compound	*Ilex* species	Plant part	References
	I. macropoda	Seed	(Koyama and Toyama, 1957 a,b)
	I. paraguariensis	Seed	(Cattaneo *et al.*, 1952)
	I. pedunculosa	Seed	(Tahara and Sakuda,1980)
	I. rotunda	Seed	(Tahara and Sakuda, 1980)
	I. serrata	Seed	(Koyama and Toyama, 1957 a,b)
Nonadecenoic acid	*I. crenata*	Seed	(Tahara and Sakuda, 1980)
	I. integra	Seed	(Tahara and Sakuda, 1980)
	I. latifolia	Seed	(Tahara and Sakuda, 1980)
Gadoleic acid	*I. aquifolium*	Leaves	(Crombie,1958; Catalano *et al.*, 1978)
Linoleic acid	*I. aquifolium*	Leaves	(Crombie, 1958; Catalano *et al.*, 1978)
	I. crenata	Seed	(Tahara and Sakuda, 1980)
	I. integra	Seed	(Koyama and Toyama, 1957 a,b; Tahara andSakuda, 1980; Hirose *et al.*, 1971)

Table 5 contd.
FATTY ACIDS

Compound	*Ilex* species	Plant part	References
	I. latifolia	Seed	(Kashimoto and Noda, 1958; Tahara and Sakuda, 1980)
	I. macropoda	Seed	(Koyama and Toyama, 1957 a,b)
	I. paraguariensis	Seed	(Cattaneo *et al.*, 1952)
	I. pedunculosa	Seed	(Tahara and Sakuda, 1980)
	I. retunda	Seed	(Tahara and Sakuda, 1980)
	I. serrata	Seed	(Koyama and Toyama, 1957 a,b)
Nonadecadienoic acid	*I. integra*	Seed	(Tahara and Sakuda, 1980)
	I. latifolia	Seed	(Tahara and Sakuda, 1980)
Eicosadienoic acid	*I. latifolia*	Seed	(Tahara and Sakuda, 1980)
Linolenic acid	*I. aquifolium*	Leaves	(Crombie, 1958; Catalano *et al.*, 1978)
	I. crenata	Seed	(Tahara and Sakuda, 1980)
	I. integra	Seed	(Tahara and Sakuda, 1980)
	I. latifolia	Seed	(Tahara and Sakuda, 1980)
	I. rotunda	Seed	(Tahara and Sak-

uda, 1980)

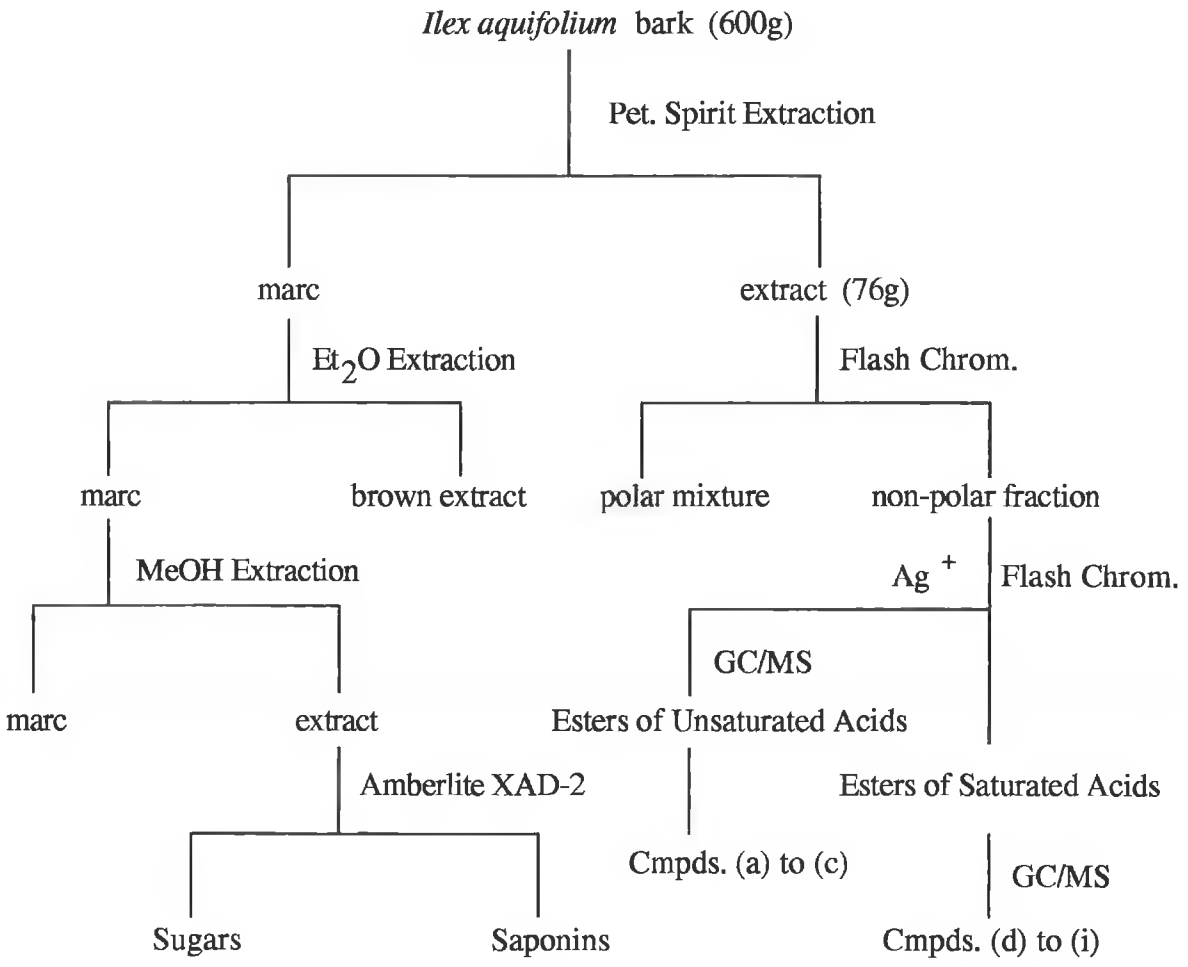
Table 6
SAPONINS

Compound	*Ilex* species	Plant part	References
Ilexosides	*I. rotunda*	Leaves	(Amimoto *et al.*, 1992, 1993a,b,c)
	I. integra	Leaves	(Yamo *et al.*, 1993)
	I. crenata	Bark & Fruits	(Kakuno *et al.*, 1992a,b; Hata *et al.*, 1992; Miyase *et al.*, 1992)
Triterpenoid Saponins	*I. cornuta*	Leaves	(Nakanishi *et al.*, 1982; Qin *et al.*, 1986)
	I. chinensis	Leaves	(Inada *et al.*, 1987)
	I. pubescens		(Hidaka *et al.*, 1987a,b; Wenjuan *et al.*, 1987)
	I. dumosa	Leaves	(Heinzmann and Schenkel, 1995)
Matesaponins	*I. paraguariensis*	Leaves	(Gosmann *et al.*, 1995; Kraemer *et al.*, 1996)
Kudinosides	*I. kudincha*	Leaves	(Ouyang *et al.*, 1996)
Dianchinenosides	*I. verticillata*	Aerial parts	(Li *et al.*, 1994)
Saponin Aglycones	*I. opaca*		(Barbosa *et al.*, 1990)

Isolation of fatty acid esters of α-amyrin

Air-dried bark was powdered and successively extracted with petroleum spirit, Et_2O and MeOH in accordance with the scheme outlined below;

***Ilex aquifolium* extraction procedure**



Evaporation of the pet. spirit extract gave a large quantity of a brown oil (12% on a dry weight basis) which auto-oxidised to a red colour on standing. TLC analysis showed the oil to consist of a major non-polar substance or mixture of substances as well as a number of minor and more polar constituents. These minor constituents form the subject-matter of a separate study. Flash chromatography over silica gel gave the major compound/s as a colourless oil. The FT-IR spectrum of the colourless oil showed ab-

sorptions characteristic of an ester group at 1731 and 1172 cm^{-1}, attributable to a carbonyl group and a C-O group respectively. The ^1H NMR (60 MHz) spectrum of the oil showed one set of peaks attributable to a pentacyclic triterpene (a distorted triplet at δ 5.12 ppm characteristic of the proton of a trisubstituted double bond, a triplet due to an R-COO-C<u>H</u> group at δ 4.44 ppm plus other signals very typical of a triterpene of the Δ^{12} ursene series).

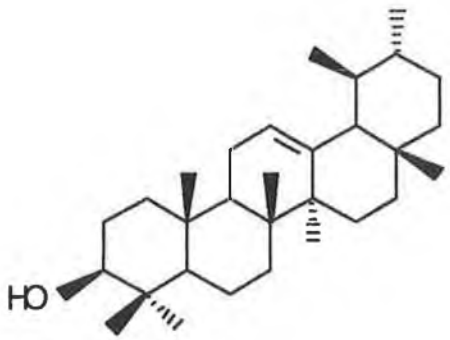
The ^1H NMR spectrum also showed signals which were typical of an unsaturated fatty acid moiety; a signal at δ 2.28 ppm assignable to a $-CH_2COO-$ group, a sharp singlet attributable to a $-(CH_2)_n-$ group occured at δ 1.26 ppm and at δ 5.32 ppm a triplet-like signal typical of a disubstituted double bond. In addition, a multiplet occurring at δ 2.00 ppm was attributable to a $-C\underline{H}_2-CH=CH-C\underline{H}_2-$ group, which formed part of an alkyl chain of a fatty acid substituent. On catalytic hydrogenation of the oil using PtO_2 in EtOAc, the double bond which produced signals at δ 5.32 and δ 2.00 ppm was reduced, showing it to be sterically unhindered.

In order to facilitate structural determination the oil was saponified with KOH in MeOH and the resulting fatty acid and triterpene alcohol moieties were studied separately. The fatty acid fraction was methylated with CH_2N_2. The derivative was analysed by $AgNO_3$ impregnated silica gel TLC plates and was found to produce two spots rather than the expected one, a result which suggested that the derivative in fact consisted of a mixture of saturated and unsaturated compounds.

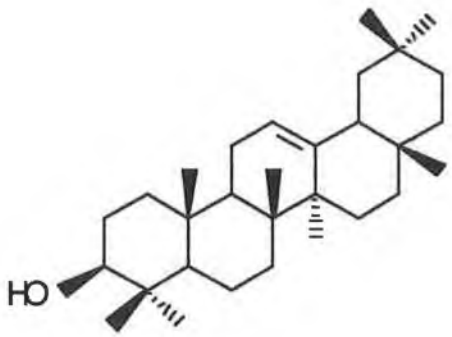
Fatty acid methyl esters (FAME's) may be separated according to their degree of unsaturation by chromatography in the presence of Ag^+ ions (Newton and Walton, 1996; Hostettmann *et al.*, 1998). A loose complex is formed between the Ag^+ ions and the π-electrons of the double bond. The lower spot would then be expected to be the unsaturated component of the mixture.

Flash chromatography of the FAME mixture over Ag^+ / silica gel afforded first the saturated FAME's (Cmpds. (d) to (i)) and next a mixture of the unsaturated FAME's (Cmpds. (a) to (c)), all of which were subsequently analysed and identified using GC/MS.

Identification of the triterpene moiety



α-AMYRIN



β-AMYRIN

The triterpene moiety, mp 198-202°, gave a positive Libermann-Burchard test for triterpenes. EIMS indicated an M^+ ion at *m/z* 426 for the alcohol and this was confirmed by an M^+ at *m/z* 468 for the corresponding acetate. The FT-IR of this compound (**Spectrum 1**) showed an OH absorption band at 3318 cm^{-1} and it lacked any peak due to a -C=O group. It also showed absorptions at 1655 and 808 cm^{-1} typical of a trisubstituted endo-olefinic group. Treatment of the triterpene with a mixture of Ac_2O and pyridine led to the formation of a monoacetate, this was confirmed by ^1H NMR (60 MHz) spectroscopy which showed a sharp singlet at δ 2.00 ppm due to an acetate methyl group and the ^{13}C NMR which showed signals at δ 171.00 and δ 21.30 ppm corresponding to the carbons of an acetyl group. In addition, the ^{13}C NMR spectrum of the derivative gave a signal at δ 80.92 ppm, indicative of a C-3 atom attached to such an acetate group (Tanaka and Matsunaga, 1988).

The presence of an endo-olefinic group was supported by two of the ^{13}C NMR signals observed in the spectrum of the acetate. These signals occurred at δ 124.29 and δ 139.61 ppm and are characteristic of olefinic carbon atoms at C-12 and C-13 in the molecule as shown in the table below. The resonances of the olefinic carbons in the triterpenes is indicative of the position of the double bond within the molecule as shown in **Table 7** (Mahato and Kundu 1994);

Table 7. Double Bond Position δ ppm

	C	CH
Δ^5	140.6	121.5
Δ^{12}	139.6	124.3
Δ^{14}	158.1	118.7
Δ^{18}	142.8	129.8

The FT-IR of the acetate derivative (**Spectrum 2**) indicated the absence of an OH function, but showed the expected carbonyl stretching vibration at 1735 cm^{-1} and a C-O absorption at 1244 cm^{-1}, thus confirming that the triterpene moiety was a monohydroxy compound. Analysis of the ^1H and ^{13}C NMR spectra of the triterpene moiety and its acetate derivative yielded the following information;

(a) The C-12 proton

A distorted triplet appeared at δ 5.12 ppm ($W_{1/2}$ = 4 Hz) corresponding to an olefinic proton of a trisubstituted double bond of the Δ^{12} ursene and oleanene series triterpenes in the ^1H NMR spectrum of the triterpene moiety (Kojima and Ogura, 1989). The resistance of the olefinic group to catalytic hydrogenation suggested that the double bond was sterically hindered and was thus part of a ring system, such behaviour is typical of pentacyclic triterpene ring systems (De Mayo, 1959).

(b) The C-3 proton

The orientation of the 3-OH group is readily established by measuring the $W_{1/2}$ of the corresponding methine proton (Richards, 1988a). A multiplet approximating to a double doublet centred at δ 3.16 ppm ($W_{1/2}$ = 16 Hz) in the ^1H NMR (60 MHz) spectrum of the triterpene moiety was attributable to a methine proton at C-3. The ^1H NMR signal for this proton was shifted upfield from its position at δ 4.45 ppm in the original ester, and as a result of an acetylation shift, it returned downfield to δ 4.44 ppm in the acetate (**Spectrum 3**). The $W_{1/2}$ for this ^1H NMR signal was 16 Hz approx. indicating the proton to be axially (α) orientated. Studies of Dreiding models showed that in this configuration the dihedral angle between the C-3 axial proton and the C-2 axial proton is 180° giving a coupling constant, J = 10 Hz approx. while between the C-3 proton and the C-2 equatorial proton it is 60° giving J = 2 Hz approx., thus resulting in a $W_{1/2}$ = 16 Hz .

A methine proton in the equatorial orientation would show the corresponding dihedral angle to be in the order of 54° with a coupling constant of J = 3 Hz approx., the methine signal then appearing as a broad singlet or distorted triplet at δ 3.70 ppm with $W_{1/2}$ = 4 Hz approx.

(c) Methyl groups

Signals attributable to eight methyl groups appeared at δ 0.80 (x 2), 0.84, 0.88, 0.92, 1.00, 1.04 and 1.10 ppm in the spectrum of the triterpene moiety and at δ 0.76, 0.80, 0.82, 0.84, 0.86, 0.96, 1.00 and 1.06 ppm in that of its acetate derivative. This pattern is characteristic of the methyl groups of triterpenes of the ursane and oleanane series. The ^{13}C NMR of the acetate derivative (**Spectrum 4**) on comparison with literature data, enabled the eight methyl groups to be assigned to the following carbon atoms; C-23, C-24, C-25, C-26, C-27, C-28, C-29 and C-30 (Mahato and Kundu 1994).

The ^{13}C NMR spectrum of the acetate is given in **Table 8**, assignments are based on a comparison made with the literature. The values given in **Table 8** for C-18, C-19, C-20, C-21, C-22, C-29 and C-30 are consistent with an assignment of α-amyrin acetate to the derivative.

Table 8. ^{13}C NMR data for α-amyrin acetate

Carbon	δ ppm	Carbon	δ ppm	Carbon	δ ppm
1	38.41	11	23.34	21	31.21
2	23.57	12	124.29	22	41.49
3	80.92	13	139.61	23	28.03
4	36.75	14	42.03	24	16.71
5	55.21	15	28.72	25	15.71
6	18.21	16	26.57	26	16.83
7	32.82	17	33.71	27	23.34
8	39.98	18	59.02	28	28.03
9	47.60	19	39.62	29	17.49
10	36.75	20	39.62	30	21.38
				OCOMe	21.30
				OCOMe	171.00

Had the compound been β-amyrin acetate then the values for those seven carbon atoms, discussed above, would have been as shown below in **Table 9** (Mahato and Kundu 1994).

Table 9. ^{13}C NMR data for β-amyrin acetate

Carbon	δ ppm
18	47.4
19	46.9
20	31.1
21	34.8
22	37.2
29	33.3
30	23.7

The EIMS of the triterpene moiety (**Spectrum 5**) gave a diagnostically significant peak at *m/z* 218, which was attributable to cleavage of the molecule in ring C by means of a retro-Diels-Alder reaction and is characteristic of the Δ^{12} ursene and oleanene series triterpenes. The EIMS of pentacyclic triterpenes has been studied extensively by Karliner and Djerassi, who have shown that the most characteristic fragmentation for triterpenes of the Δ^{12} oleanene and ursene series is the formation of an ion at *m/z* 218, as a result of a retro-Diels-Alder reaction occurring in ring C, with retention of charge by the diene (Karliner and Djerassi, 1966). Retention of charge by the olefin fragment gives a less intense peak at *m/z* 207, such an ion was visible in the EIMS of the triterpene moiety.

The formation of the other major peaks observed in the EIMS arise by subsequent fragmentation of these ions as shown in **Fig. 10.**

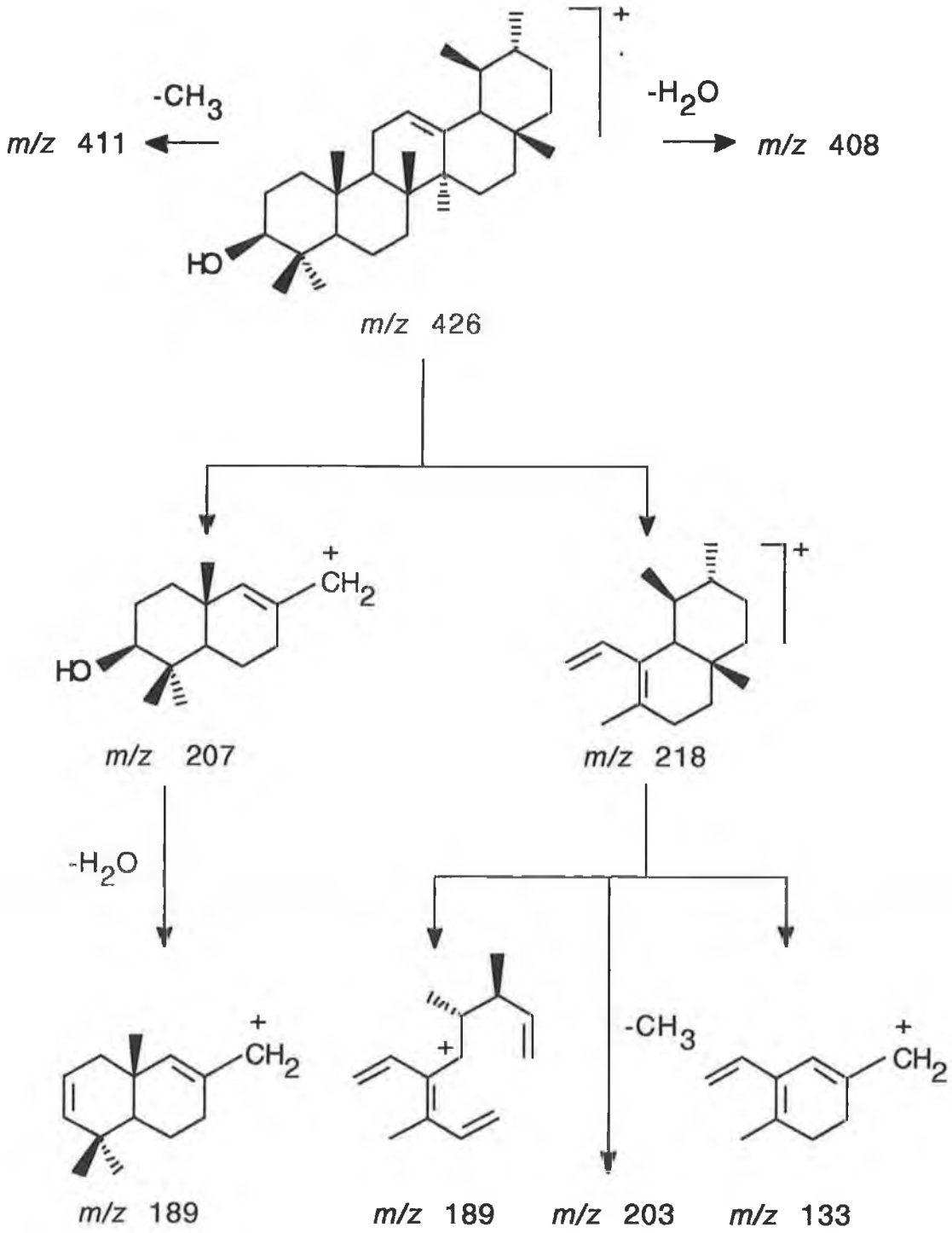

-CH3
m/z 411
-H2O
m/z 408
m/z 426
m/z 207
m/z 218
-H2O
-CH3
m/z 189
m/z 189
m/z 203
m/z 133

Fig. 10

Triterpenes with the olefinic group at other positions in the molecule give rise to a different fragmentation pattern and so the EIMS serves to distinguish between the various types of triterpenes, even where their molecular weights are the same. These results are consistent with those reported for α-amyrin (Karliner and Djerassi, 1966).

The EIMS of the acetate derivative (**Spectrum 6**) also gave a characteristic retro-Diels-Alder cleavage producing diagnostically important fragments at *m/z* 218 and 249. The loss of CH_3COOH from the molecular ion is typical of triterpenoid acetates (Tanaka and Matsunaga, 1988; Akihisa *et al.*, 1997; Anjaneyulu and Ravi, 1989). It is readily accounted for by a McLafferty rearrangement of the molecular ion giving rise to the fragment at *m/z* 408. A similar mechanism accounts for the formation of the *m/z* 189 (a) fragment due to loss of CH_3COOH from the *m/z* 249 ion. This EIMS data was also consistent with the proposed structure of the hydrolysis product. The suggested fragmentation pattern for α-amyrin acetate is shown in **Fig. 11**.

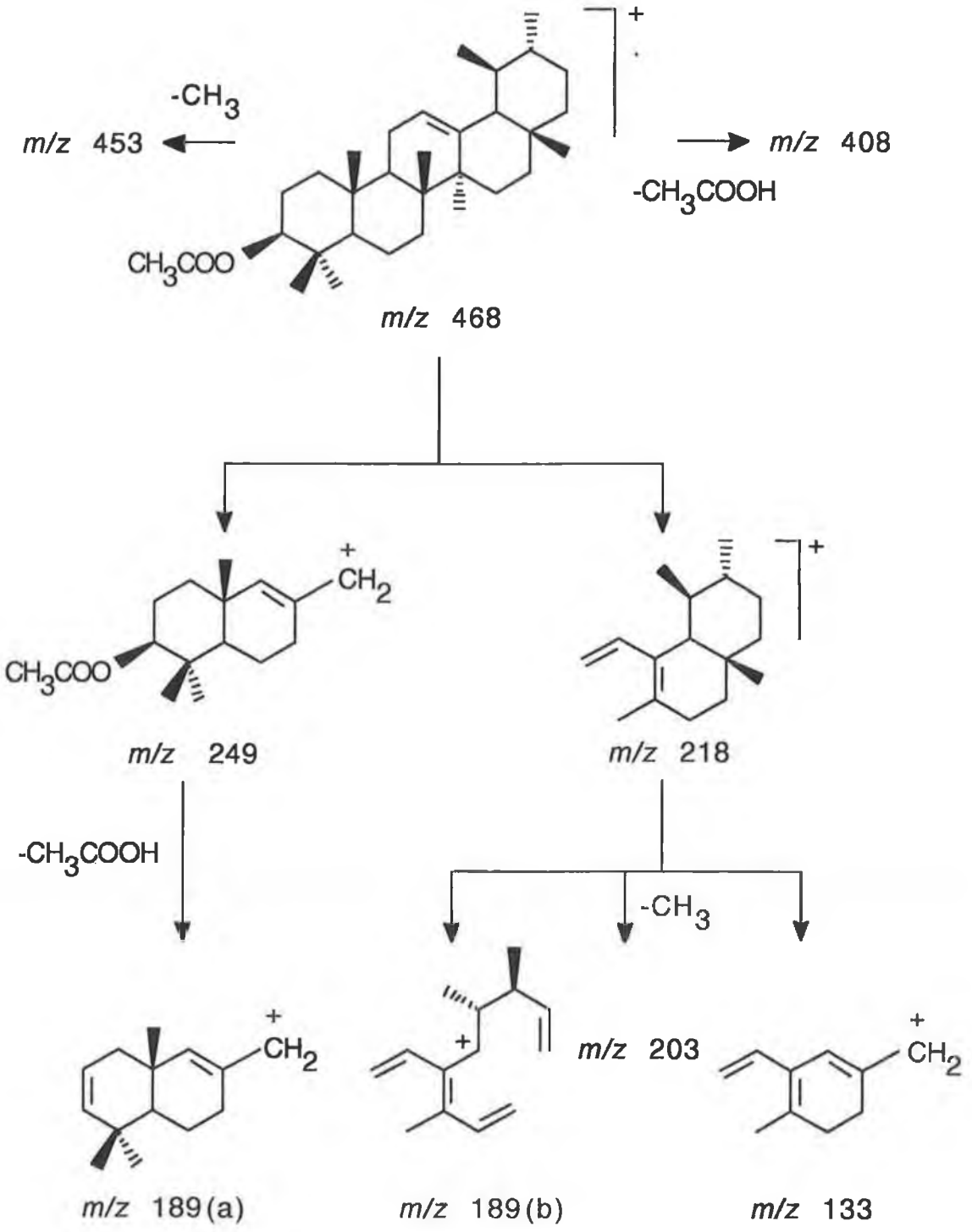

-CH3
m/z 453
m/z 408
-CH3COOH
CH3COO
m/z 468
CH2
CH3COO
m/z 249
m/z 218
-CH3COOH
-CH3
m/z 203
CH2
CH2
m/z 189(a)
m/z 189(b)
m/z 133

Fig. 11

Identification of the fatty acid moiety components

The FT-IR spectrum of the acidic moiety showed a broad OH stretching vibration between 3500 and 2300 cm^{-1}, an OH deformation at 938, a C=O absorption at 1706, a C-O vibration at 1296 cm^{-1} typical of a carboxylic acid as well as showing a *cis*-double bond absorption at 714 cm^{-1}.

The ^1H NMR spectrum exhibited signals typical of unsaturated fatty acids or a mixture of unsaturated and saturated fatty acids in the acidic moiety. These signals were a 2H broad triplet-like signal centred at δ 5.32 due to a disubstituted, *cis*-olefinic group, a doublet at δ 2.24 due to a $-CH_2COO-$ group, a multiplet at δ 2.00 due to a $-CH_2-C=C-CH_2-$ group, a singlet at δ 1.24 due to a $-(CH_2)_n-$ chain and a distorted triplet for a methyl signal at δ 0.86 ppm, due to the effects of virtual coupling.

The acidic moiety was methylated using CH_2N_2 and was then subjected to flash chromatography over Ag^+ / silica gel, as previously discussed. Subsequent GC/MS analysis showed the methylated acidic moiety to consist of a mixture of the fatty acid methyl esters of decanoic (C_{10}), myristic (C_{14}), pentadecanoic (C_{15}) (**Spectrum 7**), palmitic (C_{16}) (**Spectrum 8**), heptadectrienoic ($C_{17:3}$), heptadecanoic (C_{17}), linoleic ($C_{18:2}$), oleic ($C_{18:1}$) (**Spectrum 9**), and stearic (C_{18}) (**Spectrum 10**) acids. All of these compounds gave rise to a peak at *m/z* 74, which is characteristic of methyl esters of long chain fatty acids. This fragment is formed by a McLafferty rearrangement of the methyl esters as shown in the following **Fig. 12**;

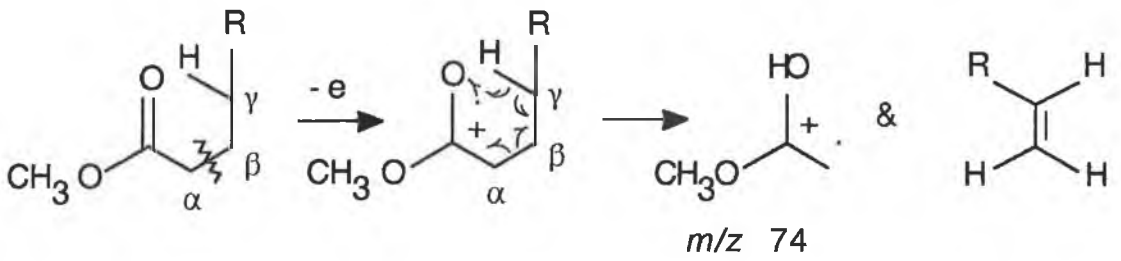


Fig. 12

Authentic FAME samples were run, their R_t's and MS were identical with those for the nine FAME's prepared from the *Ilex aquifolium* extract. The molecular ions and other, major diagnostic peaks from the mass spectra are illustrated in **Table 10**;

Table 10. GC/MS data for the FAME's

FAME	M^+	$[M-CH_3O]^+$	$[M-CH_3(CH_2)_2]^+$	$[(CH_2)_6COOCH_3]^+$
Me decanoate	: *m/z* 186	----	143	143
Me myristate	: *m/z* 242	211	199	143
Me pentadecanoate	: *m/z* 256	225	213	143
Me palmitate	: *m/z* 270	239	227	143
Me heptadectrienoate	: *m/z* 278	----	----	----
Me heptadecanoate	: *m/z* 284	253	241	143
Me linoleate	: *m/z* 294	263	----	----
Me oleate	: *m/z* 296	265	----	----
Me stearate	: *m/z* 298	267	255	143

Quantitation of the FAME's

The ^1H NMR spectrum of the FAME mixture prepared from the plant showed, amongst other signals, a 2H multiplet centred at δ 5.32 approximating to a broad triplet due to a disubstituted, *cis*-olefinic group and a singlet at δ 3.60 due to a CH_3O- group. Integration of the spectrum gave a ratio of 1 : 5 for these two signals, thus suggesting the mixture to be composed of; 30% unsaturated FAME's and 70% saturated FAME's, approximately. The GC assay of the mixture was in agreement with the ^1H NMR quantitation.

The GC assay of the FAME mixture was performed as follows; a series of authentic

standards of varying concentration, corresponding to six of the nine FAME's worked up from the petroleum spirit extract of the plant, were prepared. The standards used were; methyl myristate, methyl pentadecanoate, methyl palmitate, methyl heptadecanoate, methyl oleate and methyl stearate. These standards were used to establish the instrument's linearity of response to concentration changes and R_t's. On satisfactory completion of this preparatory work, the FAME mixture prepared from the plant was dissolved in *iso*-hexane and eluted on the GC column under identical conditions to those used to analyse the authentic standards. The following table presents the mean results obtained from six determinations of the GC assay, and they are supported by the quantitation data (**Table 11**) from the GC/MS analysis of the FAME mixture;

Table 11. FAME quantitation results

FAME		Mean % abundance
Unsaturated		
Methyl oleate	:	11
Methyl linoleate	:	16
Methyl heptadectrienoate	:	5
Saturated		
Methyl decanoate	:	3
Methyl myristate	:	≤ 1
Methyl pentadecanoate	:	≤ 1
Methyl palmitate	:	63
Methyl heptadecanoate	:	≤ 1
Methyl stearate	:	2

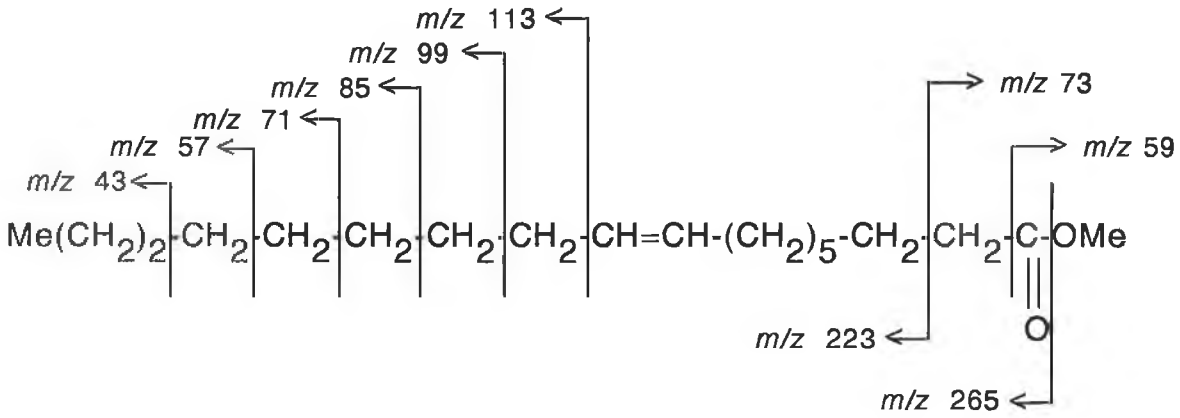
Separation and analysis of oleic acid

A sample of the mixture of the α-amyrin fatty acids obtained from the petroleum spirit extract of the plant was subjected to flash chromatography over Ag^+ / silica gel. TLC analysis showed that a separation had occurred on the basis of degree of unsaturation. The isolated α-amyrin oleate was hydrolysed with a methanolic solution of KOH and the resulting oil was subsequently analysed by TLC which showed a single spot due to the presence of oleic acid. This acid was methylated with CH_2N_2 and the resulting methyl ester derivative, which gave an M^+ ion at *m/z* 296, was analysed by FT-IR, ^1H NMR and GC/MS.

FT-IR analysis of the methyl oleate showed a characteristic C=O absorption at 1743 cm^{-1} and bands at 1650 and 723 cm^{-1} indicative of a *cis*-olefinic group. The ^1H NMR spectrum of the derivative confirmed the existance of a disubstituted *cis*-olefinic bond in the compound by indicating the presence of a triplet-like multiplet at δ 5.26 ppm due to a -C<u>H</u>=C<u>H</u>- group and a doublet at δ 2.00 ppm which was attributable to a -C<u>H</u>$_2$C=CC<u>H</u>$_2$- group. A sharp singlet at δ 3.60 ppm assignable to a methoxy group and a doublet at δ 2.22 ppm due to a -CH_2COO- group were indicative of a methyl ester function in the compound. The existence of a long aliphatic chain in the derivative was supported by the presence of a broad singlet at δ 1.24 ppm assignable to a -$(CH_2)_n$- group and a distorted triplet at δ 0.96 ppm attributable to the terminal methyl group.

The structure of the fatty acid was further supported by the GC/MS analysis of its methyl ester which in addition to the molecular ion reported above, also gave characteristic peaks at *m/z* 265 and *m/z* 264 due to the formation of $[M\text{-}OMe]^+$ and $[M\text{-}MeOH]^+$ ions respectively. Diagnostically significant peaks also occurred at *m/z* 222 and *m/z* 74 attributable to a McLafferty rearrangement, due to the presence of a methyl ester group and a γ proton in the molecule. The other important peaks in the MS were

observed at *m/z* 59 attributable to a $[COOMe]^+$ ion and at *m/z* 113, 99, 85, 71, 57 and 43. These assignments are accounted for in the following fragmentation pattern;



M^+ at *m/z* 296

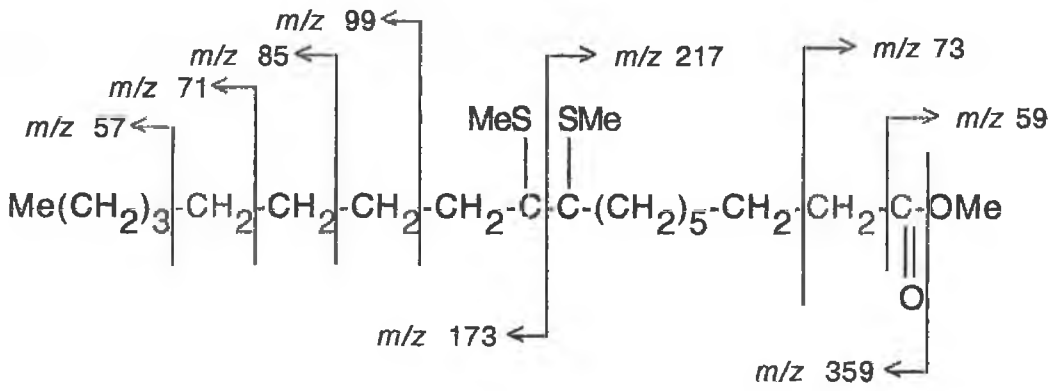
The above observations suggest that the compound was in fact oleic ($C_{18:1}$, octadecenoic) acid.

The mass spectra of underivatised olefins and unsaturated fatty acids show rearrangements of the C=C during fragmentation, making it difficult to satisfactorily establish the position of double bonds in long-chain compounds by mass spectroscopy (Vincenti *et al.*, 1987). The most common approach used to overcome these difficulties is to derivatise the double bond/s prior to analysis. Epoxidation, ozonolysis, silylation and oxidation with OsO_4 are well established derivatisation methods. More recently, reactions yielding Diels-Alder, amino alcohol or dimethyl disulphide (DMDS) adducts have provided satisfactory results. These groups direct fragmentation of the alkyl chain leading to the formation of readily recognisable ions in the mass spectrum (Vincenti *et al.*, 1987; Francis and Veland, 1981).

Therefore, in order to facilitate determination of the position of the double bond in the

fatty acid side chain of the molecule, the methyl oleate was converted to the DMDS derivative, by treating it with a mixture of $(CH_3)_2S_2$ and I_2. This derivative was then subjected to GC/MS analysis which yielded the following results; an M^+ ion at *m/z* 390, an *m/z* 359 peak due to an $[M\text{-}OMe]^+$ ion in addition to diagnostically significant peaks at *m/z* 217 and *m/z* 173 attributable to $[\text{-}CH(SCH_3)\text{-}(CH_2)_7COOCH_3]^+$ and $[CH_3(CH_2)_7\text{-}CH(SCH_3)\text{-}]^+$ ions respectively.

These latter pair of ions result from the cleavage of the molecule at its double bond. This cleavage was directed by the presence of the methylthio groups which added across the double bond during the $(CH_3)_2S_2$ derivatisation-reaction. A peak also appeared at *m/z* 74 which was readily accounted for by a McLafferty rearrangement of the molecule at its methyl ester function. Other important peaks were observed at *m/z* 59 due to a $[COOMe]^+$ ion and at *m/z* 99, 85, 71 and 57. The following fragmentation pattern for the DMDS derivative illustrates all of the cleavages discussed;



M^+ at *m/z* 390

These data indicate that the *cis*-olefinic group occurs between C-9 and C-10 in the original ester and hence confirms that the compound is in fact, oleic acid.

Phytochemical investigation of the *Betula* spp.

Introduction

The *Betula* genus is widely dispersed, for example *Betula pendula*, known as Silver Birch is found in Europe and Asia Minor. The name *Betula* probably derives from the Sanscrit *bhurga*, meaning 'a tree whose bark is used for writing upon'. The horticultural fascination with birches lies in their bark, especially for ornamental gardening purposes. Besides the presence of hybrids, there is wide variation within the species themselves. The important matter of bark colour and manner of peeling is not always specific, and may be influenced by environmental, especially soil conditions. This is exemplified by *Betula papyrifera* (Paper-bark or Canoe Birch) with its vast range, it is often referred to as the birch 'with the whitest bark of all'. But, in the wild some members of this species, and even whole populations, may always remain brown. *Betula pubescens* (the Downy or White Birch of Britain and Europe) is also very white-barked.....often peeling in a spectacular fashion like *B. papyrifera*. One of the most widespread of birches in the Far East is *Betula ermanii*, it is regarded as being a very attractive birch, its bark peeling in sheets, yellowish when newly revealed, with a pinkish tone also present (Ashburner, 1980).

Medicinal uses

Bark taken from *Betula* spp. (cortex *Betulae*) has been used as an antiseptic and as a folk-remedy in the treatment of skin diseases, malaria, dropsy and gout, and its oil was reputed to possess disinfectant properties. Because of its antiseptic properties it was used for sterilising wound plasters and bandages and finely powdered birch bark blended with KNO_3, was pelletised and burnt in a closed room, because its fumes possessed sterilising qualities. Birch tar (pix *Betulae* or *oleum rusci*) from *Betula alba* or *B. pubescens* as it is also known, was used to treat rheumatism and gout as well as being employed as a nematocide antiseptic. In veterinary medicine it found application

in the treatment of colic and mange.

The young shoots and leaves of *B. alba* secrete a resinous substance which is said to be a tonic laxative. The leaves have a bitter taste and have been infused to produce 'Birch Tea' which has been used as a treatment in gout, rheumatism and dropsy. A decoction of the leaves has been reported as efficacious for bathing skin eruptions and lesions. Bark oil is astringent and is used in the treatment of skin diseases, especially eczema. The inner bark too, is bitter and astringent and has been used to treat intermittent fevers, while the vernal sap is reputed to possess diuretic properties.

The bark of *Betula lenta* is stimulant, diaphoretic and astringent in warm infusion, in decoction or syrup form it is an excelent tonic for dysentery. *B. lenta* bark contains oil of wintergreen (methyl salicylate), which is isolated as a hydrolysis product of a precusor to it, contained in sweet birch oil. Its chief medicinal use is as a rubifacient and in former times it served as an antirheumatic.

Betula nana better known as Smooth Dwarf Birch, gives rise to the preparation of a substance termed Moxa, which is regarded as being an effective remedy in the treatment of all painful diseases (Grieve, 1992d). A decoction of the inner bark of *Betula occidentalis* or Western Red Birch as it is more commonly known, was used by the Western Indians of N. America for treating colds, coughs, and other pulmonary ailments, while 'Cones' of *Betula pumila* were heated over coals by Pillager Indians to make an incense for the relief of catarrh sufferers, and the tea prepared from these 'cones' was taken by Pillager women as a postparturition tonic, as well as an aid through dificult menses. *Betula* spp. were also employed by Amerindians in the form of bark decoctions to relieve the pain of burns and scalds and these preparations were further employed in the treatment of wounds.

In parts of the world where tooth-brushing is uncommon, the practice of cleaning teeth by means of 'chewing sticks' has been known since antiquity. The use of chewing sticks persists today amongst many African and Southern Asian communities as well as in isolated areas of tropical America and the Southern United States. A great number of the species selected for such use, have medicinal properties that may be antibacterial, antineoplastic, anti-sickle cell or analgesic. These species include *Betula lutea* and *B. lenta*, both native to the South Eastern United States and the twigs of these species have been shown to be efficacious in the treatment of gum-inflammation and tooth-ache (Lewis and Elvin-Lewis, 1997).

Betulin, a pentacyclic triterpene occurring in many of the birches, showed inhibitory effects on Epstein-Barr virus activation and bacteriostatic activities against *Escherichia coli, Salmonella typhi, Shigella flexneri* and *Staphylococcus aureus.* The compound demonstrates tumour-inhibition activity against human epidermoid carcinoma of the nasopharynx, *in vitro,* and against the Walker 256 tumour system. For betulin itself and betulin-containing herbs antiseptic, anti-inflammatory and insecticidal activities have been reported (Hayek *et al.*, 1989). Holmes demonstrated that betulin can be applied as an insecticide and will relieve itching. It was also found to have hair-care effects and therefore, can be used as an additive in shampoo formulas. Furthermore, it was considered to be a suitable ingredient for toilet powders and cosmetics (Jaackelainen, 1981).

More recently, scientists working at the French laboratory of the pharmaceutical company Rhone-Poulenc Rorer, have synthesised a number of compounds that appear to prevent HIV from invading human immune system cells *in vitro.* These synthetic compounds are derivatives of a terpenoid isolated from European Birch trees. That terpenoid is betulinic acid and it is found in particular in the bark of the European Plane tree. The synthetic derivatives from this naturally occurring triterpene appear to act by

preventing HIV from spreading to other normal cells. Research carried out by the Rega Institute for Medical Research in Belgium, in conjunction with Rhone-Poulenc Rorer, indicates that these molecules act at an early stage of the viral cycle by inhibiting the fusion of the outer coating of the virus with the membrane of the target cell. However, early research would appear to show that the compounds are only effective against the most common strains of HIV-1 and not against Zairean strains found in sub-Saharan Africa, or HIV-2 found in West Africa.

In addition to the above potential therapeutic usage for betulinic acid, is research data emanating from the University of Illinois at Chicago recently showed findings that suggest that betulinic acid isolated from White Birch bark possesses efficacy in the treatment of malignant melanoma. Pezzuto found that the compound, which is relatively non-toxic, induces a process termed apoptosis or programmed cell death and is specific for malignant cells only (*Anon.*, 1995).

The fact that the compound has been demonstrated to be non-toxic in laboratory animal studies makes it different from other anti-cancer agents such as taxol, previously discussed, which are on the whole non-specific and target all cells, both normal and malignant. Pezzuto pointed out that the disease occurs primarily due to over-exposure to the sun's harmful UV rays and thus believes that the compound will find important commercial application as an ingredient in products such as sun tan lotions, where in conjunction with a sun-screen it will help prevent the further occurence of this disease which in the USA alone is the most rapidly increasing form of cancer (*Anon.*, 1995).

Non-medicinal uses

B. alba is used in boat-building and roofing and the tree has been one of the sources from which asphyxiating gasses have been manufactured. The bark of this species yields 'Oil of Birch Tar' and the peculiar, well-known odour of 'Russia Leather' is due

to the use of the oil in the process of dressing. It imparts durability to leather, and due to its presence, books bound in 'Russia Leather' are not liable to become mouldy. In fact the production of 'Birch Tar Oil' is a Russian industry of considerable importance. The oil is also distilled in Holland and Germany, but their product is appreciably different from the Russian oil (*Oleum Rusci*). Interestingly, this oil possesses the property of being able to repel insects, it contains methyl salicylate, cresol (hydroxytoluene) and guaiacol (2-methoxyphenol).

Betulin, obtained in abundance from the *Betula* spp. is an important triterpenoid in the non-medical world, also. An important commercial source of it, is the unsaponifible fraction of crude 'tall oil' obtained from the sulphate pulping process of pine and birch. On distillation of the bark at atmospheric pressure a Russia-leather smell is detected. Betulin has a number of commercial uses, among them are its use as a light stabilizer for cellulose and wood pulp. Polyesters of betulin prepared with linseed fatty acids yield quality protective coatings. It is also used in the manufacture of resins, lacquers, emulsifiers, polyurethanes and cosmetic products.

In some parts of Europe beer, wine, spirit and vinegar are prepared from the sugary juice that flows from the wounded stem of the Birch tree. Birch wine prepared from this thin, sweet sap, collected from incisions made in the trees in March is fermented with honey, cloves and lemon peel to make a pleasant cordial. Finally, *B. lenta* which is also known as Cherry Birch, Black Birch, Sweet Birch or Mahogany Birch is an American variety and its richly-grained wood is suitable for use in cabinet making and other forms of fine woodwork. *B. papyrifera* is largely used for canoe-making in North America (Grieve, 1992c).

Chemical constituents of *Betula* spp.

In addition to betulin and betulinic acid, birch bark contains, amongst other com-

pounds, tannic acid which is used for tanning as it imparts a pale colour to the skin. Wherever there are large birch forests throughout Northern Europe, this process is employed. Betulin is also known as betulinol, trochton, birkenkampher and resinol.

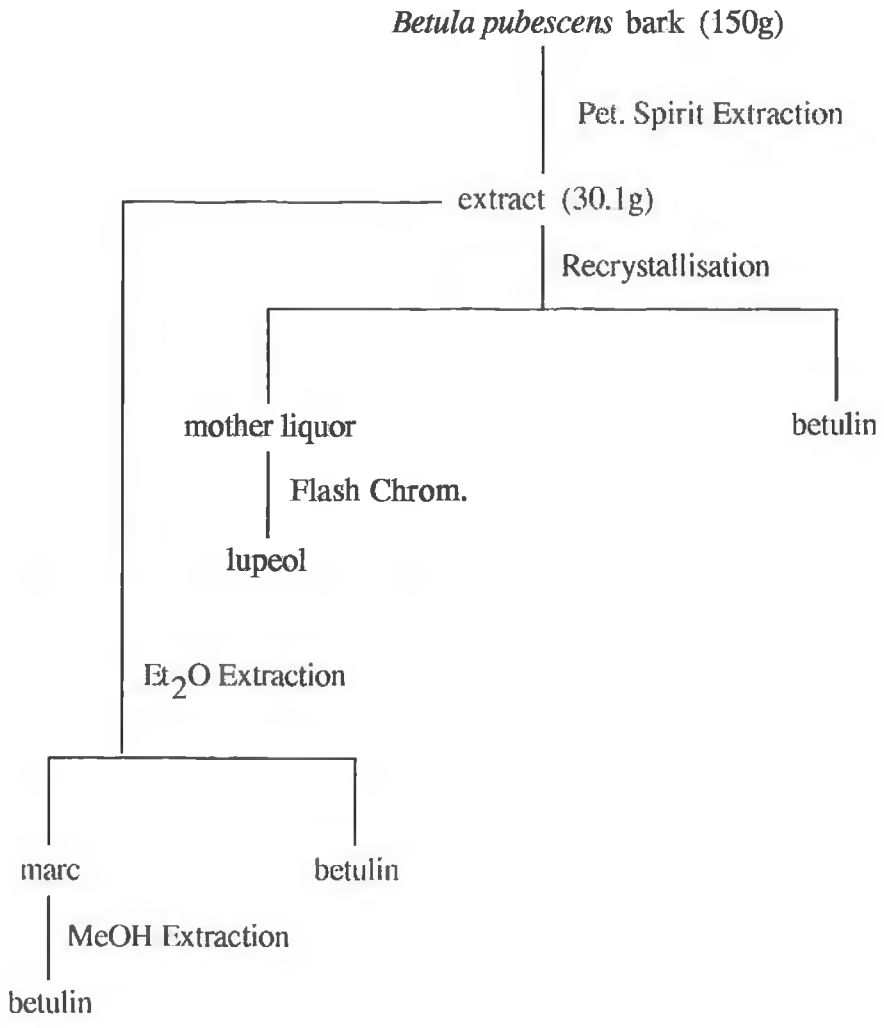
It is a widely distributed and ubiquitous pentacyclic triperpene found throughout the genus *Betula*, and is the principle extractive substance of the bark of this genus. The characteristic white colour of the bark is attributable to it. Lowitz first isolated the compound in 1788 by using a sublimation technique (Lowitz, 1788), the compound's molecular formula was confirmed in the 1920's, while its structure was elucidated at the begining of 1950. It constitutes one of the first natural products isolated from a plant source (Hayek *et al.*, 1989).

The compound possesses a lupane skeleton, the characteristic features of which are the five membered E ring, and the α-*iso*-propylene group bonded to the C-19 atom of that ring. In addition to betulin the following lupane derivatives have been isolated; lupeol, betulinic acid, betulinic aldehyde, betulonic acid and betulonic aldehyde. Betulin and lupeol are the principal components of the bark while minor amounts of the others are also present.

Isolation of betulin and lupeol

Air-dried bark was powdered and successively extracted with petroleum spirit, Et_2O and MeOH in accordance with the scheme outlined below;

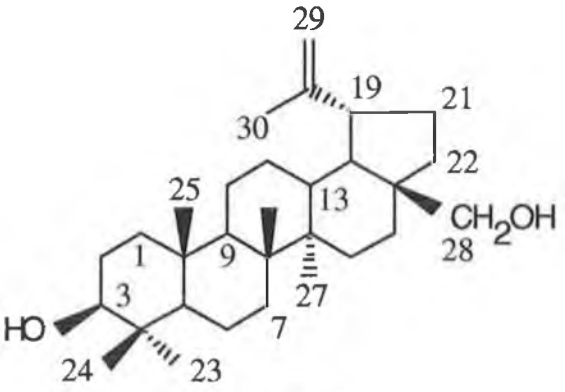
***Betula pubescens* extraction procedure**



Evaporation of the petroleum spirit extract gave a large quantity of a brown semi-solid (20% on a dry weight basis). TLC analysis showed this semi-solid to consist of a mixture of two compounds. Recrystallisation of a small quantity of the semi-solid from

$CHCl_3$-MeOH gave one of the compounds as white needle-like crystals. Flash chromatography of the mother liquor over silica gel gave the second compound as white platelets. TLC analysis showed that further extraction of the semi-solid material with Et_2O and MeOH yielded more of the first compound.

Identification of betulin



BETULIN

The polar compound isolated from the pet. spirit extract of the plant's bark, M^+ at *m/z* 442, gave a positive Liebermann-Burchard test, indicating it to be a triterpene. Under mild conditions it formed a monoacetate with Ac_2O, while under more vigorous conditions it formed a diacetate on treatment with Ac_2O and pyridine, which was indicative of a molecule having two hydroxylic groups.

The FT-IR of this compound (**Spectrum 11**) showed an OH stretching vibration at 3425 cm^{-1} which was evidence for the presence of an alcohol and in addition a C-O stretching vibration, typical of a primary OH group, appeared at 1032 cm^{-1} (Srivastava and Kulshreshtha, 1988; Nakanishi, 1974). The ^1H NMR spectrum of the compound showed two singlets at δ 2.68 and δ 3.32 ppm, these signals disappeared on addition of D_2O, thus confirming the presence of the two hydroxylic groups. Due to the location of these two OH signals the region of the spectrum in which they appeared was

made difficult to interpret accurately. There also appeared CH_2 and CH_3 stretching and bending vibrations at 2931, 1454 and 1372 cm^{-1} in the FT-IR spectrum. In addition, C=C absorptions appeared at 3060, 1642 and 879 cm^{-1} in the FT-IR of the isolated compound, which were removed by catalytic hydrogenation using Pd/C, thus indicating the presence of a sterically unhindered olefinic group. Interpretation of the ^1H and ^{13}C NMR spectra of the compound and its derivatives also provided the following information;

(a) The C-3 proton

The ^1H NMR spectrum showed a quartet-like signal at δ 3.20 ppm ($W_{1/2}$ = 16 Hz). The literature suggested that this signal was attributable to an α-methine proton in the axial orientation bonded at C-3. The ^1H NMR spectrum of the monoacetate showed the signal for the C-3 methine proton, approximating to a quartet, centred at δ 3.16 ppm ($W_{1/2}$ = 16 Hz). On formation of the diacetate (**Spectrum 12**) this multiplet underwent an acetylation shift and moved downfield to δ 4.40 ppm (Δδ = 1.2 approx.).

The formation of the diacetate was evidenced both by the appearance of two, three proton singlets, at δ 2.02 and δ 2.12 ppm in its ^1H NMR spectrum, and the presence of resonances for two methyl groups at δ 21.04 and δ 21.30 ppm with the signals for the corresponding carbonyls at δ 171.02 and δ 171.63 ppm in its ^{13}C NMR (**Spectrum 13**) (De Pascual Teresa *et al.*, 1987).

(b) The C-28 methylene protons

The ^1H NMR spectrum of the monoacetate showed a singlet at δ 2.04 ppm, assignable to the acetate methyl and an AB quartet, attributable to two protons, centred at δ 3.96, which was shifted downfield from its position at δ 3.36 in the spectrum of the original alcohol. The change in the chemical shift of the AB quartet (Δδ = 0.60 ppm) was att-

ributable to acetylation of this primary OH group and the quoted values are consistent with a -CH_2OH group at the C-28 position of the molecule (Patra *et al.*, 1988). The appearance of the AB splitting pattern supports the presence of the -CH_2OH group adjacent to a chiral centre, this fact was borne out by the values for the quartet's coupling constants (AB system, $J = 10$ Hz) which are typical (Patra *et al.*, 1988) of the coupling between geminal protons. That the methylene protons did not give a simple singlet as might be expected, was attributable to the fact that they were nonequivalent. They are said to be diastereotopic in that they are attached to a prochiral centre, which in turn is attached to a chiral centre and replacement of one of them with a different substituent would result in a pair of diastereoisomers.

(c) The olefinic methylene protons

The ^1H NMR spectrum for the diacetate, the monoacetate and the original compound showed a doublet-like signal centred at δ 4.50 ppm and was attributable to the terminal methylene of an olefinic group (Patra *et al.*, 1988). The ^{13}C NMR spectrum of the diacetate showing signals at δ 109.88 and δ 150.12 ppm were attributable to a terminal methylene carbon and a quaternary carbon atom, respectively (Patra *et al.*, 1988). The ^1H NMR spectrum of a hydrogenated derivative prepared using Pd/C, lacked a signal at δ 4.50 ppm.

(d) The methyl group protons

A singlet, attributable to a vinylic methyl group, showing some broadening due to an NOE, appeared at δ 1.62 in the ^1H NMR spectrum of the original compound, and its derivatives. The ^1H NMR also showed a set of singlets representing six methyl groups at δ 0.72, 0.78, 0.96, 1.00, 1.20 and 1.62 ppm, characteristic of the lupane series triterpenoids. A comparison of the ^{13}C NMR chemical shift values for the diacetate, with those recorded in the literature, enabled the six methyl groups to be identified as

occurring at the C-23, C-24, C-25, C-26, C-27 and C-30 positions in the molecule (Patra *et al.*, 1988). The chemical shift assignments for these carbons and all others in the compound are given in **Table 12.**

Table 12. ^{13}C NMR data for betulin diacetate

Carbon	δ ppm	Carbon	δ ppm	Carbon	δ ppm
1	38.33	11	20.76	21	29.69
2	23.66	12	25.10	22	34.09
3	80.88	13	37.51	23	27.90
4	37.76	14	42.64	24	16.46
5	55.33	15	27.01	25	16.12
6	18.13	16	29.53	26	15.99
7	34.52	17	46.26	27	14.69
8	40.85	18	48.73	28	62.78
9	50.25	19	47.68	29	109.88
10	37.02	20	150.12	30	19.06
				OCOMe	21.04
				OCOMe	21.30
				OCOMe	171.02
				OCOMe	171.63

The fragmentation pattern proposed for the EIMS of the isolated compound (**Spectrum 14**) is shown in **Fig. 13**;

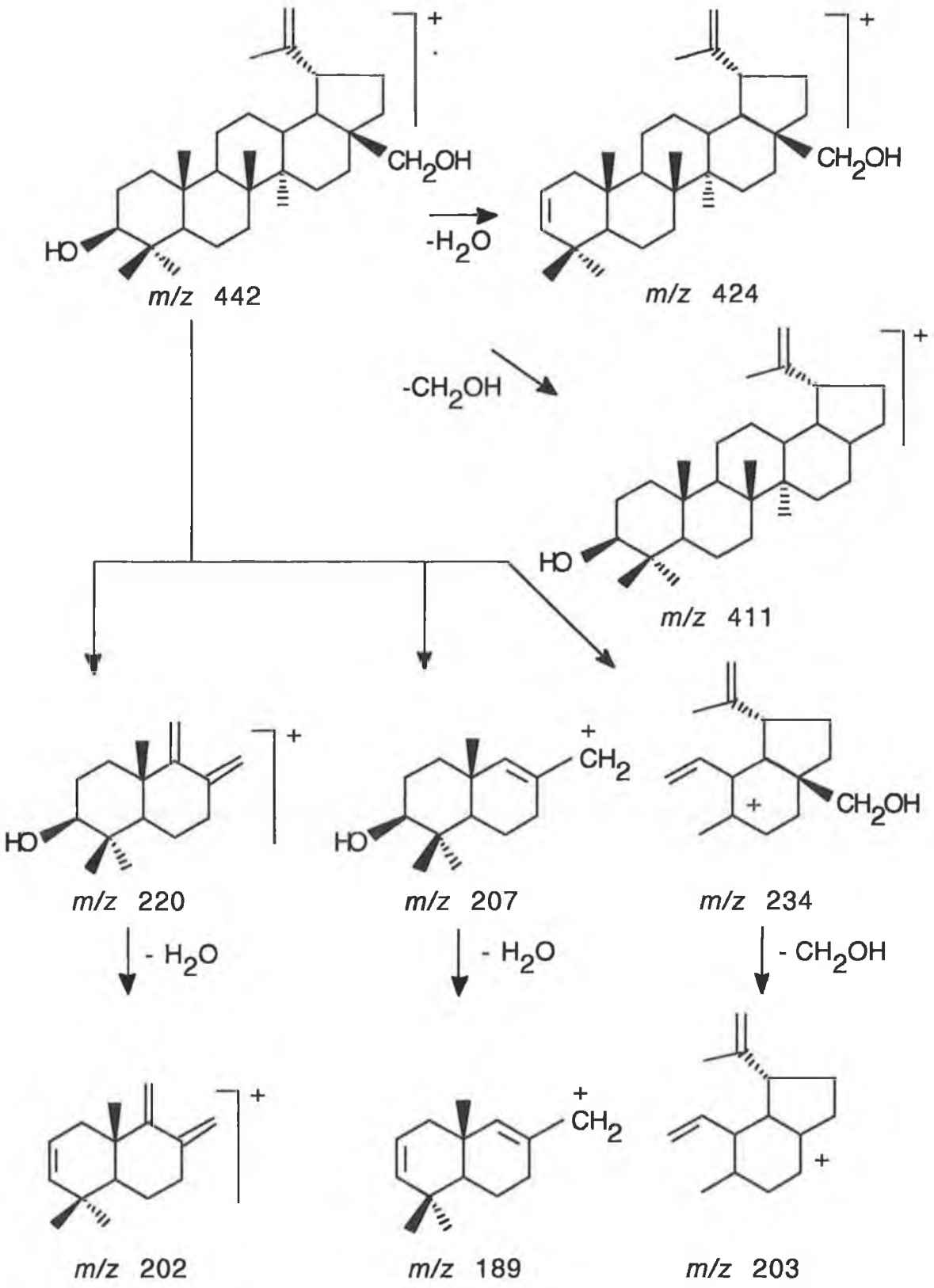
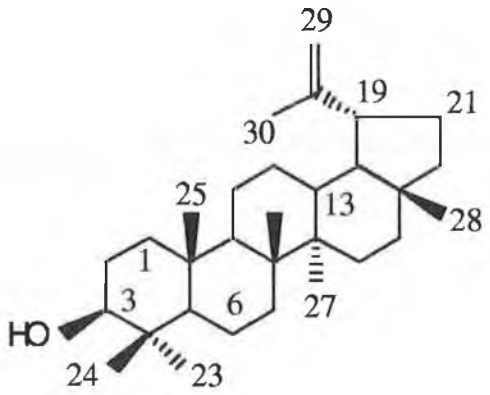

HO
CH2OH
-H2O
m/z 442
m/z 424
-CH2OH
m/z 411
m/z 220
m/z 207
m/z 234
- H2O
- H2O
- CH2OH
m/z 202
m/z 189
m/z 203

Fig. 13

The EIMS demonstrated significant peaks at *m/z* 189, 203, 207, 234, 411 and 424. These peaks are characteristic of a lupane series compound, with those at *m/z* 207 and 234 being particularly significant, as they are due to cleavage of the molecule at ring C (Patra *et al.*, 1988). Thus, the fragmentation pattern as shown, is in keeping with the proposition that the compound under investigation is in fact, betulin.

Examination of the spectra to-date, in addition to the well documented fact that all the *Betula* spp. so far investigated contain relatively high concentrations of the pentacyclic triterpene betulin, suggested that the compound isolated was betulin. The spectra of the compound and its derivatives were superimposable on those reported for an authentic sample of betulin and thus served to confirm its identity.

Identification of lupeol



LUPEOL

The FT-IR of the second compound (**Spectrum 15**) isolated showed absorptions at; 3358 cm^{-1} (OH), 2944, 1457 and 1380 cm^{-1} (CH_2's and CH_3's), 3060, 1637 and 880 cm^{-1} (C=C) and 1043 cm^{-1} (C-O). The FT-IR spectrum of the acetate prepared from Ac_2O and pyridine, showed an absence of an OH group. This spectrum showed absorptions at; 3060, 1637 and 877 cm^{-1} (C=C), 2944, 1457 and 1366 cm^{-1} (CH_2's

and CH_3's), 1733 cm^{-1} (acetate C=O) and 1248 cm^{-1} (acetate C-O). In addition to the FT-IR absorptions, the ^{13}C NMR of the acetate (**Spectrum 16**) confirmed its production by virtue of a signal which appeared at δ 80.97 ppm. This signal was typical of a C-3 atom having an acetate attached to it. In addition, acetyl signals were appearent in the ^{13}C NMR spectrum of the derivative at δ 21.32 and δ 171.04 ppm (Patra *et al.*, 1988).

The ^1H NMR spectrum of the compound showed a double doublet-like signal centred at δ 3.16 ($W_{1/2}$ = 16 Hz) which was attributable to an axial methine proton at C-3. The acetate's ^1H NMR spectrum (**Spectrum 17**) indicated that the C-3 methine proton had undergone an acetylation shift to δ 4.24 ppm (Δδ = 1.08) and that an AcO group signal had appeared at δ 1.98 ppm. These data were further evidence for the formation of the acetate derivative.

The ^1H NMR spectrum of the isolated compound and its acetate showed a triplet-like, two proton signal at δ 4.60 ppm attributable to a pair of terminal olefinic protons, similar to that found in the spectrum of betulin. Both spectra showed a sharp singlet in the region δ 1.62 to δ 1.66 ppm, which was due to a methyl group attached to an olefinic carbon atom. In addition, the ^{13}C NMR spectrum of the acetate exhibited signals at δ 150.98 and δ 109.35 ppm attributable to a terminal double bond (Marx Young *et al.*, 1997). On catalytic hydogenation of the alcohol using PtO_2 in EtOAc, the ^1H NMR signals attributable to the olefinic methylene protons and the methyl group attached to the olefinic carbon disappeared and were replaced by two new signals in the methyl region of the hydrogenated derivative's ^1H NMR spectrum at δ 0.74 and δ 0.78 ppm approx.

The presence of the vinylic methyl group singlet at δ 1.66 ppm in the ^1H NMR spectrum of the alcohol, showed some broadening due to an NOE. The assignment of

this ^{1}H NMR signal was further supported by the ^{13}C NMR spectrum of the acetate which showed a signal at δ 19.28 ppm, characteristic of a C-30 methyl group joined to an olefin (Marx Young *et al.*, 1997). Lastly, only six of the compound's seven methyl signals were clearly visible in the region δ 0.8 to 1.9 ppm of its ^{1}H NMR spectrum. The carbons of all seven methyl groups (C-23, C-24, C-25, C-26, C-27, C-28 and C-30) were, on comparison to values found in the literature, identifiable from the ^{13}C NMR spectrum of the compound's acetate (Marx Young *et al.*, 1997; Patra *et al.*, 1988). The chemical shift values for these signals and all others attributable to lupenyl acetate are listed in **Table 13.**

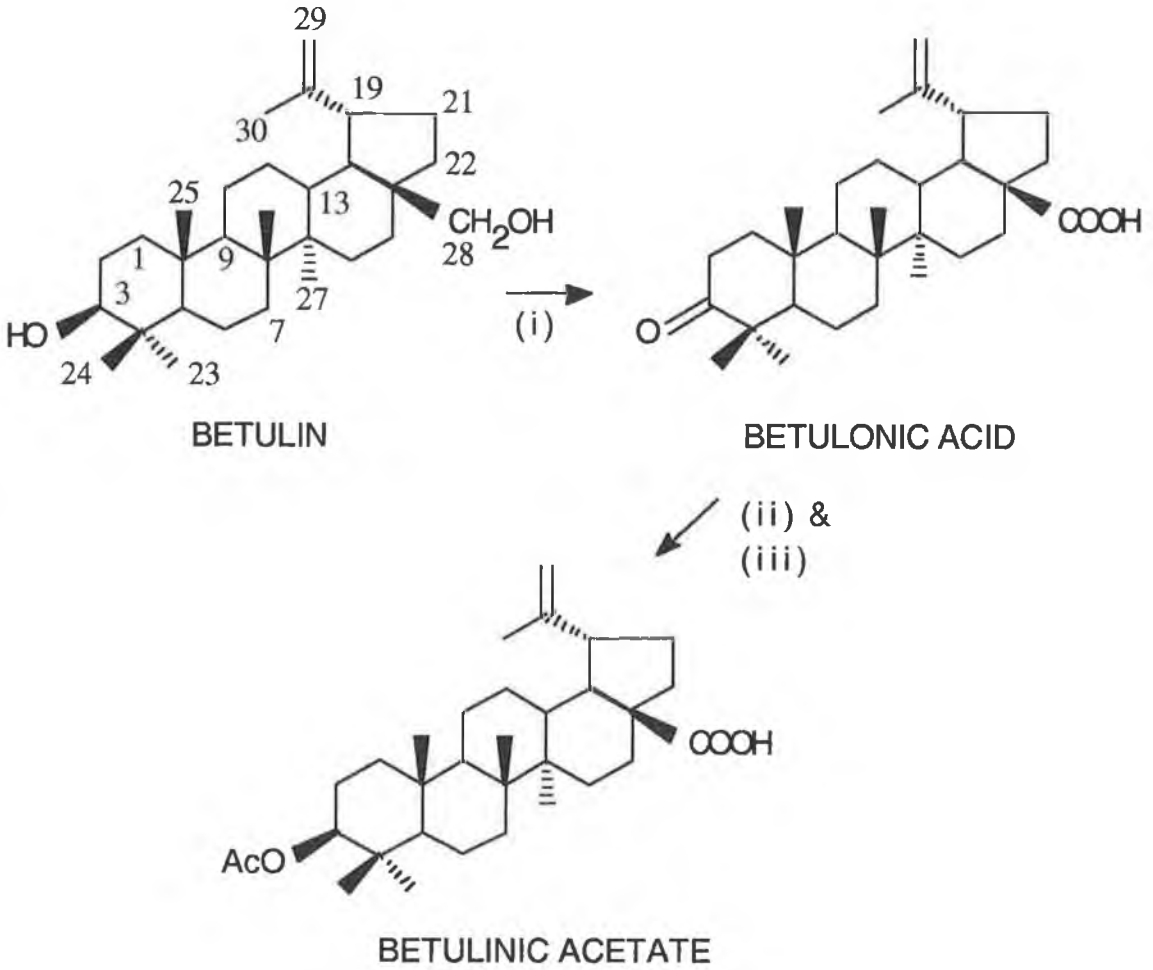
Table 13. ^{13}C NMR data for lupenyl acetate

Carbon	δ ppm	Carbon	δ ppm	Carbon	δ ppm
1	38.37	11	20.92	21	29.81
2	23.69	12	25.07	22	39.98
3	80.97	13	38.02	23	27.93
4	37.07	14	42.81	24	15.95
5	55.36	15	27.41	25	16.17
6	18.19	16	35.55	26	15.95
7	34.19	17	42.99	27	14.49
8	40.83	18	48.27	28	18.00
9	50.33	19	47.99	29	109.35
10	37.07	20	150.98	30	19.28
				OCOMe	21.32
				OCOMe	171.04

In summary, the ^{1}H NMR spectrum of this compound proved to be very similar to that

of betulin, with the exception of the AB quartet centred at δ 3.36 ppm attributable to the presence of a -CH_2OH group. This observation was further supported by the appearance of the C-28 methyl signal at δ 18.00 ppm in the ^{13}C NMR spectrum of the compound's acetate derivative, which in the corresponding betulin derivative could be expected to appear at approximately δ 62.8 ppm. Therefore, from the above data it can be inferred that the isolated compound was the lupane series triterpene, lupeol.

The conversion of betulin to betulinic acetate



BETULIN

BETULONIC ACID

BETULINIC ACETATE

Fig. 14

Reagents: (i) CrO_3 / H_2SO_4, (ii) $NaBH_4$ and (iii) Ac_2O / C_5H_5N.

Due to the economic importance and pharmacological efficacy of betulinic acid in the treatment of the skin disease, malignant melanoma, production of its acetate derivative was undertaken from betulin, isolated from *Betula pubescens*. The transformation of betulin to betulinic acetate was carried out as shown in the **Fig. 14**.

Betulin isolated from the plant was subjected to a Jones' Oxidation at a temperature of 0 ^{o}C. TLC analysis showed that this reaction yielded a mixture of compounds which included the keto-acid, betulonic acid. The reaction mixture was separated using Vacuum-Liquid Chromatography and the keto-acid was isolated in the earlier fractions. TLC analysis of the product showed it to be pure, a single spot appearing on the plate with an R_f higher than that for betulin. The FT-IR spectrum of this derivative showed absorptions characteristic of a COOH group (OH: between 3750 and 2367 cm^{-1}, C=O: at 1700 cm^{-1} and an acidic C-O stretch at 1241 cm^{-1}) as well as absorptions at 3069 cm^{-1} and 1641 cm^{-1} attributable to stretching vibrations of an olefinic group. In addition, a -CH out of plane bending vibration attributable to a terminal olefinic methylene group appeared at 882 cm^{-1}.

The ^{1}H NMR spectrum of the oxidation product showed a very broad singlet-like signal at δ 8.60 ppm attributable to an OH group, which disappeared on the subsequent addition of a few drops of D_2O. This data when considered in conjunction with a broad, distorted, doublet-like signal at δ 2.90 ppm which was attributable to a H-18 proton, and the evidence from the FT-IR in relation to the COOH absorptions, as discussed, indicates that the compound is in fact an acid. A doublet-like signal assignable to a terminal olefinic methylene group appeared at δ 4.68 and is in agreement with the FT-IR data relating to the presence of such a group in the molecule.

A further, important signal was apparent as a singlet at δ 2.20 ppm and was assignable to a C-3 keto-methylene (O=C-CH_2-) function (Srivastava and Kulshreshtha, 1988).

This assignment indicated that the compound, in addition to being an acid, was also a ketone in nature. The proposition that the compound possessed a keto group was further evidenced by virtue of the fact that the FT-IR spectrum lacked an absorption for a primary alcohol, C-O stretch at 1030 cm^{-1}. Six methyl group signals were observable in the derivative's ^1H NMR spectrum as follows; one at δ 1.70 ppm showing some broadening due to an NOE, one each at δ 0.96, δ 1.00 and δ 1.08 and two at δ 1.04 ppm. Thus, the above data suggests that the product formed, was the keto-acid derivative of betulin; betulonic acid.

The keto acid was then reduced with $NaBH_4$ in MeOH and the product acetylated with Ac_2O and pyridine yielding the monoacetate of betulinic acid. Chromatographic and spectroscopic evidence for the formation of this product is presented as follows; TLC analysis of the product showed a compound with an R_f slightly lower than that for betulin. The FT-IR (**Spectrum 18**) showed absorptions characteristic of a COOH (OH: between 3750 and 2364 cm^{-1}, and C=O: at 1700 cm^{-1}). An acetate C=O stretch was present at 1735 cm^{-1} and an acetate C-O stretch appeared at 1245 cm^{-1}. Olefinic stretching vibrations occurred at 3080, 1640 and 884 cm^{-1}, while CH_2 and CH_3 bending vibrations were evident at 1450, 1375 and 733 cm^{-1}.

The ^1H NMR (**Spectrum 19**) showed a multiplet centred at δ 4.30 ppm, assignable to a methine proton signal at C-3 (AcO-C-H) while in addition, an AcO group absorption occurred as a sharp singlet at δ 1.98 ppm. The ^{13}C NMR spectrum of the product exhibited signals characteristic of an acetate function at δ 21.31, δ 80.94 and δ 171.09 ppm as listed in **Table 14.** The spectroscopic data indicated that the compound was in fact a monoacetate.

A broad multiplet approximating to a quartet centred at δ 2.15 ppm due to a H-18 proton was also evident in the ^1H NMR spectrum (Shirasuna *et al.*, 1997). The ^{13}C

NMR spectrum showed a signal at δ 182.12 ppm, characteristic of an acid group positioned at C-28 (Patra *et al.*, 1988). These data in addition to the FT-IR COOH absorptions, previously mentioned, indicate that the product possessed an acid function.

A doublet-like signal at δ 4.60 ppm and a singlet at δ 1.65 ppm showing some broadening due to an NOE were, as previously discussed in the identifications of betulin and lupeol were attributable to an α-*iso*-propylene group. The ^{13}C NMR of the compound (**Spectrum 20**) also showed signals typical of the α-*iso*-propylene group, at δ 19.30, δ 109.72 and δ 150.35 ppm (Patra *et al.*, 1988). These data when considered in addition to the C=C stretching vibrations in the FT-IR spectrum, would suggest that the α-*iso*-propylene group, present at the C-19 position in the original betulin precursor, remained intact throughout the whole process of chemical transformation. The molecule's five other methyl group signals appeared in the usual region of the ^{1}H NMR spectrum, ranging from δ 0.80 to δ 1.00 ppm. The ^{13}C NMR spectrum on comparison with the data found in the literature, enabled these five methyl groups to be assigned to the following positions in the molecule; C-23, C-24, C-25, C-26 and C-27. The chemical shift values for these and all the other signals in the compound are provided in **Table 14.**

Table 14. ^{13}C NMR data for betulinic acetate

Carbon	δ ppm	Carbon	δ ppm	Carbon	δ ppm
1	38.37	11	20.81	21	29.64
2	23.64	12	25.37	22	37.03
3	80.94	13	38.37	23	27.90

Table 14 contd. ^{13}C NMR data for betulinic acetate

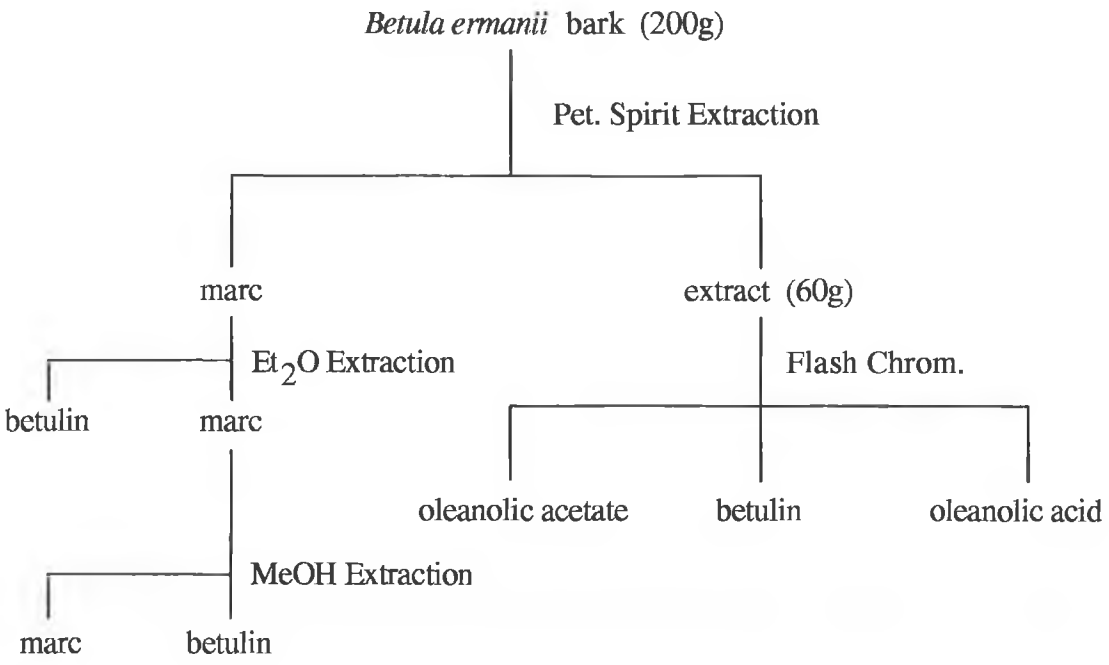
Carbon	δ ppm	Carbon	δ ppm	Carbon	δ ppm
4	37.75	14	42.38	24	16.42
5	55.37	15	30.53	25	16.12
6	18.12	16	32.10	26	15.99
7	34.17	17	56.36	27	14.61
8	40.64	18	46.90	28	182.12
9	50.35	19	49.20	29	109.72
10	37.75	20	150.35	30	19.30
				OCOMe	21.31
				OCOMe	171.09

In conclusion, the structural analysis as discussed above, would suggest that the compound formed was the monoacetate of betulinic acid.

Isolation of oleanolic acetate, betulin and oleanolic acid

Air-dried bark was powdered and successively extracted with petroleum spirit, Et_2O and MeOH in accordance with the scheme outlined below;

Betula ermanii extraction procedure

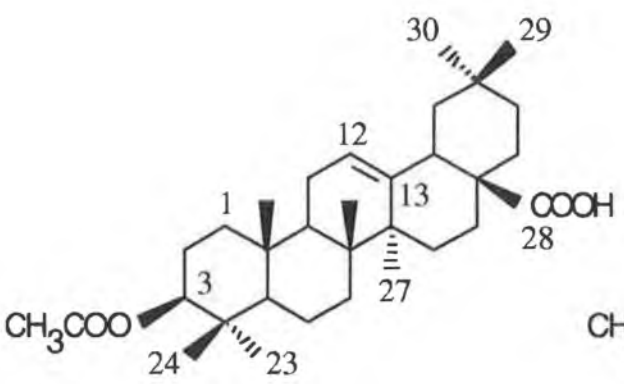


Evaporation of the petroleum spirit extract gave a large quantity of cream crystals. TLC analysis of these crystals showed them to consist of three major compounds which were separated by flash chromatography over silica gel. The Et_2O and MeOH extracts on recrystallisation from $CHCl_3$-MeOH (1:1) produced crops of white crystals. These crystals were shown by TLC analysis to be betulin.

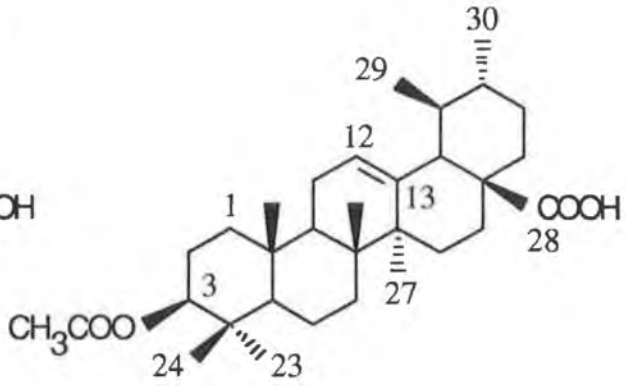
Identification of oleanolic acetate

The least polar compound isolated from the petroleum spirit extract gave a blue colour in the Libermann-Burchard test indicative of a triterpene or a steriod. The FT-IR

(**Spectrum 21**) showed absorptions characteristic of a C=C at 1681 and 817 cm^{-1}, an



ACETYLOLEANOLIC ACID



ACETYLURSOLIC ACID

ester function at 1728 and 1251 cm^{-1}, and a carboxylic acid group at 1710 and a broad peak between 3750 and 2725 cm^{-1}. The compound was readily hydrolysed by refluxing with NaOH and it formed a methyl ester on reaction with CH_2N_2. Attempts to hydrogenate the compound with Pt or Pd/C were unsuccessful indicating the sterically hindered nature of the olefinic group.

The ^{13}C NMR DEPT experiment of the methyl ester (**Spectra 22** and **23**) identified nine methyl groups including a methoxy, ten methylenes, five methines, five quaternary carbons, two carbons of a trisubstituted double bond, and two carbonyl carbons. The ^{1}H NMR spectrum (400 MHz) of the methyl ester (**Spectrum 24**) showed seven singlets for seven tertiary methyl groups typical of a pentacyclic triterpene of the Δ^{12} oleanene series. (In the 60 MHz ^{1}H NMR spectrum, which was used in the initial studies of this compound, overlap of some of these signals did not allow their unambiguous identification.)

The ester function was identified as an acetate by the singlet at δ 1.98 in the ^{1}H NMR and signals at δ 21.27 and δ 171.07 ppm in the ^{13}C NMR spectrum. A distorted triplet at δ 4.40 ($W_{1/2}$ = 16 Hz) in both the 60 and 400 MHz spectra shifted upfield to δ 3.06

in the hydrolysis product, was typical of an acetoxymethine proton at C-3. As has been discussed previously when dealing with the structure of α-amyrin, the coupling constants and the $W_{1/2}$ values are indicative of an axial orientation for the methine proton. The resonance for an olefinic proton occurred at δ 5.16 in the ^{1}H NMR, and as previously mentioned when discussing the structure of α-amyrin, this value was characteristic of the trisubstituted double bond of both the Δ^{12} oleanene and Δ^{12} ursene series of triterpenes (Kojima and Ogura, 1989) and does not allow a distinction to be drawn between these two types of compounds.

However, the two series of compounds can be readily distinguished by means of the ^{13}C NMR spectra. The Δ^{12} ursenes show the signals for C-12 and C-13 at ~ δ 125 and δ 138 ppm respectively. The C-12 is deshielded by 2 ppm and the C-13 is shielded by 5 ppm relative to the corresponding in the Δ^{12} oleanenes which occur at ~ δ 122 and δ 143. The difference in the shift values for the two series of compounds is attributed to the fact that the β-methyl group attached to the C-19 atom in the ursenes is in close proximity to the olefinic group; γ and δ to C-13 and C-12, respectively (Mahato and Kundu, 1994). The olefinic carbons of the methyl ester of the acetyloleanolic acid isolated from the birch resonated at δ 122.20 and δ 143.84 hence confirming the compound to belong to the Δ^{12} oleanene series.

The carboxylic acid group was placed at C-17 on the basis of the H-18 resonance which gave an ABX double doublet (J = 4 and 12 Hz) at δ 2.76 ppm. This signal also distinguishes the oleanolic acid derivatives from the isomeric ursolic acid compounds. In the ursolic acid derivatives, which have only one proton at C-19, the H-18 shows as a doublet at ~ δ 2.2 ppm. Isomeric mixtures of ursolic and oleanolic acid derivatives often occur together in nature, and the problem of their separation has so far not been resolved. (Both the acids themselves and their derivatives show identical chromatographic behaviour.) Since both of these signals occur separate from the other

resonances in the spectra, they are a useful indicator of the presence of mixtures of these compounds in plant extracts.

Two series of oleanolic acid derivatives are recognised on the basis of the configuration of the H-18. This proton can be either axial (β) or equatorial (α) with the most common compounds belonging to the 18β-series. Inspection of the ^{13}C NMR spectrum readily allows a distinction to be made between the two groups. The geometry of the D/E ring junction causes little change in the shielding of the carbons in the A and B rings. In ring C, C-12 and C-13 of the 18α-series undergo upfield shifts of approximately 5 and 2.5 ppm, respectively, with respect to the corresponding carbons of the 18β-series. The upfield shift of the C-12 is attributed to the strong steric interaction with the C-19. In ring D, C-16 exhibits a very significant downfield shift of 11 ppm caused by the absence of two γ-gauche interactions with C-9 and C-21 in the triterpenes of the 18α-series. This shielding effect is due to the two γ-gauche interactions with the axial hydrogens at C-19 and C-20 (Mahato and Kundu, 1994).

An unusual feature of the formation of the acetate was the formation of a relatively stable anhydride, on treatment of the oleanolic acid with a mixture of Ac_2O and pyridine, as indicated by the FT-IR spectrum of this derivative. The presence of strong, characteristic C=O absorptions at 1734 and 1813 cm^{-1}, in addition to a C-O absorption at 1245 cm^{-1} confirmed the production of the anhydride. Anhydrides give rise to two bands in the carbonyl region of the FT-IR spectrum, due to vibrational coupling of the two C=O groups (Smalley and Wakefield, 1997; Nakanishi, 1974b). The ^{1}H NMR spectrum of this derivative indicated the presence of a CH_3-CO-O-CO- group at δ 2.19 ppm (sharp singlet) (Richards, 1988b; Nakanishi, 1974c) and an AcO group at δ 2.00 ppm (sharp singlet). These signals were further confirmation for the production of the anhydride.

A comparison of the ^{13}C NMR spectrum of the methyl ester of the compound (**Table 15**) with that reported in the literature for the methyl ester of acetyloleanolic acid (Mahato and Kundu, 1994) confirmed the identity of the isolated compound.

Table 15. ^{13}C NMR DEPT data for methyl oleanolate acetate

Carbon	DEPT	δ ppm	Carbon	DEPT	δ ppm	Carbon	DEPT	δ ppm
1	CH_2	38.14	11	CH_2	23.05	21	CH_2	33.84
2	CH_2	23.51	12	CH	122.20	22	CH_2	32.51
3	CH	80.85	13		143.84	23	CH_3	27.97
4		36.95	14		41.66	24	CH_3	16.63
5	CH	55.23	15	CH_2	27.57	25	CH_3	15.30
6	CH_2	18.19	16	CH_2	23.42	26	CH_3	16.76
7	CH_2	32.62	17		46.74	27	CH_3	25.83
8		39.30	18	CH	41.21	28		178.34
9	CH	47.48	19	CH_2	45.80	29	CH_3	33.05
10		36.95	20		30.73	30	CH_3	23.57
						OCOMe		21.27
						OCOMe		171.07
						COOMe		51.48

The EIMS of the methyl ester (**Spectrum 25**) was also in agreement with this deduction. As well as the expected molecular ion at *m/z* 512, diagnostically significant peaks were observed at *m/z* 452 [M^+ - CH_3COOH], *m/z* 249 and *m/z* 262. The latter peak is due to the retro-Diels-Alder reaction in C; the charge being retained by the diene. The proposed fragmentation pattern is given in **Fig. 15**;

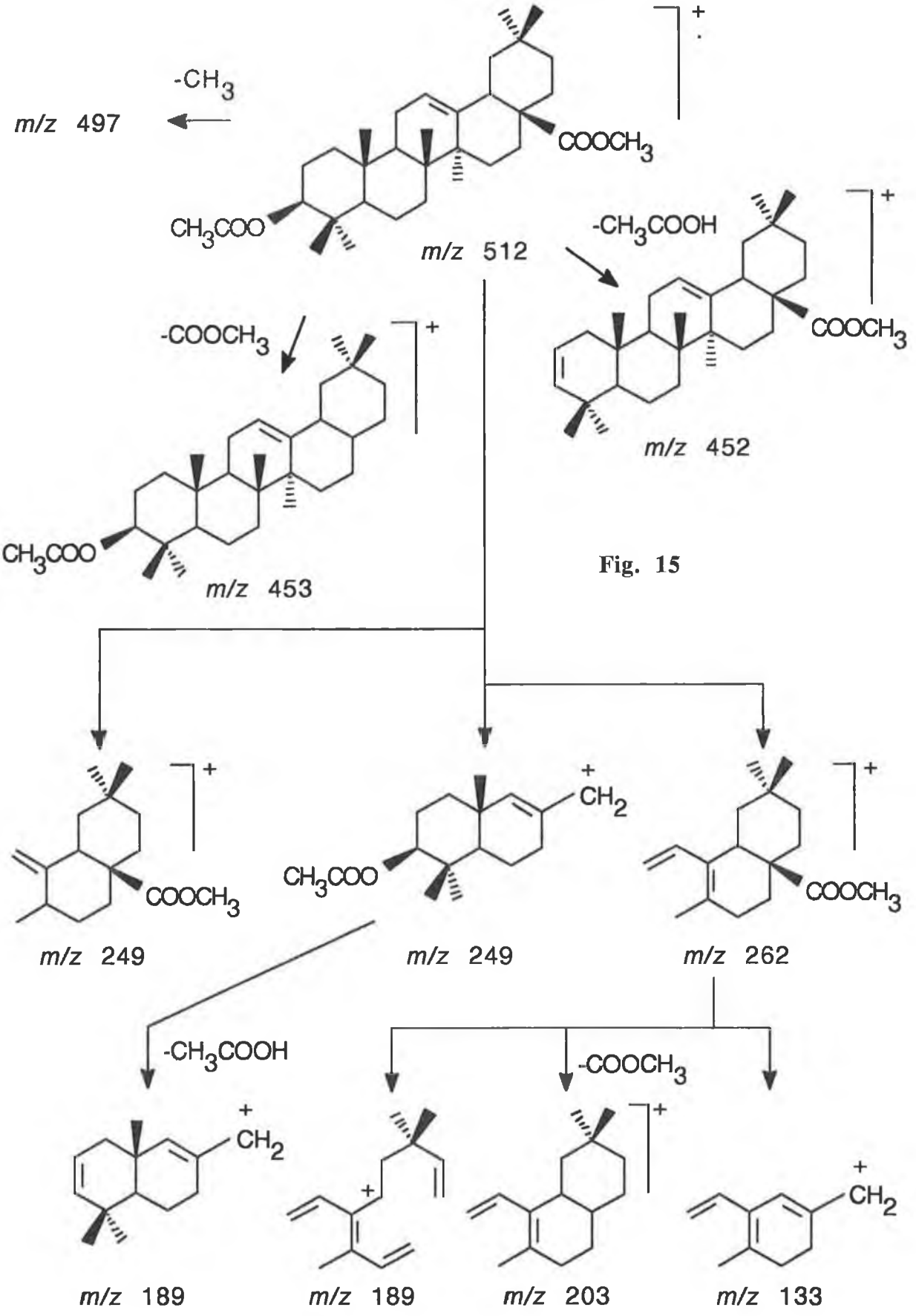
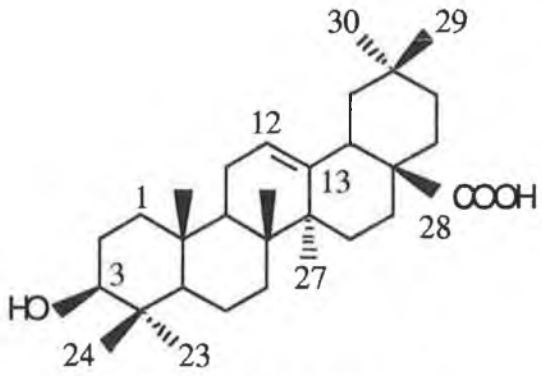

-CH3
m/z 497
COOCH3
CH3COO
m/z 512
-CH3COOH
COOCH3
m/z 452
-COOCH3
CH3COO
m/z 453
COOCH3
m/z 249
CH2
CH3COO
m/z 249
COOCH3
m/z 262
-CH3COOH
CH2
m/z 189
-COOCH3
m/z 189
m/z 203
CH2
m/z 133

Fig. 15

Identification of betulin

The moderately polar compound on isolation from the pet. spirit extract of the bark produced chromatographic and spectroscopic data identical to that reported for betulin, previously isolated from *Betula pubescens.*

Identification of oleanolic acid



OLEANOLIC ACID

The polar compound isolated from the pet. spirit extract produced a spot on TLC which had an R_f identical to that produced by oleanolic acid obtained from the hydrolysis of oleanolic acetate. The FT-IR spectrum of the compound showed stretching vibrations for an OH group at 3356 cm^{-1}, a C=O at 1695 cm^{-1} and a C-O at 1261 cm^{-1}. This compound was methylated using CH_2N_2, and the resulting derivative gave an R_f identical to that of the methyl ester of oleanolic acid.

The ^1H NMR spectrum of the methylation product was the same as that for methyl oleanolate, it showed a broad signal approximating to a triplet centred at δ 5.12 ($W_{1/2}$ = 6 Hz) attributable to the proton of a trisubstituted olefinic group at the C-12 position. A sharp singlet appeared at δ 3.52 ppm assignable to a methoxyl group and a multiplet approximating to a quartet centred at δ 3.22 approx. ($W_{1/2}$ = 20 Hz) was due to an axial methine proton.

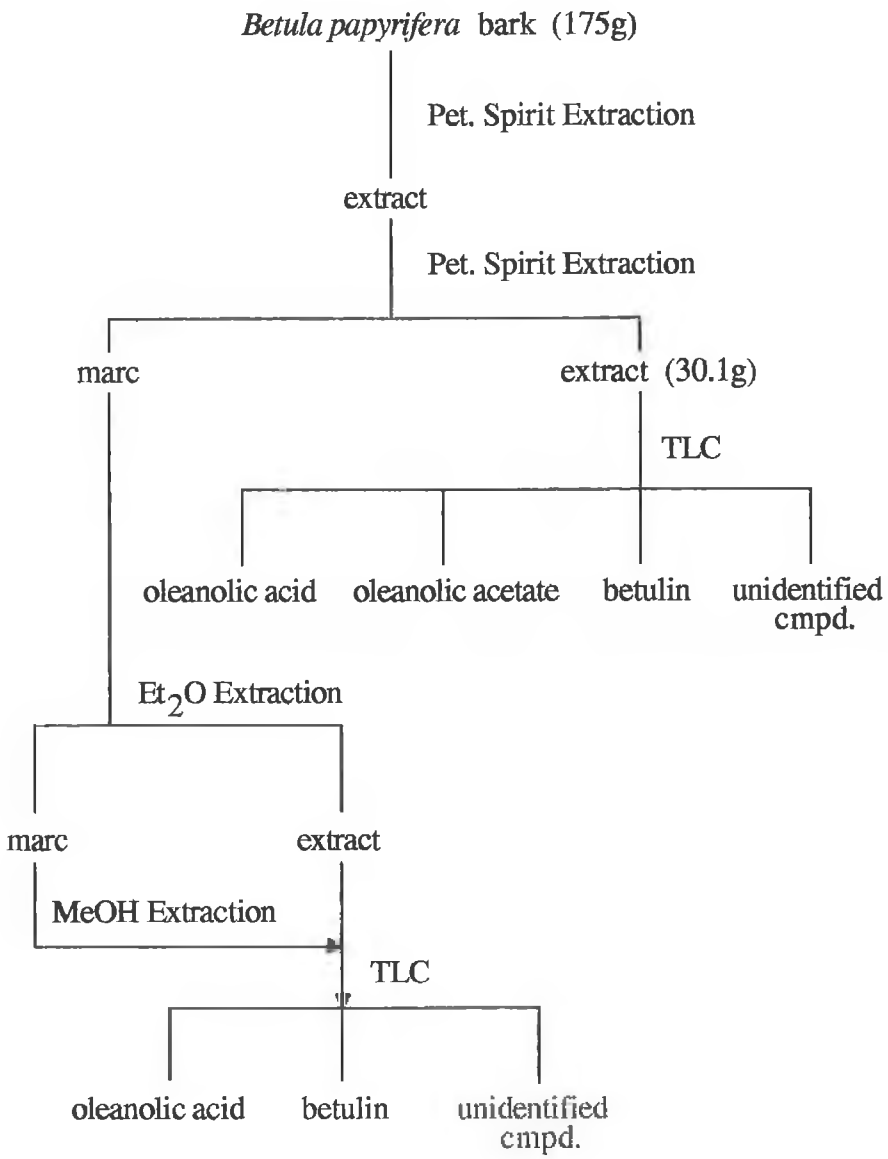
Centred at δ 2.62 ppm and overlapping with the δ 3.22 signal, was a multiplet approximating to a triplet, which was attributable to the diagnostically significant C-18 proton of a triterpene belonging to the oleanane series. Methyl proton signals typical of the oleanane series occurred at δ 1.10 (Me-27), δ 0.96 (Me-25), δ 0.88 (Me's-23, 29 and 30), δ 0.76 (Me-24) and δ 0.72 ppm (Me-26) (Ageta *et al.*, 1974; Karliner and Djerassi, 1966). The methyl ester was acetylated using Ac_2O and pyridine, the ^1H NMR and ^{13}C NMR spectra of this derivative were identical to that of methyl oleanolate acetate, previously discussed. These data indicate the compound to be oleanolic acid.

Betula papyrifera

Isolation of oleanolic acid, oleanolic acetate and betulin

Bark from this species was air-dried, powdered and successively extracted with petroleum spirit, Et_2O and MeOH according to the scheme outlined below;

***Betula papyrifera* extraction procedure**



Evaporation of the petroleum spirit extract gave a sticky brown ppt. which was further extracted with petroleum spirit. On evaporation of the solvent from this extraction a

crop of white crystals resulted. TLC analysis of these crystals showed them to consist of a mixture compounds. Three of the compounds had the same R_f's as oleanolic acid, oleanolic acetate and betulin, respectively. A fourth unidentified compound was also present. This mixture was separated by means of flash chromatography over silica gel which gave the individual compounds as white crystals.

Identification of oleanolic acid, oleanolic acetate and betulin

The identity of the first three compounds were confirmed by virtue of the fact that their ^{1}H NMR and FT-IR spectra were identical to and superimposable on those for oleanolic acid, oleanolic acetate and betulin, previously isolated and identified from the other *Betula* spp examined.

Subsequent extraction of the marc from the petroleum spirit extraction led to the isolation of further quantities of oleanolic acid, betulin and the unidentified compound. Again the R_f's, ^{1}H NMR and FT-IR of these compounds were in agreement with those for samples of these compounds previously isolated and identified.

EXPERIMENTAL

Plant Material

Outer bark from the common holly species (*Ilex aquifolium*) and a species of native birch (*Betula pubescens*) were collected in early Autumn from trees growing on limestone soil in the region of the Eastern bank of Lough Corrib, Co. Galway. In addition outer bark from exotic birch species (*Betula ermanii* and *Betula papyrifera*) were gathered at the same time of year from the woodlands of Florencecourt Estate, Co. Fermanagh. All the plant material used in this study was identified by the Life Sciences Department of the Galway-Mayo Institute of Technology.

Analytical Data

^{1}H NMR spectra were recorded at 60 MHz on a Jeol PMX 60 SI spectrophotometer and at 400 MHz on a Joel JNM-LA400 FT-NMR System in $CDCl_3$ or $CDCl_3$-DMSO using TMS as an internal standard. **^{13}C NMR spectra** were recorded at 400 MHz on a Joel JNM-LA400 FT-NMR System in $CDCl_3$ using TMS as an internal standard. ^{1}H and ^{13}C Chemical shifts were measured in δ (ppm) downfield from TMS. ^{13}C NMR spectra were used to determine carbon atom type by employing a combination of broad band decoupled and DEPT experiments.

FT-IR spectra were recorded in KBr discs or neat between NaCl plates using a Perkin Elmer 1600 Series FTIR spectrophotometer. Direct probe EIMS were measured on a Kratos Profile HV-4 double focusing, high resolution mass spectrophotometer. **GC/MS** and **GC** samples were run in *iso*-hexane (High Purity Grade) on a Varian Saturn GC/MS/MS 4D (column: Rtx-1, 30 m x 0.25 mm ID x 0.25µm df), and a Varian 3400 gas-chromatograph (column: DB-WAX, 30 m x 0.53 mm ID x 1 µm df, detector: FID, carrier gas: He and flow rate: 30 $cm^3.min^{-1}$), respectively. All gas chromatography injections were performed with a SuperfleX 10 µl glass syringe.

TLC analysis was performed using silica gel HF_{254} plates, visualisation was achiev-

ed with 10% aqueous H_2SO_4 and heating in the case of triterpenoid analysis. Unsaturated FAME-TLC analysis was performed using argentated silica gel HF_{254} plates which were developed in chromatography tanks wrapped in black polythene in order to exclude light, then sprayed with 2,7-dichlorofluorescein stain and visualised under UV light. TLC analysis of FFA mixtures was carried out using silica gel HF_{254} plates followed by visualisation with bromocresol green stain or the vapours from I_2 crystals.

Flash chromatography was carried out in chromatography columns (150 mm x 25 mm ID) packed with silica gel (Merck, grade 9385, 230-400 mesh, 60 Angstrom) slurries which were compacted by tapping the side of the column as solvent flowed through. Similar chromatography columns, wrapped in black polythene in order to exclude light, were used for argentation flash chromatography. Layers of acid washed silver sand and 25 mm diameter filter papers were used to protect the top and bottom of all packed columns.

Vacuum-liquid chromatography was carried out in a sintered glass funnel packed with a slurry of silica gel (TLC grade), which was protected top and bottom with filter paper. The sample to be eluted was mixed with a 'Celite 521' slurry which was subsequently rotary evaporated in order to homogenously coat the surface of the celite particles with the sample. This sample impregnated celite was then evenly applied over the surface of the filter paper, protecting the top of the silica gel stationary phase in the glass funnel. In turn, a protecting filter paper and covering of acid-washed silver sand were placed on top of the celite-sample layer. Elution of the sample components was performed under low-suction.

The Liebermann-Burchard test, which is a colourimetric test for steroids and triterpenoids, was employed as follows; a solution of the test sample was prepared in

$CHCl_3$ and treated with a few drops of H_2SO_4 conc. and Ac_2O. The presence of triterpenoid is indicated by the production of a red-green or violet colour in the test tube.

Extraction of *Ilex aquifolium* bark

Petroleum spirit extraction

Bark collected in early October was air-dried and powdered in a Waring blender. The dried bark (600 g) was extracted with petroleum spirit (boiling fraction: 40 - 60 °C) for approximately 12 hrs. in a Soxhlet apparatus, giving a yellowish solution. The soln. was filtered, concentrated to 100 cm^3 and, on standing overnight at ambient temperature, a cream coloured ppt. formed. This was filtered off, dried, weighed (60 g) and analysed by TLC which showed it to be impure. The filtrate produced a red oil (76 g) on evaporation of the petroleum spirit to dryness. TLC of this oil showed a major non-polar compound as well as traces of minor constituents.

FT-IR ν_{max}^{NaCl} cm^{-1}: 3000-2850 (C-H stretching), 1731 (C=O), 1500-1300 (C-H bending) and 1172 (C-O);

^{1}H NMR (60 MHz, $CDCl_3$): δ 0.80, 0.84, 0.98, 1.00 and 1.06 (3H, s, 5 x Me), 0.88 (9H, br s, 3 x Me), 1.26 (br s, $-(CH_2)_n-$), 2.18 (2H, d, CH_2COO-), 4.42 (1H, t, $W_{1/2}$ = 14 Hz, H-COOC-), 5.12 (1H, m, $W_{1/2}$ = 8 Hz, H-12) and 5.24 (2H, t, $W_{1/2}$ = 8.5 Hz, -CH=CH-).

Et_2O extraction

The bark residue was extracted with Et_2O, producing a green coloured extract which was rotary evaporated and analysed by TLC. This analysis showed the green coloured material to be impure. The extract was recrystallised from MeOH and it yielded a white

ppt., ^{1}H NMR and FT-IR were recorded. The residue was triturated with petroleum spirit, a semi-solid formed and was recrystallised from EtOAc. On cooling in an ice-bath, the recrystallisation produced a gelatinous substance, this was reheated and cooled slowly and a white ppt. formed which was collected and dried.

MeOH extraction

The marc was then extracted with MeOH producing a colourless solution in the Soxhlet extractor, on standing a fine cream coloured ppt. formed. The solvent was subsequently rotary evaporated yielding a mixture of a yellowish powder and a green coloured resin. Subsequent washing with Me_2CO removed some of the green coloured material. TLC, FT-IR and ^{1}H NMR analysis showed this material to be composed of saponins and sugars.

Flash chromatography

A flash chromatography column (150 mm x 25 mm ID) was packed with a silica gel-petroleum spirit slurry. The red oil from the petroleum spirit extract (3.6 g) was dissolved in 10 cm^3 of an EtOAc-petroleum spirit (3:7) mixture and was eluted with petroleum spirit as solvent system. This produced oily fractions which were analysed by TLC, using EtOAc-petroleum spirit (1:9) and toluene-petroleum spirit (3:17) as mobile phases. This analysis showed the presence of a major non-polar substance as a pure compound in fractions 1 to 5.

Saponification of the purified red oil

The purified red oil (0.2 g) from the column chromatography was dissolved in MeOH (25 cm^3) and KOH (0.6 g) was added. This reaction mixture was refluxed for 1 hr. The oil was not very soluble in the MeOH, therefore absolute EtOH (20 cm^3) was added with more KOH (0.6 g). The mixture was refluxed for a further 6 hrs. and the product was analysed by TLC. H_2O was added to the reaction mixture and a white ppt.

of α-amyrin (0.1 g), melting point 184 - 186 °C, formed. The α-amyrin ppt. was analysed by TLC using CH_2Cl_2-petroleum spirit (1:2) as mobile phase, this analysis showed the compound to be pure.

EIMS (probe), *m/z* (rel. int.): 426 $[M]^+$, 411 $[M-CH_3]^+$, 408 $[M-H_2O]^+$, 218, 207, 203, 189 and 133.

FT-IR ν_{max}^{KBr} cm^{-1}: 3600-3070 (OH), 3000-2850 (C-H stretching), 1654 (C=C), 1500-1320 (C-H bending) and 880 (C=C);

^{1}H NMR (60 MHz, $CDCl_3$): δ 0.80 (9H, s, 3 x Me), 0.84, 0.92, 1.00, 1.04 and 1.10 (each 3H, s, 5 x Me), 3.16 (1H, m, $W_{1/2}$ = 16 Hz, H-C-OH) and 5.12 (1H, t, $W_{1/2}$ = 4 Hz, H-12).

The mother liquor was acidified with HCl dil. and a colourless oil layer formed. This layer was extracted into Et_2O which was evaporated and the resulting oil was analysed by TLC using CH_3CN-Me_2CO (1:4) as mobile phase. The oil was subsequently shown to be a mixture of saturated and unsaturated fatty acids by FT-IR, ^{1}H NMR and GC/MS.

FT-IR ν_{max}^{NaCl} cm^{-1}: 3625-2400 (OH), 3000-2850 (C-H stretching), 1706 (C=O stretching), 1500-1400 (C-H bending), 1295 (C-O), 937 (OH deformation) and 714 and 667 (*cis* C=C);

^{1}H NMR (60 MHz, $CDCl_3$): δ 0.86 (3H, distorted t, $Me(CH_2)_n$-), 1.24 (br s, $-(CH_2)_n$-), 2.00 (4H, m, $-CH_2$ C=C CH_2-), 2.24 (2H, d, $-CH_2$ C00-) and 5.28 (2H, t, -CH=CH-).

Methylation of the free fatty acids

1. Preparation of nitrosomethylurea

Acetamide (11.8 g) was dissolved in Br_2 liquid (17.6 g) and 25% aqueous NaOH (32 cm^3) was added to this solution. The mixture was heated on a steam-bath until it effervesced and then heating was continued for a further 3 mins. The soln. changed colour from yellow to red and white crystals then formed. The soln. was cooled in an ice-bath for 1 hr. and the product was filtered, washed with H_2O and dried. The product (acetylmethylurea) (7.92 g) was dissolved in HCl conc. (10 cm^3) and heated on a steam-bath for 12 mins. H_2O (10 cm^3) was added to the mixture, which was cooled below 10 ^oC in an ice-bath and 76% w/v $NaNO_2$ soln. (10 cm^3) was added dropwise to the cooled mixture. On standing in the ice-bath for a further 5 mins. nitrosomethylurea precipitated from soln. It was washed with cold H_2O, filtered, and dried in an evacuated desiccator.

2. Preparation of diazomethane

50% aqueous KOH (2 cm^3) and Et_2O (9 cm^3) were mixed and cooled to 5 ^oC in an ice-bath, nitrosomethylurea (0.3 g) was added and the mixture was distilled, producing a solution of CH_2N_2 in cooled Et_2O.

3. Methylation of the free fatty acids using diazomethane

The free fatty acid mixture (0.3 g) was dissolved in Et_2O (10 cm^3), cooled in an ice-bath and treated with a solution of CH_2N_2 in Et_2O. TLC analysis was used to follow the course of the reaction. On completion the reaction mixture was refrigerated for 8 hrs. and then the Et_2O was evaporated. The mixture was dissolved in *iso*-hexane and GC/MS analyses were carried out.

Silver ion chromatography of the FAME mixture

Silica gel (100 g) and $AgNO_3$ (10 g) were mixed together in CH_3CN (100 cm^3). This

mixture was then rotary evaporated to remove the CH_3CN solvent. A chromatography column (150 mm x 25 mm ID), wrapped in black polythene to exclude light was packed with an EtOAc-hexane slurry of the argentated silica gel. The FAME mixture (0.5 g) isolated from the petroleum spirit extract, was carefully pipetted onto the column and eluted under air-pressure of 2 $kg.cm^{-2}$ with EtOAc-hexane (1:19). 20 x 25 cm^3 fractions were collected and analysed by TLC on argentated silica gel HF_{254} plates using MeOH-$CHCl_3$ (1:9) as mobile phase. This analysis showed that fractions 2 to 8 contained saturated FAME's, while fractions 10 to 15 contained unsaturated FAME's.

GC/MS

GC conditions

Injector temp. 260 °, transfer line 260 °, initial temp. 110 °, ramp rate 10 $°.min^{-1}$ and final temp. 210 °C.

MS *m/z* (rel. int.)

Methyl decanoate : 186 $[M]^+$, 167 $[M-H_20]^+$, 143 $[M-Me(CH_2)_2]^+$, 112, 74, 43, 32 and 28,

Methyl myristate: 242 $[M]^+$, 211 $[M-OMe]^+$, 199 $[M-Me(CH_2)_2]^+$, 185, 171, 157, 143, 129, 115, 101, 87 and 74,

Methyl pentadecanoate : 256 $[M]^+$, 225 $[M-OMe]^+$, 213 $[M-Me\ (CH_2)_2]^+$, 157, 143, 129, 115, 101, 74, 55, and 41,

Methyl palmitate : 270 $[M]^+$, 239 $[M-OMe]^+$, 227 $[M-Me(CH_2)_2]^+$, 199, 185, 171, 157, 143, 129, 115, 101, 87, 74 and 43,

Methyl heptadectrienoate : 278 [M]+, 223 $[M-MeCH_2CH=CH-]^+$, 205 [M-MeOOC-

CH_2-]$^+$, 149 [M-MeOOC-$(CH_2)_5$-]$^+$, 121, 74, 55, 32 and 28,

Methyl heptadecanoate : 284 [M]$^+$, 253 [M-OMe]$^+$, 241 [M-Me$(CH_2)_2$]$^+$, 227, 213, 199, 185, 171, 157, 143, 129, 115, 101, 87, 74 and 43,

Methyl linoleate : 294 [M]+, 263 [M-MeO]$^+$, 234 [M-MeCOOH]$^+$, 220 [M-MeCOOMe]$^+$, 74, 59, 43, 32 and 28,

Methyl oleate : 296 [M]$^+$, 281 [M-Me]$^+$, 265 [M-OMe]$^+$, 264 [M-MeOH]$^+$, 246, 222, 207, 193, 96, 74 and 43,

Methyl stearate : 298 [M]$^+$, 267 [M-OMe]$^+$, 255 [M-Me$(CH_2)_2$]$^+$, 241, 227, 213, 199, 185, 171, 157, 143, 129, 101, 87, 74 and 43.

Acetylation of α-amyrin

The α-amyrin (0.2 g) resulting from the alkaline hydrolysis of the α-amyrin fatty acid esters was collected and dried. It was dissolved in Ac_2O (3 cm^3) and pyridine (3 cm^3) and the reaction mixture was left to stand at ambient temperature for 36 hrs. The mixture was then poured onto crushed ice and the resulting white ppt. was filtered off and dried. The product was analysed by TLC using EtOAc-petroleum spirit (3:7) as mobile phase, which showed it to be pure.

EIMS (probe), *m/z* (rel. int.): 468 [M]$^+$, 453 [M-CH_3]$^+$, 408 [M-CH_3COOH]$^+$, 249, 218, 203, 189 and 133.

FT-IR ν_{max}^{KBr} cm^{-1}: 3000-2850 (C-H stretching), 1735 (C=O), 1500-1325 (C-H bending), 1244 (C-O) and 826 (C=C);

^{1}H NMR (60 MHz, $CDCl_3$): δ 0.76, 0.80, 0.82, 0.96, 1.00 and 1.06 (each 3H, s, 6 x Me), 0.84 (6H, s, 2 x Me), 2.00 (3H, ss, OAc), 4.44 (1H, m, $W_{1/2}$ = 14 Hz, H-C-OAc) and 5.12 (1H, t, $W_{1/2}$ = 6 Hz, H-12);

^{13}C NMR (400 MHz, $CDCl_3$): refer to **Table 8.**

Attempted hydrogenation of α-amyrin

α-Amyrin (0.139 g) was dissolved in EtOH (30 cm^3) and 5% Pd/C (0.1 g) was added. This reaction mixture was placed in a Parr hydrogenator at a pressure of 3.5 $kg.cm^{-2}$ of H_2 for 30 hrs., it was filtered and the solvent evaporated. The product was collected, dried and recrystallised from $CHCl_3$, ^{1}H NMR showed the presence of an olefinic proton thus indicating that the reduction had not occurred.

^{1}H NMR (60 MHz, $CDCl_3$): δ 0.80 (9H, s, 3 x Me), 0.84, 0.92, 1.00, 1.04 and 1.10 (each 3H, s, 5 x Me), 3.16 (1H, m, $W_{1/2}$ = 16 Hz, H-C-OH) and 5.12 (1H, t, $W_{1/2}$ = 4 Hz, H-12).

Quantitation of the FAME mixture

A gas chromatograph having a flame ionisation detector was used to quantify the FAME mixture prepared from the plant. Serial dilution with *iso*-hexane (High Purity Grade) was used to make up 1, 5 and 10 ppm test solutions of the following authentic standards; methyl myristate, methyl pentadecanoate, methyl palmitate, methyl heptadecanoate, methyl oleate and methyl stearate.

These standards were injected (1μl aliquots) onto the capillary column of the chromatograph in order to verify the detector's linearity of response to concentration changes and to perform a sensitivity calibration on the instrument. In addition, R_t's were established which were cross-referenced to the results obtained from the GC/MS

analysis.

GC

GC conditions

Injector temp. 250 °, detector temp. 280 °, initial temp. 100 °, ramp rate 2 °.min^{-1} and final temp. 180 °C.

Silver ion chromatography of the α-amyrin fatty acid esters

Silica gel (100 g) and $AgNO_3$ (10 g) were mixed together in CH_3CN (100 cm^3). This mixture was then rotary evaporated to remove the CH_3CN solvent. A chromatography column (150 mm x 25 mm ID), wrapped in black polythene to exclude light was packed with an EtOAc-hexane slurry of the argentated silica gel. The mixture of α-amyrin fatty acid esters (1 g) obtained from the petroleum spirit extract, was carefully pipetted onto the column and eluted under air-pressure of 2 kg.cm^{-2} with EtOAc-hexane (1:19). 22 x 25 cm^3 fractions were collected and analysed by TLC on argentated silica gel HF_{254} plates using MeOH-$CHCl_3$ (1:9) as mobile phase. This analysis showed that fractions 19 to 24 contained the α-amyrin oleate.

^{1}H NMR (60 MHz, $CDCl_3$): δ 0.80, 0.84, 0.98, 1.02, 1.08 (each 3H, s, 5 x Me), 0.88 (9H, br s, 3 x Me), 1.26 (br s, -$(CH_2)_n$-), 2.00 (6H, br s, -CH_2CH=$CHCH_2$-), 2.24 (2H, d, -CH_2COO-), 4.50 (1H, m, $W_{1/2}$ = 15 Hz, H-COOC-R), 5.10 (1H, m, $W_{1/2}$ = 9 Hz, H-12) and 5.32 (2H, t, $W_{1/2}$ = 3 Hz, -CH=CH-).

Hydrolysis of the α-amyrin oleate

Isolated α-amyrin oleate (0.15 g) from the Ag^+ flash chromatography was hydrolysed by refluxing it in a methanolic soln. of KOH for 14 hrs. On cooling to ambient temperature the reaction mixture was poured onto crushed ice and the resulting ppt. of α-amyrin was filtered off. The supernatant was acidified with HCl dil. and the result-

ing oily layer was extracted into Et_2O. On evaporation of this solvent the oleic acid appeared as a colourless oil, TLC analysis was carried out on argentated silica gel HF_{254} plates using MeOH-$CHCl_3$ (1:9) as mobile phase, which showed the oil to be pure.

Methylation of the oleic acid

The oleic acid (0.01 g) was methylated in a soln. of CH_2N_2 in Et_2O using the method previously described.

FT-IR ν_{max}^{NaCl} cm^{-1}: 3000-2850 (CH stretching), 1743 (C=O stretching), 1650 (C=C stretching), 1550-1400 (CH bending) and 723 (C=C bending);

^{1}H NMR (60 MHz, $CDCl_3$): δ 0.96 (3H, distorted t, $Me(CH_2)_n$-), 1.24 (br s, -$(CH_2)_n$-), 2.00 (4H, d, -CH_2C=CCH_2-), 2.22 (2H, d, -CH_2COO-), 3.60 (3H, s, MeO) and 5.26 (2H, m, -CH=CH-);

GC/MS

GC conditions

Injector temp. 250 °, transfer line 270 °, initial temp. 100 °, ramp rate 10 °.min^{-1} and final temp. 210 °C.

MS *m/z* (rel. int.)

Methyl oleate: 296 $[M]^+$, 265 $[M\text{-}OMe]^+$, 264 $[M\text{-}MeOH]^+$, 246, 222, 207, 193, 96, 74 and 43.

Preparation of the α,β-*bis* (methylthio) adduct of methyl oleate

The methyl oleate (0.04 g) was treated with $(CH_3)_2S_2$ (0.5 cm^3) in the presence of a catalytic amount of I_2 (0.003 g). This reaction mixture was heated in a water-bath at

50 °C for 6 hrs. in small-volume sealed vials. H_2O was subsequently added to the reaction mixture followed by extraction with Et_2O. The two layers were separated in a separatory funnel and the organic layer was washed with 10% aqueous $Na_2S_2O_3$ to quench the reaction by removing the excess I_2 and was subsequently dried over Na_2SO_4 anhyd. On evaporation of the Et_2O, the α,β-*bis* (methylthio) adduct appeared as a waxy substance. This derivative was dissolved in *iso*-hexane and subjected to GC/MS analysis in order to establish the position of the double bond in the original fatty acid moiety.

GC/MS

GC conditions

Injector temp. 260 °, transfer line 270 °, initial temp. 100 °, ramp rate 15.9 °.min^{-1} and final temp. 215 °C.

MS *m/z* (rel. int.)

α,β-*bis (methylthio) methyl oleate*: 390 $[M]^+$, 359 $[M\text{-}OMe]^+$, 343, 217 $[\text{-}(MeS)C\text{-}(CH_2)_7COOMe]^+$, 185, 173 $[Me(CH_2)_7C(SMe)\text{-}]^+$, 157, 143, 137, 125, 87 and 61.

Extraction of *Betula pubescens* bark

Petroleum spirit extraction

The air-dried bark (150 g) was powdered in a Waring blender and successively extracted with petroleum spirit, Et_2O and MeOH (2.5 dm^3 of each solvent) in a Soxhlet extractor for 3 hrs. per extraction. The petroleum spirit extract was analysed by TLC, which showed the presence of two major spots and a number of minor ones. The extract was subsequently evaporated to dryness affording a brown semi-solid mass (30.1 g representing a 20% yield).

A mixture of $CHCl_3$-MeOH (1:1) was used to recrystallise the extract, betulin in white crystalline form, melting point 256 - 258 °C, was recrystallised. The betulin was dried and analysed by TLC using EtOAc-petroleum spirit (1:3) as mobile phase, which showed it to be virtually pure. The crystals were further recrystallised from the same solvent, yielding fine needle-like crystals of betulin which were dried and analysed by EIMS, ^1H NMR and FT-IR. The mother liquor on concentration yielded further crops of betulin.

EIMS (probe), *m/z* (rel. int.): 442 $[M]^+$, 424 $[M-H_2O]^+$, 411 $[M-CH_2OH]^+$, 234, 220, 203 and 189;

FT-IR ν_{max}^{KBr} cm^{-1}: 3600-3100 (OH), 2940-2840 (C-H stretching), 1642 (C=O stretching), 1150-900 (C-O) and 880 ($C=CH_2$);

^1H NMR (60 MHz, $CDCl_3$): δ 0.72, 0.78 and 0.96 (each 3H, s, 3 x Me), 1.00 (9H, s, 3 x Me), 1.62 (3H, s, C=C-Me), 3.20 (2H, AB q, J_{AB} = 8 Hz, CH_2OH) and 4.56 (2H, d, -C(Me)=CH_2).

The TLC analysis of the petroleum spirit extract also showed the presence of a non-polar compound with a higher R_f than betulin. Flash chromatography was performed on 1 g of the extract using EtOAc-hexane (1:9) as mobile phase. This yielded white crystals of the pentacyclic triterpene lupeol, melting point 213 - 215 °C, which on analysis by TLC were shown to be pure.

FT-IR ν_{max}^{KBr} cm^{-1}: 3600-3100 (OH), 3060 (C=C), 3000-2800 (C-H stretching), 1636 (C=C stretching), 1500-1300 (C-H bending), 1043 (C-O) and 880 (C=C);

^1H NMR (60 MHz, $CDCl_3$): δ 0.76, 0.78, 0.84 and 1.04 (each 3H, s, 3 x Me), 0.98

(6H, br s, 2 x Me), 1.68 (3H, br s, Me-C=C), 3.16 (1H, dd, $W_{1/2}$ = 16 Hz, H-C-OH) and 4.60 (2H, m, H_2C=C-).

Et_2O and MeOH extractions

Subsequent extraction with these solvents, followed by recrystallisation yielded further crops of pure betulin which were analysed by TLC, FT-IR and ^{1}H NMR.

Betulin monoacetate

Betulin (0.3082 g) was dissolved in Ac_2O (10 cm^3) and was left to stand at ambient temperature for 48 hrs. H_2O was added to the mixture and the resulting crystalline product was collected and dried.

^{1}H NMR (60 MHz, $CDCl_3$): δ 0.76, 0.84 and 1.04 (each 3H, s, 3 x Me), 1.00 (9H, s, 3 x Me), 1.68 (3H, br s, Me-C=C), 2.04 (3H, s, OAc), 3.16 (1H, m, $W_{1/2}$ = 16 Hz, H-C-OH), 3.96 (2H, AB q, J_{AB} = 10 Hz, -CH_2OAc) and 4.56 (2H, d, -C(Me)=CH_2).

Betulin diacetate

Betulin (0.3035 g) was dissolved in pyridine (5 cm^3) and Ac_2O (5 cm^3) and was left to stand at ambient-temperature for 14 hrs. The reaction mixture was then poured onto crushed ice and a white ppt. filtered. The product was recrystallised from MeOH-H_2O and the resulting crystals, after collection and drying, gave a melting point 224 - 225 ^oC.

^{1}H NMR (60 MHz, $CDCl_3$): δ 0.78, 0.96 and 1.00 (each 3H, s, 3 x Me), 0.84 (9H, s, 3 x Me), 2.02 (3H, s, OAc), 2.12 (3H, s, OAc), 3.93 (2H, AB q, J_{AB} = 10 Hz, CH_2OAc), 4.40 (1H, m, H-C-OAc) and 4.52 (2H, d, C(Me)=CH_2);

^{13}C NMR (400 MHz, $CDCl_3$): refer to **Table 12.**

Hydrogenation of betulin

Betulin (0.24 g) was dissolved in EtOH (20 cm^3), 5% Pd/C catalyst (0.08 g) was added and the mixture was placed in a Parr hydrogenator at a pressure of 3.5 $kg.cm^{-2}$ of H_2 at ambient-temp for 8 hrs. The reaction mixture was filtered to remove the catalyst and the solvent was evaporated. TLC analysis showed the product to be impure and so it was recrystallised from an EtOH-EtOAc mixture yielding white crystals which TLC analysis showed to be pure.

FT-IR ν_{max}^{KBr} cm^{-1}: 3600-3200 (OH), 3000-2850 (C-H stretching), 1500-1300 (CH_3 assymetric and symmetric stretching) and 1030 (C-O);

^1H NMR (60 MHz, $CDCl_3$): δ 0.72, 0.80 and 1.00 (each 3H, s, 3 x Me), 0.92 (9H, s, 3 x Me), 3.16 (1H, m, H-C-OH) and 3.64 (2H, AB q, $W_{1/2}$ =16 Hz, $-CH_2OH$).

Acetylation of lupeol

Lupeol (0.25 g) was dissolved in a mixture of Ac_2O (4 cm^3) and pyridine (4 cm^3) and the reaction mixture was left to stand at ambient temperature for 40 hrs. It was then poured onto crushed ice and a white ppt. formed which was filtered off and dried. The white crystals of lupeol acetate were analysed by TLC using EtOAc-petroleum spirit (3:7) as mobile phase, this analysis showed them to be pure.

EIMS (probe), *m/z* (rel. int.): 468 $[M]^+$, 453 $[M-CH_3]^+$, 408 $[M-HOAc]^+$, 249, 218 and 204;

FT-IR ν_{max}^{KBr} cm^{-1}: 3060 (C=C), 3000-2870 (C-H stretching), 1732 (C=O stretching), 1500-1300 (C-H bending), 1247 (C-O) and 876 ($C=CH_2$);

^{1}H NMR (60 MHz, $CDCl_3$): δ 0.74, 0.88 and 1.0 (each 3H, s, 3 x Me), 0.80 (9H, br s, 3 x Me), 1.62 (3H, br s, -C=C-Me), 1.98 (3H, s, OAc), 4.24 (1H, m, H-C-OAc) and 4.48 (2H, d, -C(Me)=CH_2);

^{13}C NMR (400 MHz, $CDCl_3$): refer to **Table 13.**

Hydrogenation of lupeol

Lupeol (0.30 g) was dissolved in EtOAc (25 cm^3), PtO_2 catalyst (0.10 g) was added and the mixture was placed in a Parr hydrogenator at a pressure of 4.0 $kg.cm^{-2}$ of H_2 at ambient-temp for 10 hrs. The reaction mixture was filtered to remove the catalyst and the solvent was evaporated. TLC analysis showed the product to be impure, it was recrystallised from a mixture EtOH-EtOAc yielding white crystals which TLC analysis showed to be pure.

Jones' oxidation of betulin

Jones' reagent was prepared by dissolving CrO_3 (2.67 g) in H_2O (10 cm^3). This solution was then cooled and H_2SO_4 conc. (2.3 cm^3) was cautiously added, dropwise with stirring. Betulin (2.94 g) dissolved in Me_2CO (40 cm^3) was titrated with the Jones' reagent at a temperature between 0 - 2 °C. The reaction mixture separated into a green lower layer of Cr (VI) salts and an upper layer which was an Me_2CO soln. of oxidation products. The immiscible layers were placed in a separatory funnel and separated. The Me_2CO was evaporated and a complex mixture (as shown by TLC) was recovered.

Vacuum-Liquid Chromatography

A sintered glass funnel was packed with a slurry of silica gel in petroleum spirit. The complex mixture from the Jones' oxidation was slurried with celite in CCl_4. This was rotary evaporated and was evenly layered on the silica gel in the glass funnel. The mix-

ture was eluted under low vacuum using EtOAc-petroleum spirit (1:5) as mobile phase. 22 x 30 cm^3 fractions were collected and analysed by TLC. Fractions 8 and 9 having the same R_f were combined. The solvent was evaporated, yielding white crystals of betulonic acid. TLC analysis indicated this derivative to be pure.

FT-IR ν_{max}^{KBr} cm^{-1}: 3625-2400 (OH), 3070, 1641 and 883 (C=C stretching and bending), 3000-2850 (C-H stretching), 1700 (C=O stretching), 1500-1350 (C-H bending) and 1242 (C-O);

^{1}H NMR (60 MHz, $CDCl_3$): δ 0.96, 1.00 and 1.08 (each 3H, s, 4 x Me), 1.04 (6H, s, 2 x Me), 1.70 (3H, br s, Me-C=C), 2.20 (2H, m, $-CH_2-C=O$), 2.90 (1H, br m, H-18) and 4.68 (2H, d, $-C(Me)=CH_2$).

Reduction and acetylation of betulonic acid

Betulonic acid (0.40 g) was dissolved in a solution of $NaBH_4$ (0.10 g) in MeOH (25 cm^3). This reaction mixture was left to stand for 3 hrs. at ambient temperature and was then acidified to litmus, by the dropwise addition of HCl dil. A white crystalline ppt. of inorganic Na salts formed and were filtered off. The resulting filtrate was evaporated yielding impure crystals of betulinic acid, as shown by TLC.

The betulinic acid (0.360 g) was subsequently treated with a mixture of Ac_2O (6 cm^3) and pyridine (6 cm^3) and was left to stand for 10 hrs. at ambient temperature. H_2O was then added to this reaction mixture and the resulting betulinic acetate was filtered and dried under high vacuum. TLC analysis showed this derivative to be pure.

FT-IR ν_{max}^{KBr} cm^{-1}: 3625-2400 (OH), 3080, 1641 and 884 (C=C stretching and bending), 3050-2850 (C-H stretching), 1735 (C=O stretching), 1500-1350 (C-H bending) and 1245 (C-O stretching);

^{1}H NMR (60 MHz, $CDCl_3$): δ 0.84 (9H, br s, 3 x Me), 0.92 (6H, br s, 2 x Me), 1.65 (3H, br s, Me-C=C), 1.98 (3H, s, OAc), 2.15 (1H, br m, H-18), 4.30 (1H, m, H-C-OAc) and 4.60 (2H, d, -C(Me)=CH_2);

^{13}C NMR (400 MHz, $CDCl_3$): refer to **Table 14.**

Extraction of *Betula ermanii* bark

Petroleum spirit extraction

The bark was air-dried, powdered and Soxhlet extracted with petroleum spirit. Evaporation of the extract produced crystals which were dried and recrystallised from MeOH-$CHCl_3$ (1:1) and then further recrystallised from EtOAc-petroleum spirit (3:7). They were analysed by TLC which showed the presence 3 major compounds which were separated by means of flash chromatography.

Flash chromatography

A flash chromatography column (150 mm x 25 mm ID) was packed with a silica gel-petroleum spirit slurry. EtOAc-petroleum spirit (3:7) was used as mobile phase. The petroleum spirit extract (0.44 g) was dissolved in mobile phase (20 cm^3) and was pipetted onto the column and eluted under air-pressure of 2 $kg.cm^{-2}$. 20 x 25 cm^3 fractions were collected and these were analysed by TLC. Fractions having similar R_f's were combined and the solvent was evaporated.

All crystals collected in this manner were recrystallised from column mobile phase, followed by further recrystallisation from EtOAc, then they were filtered and analysed by TLC. This analysis showed that the early fractions contained oleanolic acid acetate, melting point 278 - 280 °C, the mid-range fractions gave betulin, melting point 256 -

258 °C, while the later fractions afforded oleanolic acid, melting point 316 - 318 °C. The yields of oleanolic acid and its monoacetate were low and TLC analysis of them and their mother liquors was carried out. The analysis indicated the compounds to be pure.

Et_2O extraction

The marc remaining after petroleum spirit extraction was extracted with Et_2O, evaporation of the solvent gave a crop of crystals and these were dried and analysed by TLC. This analysis showed the crystals to be impure. The crystals were recrystallised from MeOH-$CHCl_3$ (1:1) and were further recrystallised from EtOAc-petroleum spirit (3:7). However they were not fully soluble in the EtOAc-petroleum spirit (3:7) mixture and insoluble material was removed by filtration while dissolved material was recrystallised from the solvent mixture.

MeOH Extraction

The marc after Et_2O extraction was powdered in a blender and infused with boiling MeOH (600 cm^3). The extract was concentrated and left to cool, the resulting ppt. was collected and dried. Recrystallisation of the ppt. was carried out using $CHCl_3$-MeOH (1:1) which yielded white crystal. These were analysed by TLC, which showed them to be impure and so were further recrystallised from EtOAc-petroleum spirit (3:7). The resulting white crystals were collected and dried and were shown to be pure by TLC analysis. Crops which gave the same R_f on TLC were combined and recrystallised from Me_2CO to give pure Betulin. FT-IR and ^1H NMR values were similar to those previously reported.

The mother liquor was concentrated and further crops of betulin were obtained, TLC analysis was performed which showed the compound to be pure.

Treatment of oleanolic acid acetate with acetic anhydride

Oleanolic acid acetate (0.106 g) was dissolved in Ac_2O (3 cm^3) and pyridine (3 cm^3) and was left to stand at ambient temperature for 7 hrs. The reaction mixture was then poured onto crushed ice and a white ppt. formed. This was collected, dried and recrystallised from Me_2CO. TLC, ^1H NMR and FT-IR analysis were performed and showed that the reaction had not gone to completion. The compound was returned to pyridine (1 cm^3) and Ac_2O (1 cm^3) and left to stand at ambient temperature for a further 18 hrs. The mixture was then poured onto crushed ice, filtered and dried.

A recrystallisation from Me_2CO was performed and TLC of the recrystallised product showed the reaction had still not gone to completion. The reaction was repeated using 0.1027 g of the compound in pyridine (3 cm^3) and Ac_20 (3 cm^3) and this was refluxed for 3hrs. The reaction mixture was then poured onto crushed ice and the resulting crystals were filtered and dried in an evacuated desiccator. They were recrystallised from EtOH, and TLC, ^1H NMR and FT-IR showed the derivative had formed.

FT-IR ν_{max}^{KBr} cm^{-1}: 3000-2800 (C-H stretching), 1813 (anhydride C=O), 1734 (acetate C=O), 1500-1400 (C-H bending), 1245 (C-O) and 989 (C=C);

^1H NMR (60 MHz, $CDCl_3$): δ 0.80 and 1.12 (each 3H, s, 2 x Me), 0.84 (9H, br s, 3 x Me), 0.88 (6H, br s, 2 x Me), 2.00 (3H, s, OAc), 2.19 (3H, s, -COO-CO-Me), 2.78 (1H, ABX q, $J_{AX}+J_{BX}$ = 16.5 Hz, H-18), 4.44 (1H, t, $W_{1/2}$ = 14 Hz, H-C-OAc) and 5.24 (1H, br s, $W_{1/2}$ = 6.5 Hz, H-12).

Attempted hydrogenation of oleanolic acid acetate

Oleanolic acid acetate (0.139 g) was dissolved in EtOH (30 cm^3) and 5% Pd/C (0.1 g) was added. This reaction mixture was placed in a Parr hydrogenator at a pressure of 3.5 $kg.cm^{-2}$ of H_2 for 60 hrs., it was filtered and the solvent evaporated. The product

was collected, dried and recrystallised from $CHCl_3$, ^1H NMR showed the presence of an olefinic proton thus indicating that the reduction had not occurred.

Methylation of oleanolic acid acetate

Oleanolic acid acetate (0.1 g) was dissolved in Et_2O (10 cm^3) and cooled in an ice-bath. The solution was treated with CH_2N_2 dissolved in Et_2O and TLC analysis was used to follow the course of the reaction. On completion the reaction mixture was refrigerated for 48 hrs. and then the Et_2O was evaporated. The methylated product was recrystallised from an EtOH-Me_2CO mixture, insoluble solids were filtered while the solution was hot and it was left to recrystallise at ambient temperature. Two crops of methyl oleanolate acetate crystals (melting point 236 - 238 ^oC) formed, these were collected, dried and recrystallised from EtOH.

EIMS (probe), *m/z* (rel. int.): 512 $[M]^+$, 497 $[M-CH_3]^+$, 452 $[M-AcOH]^+$, 453 $[M-COOMe]^+$, 262, 249, 203, 189 and 133;

FT-IR ν_{max}^{KBr} cm^{-1}: 3000-2800 (C-H stretching), 1734 (C=O), 1500-1300 (C-H bending), 1261 (C-O) and 801 (C=C);

^1H NMR (60 MHz, $CDCl_3$): δ 0.70 and 1.08 (each 3H, s, 2 x Me), 0.84 (6H, br s, 2 x Me), 0.90 (9H, br s, 3 x Me), 1.99 (3H, s, OAc), 2.80 (1H, ABX q, $J_{AX}+J_{BX}$ = 14.5 Hz, H-18), 3.46 (3H, s, OMe), 4.39 (1H, t, $W_{1/2}$ = 14 Hz, H-C-OAc) and 5.21 (1H, m, $W_{1/2}$ = 7 Hz, H-12);

^{13}C NMR DEPT (400 MHz, $CDCl_3$): **Table 15.**

Hydrolysis of oleanolic acid acetate

Oleanolic acid acetate (0.1 g) was dissolved in 5% methanolic KOH (4 cm^3) and was

refluxed for 3 hrs. On completion of the reaction the mixture was poured onto crushed ice, as a result of which the oleanolic acid formed a white ppt. which was filtered and dried under vaccuum.

FT-IR ν_{max}^{KBr} cm^{-1}: 3750-3100 (OH), 1695 (C=O), 3000-2800 (C-H stretching), 1500-1330 (C-H bending) and 803 (C=C);

^{1}H NMR (60 MHz, $CDCl_3$): δ 0.76 and 1.10 (each 3H, s, 2 x Me), 0.90 (9H, br s, 3 x Me), 0.94 (6H, br s, 2 x Me), 2.68 (1H, m, W1/2 = 10 Hz, H-18), 3.06 (1H, dd, $W_{1/2}$ = 18 Hz, H-C-OH) and 5.21 (1H, m, $W_{1/2}$ = 6 Hz, H-12).

Methylation of oleanolic acid

Oleanolic acid (0.1 g) was dissolved in Et_2O (10 cm^3), cooled in an ice-bath and treated with a solution of CH_2N_2 in Et_2O, as previously described. The resulting derivative gave a melting point in the range 200 - 203 °C.

EIMS (probe), *m/z* (rel. int.): 470 $[M]^+$, 452 $[M-H_2O]^+$, 410 $[M-CH_3OOCH]^+$, 262, 249, 203, 189 and 133;

FT-IR ν_{max}^{KBr} cm^{-1}: 3650-3100 (OH), 3000-2850 (C-H stretching), 1727 (ester C=O), 1490-1300 (C-H bending), 1261 (C-O stretching) and 754 (C=C);

^{1}H NMR (60 MHz, $CDCl_3$): δ 0.72, 0.76, 0.96 and 1.10 (each 3H, s, 4 x Me), 0.88 (9H, br s, 3 x Me), 2.62 (1H, m, H-18), 3.22 (1H, m, $W_{1/2}$ = 20 Hz, H-C-OH), 3.52 (3H, s, -COOMe) and 5.21 (1H, m, $W_{1/2}$ = 6 Hz, H-12).

Acetylation of oleanolic acid

Oleanolic acid (0.1025 g) was dissolved in Ac_2O (3 cm^3) and pyridine (3 cm^3) and was

left to stand for 14 hrs. at ambient temperature. The reaction mixture was then poured onto crushed ice and a white ppt. formed. This was collected, dried and recrystallised from Me_2CO yielding white crystals, melting point 277 - 279 °C. TLC, ^1H NMR and FT-IR analysis showed the product to be identical with the acetate isolated from the plant.

Extraction of *Betula papyrifera* bark

Petroleum spirit extraction

The dried and powdered bark was Soxhlet extracted with petroleum spirit, evaporation of this solvent yielded a sticky brown ppt. Acid-washed sand was mixed with the sticky brown ppt. in order to facilitate it being powdered. The dried mixture was ground with a mortar and pestle and was further extracted with petroleum spirit. On completion of the extraction, the solvent was evaporated and the resulting crystals were collected, dried and analysed by TLC. This analysis showed the mixture to contain oleanolic acid, betulin and oleanolic acid acetate. An unidentified compound was also shown to be present.

Et_2O extraction

An Et_2O extraction of the marc resulting from the petroleum spirit extraction was performed. This was followed by solvent evaporation and the resultant extract was recrystallised from Me_2CO producing crystals which were collected and dried. A TLC analysis showed these crystals to be impure. They were recrystallised from $CHCl_3$-MeOH (1:1) followed by a further recrystallisation from Me_2CO and the resulting crystals were dried. TLC analysis showed them to be a mixture having the same R_f's as oleanolic acid and betulin. An unidentified compound was also present.

MeOH extraction

The marc from the previous extraction was further extracted with MeOH. An approx. yield of 40 g of green coloured crystals was obtained, which were collected, dried and analysed by TLC. This analysis showed them to be impure and so they were recrystallised from MeOH-$CHCl_3$ (1:1) and the green product produced was collected and dried. The MeOH mother liquor from the extract produced a second crop of these crystals which were also collected. All fractions obtained were shown to have the same R_f on TLC and so were combined and a recrystallisation from MeOH was carried out. This yielded a brown, sticky ppt. which was dissolved in Me_2CO and a further recrystallisation attempted, but this proved unsuccessful. TLC analysis showed this material to be a mixture of oleanolic acid, betulin and an unidentified compound.

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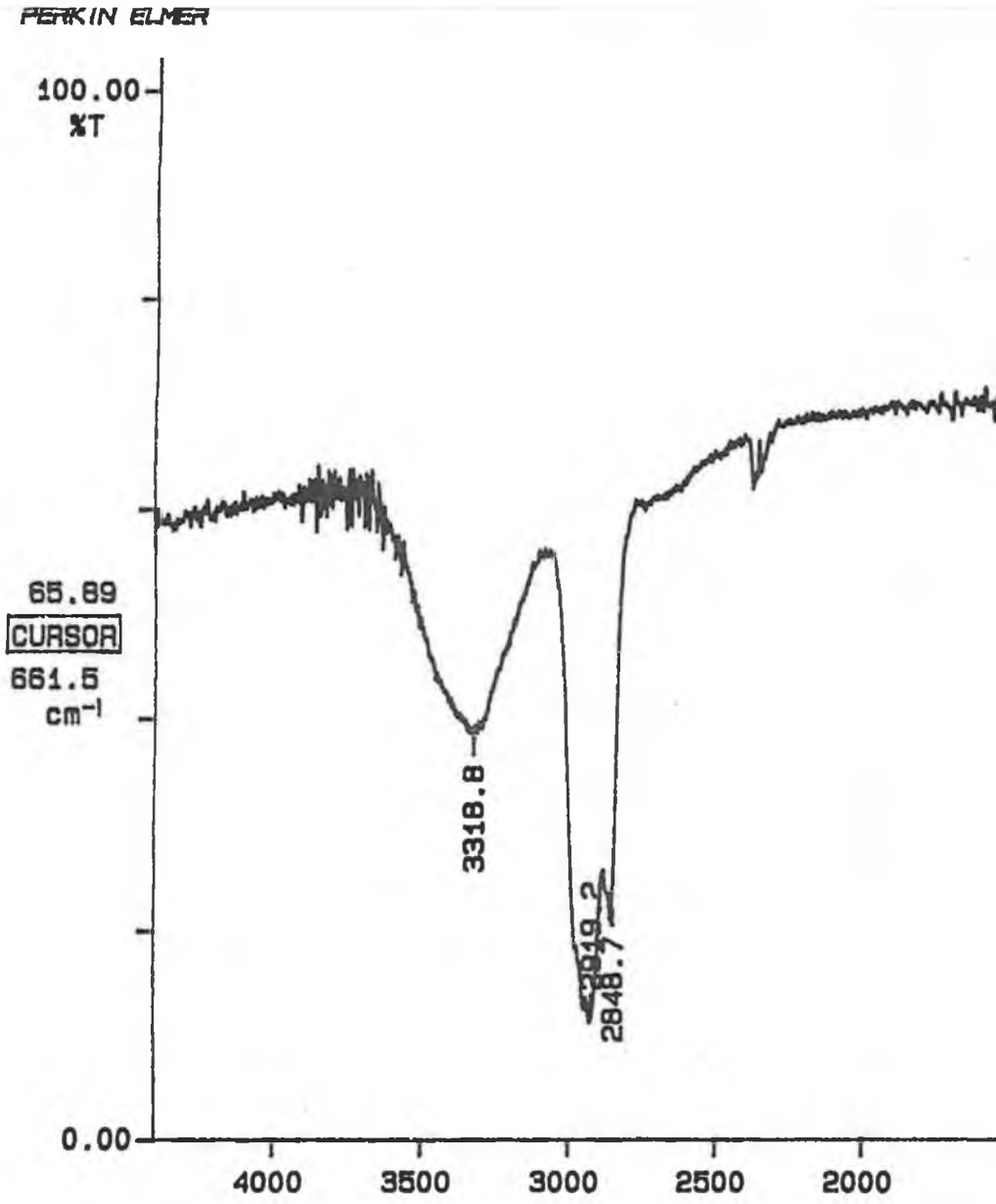
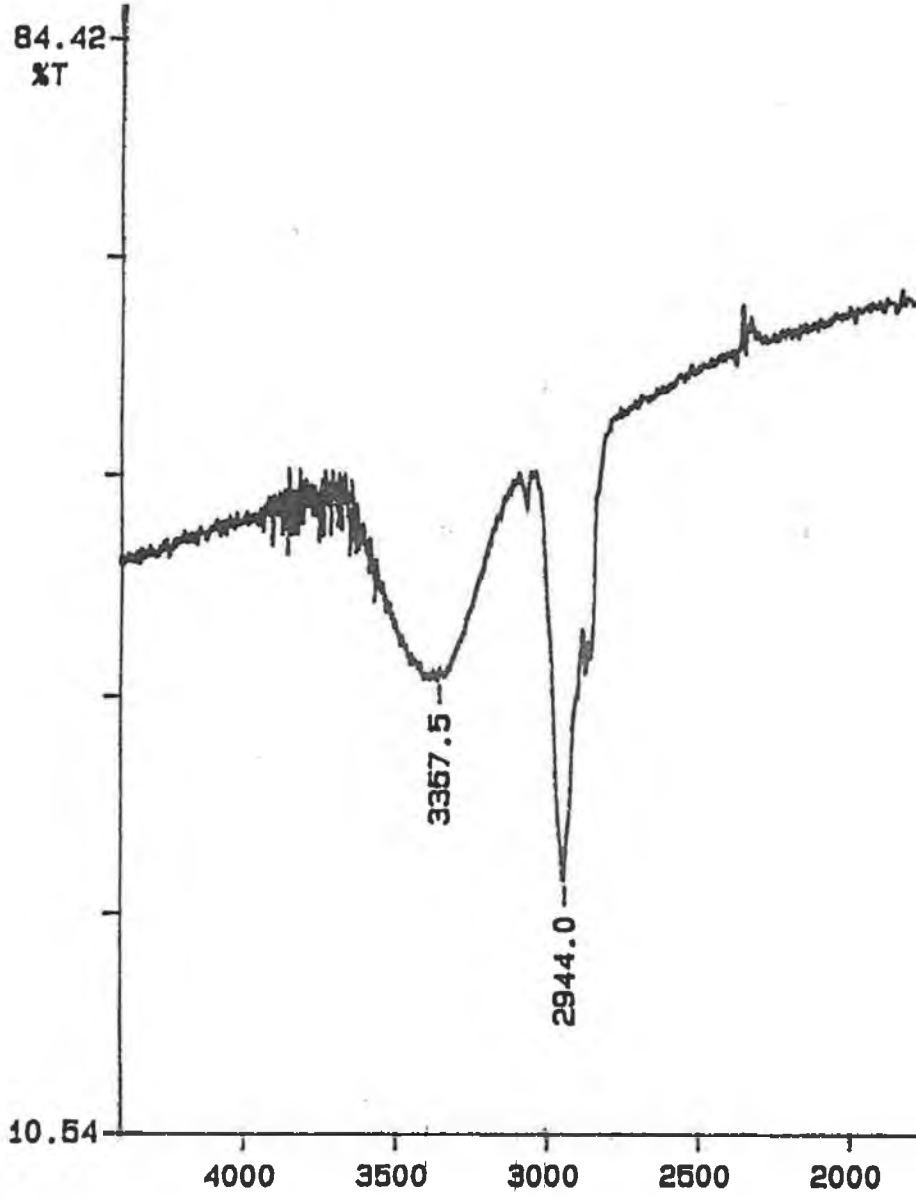
SPECTRA



95/11/15 09: 47
X: 1 scan, 4.0cm-1

1654.6
1560.6
1460.7
1372.5
1090.5
1037.6
990.6
808.4
664.5
1500
1000
cm⁻¹
500

SPECTRUM 1



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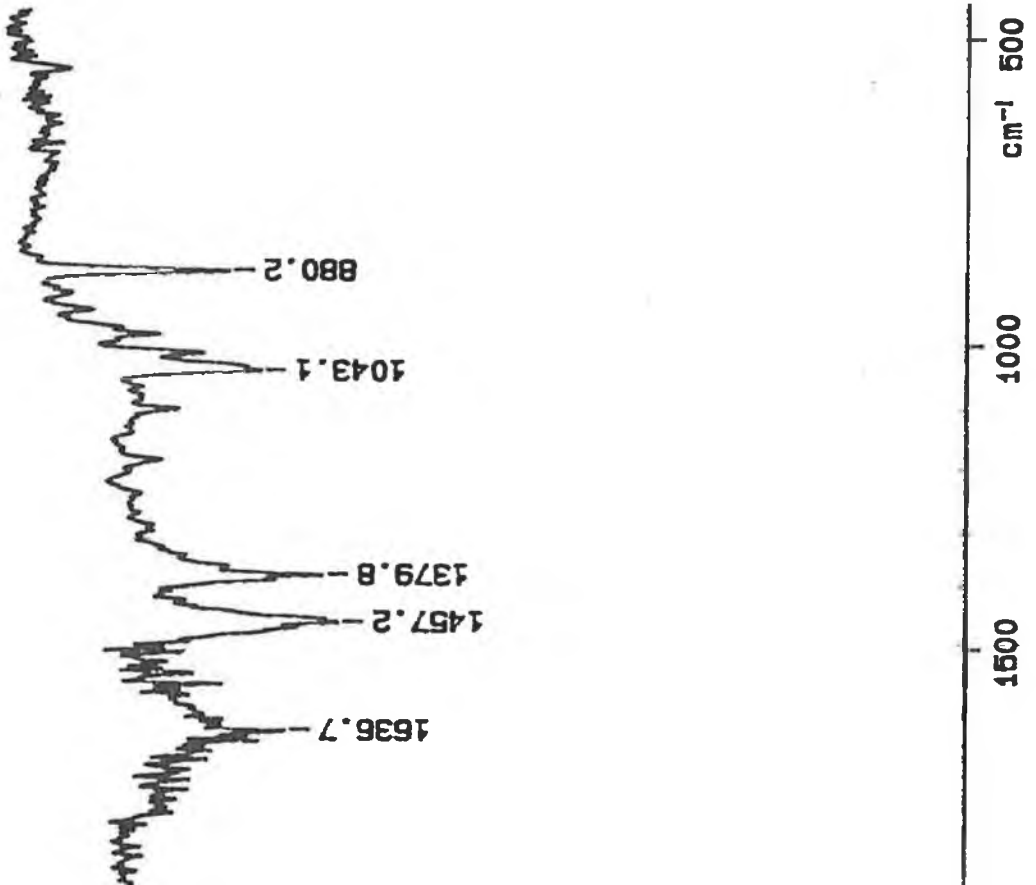
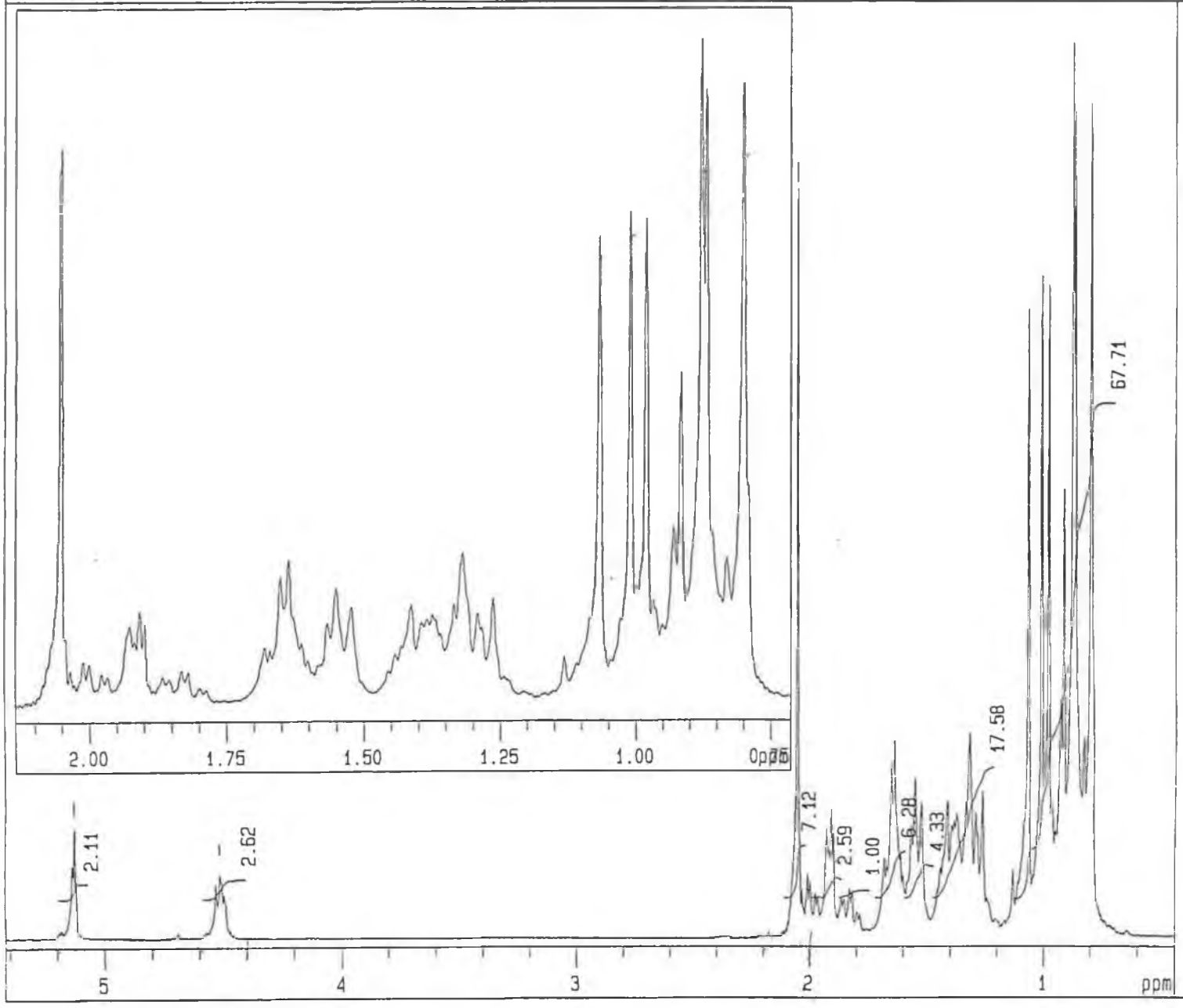
SPECTRUM 2



1H Line



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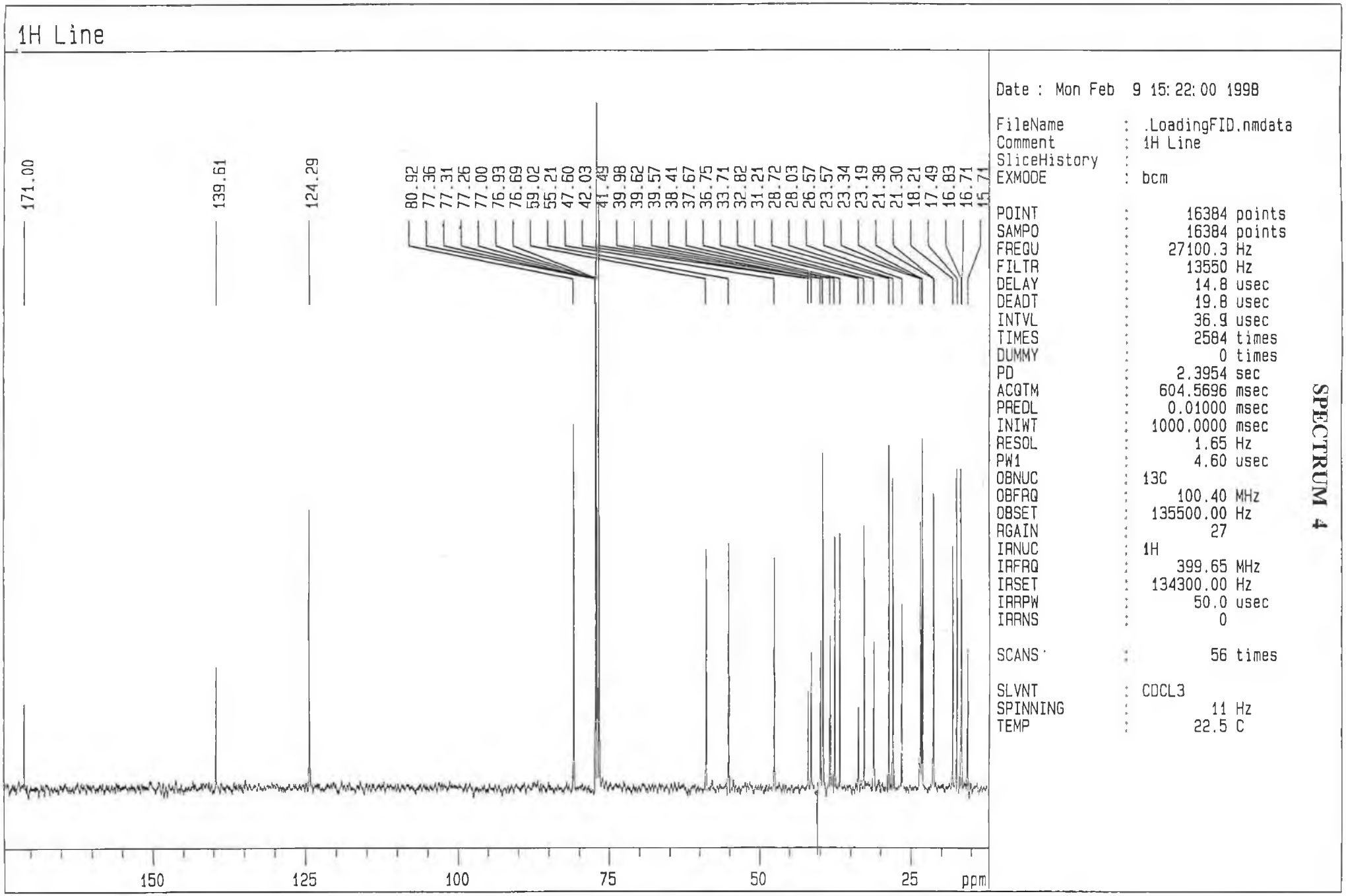
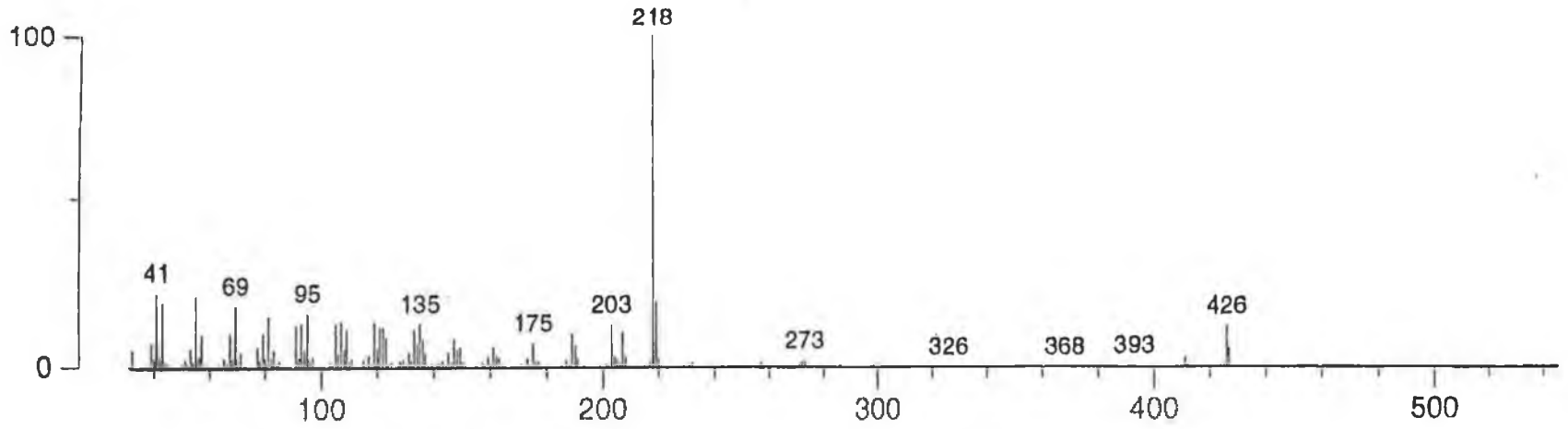
SAMPO	:	16384 points
FREQU	:	7993.6 Hz
FILTR	:	4000 Hz
DELAY	:	50.0 usec
DEADT	:	72.2 usec
INTVL	:	125.1 usec
TIMES	:	2584 times
DUMMY	:	0 times
PD	:	4.9504 sec
ACQTM	:	2049.6384 msec
PREDL	:	0.01000 msec
INIWT	:	1000.0000 msec
RESOL	:	0.49 Hz
PW1	:	5.67 usec
OBNUC	:	1H
OBFRQ	:	399.65 MHz
OBSET	:	134300.00 Hz
RGAIN	:	14
SCANS	:	3 times
SLVNT	:	CDCL3
SPINNING	:	11 Hz
TEMP	:	21.1 C

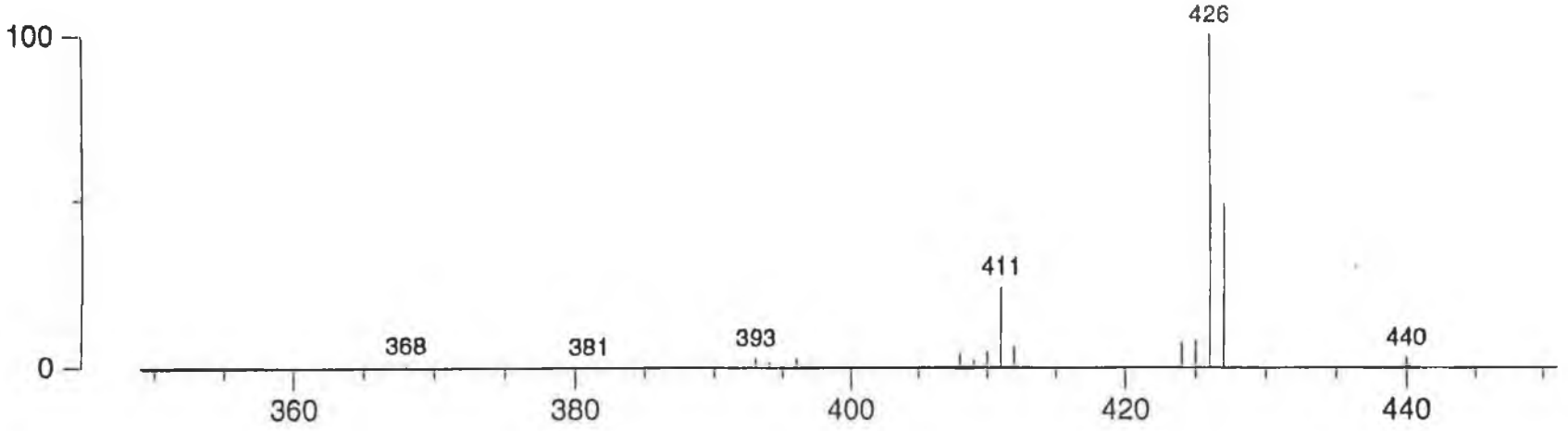
SPECTRUM 3


1H Line
171.00
139.61
124.29
80.92
77.36
77.31
77.26
77.00
76.93
76.69
59.02
55.21
47.60
42.03
41.49
39.98
39.62
39.57
38.41
37.67
36.75
33.71
32.82
31.21
28.72
28.03
26.57
23.57
23.34
23.19
21.38
21.30
18.21
17.49
16.83
16.71
15.71
150
125
100
75
50
25
ppm
Date : Mon Feb 9 15:22:00 1998
FileName : .LoadingFID.nmdata
Comment : 1H Line
SliceHistory :
EXMODE : bcm
POINT : 16384 points
SAMPO : 16384 points
FREQU : 27100.3 Hz
FILTR : 13550 Hz
DELAY : 14.8 usec
DEADT : 19.8 usec
INTVL : 36.9 usec
TIMES : 2584 times
DUMMY : 0 times
PD : 2.3954 sec
ACQTM : 604.5696 msec
PREDL : 0.01000 msec
INIWT : 1000.0000 msec
RESOL : 1.65 Hz
PW1 : 4.60 usec
OBNUC : 13C
OBFRQ : 100.40 MHz
OBSET : 135500.00 Hz
RGAIN : 27
IRNUC : 1H
IRFRQ : 399.65 MHz
IRSET : 134300.00 Hz
IRRPW : 50.0 usec
IRRNS : 0
SCANS : 56 times
SLVNT : CDCL3
SPINNING : 11 Hz
TEMP : 22.5 C
SPECTRUM 4

trg_mk0010 Scan 1 (Av 38-43 Acq) 100%=19995 mv 1 May 96 10:13
HRP +EI ----- SAMPLE CODE: MK 6. ---- QUEUE NO. 3017 ----

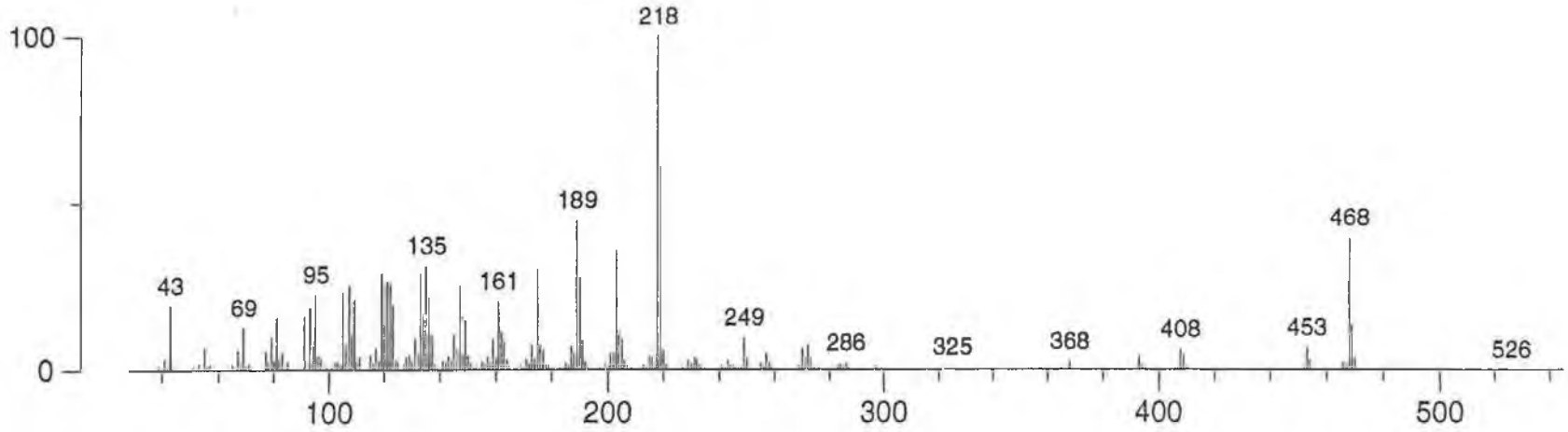


100% = 248637 ADC

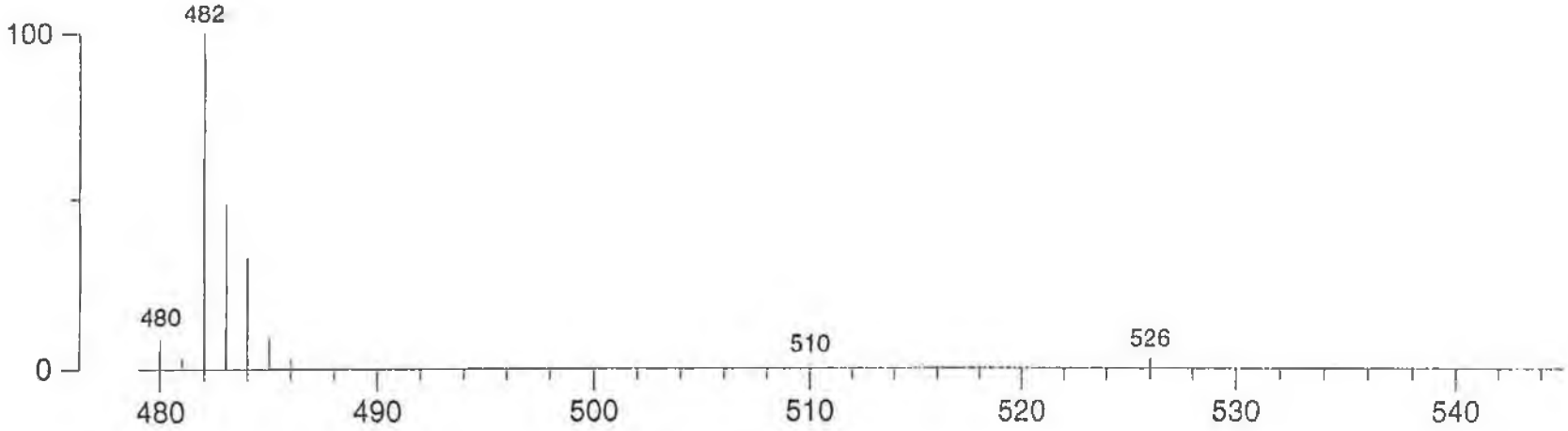


SPECTRUM 5

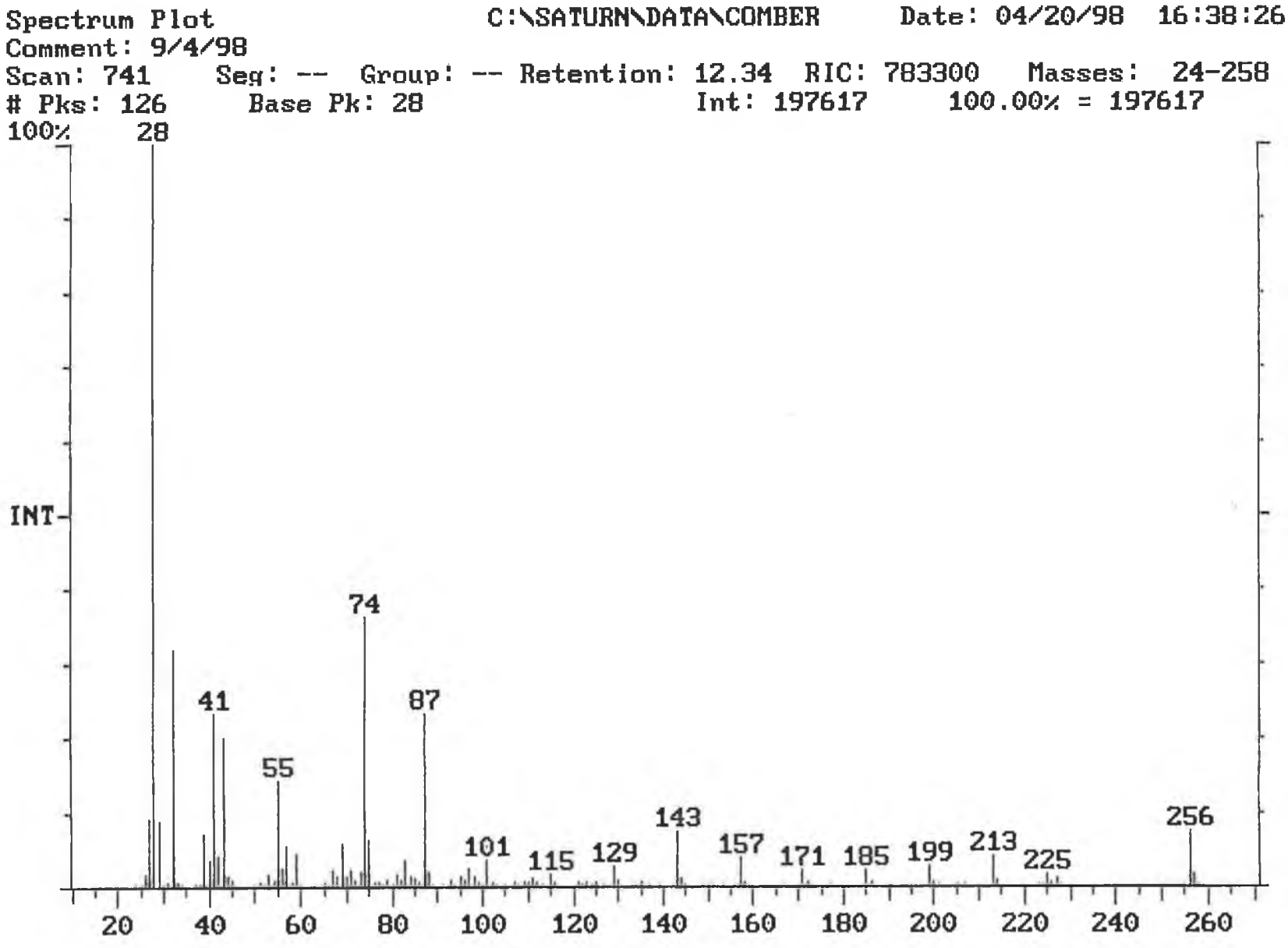
trc_mk0007 Scan 1 (Av 38-40 Acq) 100%=259204 mv 26 Feb 96 10:54
HRP +EI ----- SAMPLE CODE: KF/2/7 ---- QUEUE NO. 2734 -----



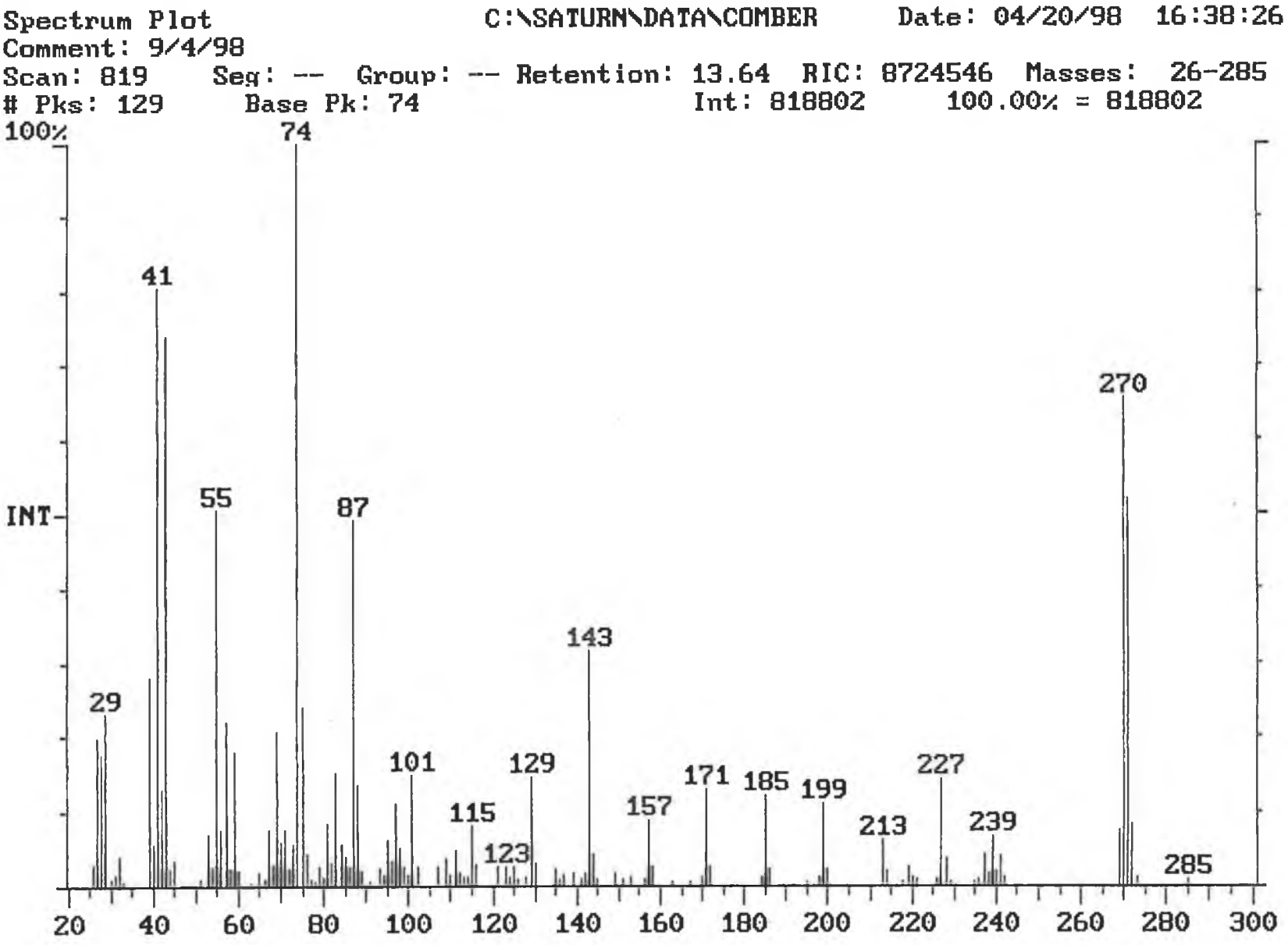
100% = 178214 ADC



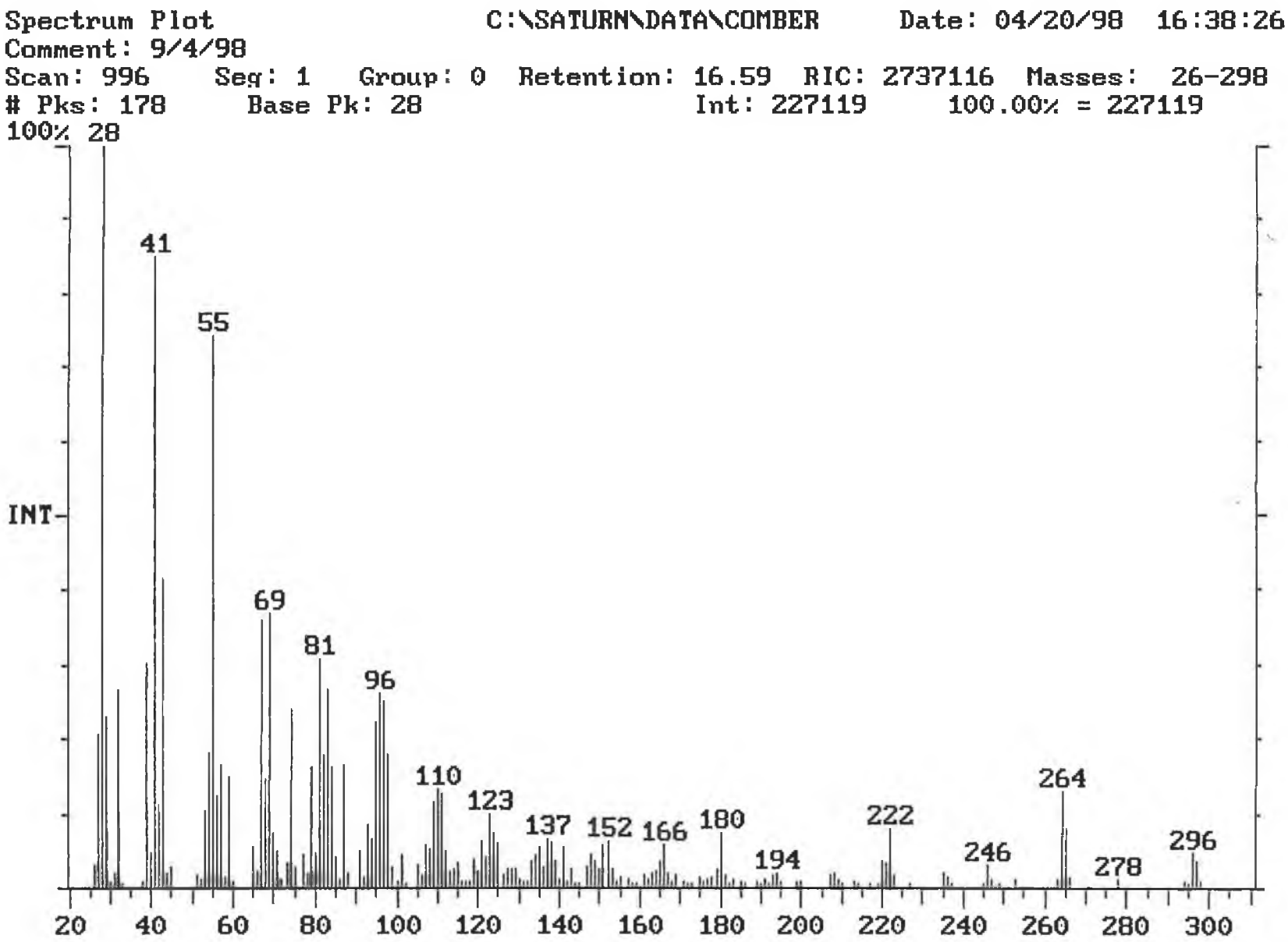
SPECTRUM 6


Spectrum Plot
C:\SATURN\DATA\COMBER
Date: 04/20/98 16:38:26
Comment: 9/4/98
Scan: 741 Seg: -- Group: -- Retention: 12.34 RIC: 783300 Masses: 24-258
Pks: 126 Base Pk: 28
Int: 197617 100.00% = 197617
100%
28
INT
74
41
87
55
143
256
101
115
129
157
171
185
199
213
225
20
40
60
80
100
120
140
160
180
200
220
240
260

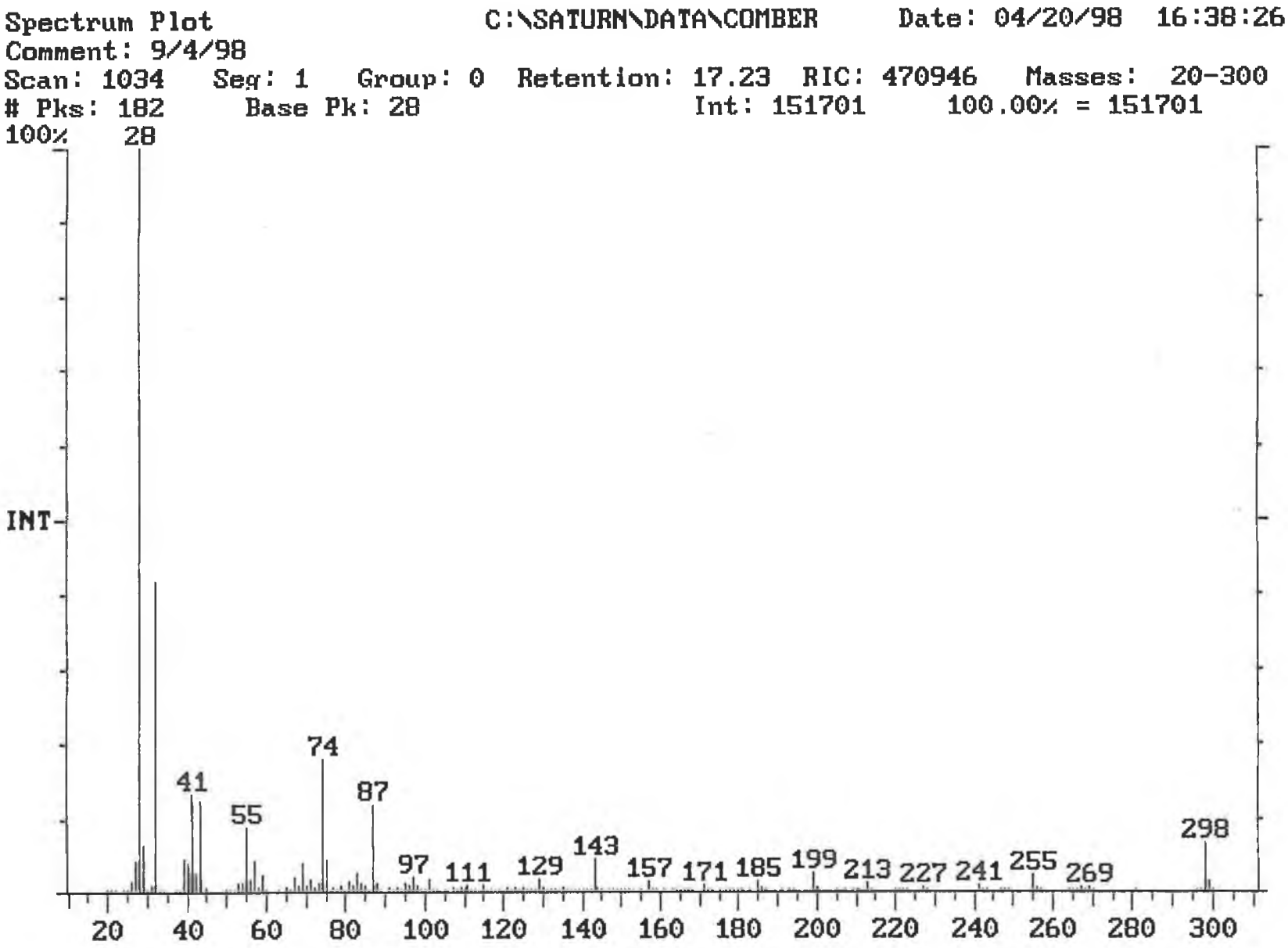
SPECTRUM 7


Spectrum Plot
C:\SATURN\DATA\COMBER
Date: 04/20/98 16:38:26
Comment: 9/4/98
Scan: 819 Seg: -- Group: -- Retention: 13.64 RIC: 8724546 Masses: 26-285
Pks: 129 Base Pk: 74
Int: 818802 100.00% = 818802
100%
INT
74
41
55
87
270
143
29
101
129
115
157
171
185
199
227
213
239
123
285
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300

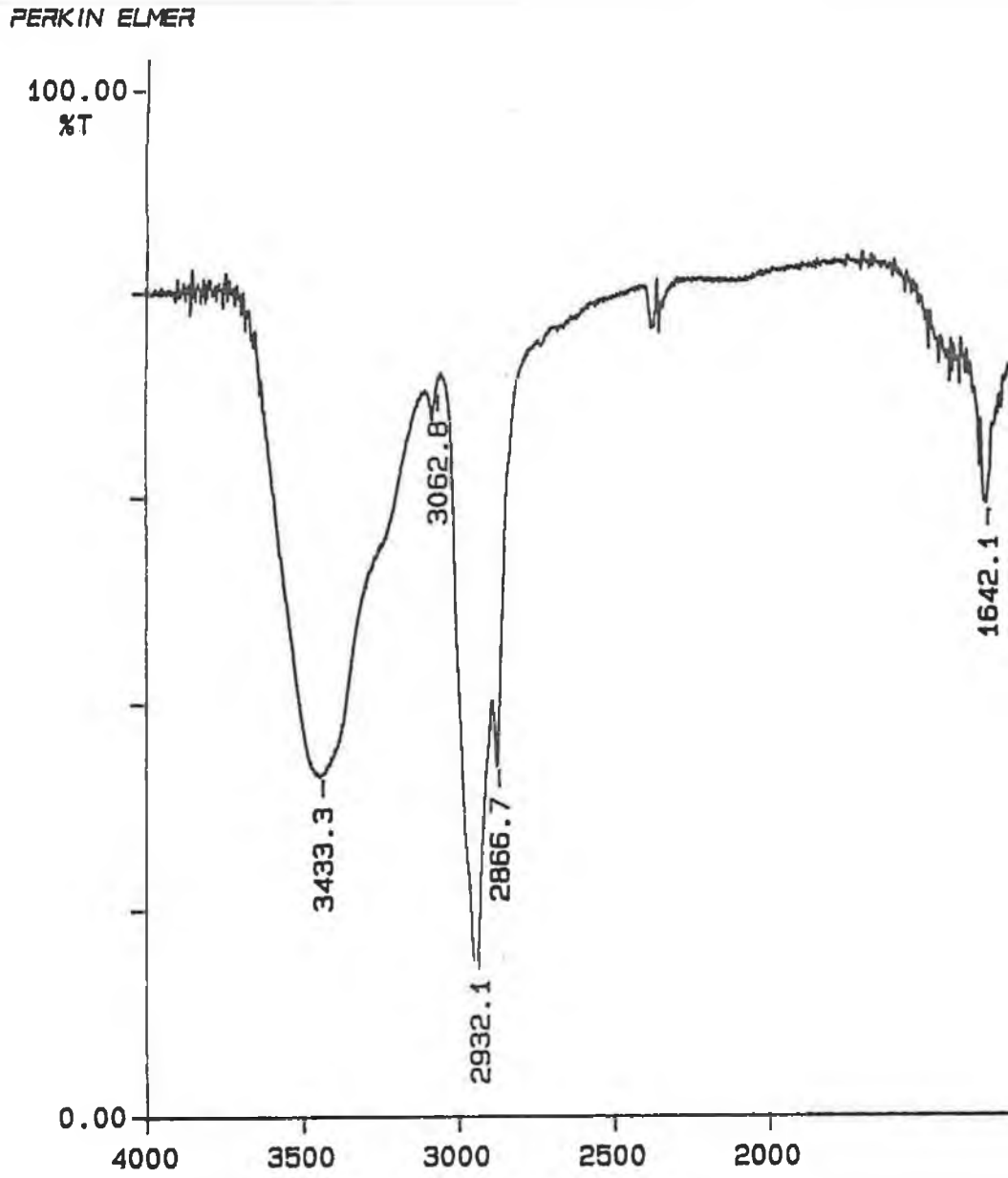
SPECTRUM 8


Spectrum Plot C:\SATURN\DATA\COMBER Date: 04/20/98 16:38:26
Comment: 9/4/98
Scan: 996 Seg: 1 Group: 0 Retention: 16.59 RIC: 2737116 Masses: 26-298
Pks: 178 Base Pk: 28 Int: 227119 100.00% = 227119
100% 28
41
55
INT
69
81
96
110
123
137
152
166
180
194
222
246
264
278
296
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300

SPECTRUM 9

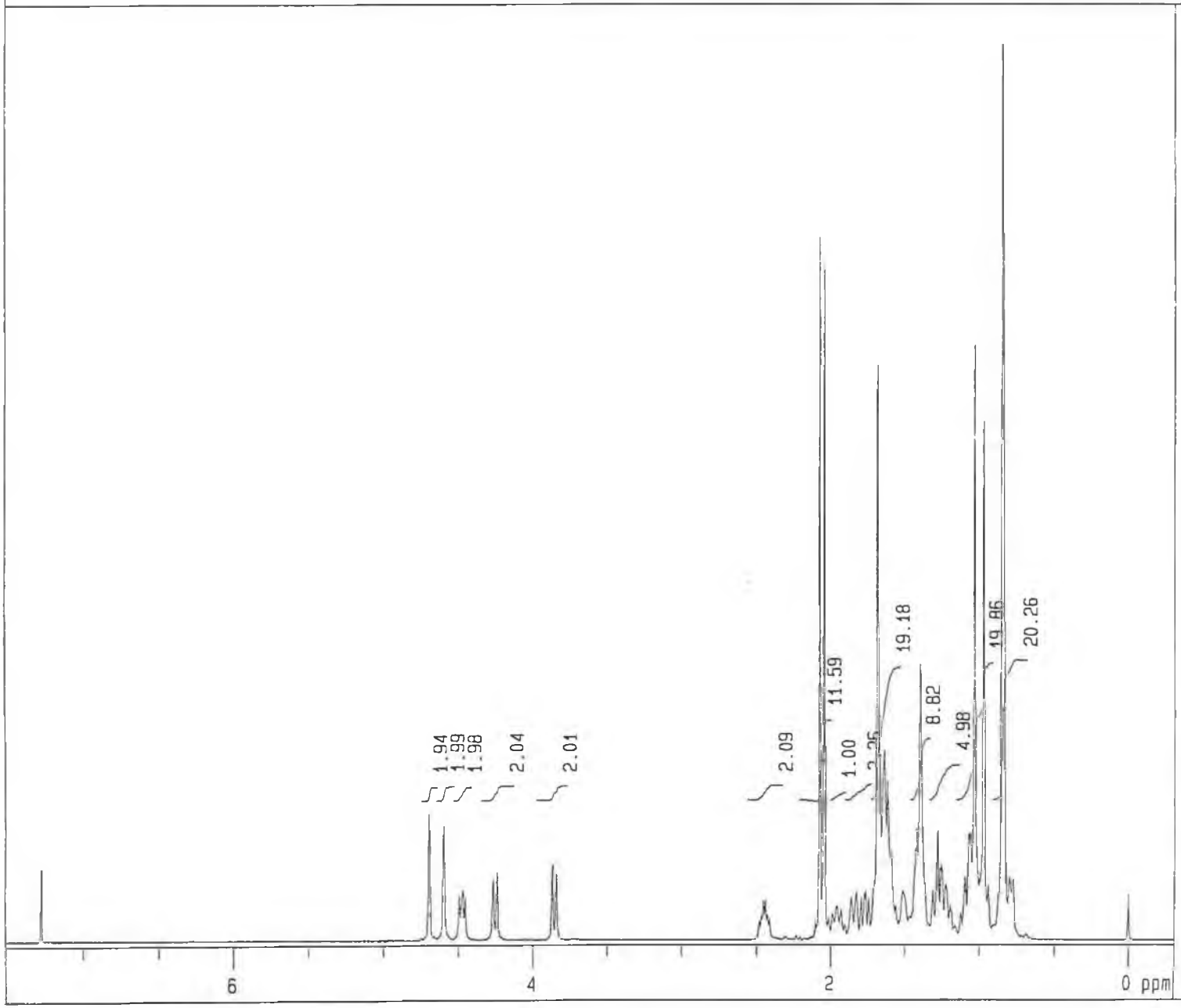

Spectrum Plot
C:\SATURN\DATA\COMBER
Date: 04/20/98 16:38:26
Comment: 9/4/98
Scan: 1034 Seg: 1 Group: 0 Retention: 17.23 RIC: 470946 Masses: 20-300
Pks: 182 Base Pk: 28
Int: 151701 100.00% = 151701
100%
28
INT
74
41
87
55
298
97
111
129
143
157
171
185
199
213
227
241
255
269
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300

SPECTRUM 10


PERKIN ELMER
100.00
%T
0.00
4000
3500
3000
2500
2000
3433.3
3062.8
2932.1
2866.7
1642.1

SPECTRUM 11

1H Line



Date : Tue Feb 10 09:11:08 1998

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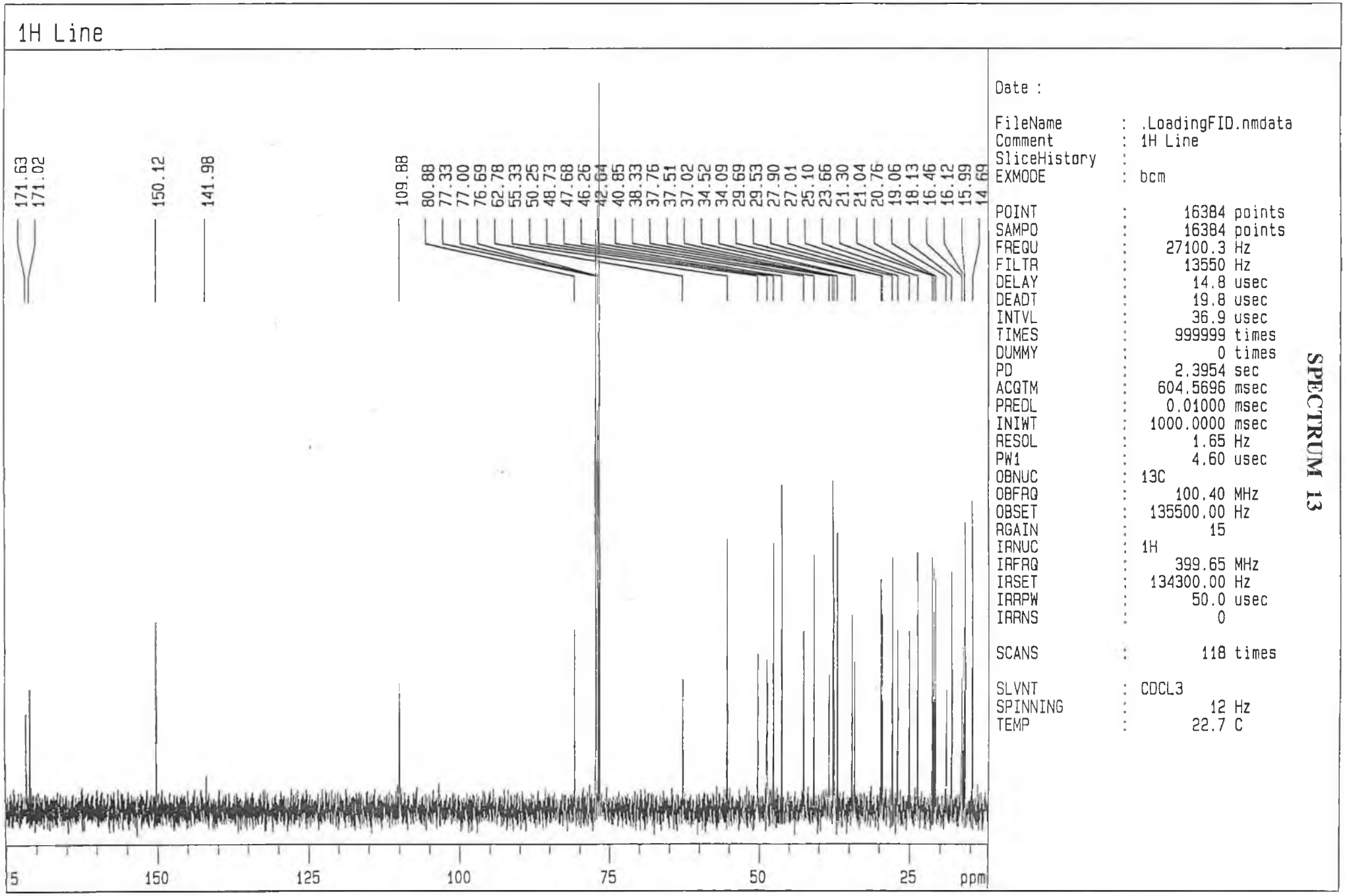
SAMPO	:	16384 points
FREQU	:	7993.6 Hz
FILTR	:	4000 Hz
DELAY	:	50.0 usec
DEADT	:	72.2 usec
INTVL	:	125.1 usec
TIMES	:	999999 times
DUMMY	:	0 times
PD	:	4.9504 sec
ACQTM	:	2049.6384 msec
PREDL	:	0.01000 msec
INIWT	:	1000.0000 msec
RESOL	:	0.49 Hz
PW1	:	5.67 usec
OBNUC	:	1H
OBFRQ	:	399.65 MHz
OBSET	:	134300.00 Hz
RGAIN	:	15
SCANS	:	3 times
SLVNT	:	CDCL3
SPINNING	:	10 Hz
TEMP	:	21.2 C

SPECTRUM 12


1H Line
171.63
171.02
150.12
141.98
109.88
80.88
77.33
77.00
76.69
62.78
55.33
50.25
48.73
47.68
46.26
42.64
40.85
38.33
37.76
37.51
37.02
34.52
34.09
29.69
29.53
27.90
27.01
25.10
23.66
21.30
21.04
20.76
19.06
18.13
16.46
16.12
15.99
14.69
5
150
125
100
75
50
25
ppm
Date :
FileName : .LoadingFID.nmdata
Comment : 1H Line
SliceHistory :
EXMODE : bcm
POINT : 16384 points
SAMPO : 16384 points
FREQU : 27100.3 Hz
FILTR : 13550 Hz
DELAY : 14.8 usec
DEADT : 19.8 usec
INTVL : 36.9 usec
TIMES : 999999 times
DUMMY : 0 times
PD : 2.3954 sec
ACQTM : 604.5696 msec
PREDL : 0.01000 msec
INIWT : 1000.0000 msec
RESOL : 1.65 Hz
PW1 : 4.60 usec
OBNUC : 13C
OBFRQ : 100.40 MHz
OBSET : 135500.00 Hz
RGAIN : 15
IRNUC : 1H
IRFRQ : 399.65 MHz
IRSET : 134300.00 Hz
IRRPW : 50.0 usec
IRRNS : 0
SCANS : 118 times
SLVNT : CDCL3
SPINNING : 12 Hz
TEMP : 22.7 C
SPECTRUM 13

trg_mk0008 Scan 1 (Av 51-59 Acq) 100%=526 mv 28 Nov 95 15:36
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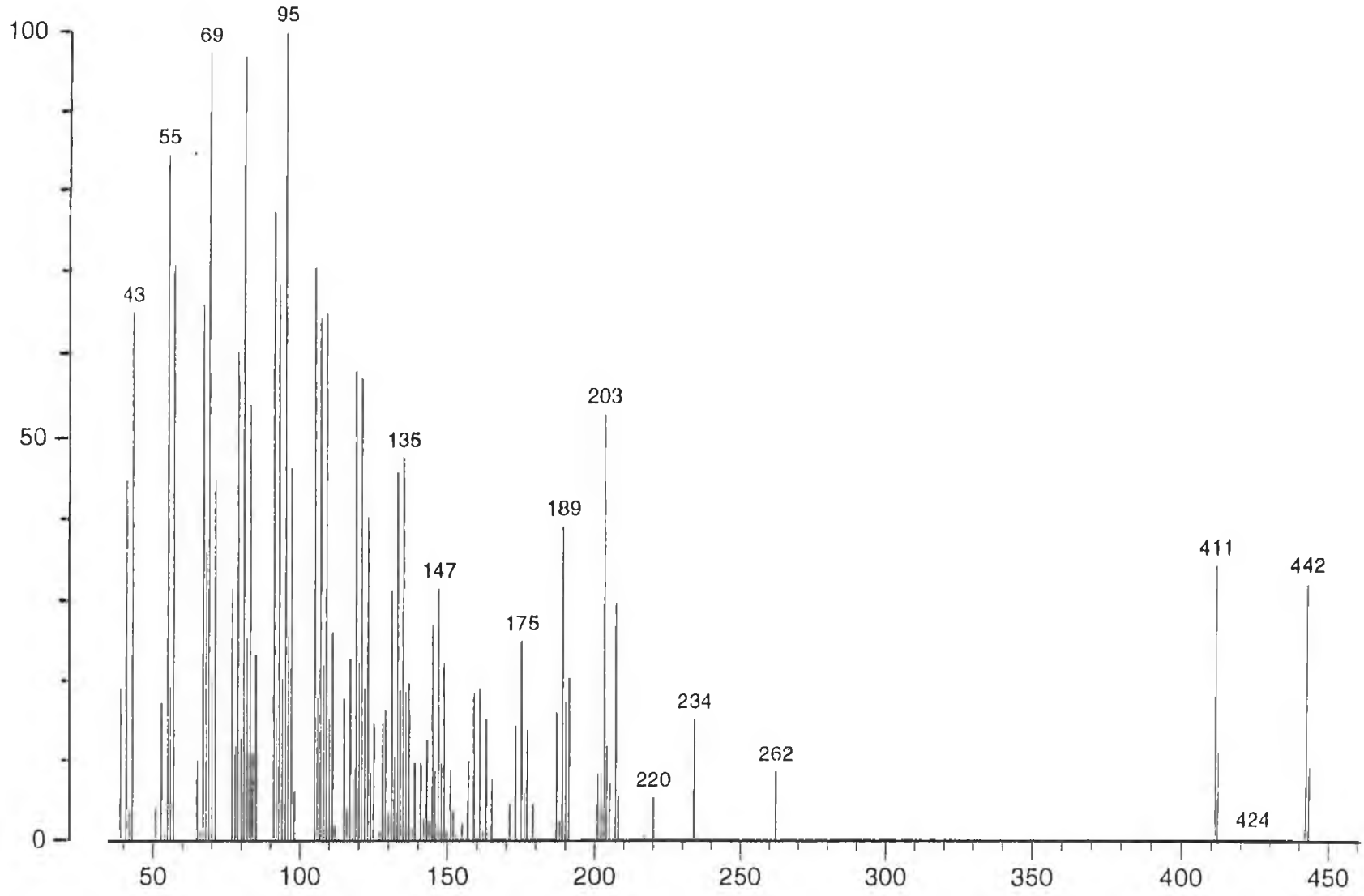
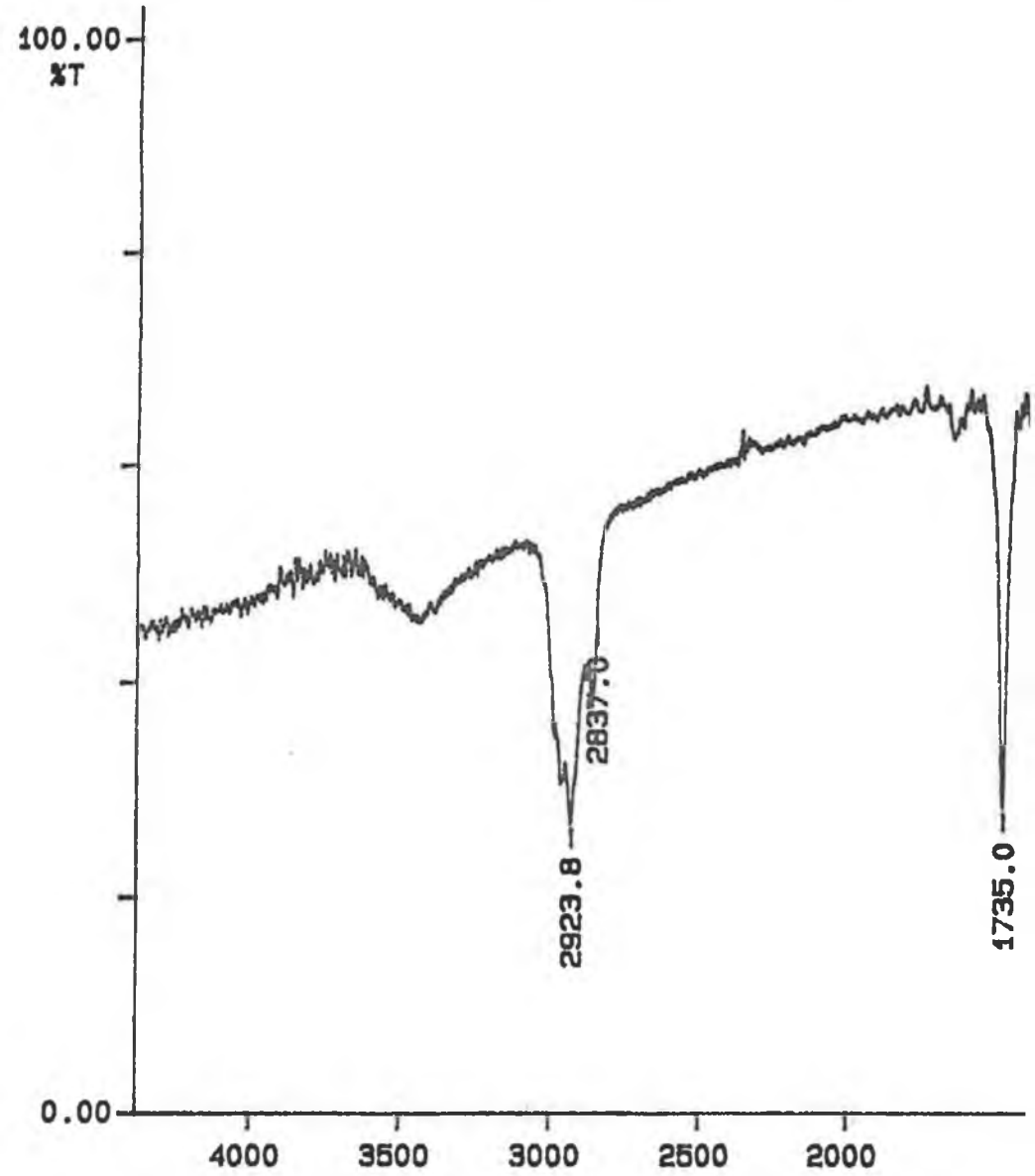
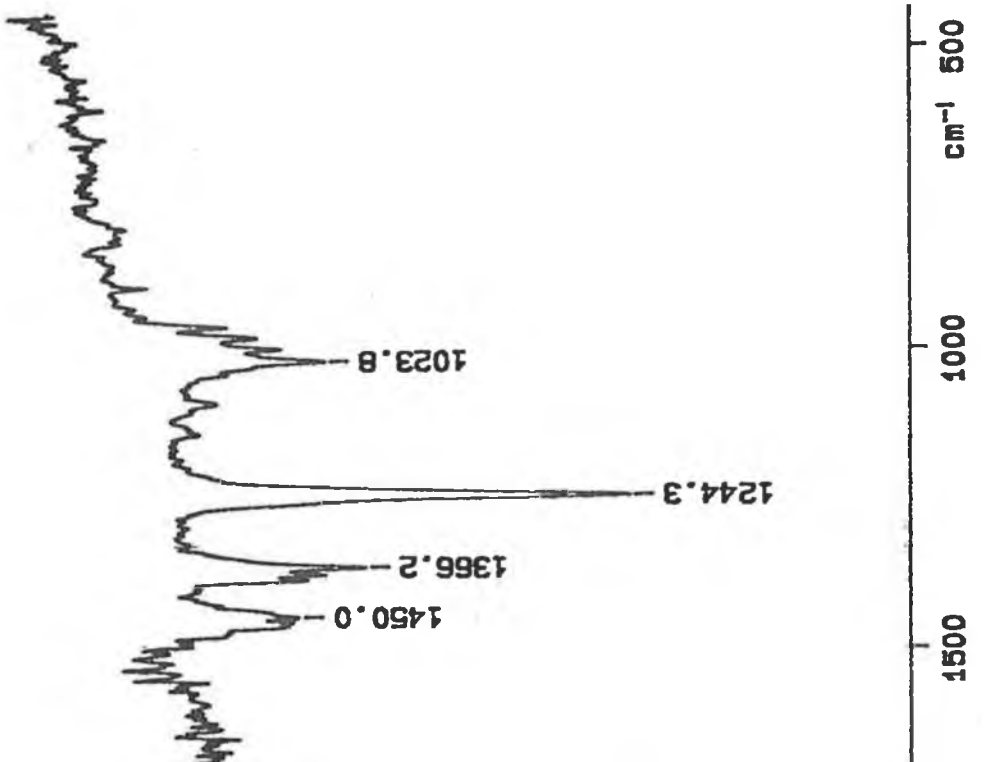


SPECTRUM 14

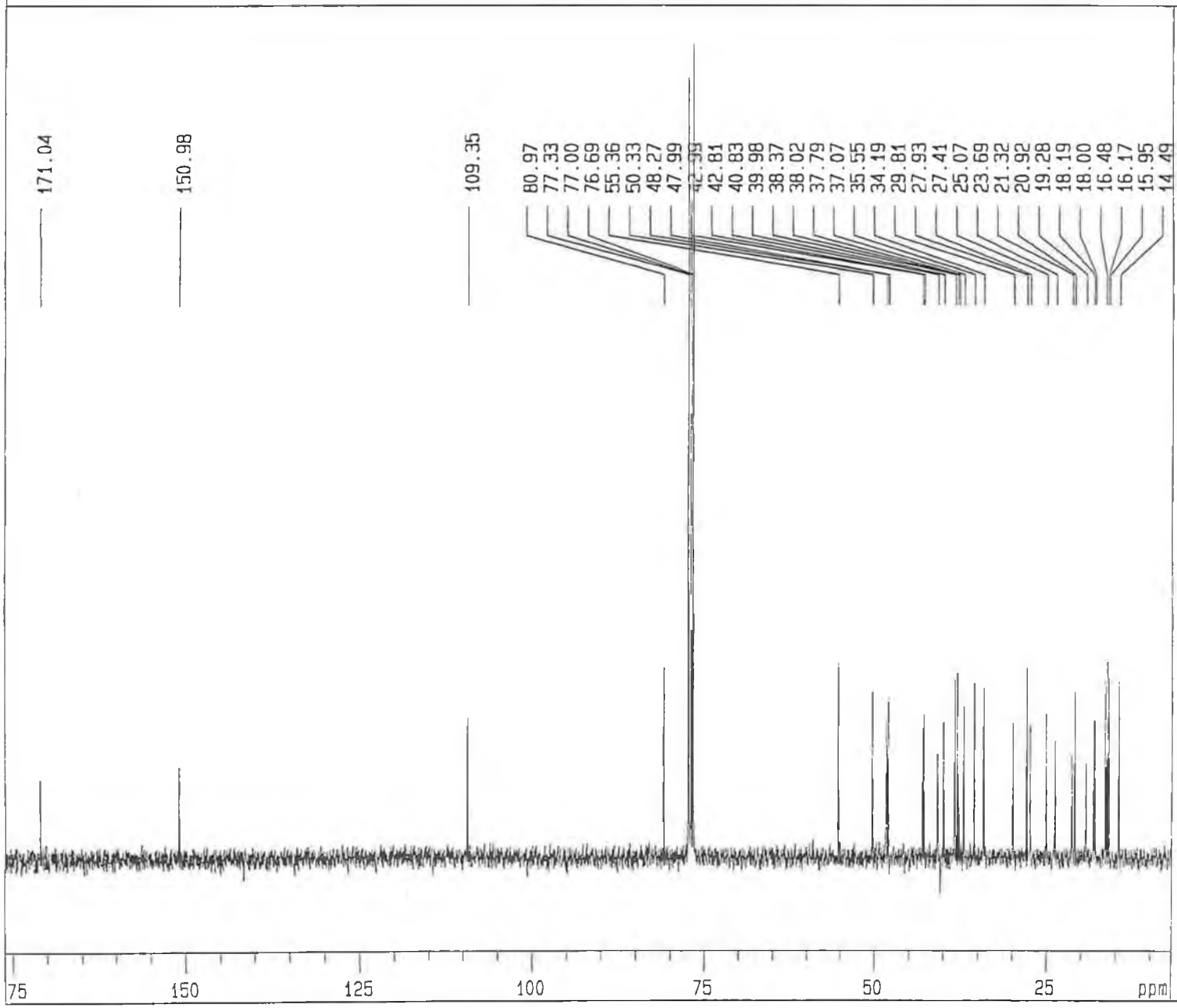


95/11/20 12:28
X: 1 scan, 4.0cm-1

SPECTRUM 15



1H Line



Date : Mon Feb 9 11:28:42 1998

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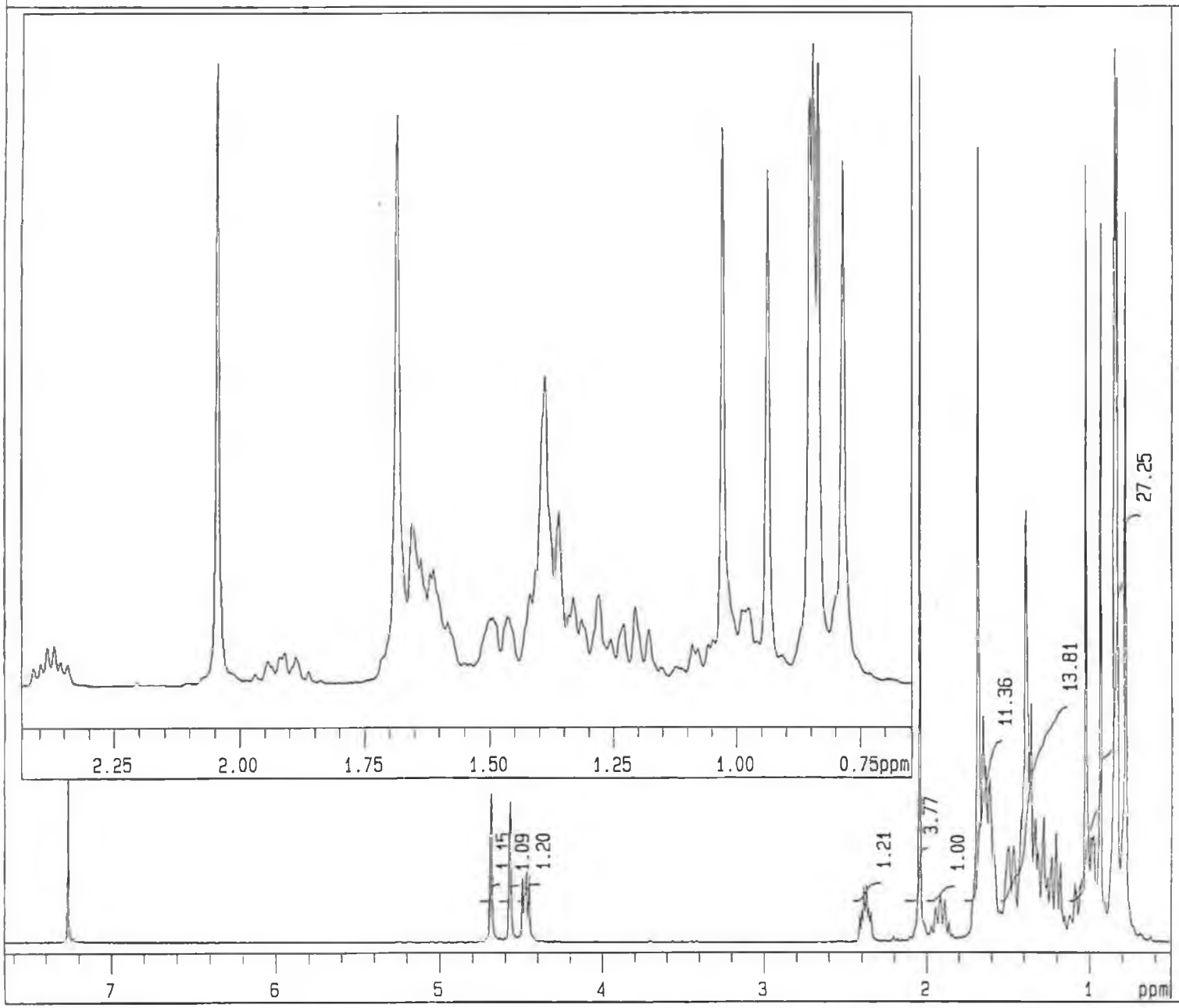
POINT	:	16384 points
SAMPO	:	16384 points
FREQU	:	27100.3 Hz
FILTR	:	13550 Hz
DELAY	:	14.8 usec
DEADT	:	19.8 usec
INTVL	:	36.9 usec
TIMES	:	2584 times
DUMMY	:	0 times
PD	:	2.3954 sec
ACQTM	:	604.5696 msec
PREDL	:	0.01000 msec
INIWT	:	1000.0000 msec
RESOL	:	1.65 Hz
PW1	:	4.60 usec
OBNUC	:	13C
OBFRQ	:	100.40 MHz
OBSET	:	135500.00 Hz
RGAIN	:	28
IRNUC	:	1H
IRFRQ	:	399.65 MHz
IRSET	:	134300.00 Hz
IRRPW	:	50.0 usec
IRRNS	:	0
SCANS	:	383 times
SLVNT	:	CDCL3
SPINNING	:	10 Hz
TEMP	:	22.9 C

SPECTRUM 16

1H Line

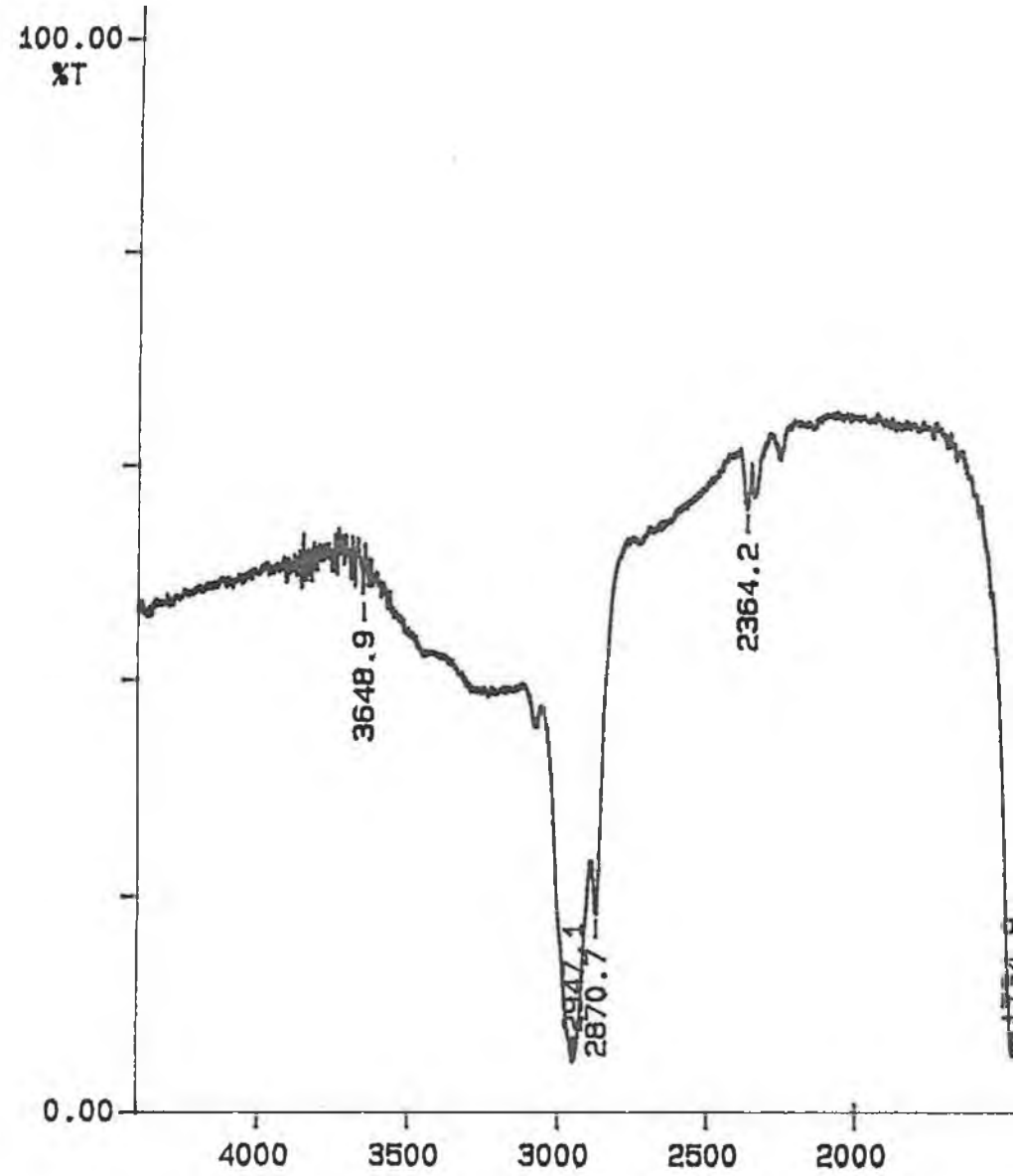


Date : Mon Feb 9 11:08:36 1998

FileName	:	.LoadingFID.nmdata
Comment	:	1H Line
SliceHistory	:	
EXMODE	:	non
POINT	:	16384 points
SAMPO	:	16384 points
FREQU	:	7993.6 Hz
FILTR	:	4000 Hz
DELAY	:	50.0 usec
DEADT	:	72.2 usec
INTVL	:	125.1 usec
TIMES	:	2584 times
DUMMY	:	0 times
PD	:	4.9504 sec
ACQTM	:	2049.6384 msec
PREDL	:	0.01000 msec
INIWT	:	1000.0000 msec
RESOL	:	0.49 Hz
PW1	:	5.67 usec
OBNUC	:	1H
OBFRQ	:	399.65 MHz
OBSET	:	134300.00 Hz
RGAIN	:	17
SCANS	:	9 times
SLVNT	:	CDCL3
SPINNING	:	11 Hz
TEMP	:	21.3 C

SPECTRUM 17

PERKIN ELMER

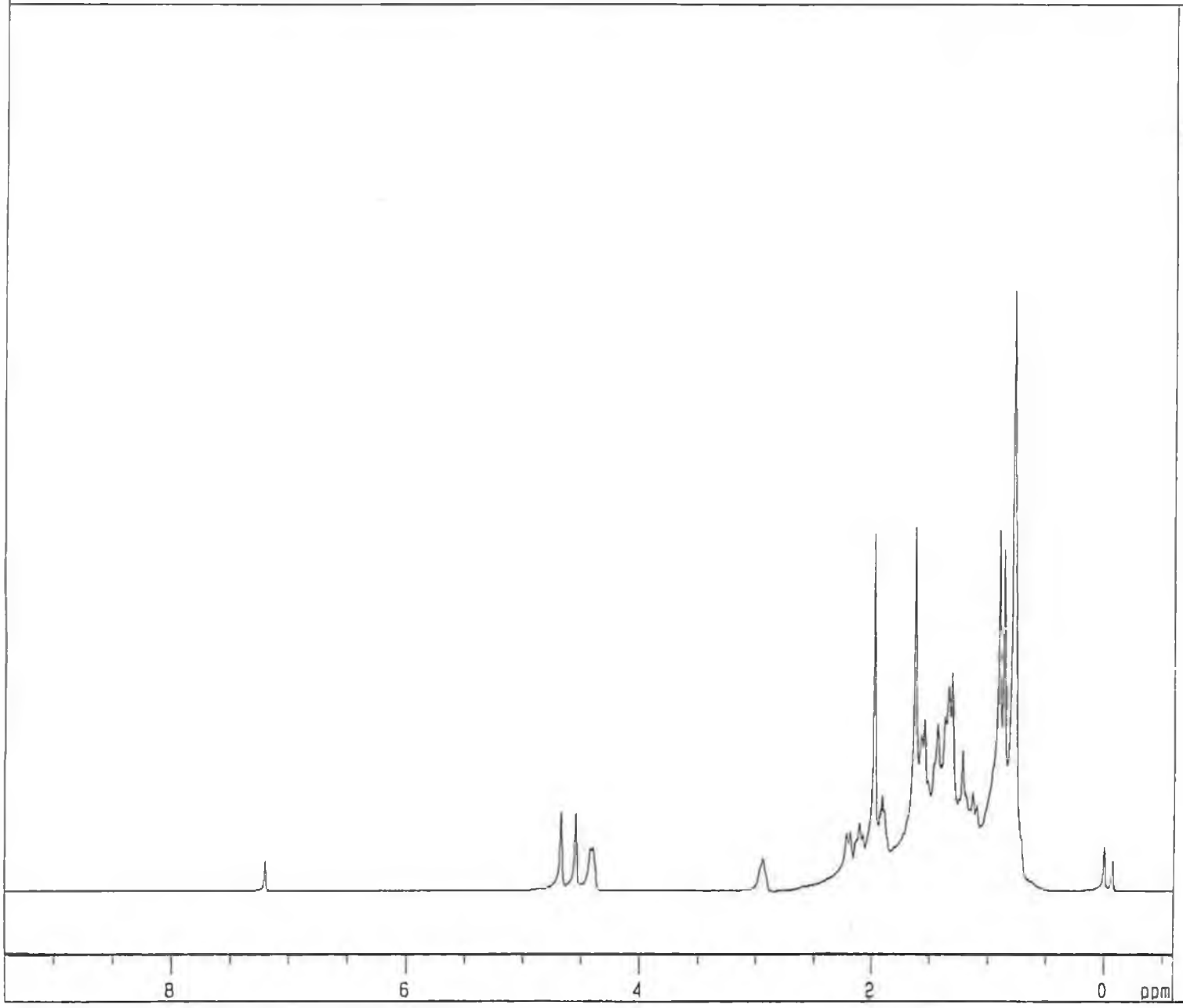


98/03/27 17:54
X: 4 scans, 4.0cm-1
Betulinic Acid

1640.9
1450.8
1375.0
1245.0
1028.8
979.7
884.0
733.2
1500
1000
cm-1
500

SPECTRUM 18

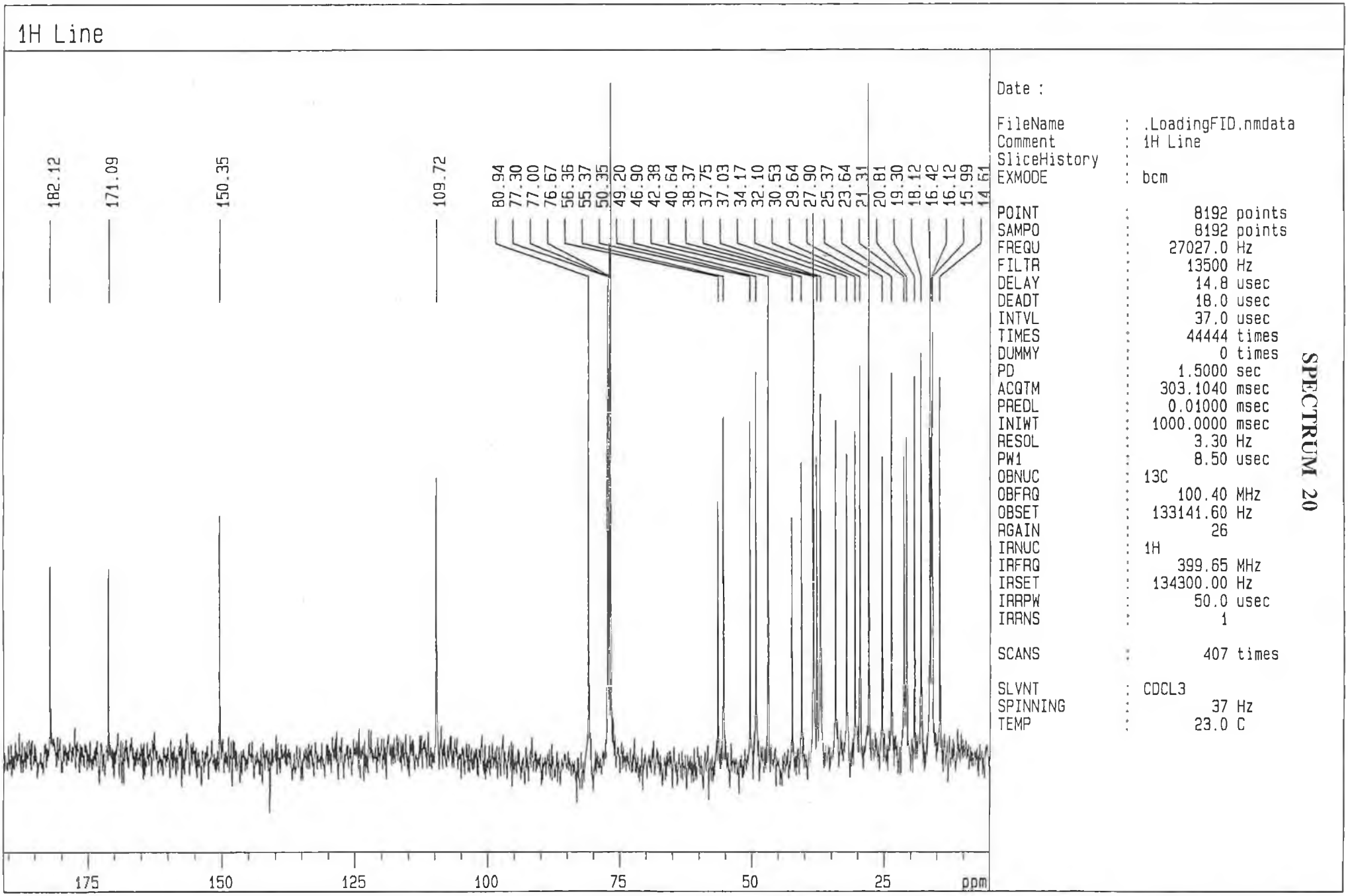
1H Line

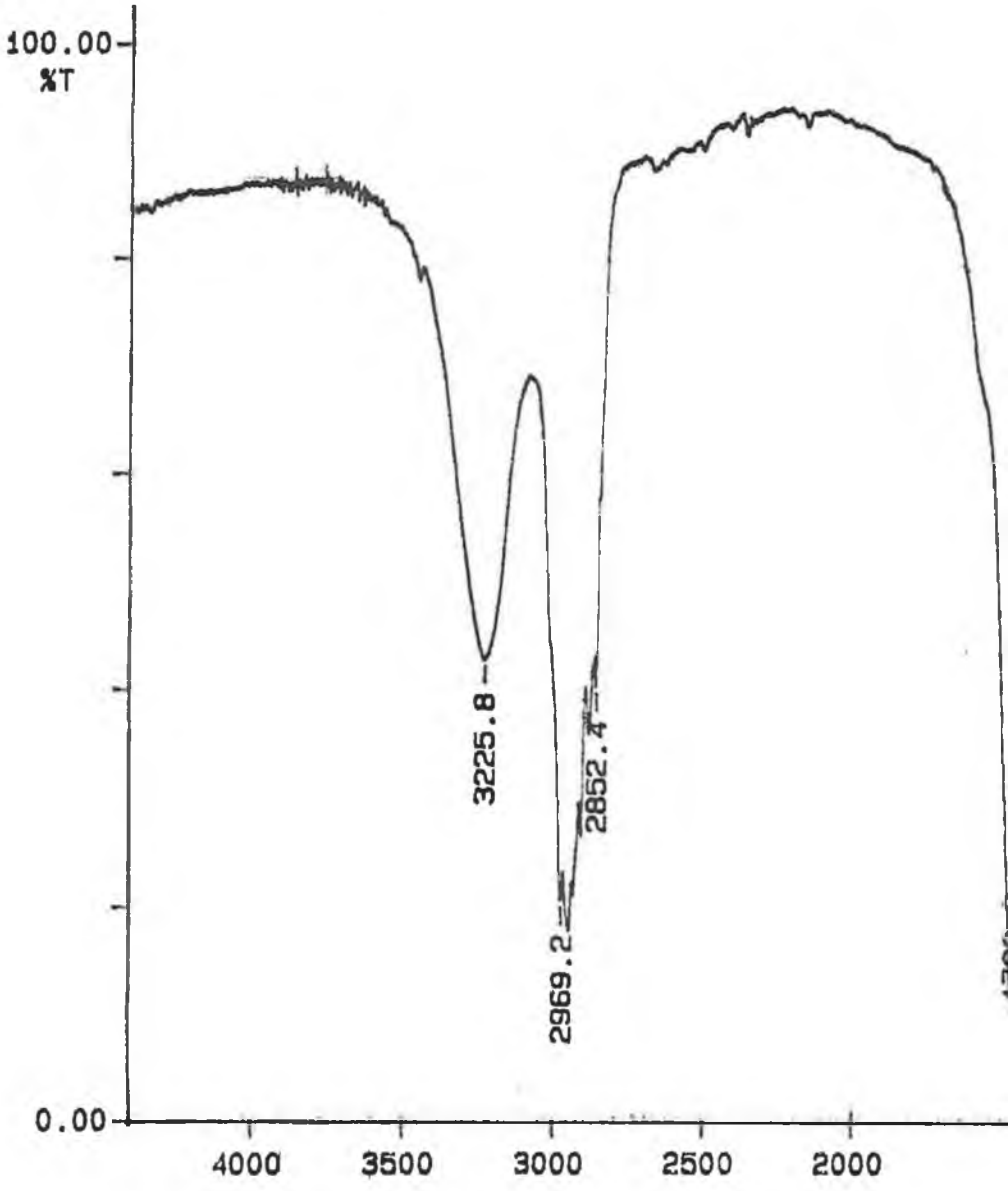


Date : Tue May 12 20:15:19 1998

FileName	:	.LoadingFID.nmdata
Comment	:	1H Line
SliceHistory	:	
EXMODE	:	non
POINT	:	8192 points
SAMPO	:	8192 points
FREQU	:	4000.0 Hz
FILTR	:	2000 Hz
DELAY	:	100.0 usec
DEADT	:	144.3 usec
INTVL	:	250.0 usec
TIMES	:	24 times
DUMMY	:	0 times
PD	:	1.9504 sec
ACQTM	:	2048.0000 msec
PREDL	:	0.01000 msec
INIWT	:	1000.0000 msec
RESOL	:	0.49 Hz
PW1	:	11.50 usec
OBNUC	:	1H
OBFRQ	:	399.65 MHz
OBSET	:	134300.00 Hz
RGAIN	:	14
SCANS	:	24 times
SLVNT	:	CDCL3
SPINNING	:	36 Hz
TEMP	:	22.3 C

SPECTRUM 19


1H Line
182.12
171.09
150.35
109.72
80.94
77.30
77.00
76.67
56.36
55.37
50.35
49.20
46.90
42.38
40.64
38.37
37.75
37.03
34.17
32.10
30.53
29.64
27.90
25.37
23.64
21.31
20.81
19.30
18.12
16.42
16.12
15.99
14.61
175
150
125
100
75
50
25
ppm
Date :
FileName : .LoadingFID.nmdata
Comment : 1H Line
SliceHistory :
EXMODE : bcm
POINT : 8192 points
SAMPO : 8192 points
FREQU : 27027.0 Hz
FILTR : 13500 Hz
DELAY : 14.8 usec
DEADT : 18.0 usec
INTVL : 37.0 usec
TIMES : 44444 times
DUMMY : 0 times
PD : 1.5000 sec
ACQTM : 303.1040 msec
PREDL : 0.01000 msec
INIWT : 1000.0000 msec
RESOL : 3.30 Hz
PW1 : 8.50 usec
OBNUC : 13C
OBFRQ : 100.40 MHz
OBSET : 133141.60 Hz
RGAIN : 26
IRNUC : 1H
IRFRQ : 399.65 MHz
IRSET : 134300.00 Hz
IRRPW : 50.0 usec
IRRNS : 1
SCANS : 407 times
SLVNT : CDCL3
SPINNING : 37 Hz
TEMP : 23.0 C
SPECTRUM 20

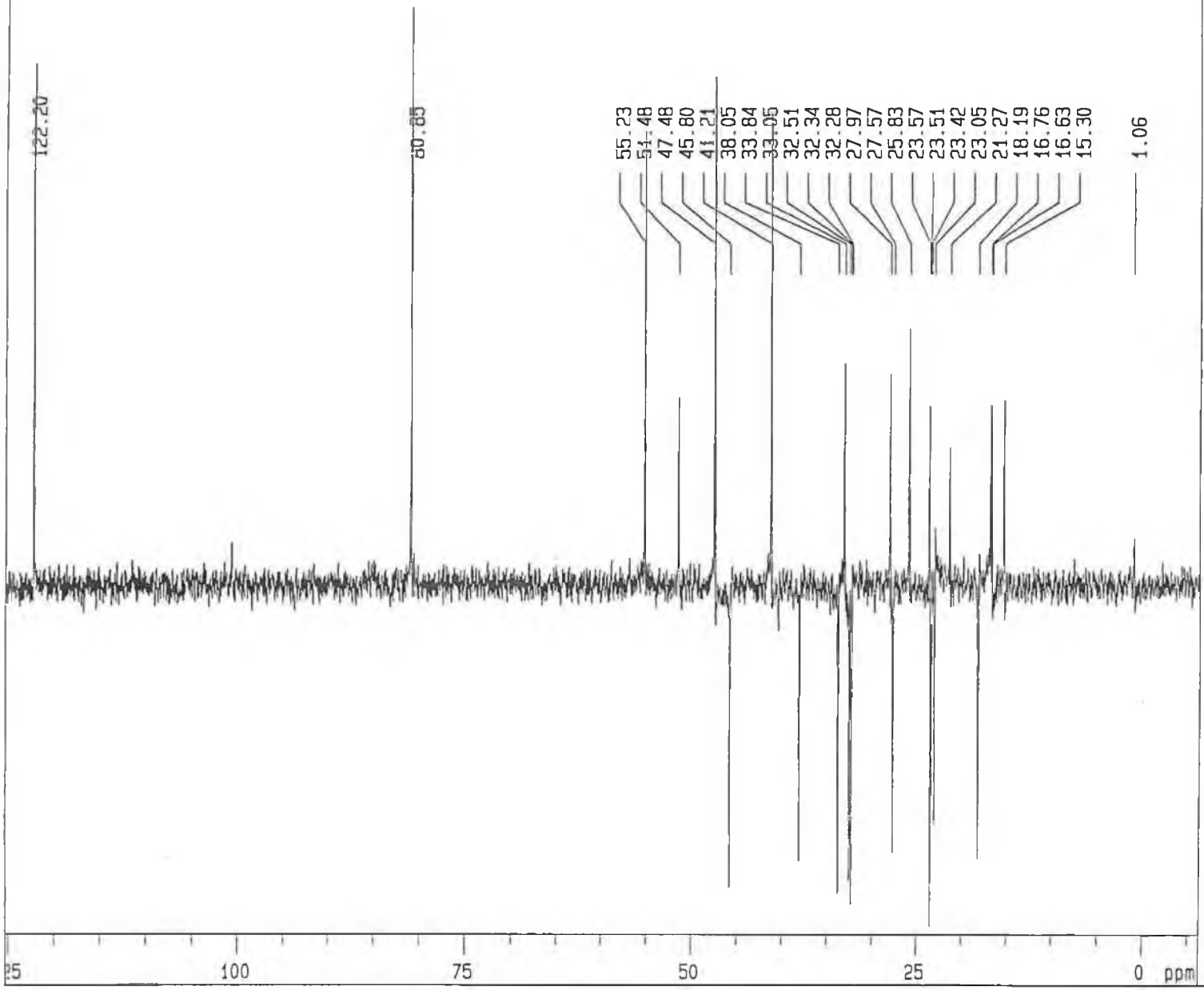


95/02/15 14:35
X: 16 scans, 4.0cm-1

1681.3
1466.3
1369.2
1281.7
1179.7
1009.8
817.0
740.5
756.4
637.9
1500
1000
cm-1
500

SPECTRUM 21

1H Line

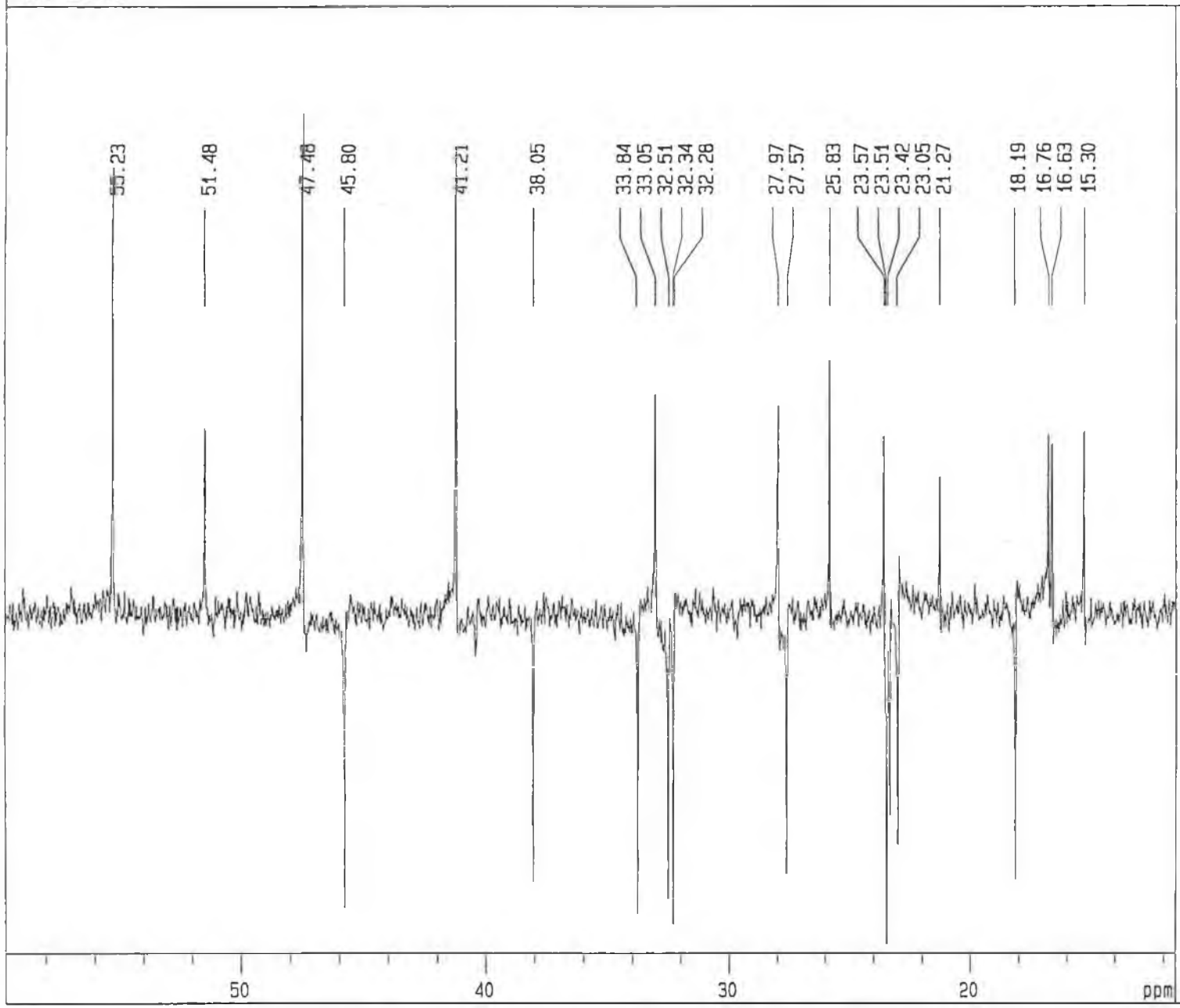


Date :

FileName	:	.LoadingFID.nmdata
Comment	:	1H Line
SliceHistory	:	
EXMODE	:	dept
POINT	:	16384 points
SAMPO	:	16384 points
FREQU	:	27100.3 Hz
FILTR	:	13550 Hz
DELAY	:	14.8 usec
DEADT	:	17.5 usec
INTVL	:	36.9 usec
TIMES	:	2584 times
DUMMY	:	0 times
PD	:	1.5000 sec
ACQTM	:	604.5696 msec
PREDL	:	0.01000 msec
INIWT	:	1000.0000 msec
RESOL	:	1.65 Hz
PW1	:	9.20 usec
OBNUC	:	13C
OBFRQ	:	100.40 MHz
OBSET	:	135500.00 Hz
RGAIN	:	28
PW2	:	17.10 usec
PW3	:	11.40 usec
JCNST	:	145.00 Hz
IRNUC	:	1H
IRFRQ	:	399.65 MHz
IRSET	:	134300.00 Hz
IRRPW	:	50.0 usec
IRRNS	:	0
SCANS	:	115 times
SLVNT	:	CDCL3
SPINNING	:	12 Hz
TEMP	:	21.9 C

SPECTRUM 22

1H Line

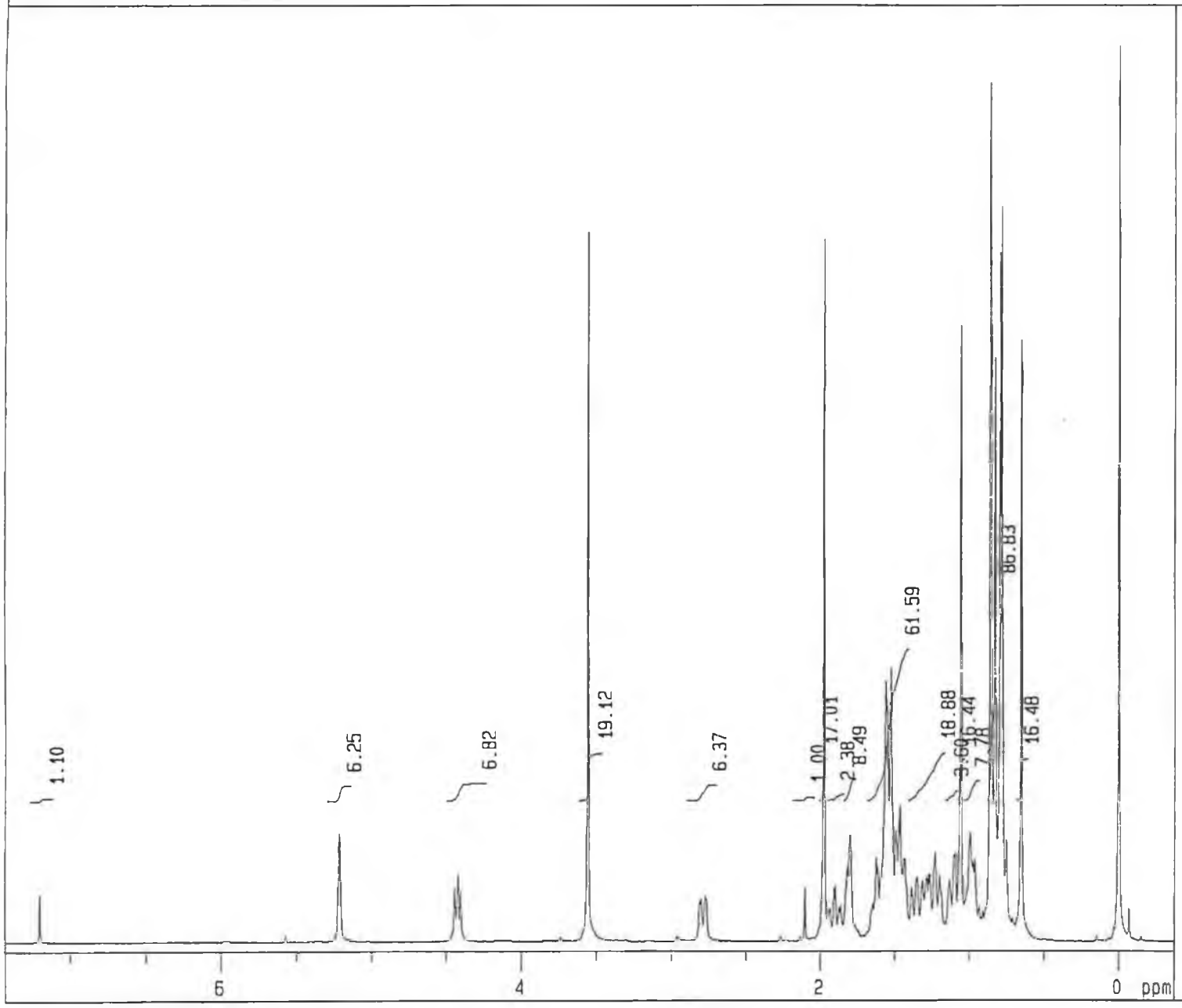


Date :

FileName	:	.LoadingFID.nmdata
Comment	:	1H Line
SliceHistory	:	
EXMODE	:	dept
POINT	:	16384 points
SAMPO	:	16384 points
FREQU	:	27100.3 Hz
FILTR	:	13550 Hz
DELAY	:	14.8 usec
DEADT	:	17.5 usec
INTVL	:	36.9 usec
TIMES	:	2584 times
DUMMY	:	0 times
PD	:	1.5000 sec
ACQTM	:	604.5696 msec
PREDL	:	0.01000 msec
INIWT	:	1000.0000 msec
RESOL	:	1.65 Hz
PW1	:	9.20 usec
OBNUC	:	13C
OBFRQ	:	100.40 MHz
OBSET	:	135500.00 Hz
RGAIN	:	28
PW2	:	17.10 usec
PW3	:	11.40 usec
JCNST	:	145.00 Hz
IRNUC	:	1H
IRFRQ	:	399.65 MHz
IRSET	:	134300.00 Hz
IRRPW	:	50.0 usec
IRRNS	:	0
SCANS	:	115 times
SLVNT	:	CDCL3
SPINNING	:	12 Hz
TEMP	:	21.9 C

SPECTRUM 23

1H Line

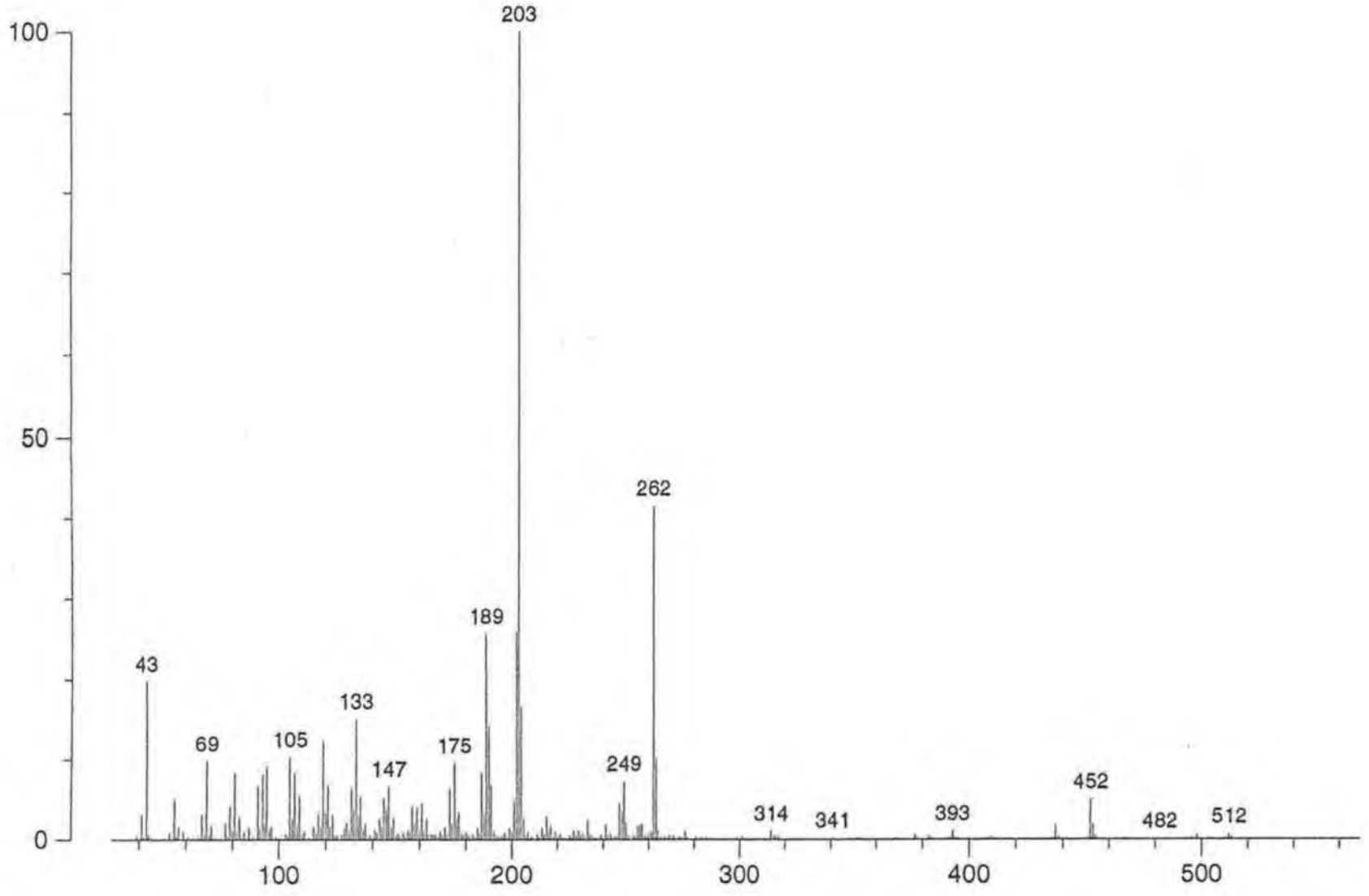


Date : Mon Feb 9 11:04:53 1998

FileName	:	.LoadingFID.nmdata
Comment	:	1H Line
SliceHistory	:	
EXMODE	:	non
POINT	:	16384 points
SAMPO	:	16384 points
FREQU	:	7993.6 Hz
FILTR	:	4000 Hz
DELAY	:	50.0 usec
DEADT	:	72.2 usec
INTVL	:	125.1 usec
TIMES	:	2584 times
DUMMY	:	0 times
PD	:	4.9504 sec
ACQTM	:	2049.6384 msec
PREDL	:	0.01000 msec
INIWT	:	1000.0000 msec
RESOL	:	0.49 Hz
PW1	:	5.67 usec
OBNUC	:	1H
OBFRQ	:	399.65 MHz
OBSET	:	134300.00 Hz
RGAIN	:	13
SCANS	:	6 times
SLVNT	:	CDCL3
SPINNING	:	12 Hz
TEMP	:	21.6 C

SPECTRUM 24

trg_mk0001 Scan 2 (Av 37, 37 Acq) 100%=132665 mv 25 Oct 95 12:59
HRP +EI ----- SAMPLE CODE: MK1 --- QUEUE NO. 2314 -----



SPECTRUM 25