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NOVEL NATURAL PRODUCT BASED ANTI-ANXIETY THERAPY AND NATURAL INSECTICIDES

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M.Sc., Monash University, Australia, 1997; B.Sc., Massey University, New Zealand, 1990.

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Dedication

This thesis is dedicated to my parents Sela and Tevita 'Amanaki Puniani and to my whole family, as well as to my Lord and Saviour Jesus Christ, for all their love, provisions and prayers…

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LIST OF ABBREVIATIONS AND SYMBOLS

Ac	acetyl
Ac_2O	Acetic anhydride
br	broad
br s	broad singlet
°C	degress celcius
CBT	Cognitive Behaviour Therapy
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
CH_2Cl_2	methylene chloride
CH_2N_2	diazomethane
cm^{-1}	wavenumber
COSY	Correlation Spectroscopy
δ	chemical shift
d	doublet
DBU	1,8-Diazabicylo[5.4.0]undec-7-ene
DCC	Dicyclohexylcarbodiimide
dd	doublet of doublets
DDT	*p,p'*-Dichlorodiphenyl Trichloroethane
DEPT	Distortionless Enhancement Polarization Transfer
DHP	Dihydropyran
DMAP	4-Dimethylaminopyridine
DMF	Dimethyl formamide

DMS	Dimethyl sulfide
DMSO	Dimethyl sulfoxide
dt	doublet of triplets
EC_{50}	Effective concentration for 50% mortality
ECB	European Corn Borer
EI	electronic ionization
EPM	Elevated Plus-Maze
equiv.	equivalents
Et	ethyl
Et_3N	triethylamine
Et_2O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
HRMS	high resolution mass spectroscopy
h	hours
HLE	Human Leucocyte Elastase
HMQC	Heteronuclear Multiple Quantum Coherence
^1H NMR	Proton Nuclear Magnetic Resonance
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectroscopy
HWE	Horner-Wadworth-Emmons
Hz	hertz
IC_{50}	concentration to inhibit growth by 50%

IR	infrared
J	coupling constant
liq.	liquid
m	multiplet
$[M]^+$	molecular ion
MDP	methylenedioxyphenyl
Me	methyl
MeOH	methanol
min	minutes
mL	milliliter
m.p.	melting point
MS	low resolution mass spectroscopy
MW	molecular weight
m/z	mass to charge ratio
N	normality
NMR	Nuclear Magnetic Resonance
OAc	acetoxy
PCC	Pyridinium chlorochromate
PDC	Pyridinium dichromate
Ph	phenyl
PLA_2	Phospholipase A_2
PMSO	Polysubstrate monooxygenase
ppm	parts per million

PPTS	Pyridinium *p*-toluenesulfonate
q	quartet
RDA	Retro-Diels-Alder
rt	room temperature
rTMS	Repetitive Transcranial Magnetic Stimulation Therapy
s	singlet
SARS	Structure-Activity Relationship Study
spp.	species
SS-01	Betulinic acid
SS-05	Betulinic acid methyl ester or methyl betulinate
SS-CE	Crude EtOH plant extract
SS-ME	Betulinic acid methyl ester or methyl betulinate
t	triplet
TEA	Triethylamine
THF	Tetrahydrofuran
THP	Tetrahydropyranyl
TI	Therapeutic Index
TLC	Thin Layer Chromatography
Ts	Toluene sulfonate (Tosylate)
WHO	World Health Organization
W-M	Wagner-Meerwein rearrangements

ABSTRACT

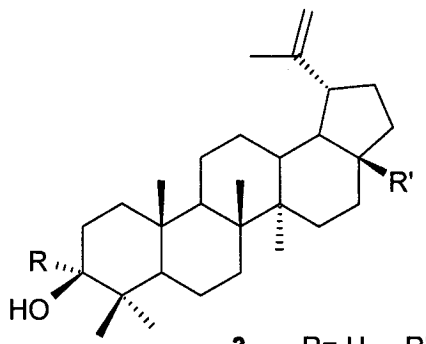
The EtOH extracts of the leaves of Margraviaceae, a relatively rare Central American vine for which ethnobotanical reports suggested possible anti-anxiety properties, showed significant anti-anxiety activity in animal models for anxiety. Subsequent bioassay-guided fractionation of these extracts yielded an EtOAc active fraction (f1). Further bioassay-directed chromatography of (f1), led to the isolation of betulinic acid (**3**) as the bioactive constituent in 0.01% of dry weight. Six known pentacyclic triterpenoids [(**1a**), (**1b**), (**2a**), (**2b**), (**4**), (**5a**)], six known flavonoids [(**6**), (**7**), (**8a**), (**8b**), (**9**), (**10**)], chondrillasterol (**11**), linolenic acid (**12**) and a porphyrin type compound (**13**) were also isolated.

When (**3**) was administered at 0.5 mg per kg (possibly less) in a variety of rat and mouse model assays, the activity of (**3**) was comparable to that of Valium, the most famous member of the benzodiazepines family.

Synthetic derivatives of (**3**) were prepared and evaluated for anti-anxiety activity. Several of the simple esters appear to have ideal properties as new drug candidates. In particular, betulinic acid methyl ester or methyl betulinate (**3a**) exhibited anti-anxiety activity superior to (**3**). The activity profile of (**3a**) is such that (**3a**) can be considered a viable drug candidate.

An excellent relay synthesis of (**3**) from another closely related natural product betulin (**14**), that is abundantly available in Eastern Ontario, was developed.

Radioactive ^{3}H-labelled betulinic acid methyl ester (**3a"**) was also prepared in order to facilitate identification of relevant anti-anxiety receptors and the mechanism of action of the compound. This is important since (**3**) showed no significant binding to any of the 40 anti-anxiety receptors currently implicated in anxiety. Therefore, it appears to act as an anti-anxiety agent via a new mode of action.



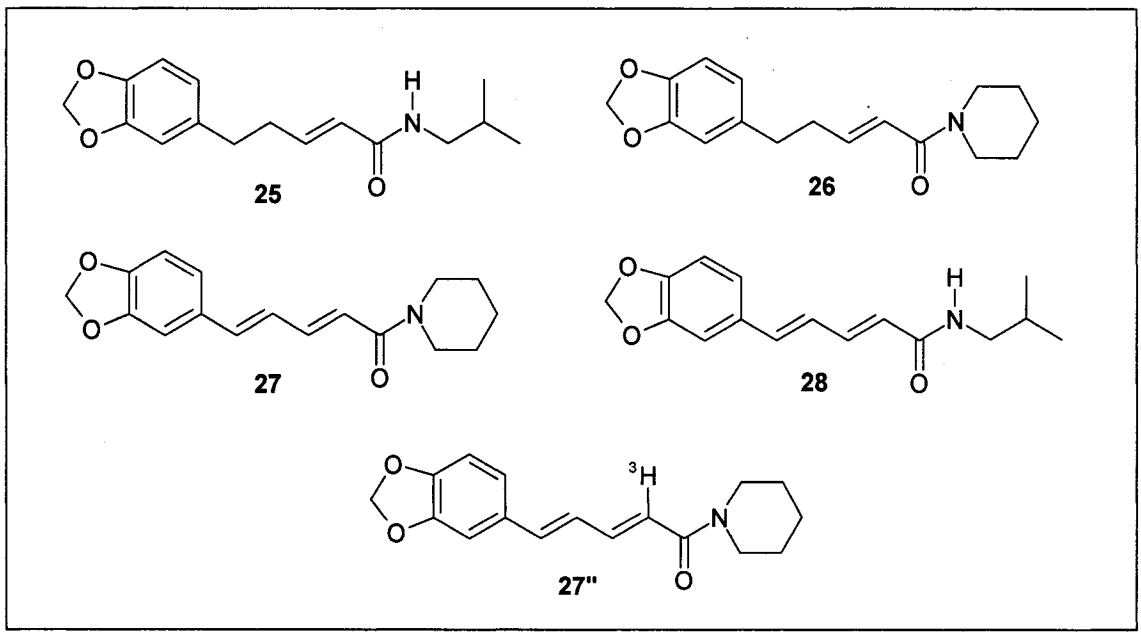
3, R= H, R'=CO_2H
3a, R= H, R'=CO_2Me
3a", R= ^3H, R'=CO_2Me
17, R= H, R'=CH_2OH

In a second project, the active components of a member of the Piperaceae or Pepper family (*P. tuberculatum*) from Costa Rica, were isolated and their structures characterized as 5,6-dihydropiperlonguminine (**25**), 5,6-dihydropiperine (**26**), piperine (**27**) and piperlonguminine (**28**).

Extracts from this neotropical plant had been previously demonstrated by our biology collaborators, Professor Arnason's group, to be strongly insecticidal towards a variety of pests including mosquitoes, earwigs and white grubs. Moreover, the *P. tuberculatum* extracts were as effective as the well-documented Asian (*P.nigrum*) and African (*P.guineense*) *Piper* species.

Piperamides (**25**)-(**28**) were synthesized in sufficient amounts to allow extensive evaluation of their insecticidal properties. Experiments with these piperamides showed that the tertiary and quaternary mixtures have greater-than-additive toxicity compared to single compounds or binary mixtures. That is, these piperamides synergize each other. Compound (**25**) was the most acutely toxic in mosquito larvae bioassays. The field trials to date indicate a high potential for the development of an effective, relatively inexpensive botanically based insecticide.

Radioactive ^3H-labelled piperine (**27"**) was also synthesized for toxicokinetic studies.


25
26
27
28
27"

CHAPTER 1: BACKGROUND

1.1. Natural Product Chemistry

Throughout history, mankind has always been interested in the exploration of natural products due to the plethora of their applications in various aspects of human lives.[1] The use of natural products, in particular plant derived materials in order to improve health and the quality of life is an integral part of mankind's history.[2] Hence, plant-based natural products have played a key role in the development of medicinal chemistry.[3-5]

References abound in the earliest recorded history of essentially all cultures.[6] The birth of the pharmaceutical industry in the late 15th century and its tremendous growth especially since the mid twentieth century spurred the search for natural products possessing, for example, anti-bacterial activity, anti-viral, cytotoxic, fungicidal, insecticidal or pesticidal activity This has led to a broad field of study known as Natural Product Chemistry.[4,7]

Natural Product Chemistry was considered a very demanding area of chemistry until about 1950.[4] Prior to that date, the isolation of pure components from the complex extracts was quite difficult since chromatographic method were still in its early stages of development. Structure assignments were even more difficult due to the absence of today's powerful spectroscopic tools especially nuclear magnetic resonance (NMR), mass spectrometry and the ultimate tool, single crystal x-ray structure determination. Structure determinations were based on chemical degradation studies.[8] These required not only substantial amounts of material, but also very careful analysis of the results. Many times a structure determination of what we now consider a rather simple natural product, for example camphor, required decades of dedicated work.[4] Today, such structure determinations are made within days or even hours.

The introduction of spectroscopic techniques beginning with ultraviolet (UV) visible, infrared (IR), NMR and eventually X-ray structure determination removed much of the "intellectual challenge" involved in structure elucidation

from the organic chemist. Leading figures in the field began to turn their attention to physical organic chemistry, development of new synthetic methods and to the synthesis of complex structures including the known or newly discovered natural products.[9] From the main stream organic chemistry point of view, natural products went into decline as a discipline.

Nevertheless, the field survived and began to flourish again due to the interests of scientists in related fields especially biology and biochemistry. Scientists in these fields brought to the table numerous bioassays for antibiotic, anti-cancer, anti-fungal and insect anti-feedant activity. These bioassays when combined with major advances in separation techniques and structure elucidation, resulted in the isolation and structure determination of an enormous number of biologically active natural products through the "bio-assay" guided approach.[10] The field is now much more interdisciplinary than earlier, and with the advent of pharmacogenomics and gene-chips, the potential of natural products will continue to grow.[11]

Natural products represent a rich and an unparalled source of molecular diversity in the discovery and development of novel therapeutic and pesticidal agents which often prove to be economically and commercially beneficial.[12] Today, the search for new medicines is big business. The search for natural chemicals that can be used in pest management is modest compared to the search for medicines but is no less important.[13]

Many phytochemical studies make use of accumulated ethnobotanical knowledge of traditional or empirical local practioners.[14] In fact, modern researchers seek to identify new bioactive natural products by tapping into the knowledge base of indigenous people in the developing world and have moved to areas of the earth that are species and culture rich like South America.[5] It is estimated that only 5-15% of the approximately 250,000 species of higher plants have been systematically investigated for the presence of bioactive phytochemicals.[15] So, the world of plants is still virtually an untapped reservoir of novel bioactive agents.

The use of natural product based pesticides is now emerging as one of the prime means to protect crop produce and the environment from synthetic pesticidal pollution which is a global problem.[13] This renewed interest in botanical pesticides is also due to the growth in organic growing practices and the recent registration of new anti-feedants from botanical sources.[16]

Why should a plant produce a compound that is useful for the treatment of anxiety is subject to speculation. However, it is sufficient to point out that numerous drugs used in modern day medical practices are plant-derived drugs. It has been estimated that at least 119 drugs derived from 90 different plant species are currently in use in one or more countries, with 77% of these being derived from plants used in traditional medicine.[15] Of the 500 million prescriptions written each year in the US, approximately 125 million involve a pharmaceutical preparation from a plant.[12] The World Health Organisation (WHO) estimated that about 80% of the population in the developing nations relies on plant-based traditional medicine for primary health care.[12,17] Plants offer a cheaper alternative treatment for native people who cannot afford, or have no access to commercially available drugs.

Many of the key and clinically used anti-cancer drugs are natural product based, for example taxol, podophyllotoxin, adriamycin (from a fungus), the vinca alkaloids (vincristine, vinblastine). These successes as well as the increasing public demand for alternative medicine has added impetus for research in this area.[3,12] Approximately 62% of the 87 approved cancer drugs are of natural origin.[15]

These estimates provide some indication of the historical importance of plant natural products as sources of novel therapeutic agents.

The design of this thesis involves studying two different plant sources in the quest for biologically active natural products. It first describes the relatively unknown *Margraviaceae* family,[18] having important anti-anxiety therapeutic properties in chapter 2. The bioactive principles that are responsible for the insecticidal activity of a neoptropical *Piper* species previously evaluated here at the University of Ottawa[19] are reported in chapter 3.

1.2. Anxiety disorders

Anxiety disorder is defined as any anxiety that persists to the point that it interferes with one's life.[20] Anxiety disorders are the most common psychiatric illnesses affecting as many as 87 million people in the world's seven major pharmaceutical markets (G-7) and > 490 million people worldwide.[21,22] A study sponsored by National Institutes of Health estimated that 19.1 million Americans 18-54 years old suffer from anxiety disorders (representing 13.3% of this age group), and as many as 35 million Americans suffer from depression.[23] Many of the people who suffers from depression also suffers from anxiety and vice versa. However, less than 25% receive any form of treatment.

These figures reveal that there is a real and urgent need for new therapeutic preparations that will reduce the incidence of anxiety.

Anxiety is characterized by an excessive and persistent sense of apprehension, uncertainty or tension stemming from the anticipation of an imagined or real threat. The physical symptoms include tachycardia, palpitation, sweating, disturbed breathing, trembling or even paralysis.[20]

Anxiety disorder manifests itself in many distinct but related forms.[24] The five most common forms are: Panic disorder (recurrent, unexpected attacks of acute anxiety or panic attacks peaking within 10 minutes); Specific phobia (consuming fear of an object or situation that is not harmful under general conditions); Obsessive-compulsive disorder (pre-occupation with specific irrational thoughts, images, impulses accompanied by elaborate or bizarre rituals); Post-traumatic stress disorder (repeated, anxious reliving of an event over an extended period of time) and Generalized anxiety disorder (ongoing anxiety or uncontrollable worry lasting at least 6 months).

Chronic anxiety disorders are associated with a high prevalence of co-morbidity and suicide risks. They are also strongly linked with impairment and disability in multiple spheres, high rates of school drop-out, marital instability, absenteeism from work, high utilization of primary care services and dependence on social welfare.[25]

While little information is available regarding the economic impact of anxiety disorders in Canada, US data indicate that annual cost of anxiety disorder is approximately $46.6 billion in 1990, nearly one-third of the nation's total mental health bill of $148 billion.[26] Such data verify that anxiety disorders are serious mental illnesses with tragic costs to the individual, the family and the society.

Anxiety disorders appear to develop from a complex set of risk factors such as genetics, brain chemistry, personality and life experiences.[27] They also seemed to be more prevalent in women.[28] Life experiences such as long-term exposure to abuse, violence or poverty may affect individuals' susceptibility to these illnesses.

Early this year, an "anxiety gene" (Pet 1-gene) was discovered in mice.[29] Pet 1-gene appears to be the first to affect adult emotional behaviour by regulating the levels of serotonin in humans. When this gene was deleted by knockout technique, it resulted in increased anxiety.

According to Professor Quirk (Puerto Rico), the prefrontal cortex in front of the brain (also known as the infralimbic area), plays a role in abolishing anxiety in laboratory rats.[30] Animals with the most activity in their prefrontal cortex are less likely to show anxiety. It was anticipated that future studies may produce anxiety medication that boost activity in this area and thus, help people with anxiety disorder especially post-traumatic stress disorders.

1.3. Treatment of anxiety disorders

Although each condition of anxiety disorder has its own unique characteristics, the full spectrum of anxiety disorders can be often treated effectively with psychological and pharmacological interventions prescribed alone or in combination.

The most effective psychological intervention appears to be Cognitive Behaviour Therapy (CBT).[31] This require 12-16 one-hour sessions with experts who have received specialized training. Two major disadvantages of CBT is the limited availability of trained therapists especially in rural areas and the relatively slow treatment response time.

Repetitive Transcranial Magnetic Stimulation Therapy (rTMS) has recently been demonstrated to have a therapeutic effect on anxiety disorders and depression.[32] In this treatment, part of the brain is stimulated by magnetic pulses.

1.3.1. Medication

Drugs which have demonstrated pharmacological efficacy in treating the core symptoms of anxiety disorders include benzodiazepines (e.g. Valium or Diazepam, Ativan or Lorazepam), azaspirodecanediones (e.g. Buspirone or Buspar) and several classes of antidepressants such as SSRI's (Selective Serotonin Reuptake Inhibitors, e.g. Prozac or Fluoxetine, Paxil), (**Figure 1.3.1.**).[33]

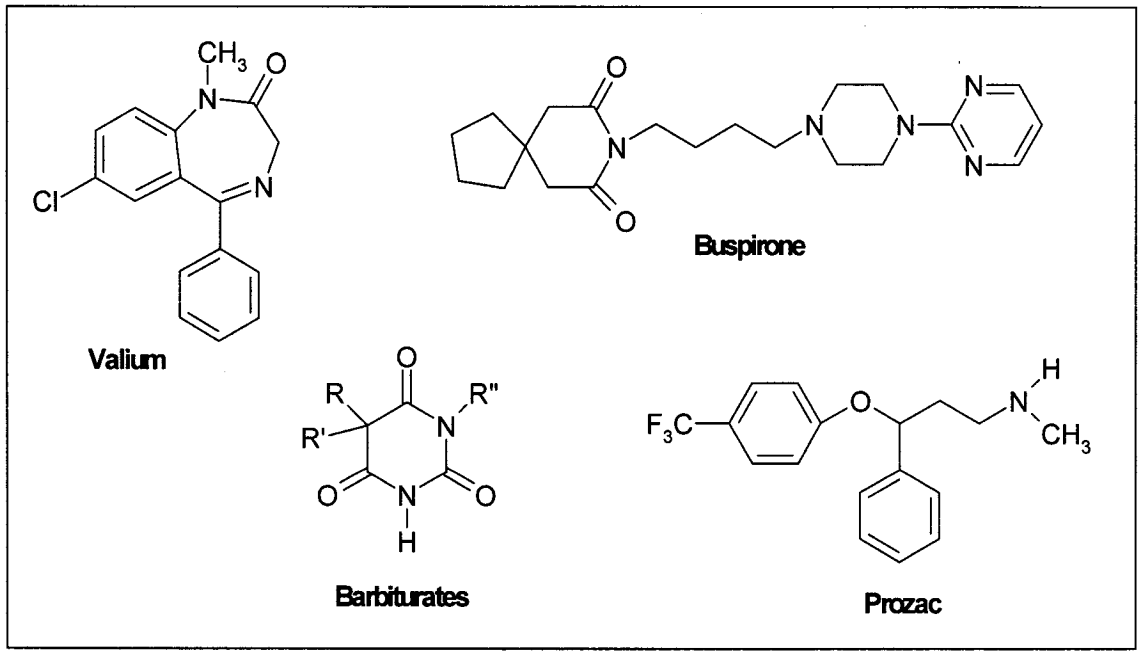


Figure 1.3.1: Common anti-anxiety and anti-depressant drugs.

The older sedative-hypnotics (e.g. Barbiturates, **Figure 1.3.1**) were discontinued due to severe side effects such as development of tolerance (whereby increasing doses are required to produce the same effect often resulting in complete loss of clinical efficacy) and liability of becoming dependent on the drug (where one would experience withdrawal symptoms upon discontinuation of drug treatment). The barbiturates are also potentially lethal when taken with alcohol or in large doses.

Librium and Valium, two member of the benzodiazepines were introduced in the 1960's to replace barbiturates. The benzodiazepines Valium are also fraught with similar side effects but these drugs continue to be heavily prescribed due to the increasing need for hypnotic and anti-anxiety drugs. The benzodiazepines (Ativan) have also been associated with vision losts, seizure and hallucination when used for a long time. Buspirone has different side effects, causing insomnia, nervousness, upset stomach, nausea, diarrhoea and headaches. The most common downsides of SSRI's are severe gastrointestinal discomfort, mild nausea and sexual dysfunction, primarily ejaculatory delay.[34]

Prescription drugs to treat anxiety disorders topped $2 billion in 1999 with an estimate of $3 billion in 2009.[21] Since the 2001 September 11 terrorist attack at the World Trade Centre, the new prescription of anti-anxiety drugs rose 25%.[35] So, there remains a strong market for synthetic pharmaceutical drugs with reduced side effect profiles.

1.3.2. Alternative medicines

"Alternative medicines" or "Complementary medicines" are original natural products which are also categorized as botanicals, herbal medicines, phytomedicinal, and phytopharmaceuticals.[12,17,36]

There is a phenomenal consumer demand for "alternative medicines" and natural product-based drugs throughout the industrialized world. A national survey estimated 629 million visits to alternative medicine practitioners in 1997 in the US, costing $27 billion in out-of-pocket costs which is more than is spent on conventional care.[36] This represents a 50% increase in the number of visits and 100% in dollars during a period of seven years.

In Canada, a total of $3.8 billion was spent on alternative/complementary health care from 1996-1997. A 1999 study reported 70% of Canadians had used one or more natural health products in the preceding six months. WHO estimates that 4 billion or 80% of the world's population presently use herbal medicine.[17]

Research on alternative medicines has also escalated during the past several years. Federal research funds through the National Centre for Complementary and Alternative medicines grew from $2 million in 1992 to $68.7 billion in 2000.[36] Major pharmaceutical firms are now engaged in a diversity of research projects dealing with alternative medicines. Hence, alternative medicines have emerged as part of the mainstream practise of medicine in the past twenty years.

The magnitude of the demand for alternative therapy is noteworthy, in the light of little or no insurance coverage for these services and products. Additionally, many of the herbal products are largely unmonitored and their quality uncontrolled. However, individuals consider "alternative medicines" to be safer and would rather use such products than conventional pharmaceutical preparations.

The latest North American figures show clearly that the use of botanicals for psychiatric diseases continues to grow greatly. The current market for anti-depression herbs like St. John's Wort is $200 million.[36] These botanicals are available in the market as dietary supplements (medicinal herbs, nutraceuticals, dietary or nutritional supplements) and not as drugs. In Canada, these therapeutic alternatives undergo a more rapid regulatory approval process as "natural health products" compared to the pharmaceutical products.[12]

The better known anxiolytic botanicals include St. John's Wort, Gotu Kola and Kava Kava. It is interesting to note that despite their widespread use their chemistry and pharmacological profile remain relatively unknown. However, the active ingredients in these botanicals have been attributed to: Phenolic and naphthodianthrone compounds like Hypericin and Hyperforin in St. Johns Worts; Asiaticoside, a triterpene acid in Gotu Kola and lactones such as methysticin and yangonin in Kava Kava.[3] In several European countries, Kava Kava has recently been banned due to its potential to induce hepatotoxicity (liver damage).[37]

In the area of neurological diseases, only a very few plant-derived anti-anxiety related drugs are known (e.g. Cabergoline and Galanthamine or Nivalin), (**Figure 1.3.2**).[14]

Therefore, a new effective treatment especially a botanical or a pharmaceutical preparation derived from a natural product would be of great interest in the alleviation of anxiety. The current market demands fast acting alternatives to the existing drugs without the various side effects such as motor impairment, memory loss, addiction liability, rapid tolerance development, drowsiness and respiratory depression.[33]

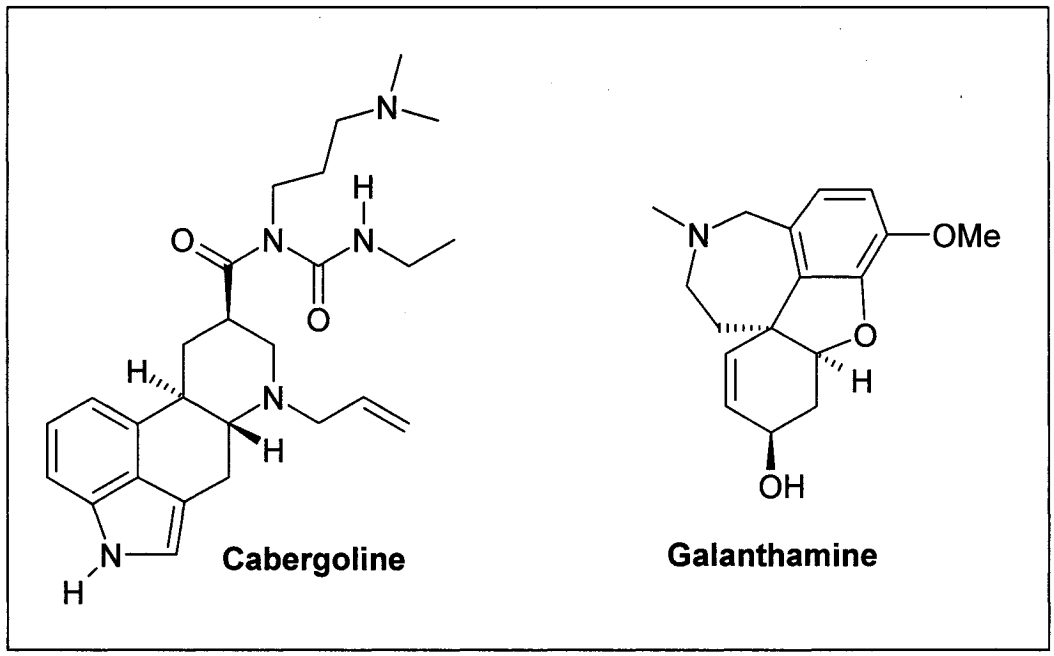


Figure 1.3.2: Plant-derived anti-anxiety drugs.

1.4. The Margraviaceae family

Margraviaceae is a small family of neotropical shrubs, often epiphytic, frequently with dimorphic foliage (5 genera and 125 species) native to Central and South America and the West Indies that we targetted in our phytochemical discovery programme mainly because it was rare, almost unknown in the literature and chemically uninvestigated.

Almost accidently, Professor Arnason from the biology department here at the University of Ottawa, discovered that tea made from leaves of members of this family were used by a few groups of the natives of these regions to relieve anxiety and fear-related stress as well as to induce sleep.[18]

For example, the Kubuyari Indians and the Barasanas on the Rio Piraparana boil the dry leaves in water in order to prepare a drink that calms extremely nervous members of the tribe who suffer from 'susto', a psychological condition of fear or apprehension resulting from the belief that hexing by an enemy has been successful.

Similarly, the Kubeos prepare a tea from the fleshy leaves and flower of this plant which is believed to hasten sleep onset. The Karijona residing in the upper Rio Vaupes also value a tea of the leaves of this plant as a tranquilizing medicine.

A few medicinal uses of Margraviaceae are also known. For instance, the Indians of Rio Apaporis apply crushed flowers to resistant wounds and abnormal skin conditions. In Venezuela, a small piece of bark is placed in the wound to stop bleeding and in Panama, infusion from ground stem and hot water is used to treat diarrohea.

The two plant species employed in this investigation belong to the genus *Souroubea, S.gilgi* AUBL. (Margraviaceae) and *S. sympetala* AUBL. (Margraviaceae). *Souroubea* is a genus of two dozen species whose chemistry was unknown. These plant species are not known in North America and Europe so this presents the possibility of introducing a "new plant" based on traditional use and scientific verification into the growing market of anti-anxiety drugs. (**Figure 1.4.1**).

Figure 1.4.1: *Souroubea sympetala* AUBL. of the Magraviaceae family.

1.5. Natural insecticides

Scientific interest and utilization of botanical insecticides declined precipitously following the introduction of DDT (p,p'-dichlorodiphenyl trichloroethane), parathion and other synthetic insecticides in the late 1940's and early 1950's.[38] These compounds had tremendous advantages over the existing botanical insecticides. Most importantly, they were extremely effective, they were inexpensive to produce and due to their chemical structures they were persistant.

DDT and the other chlorinated insecticides such as Aldrin and Dieldrin had immediate positive effects on food production, grain storage and communal health.[2] DDT for example, greatly reduced mosquito populations that are the vectors for diseases such as malaria, dengue fever. In many areas such as Southern Europe, Southern USA, Australia and parts of Asia, malaria was essentially eliminated. In other tropical countries especially India, the incidence of malaria was greatly reduced. Indeed, the usefulness of DDT in public health purposes led to the award of the Noble Prize for Physiology and Medicine to the discoverer of DDT, Dr. Paul Herman Muller in 1948.[39]

Despite these successes the long half-lives of these synthetic pesticides and their biomagnification to toxic levels in the foodchain led to heightened public concern for human health and for the environment. The detrimental effects of many of these synthetic insecticides and pesticides triggered renewed interests in the search for alternative pest control products.[13]

The early 1980's saw a substantial increase in the investigations into botanical insecticides as more environmentally acceptable pest control agents. One plant species that is almost entirely responsible for this revival of interest and use of botanical insecticides, was the Indian neem tree, *Azadirachta indica A. Juss.,* due to its potent insect antifeeding properties.[38] The successful commercialization and industrial acceptance of neem-based pest control products also provided a new paradigm for the development of pest management products from plants.

In 1997, it was demonstrated by Professor Arnason's group (University of Ottawa) that a group of *Piper* species from the neotropics (Costa Rica) belonging to the Piperaceae family of plants have insecticidal activities comparable to the well-documented Asian and African *Piper* species.[19] Among the 12 different *Piper* species tested for insecticidal activity against European Corn Borer (ECB, *Ostrinia nubilalis*), *P. tuberculatum* extracts were found to be the most active. These findings prompted us to investigate the chemical constituents of this plant (**Figure 1.6**).

1.6. The Piperaceae family

The Piperaceae or Pepper family is comprised of 4 genera represented by over 1000 species with tropical and subtropical distributions.[41] They are erect or scandent herbs, shrubs or small trees, and more or less woody pantropical vines with conspicuous stem nodes, (**Figure 1.6**). Plants of the genus *Piper* have been valued from antiquity to the present day for their organoleptic, medicinal and pesticidal properties.[42,43] This means that they have high commercial and economical implications.

Figure 1.6: *Piper tuberculatum* of the Piperaceae family.

1.6.1. Organoleptic properties of Piperaceae

The special organoleptic properties of the Piperaceae have resulted in black pepper prepared from the dried fruit of *P. nigrum* becoming virtually an universal spice in the modern world. The characteristic flavour of black pepper is produced by a complex array of compounds. The amides piperine and piperylin (or trichostachine) have been held largely responsible for the sharp pungent taste of the spice while pellitorine contributes significantly to its aroma, (**Figure 1.6.1a**).[42]

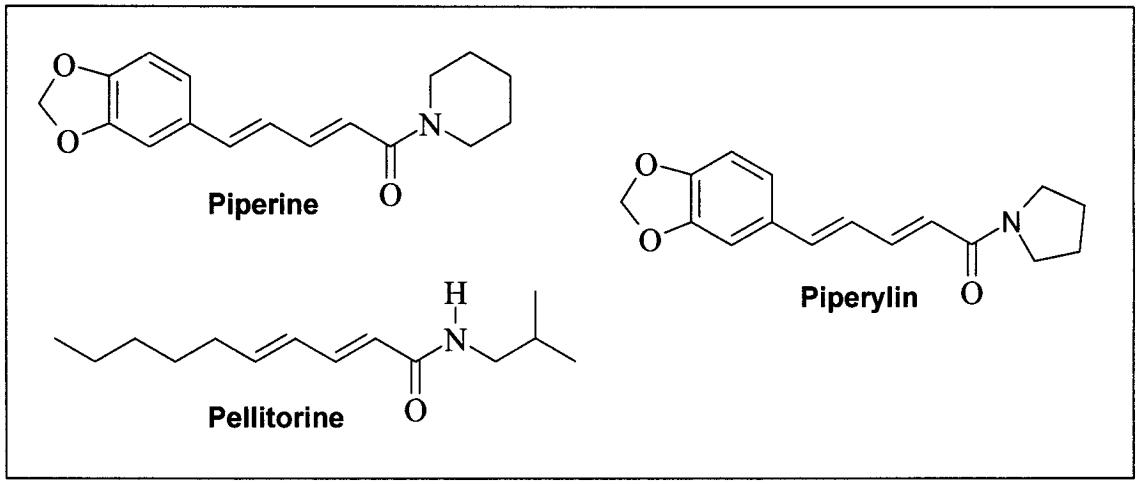


Figure 1.6.1a: Piperamides responsible for the sharp pungent taste of spice and its aroma.

The fruit of the West African *P. guineense* has also been used as a flavorant. In Southeast Asia *P. betle* is a source of aromatic essences. *P. cubeba* has long been used as a fragrance in India. The pleasant fragrance of several species have been attributed to monolignans like safrole, dillapiol,and eugenol and monoterpenes such as menthol. This may have contributed to their selection as pharmaceuticals by traditional healers, (**Figure 1.6.1b**).[41]

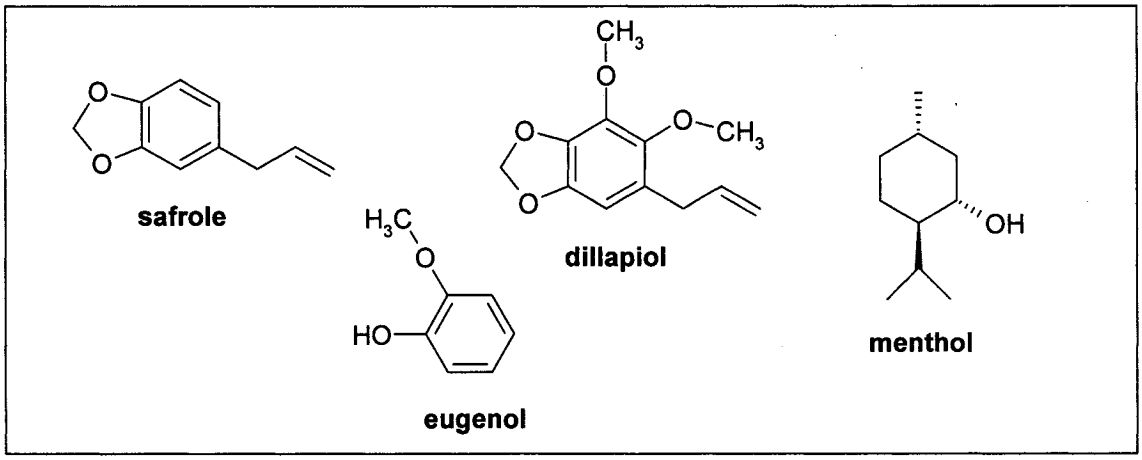


Figure 1.6.1b: Monolignans and monoterpenes responsible for the pleasant fragrance in piper species.

1.6.2. Medicinal uses of Piperaceae

The Piperaceae family has found a wide range of applications in the traditional pharmacopoeia of several cultural groups.[42,43] It is renowned for its medicinal properties in the Indian Ayurvedic system, in the folklore of Africa, Latin America and the West Indies as well as in Chinese herbal medicines. In the South Pacific Islands, *P. methysticum* (kava) is a well known source of psychoactive drug.

The range of diseases treated by *Piper species* can be broadly categorized into eight major disease areas: Respiratory (asthma, bronchitis, coughs, tuberculosis); Digestive (abdominal pain, diarrhoea, stomach disorders); Reproductive (oxytocic, abortofacient, anti-fertility); Cardiovascular (coronary vasodilating, hypotension, hypertention, chest pain); Analgesic (local anaesthetic, tongue numbing); Anti-microbial (antibacterial, antifungal); Gum and tooth problems (toothache, sore gum) and Sedative (hypnotic, anti-convulsive).

Miscellanous medicinal uses of *Piper* species include treatments of cholera, paralytic and arthritic disorders, diuretic, diaphoretic, anthelminthic, fever, hemorrhoids, tumor-inihibitory, gonorrhoea, veneral diseases, rheumatism, snake venom antidote, counterirritant, cytotoxic, anti-inflammatory, reduction of blood sugar, expectorant, beri-beri, leprosy and malaria.

1.6.3. Pest control and the *Piper* species

Piper plants have also been used traditionally against pests.[41-43] For example, *P. guineense* and *P. nigrum* have long been used as insecticides and molluscicides in several parts of Africa. The Amazonian species *P. rotundistipulun* is used locally as an insecticide and a fish poison. The Indian species *P. longum*, P. *betle*, *P. peepuloides*, *P. cubeba*, *P. falconeri* and *P. acutisleginum* have demonstrated insecticidal activity against mosquitoes and flies and were shown to repel grain pests. For instance, cubeb oil from *P. cubeba* repelled *Blattella germanica* adults and killed 100% of the mosquito larvae treated with it.

In Jamaica and Haiti, *P. aduncum* and *P. hispidum* are used as insect repellents. It has been estimated that 8 species of Piperaceae out of the 11 reported in the literature exhibit "satisfactory repellent" activity.[41] The leaves of *P. futokadsura* from Taiwan and Japan are feeding deterrent to the larvae of *Spodoptera litura*. The leaves of *P. umbellatum*, *P. hispidum* and *P. auritum* that are native to Central America and the Northwest Amazonian basin are used by indigenous people to remove headlice. More than 15 species of *Piper* have been reported to have insecticidal activity.[19]

The knowledge of the compounds responsible for all these pesticidal activities is limited, so their individual chemistry appears to be an essential area to investigate. However, it is known that the amides that are frequently isolated from the Piperaceae are toxic to fruit flies, adzuki bean weevils, cockroaches and other insect species by acting as neurotoxins.

1.6.4. Phytochemistry of *Piper* species

Of almost 1000 known *Piper* species, only about 12% have been phytochemically investigated, so this genus is still worthy of investigation.[43] The various compounds isolated from different *Piper* species can be classified under

12 categories, viz. alkaloids/amides, propenylphenols, lignans, neolignans, terpenes, steroids, kawapyrones, piperolides, chalcones and dihydrochalcones, flavones, flavanones and miscellaneous compounds. Out of the 592 compounds isolated from *Piper* species, 145 were alkaloids/amides, 89 terpenes, 70 neolignans and 47 lignans.

1.7. Piperamides as insecticides

In 1949-1953, Jacobson and Crombie first drew attention to the insecticidal activity of unsaturated amides found in *Piper* species with their syntheses of piperamide pellitorine.[42] However, the insecticidal use of aliphatic amides such as pellitorine is limited by their extreme instability.

More recently, Miyakado and co-workers at the Sumitomo Company in Japan who have been active in this field, observed that the insecticidal activity of a pure synthetic sample of the amide pipercide isolated from *P. nigrum* against the adzuki bean weevil (*Callosobruchus chinensis*) was only one-third that of the crude specimen.[44-46] A closer examination of the crude extract revealed the presence of two minor piperamides, dihydropipercide and guineensine, (**Figure 1.7**).

These amides generated great interest as a result of their potent insecticidal activity. Substantial synergistic effects were observed when these piperamides were mixed in various combinations such that a mixture of equal parts of all three piperamides exceeded pyrethrin in its toxicity to adult male adzuki bean weevils. The presence of an aromatic ring at the distal end of a dienoic acid isobutylamide of suitable length confers increased stability and enhanced insect toxicity.

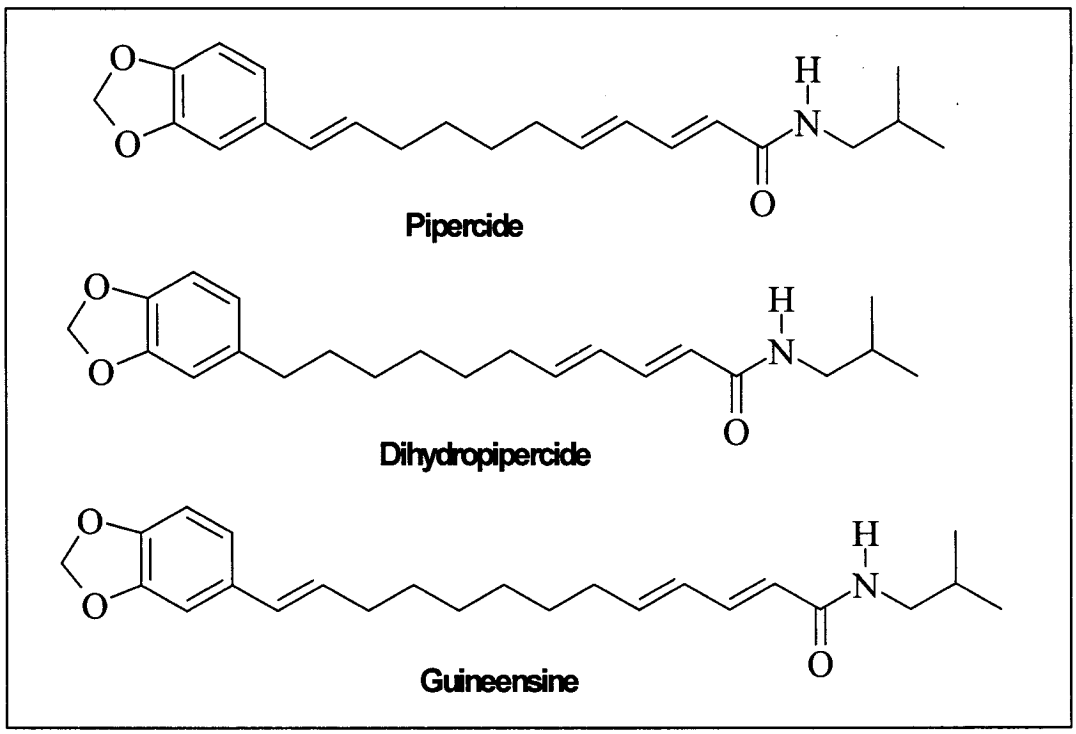


Figure 1.7: Piperamides with potent insecticidal activity.

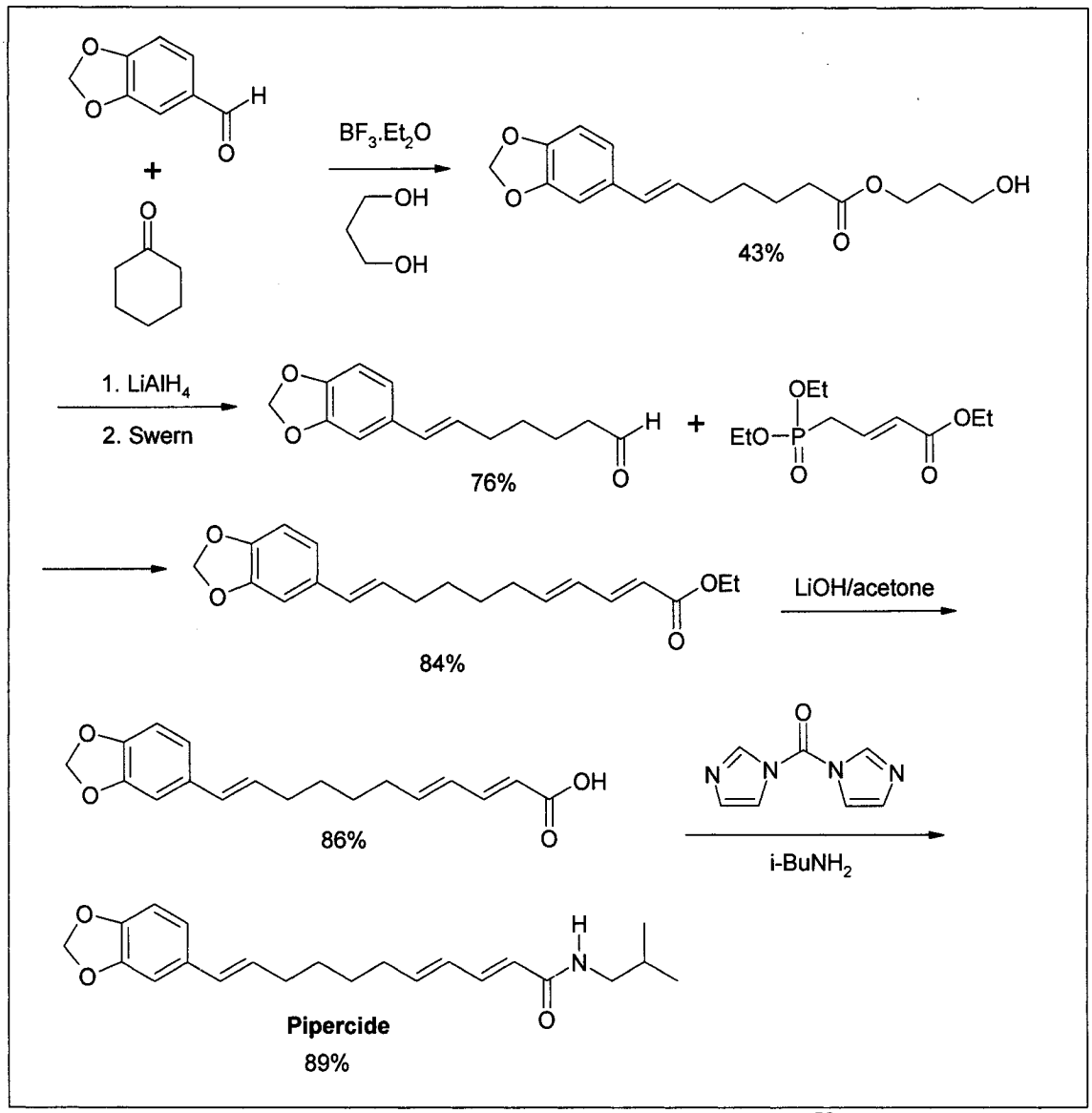
1.7.1. Structure-activity studies

Structure-activity studies based on dihydropipercide (**Figure 1.7**) and *C. chinensis* males as the test insects led to the following general conclusions; the 2E, 4E-dienamide is important for activity, isobutylamine is the optimum amine among the amide moieties, appropriate chain length is important in determining the extent of insect toxicity, with 11 to 13 carbons between the aromatic group and the nitrogen atom showing the strongest activity and the terminal methylenedioxyphenyl (MDP) can be substituted by other groups without causing significant loss of activity.[42]

A unique feature of these piperamides is the combination of an amide functionality with the MDP unit. Gbewonyo *et. al.* showed that the MDP group influenced the toxicity of these molecules.[47] This was thought to occur because the MDP group interferes with the action of the polysubstrate monooxygenases (PMSO) that detoxify insecticides.

1.7.2. Synthesis of Pipercide

Pipercide has been synthesized by several routes in 9-12 steps in fairly modest yields.[44,48,49] Recently, Strunz and Finlay reported a more concise and efficient synthesis of pipercide in 21% overall yield.[50] The key step was a modified Sakai procedure[51] for an Aldol condensation Grob-type fragmentation sequence in the reaction of piperonal and cyclohexanone under boron trifluoride catalysis with subsequent addition of 1,3-propane diol, (**Scheme 1.7.2**).



Scheme 1.7.2: Synthesis of Pipercide by Strunz and Finlay.[50]

1.7.3. *Piper tuberculatum*

Several studies have suggested that piperamides could have practical insecticidal potential because of the good efficacy and knockdown effects. It has been shown that *P. tuberculatum* extracts were the most active of 12 extracts of neotropical *Piper* species tested against ECB, with activity comparable to *P. nigrum* and *P. guineense*. Since its chemistry was still quite unknown, this *Piper* species (**Figure 1.6**) was our target of investigation.[19,41]

Prior to our study, only two reports on *P. tuberculatum* were recorded in the literature.[52,53] In 1981, a piplartine-dimer was isolated from the root bark of *P.tuberculatum* (**Figure 1.7.3a**).[52] In 1997, Araujo-Junior *et. al.* isolated piperdarine from the stems of *P. tuberculatum* (**Scheme 1.7.3b**).[53]

Subsequent to our study, two more reports on *P. tuberculatum* appeared in the literature.[54,55]

In the year 2000, Navickiene *et. al.,* isolated seven amides from the seeds of *P. tuberculatum* collected in Peru which were shown to have antifungal activity.[54] These piperamides included piperine (**27**), 5,6-dihydropiperine (**26**) and 5,6-dihydropiperlonguminine (**25**) which were isolated in our own work with the fruits of *P. tuberculatum* (**Figure 1.7.3c**).

Recently, Navickiene *et. al.*, again reported the isolation of four piperamides from the leaves of *P. tuberculatum* (**Figure 1.7.3d**).[55]

Most of these piperamides were active against the fungus *Cladosporium sphaerospermum*.[54,55]

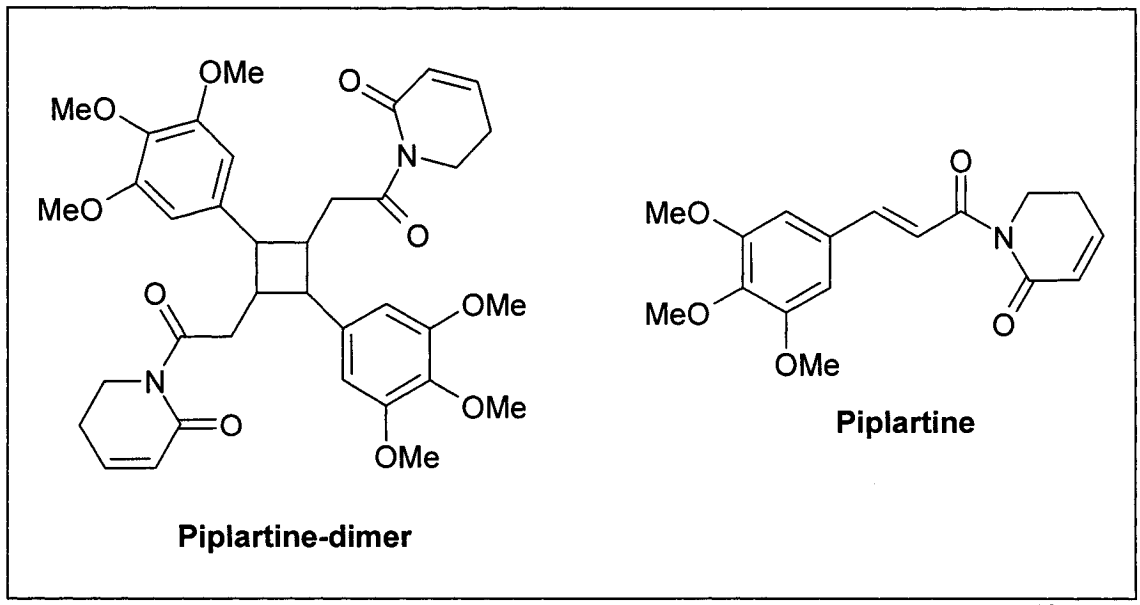
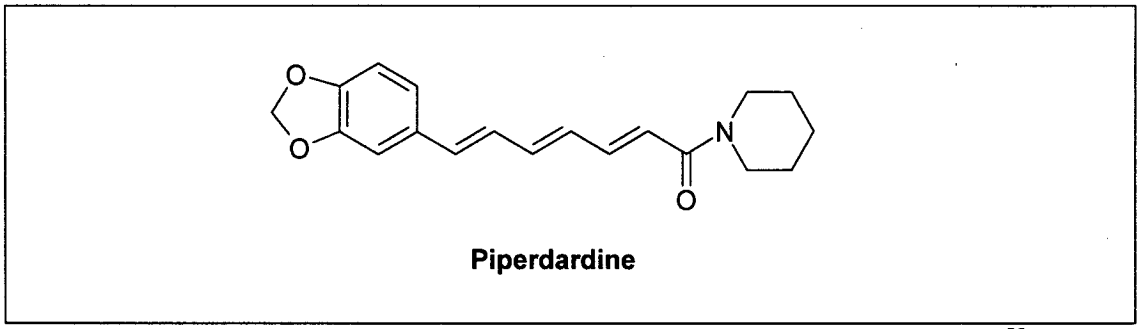


Figure 1.7.3a: Piplartine-dimer isolated from the roots of *P. tuberculatum*.[52]



Piperdardine

Figure 1.7.3b: Piperdardine isolated from the stems of *P. tuberculatum*.[53]

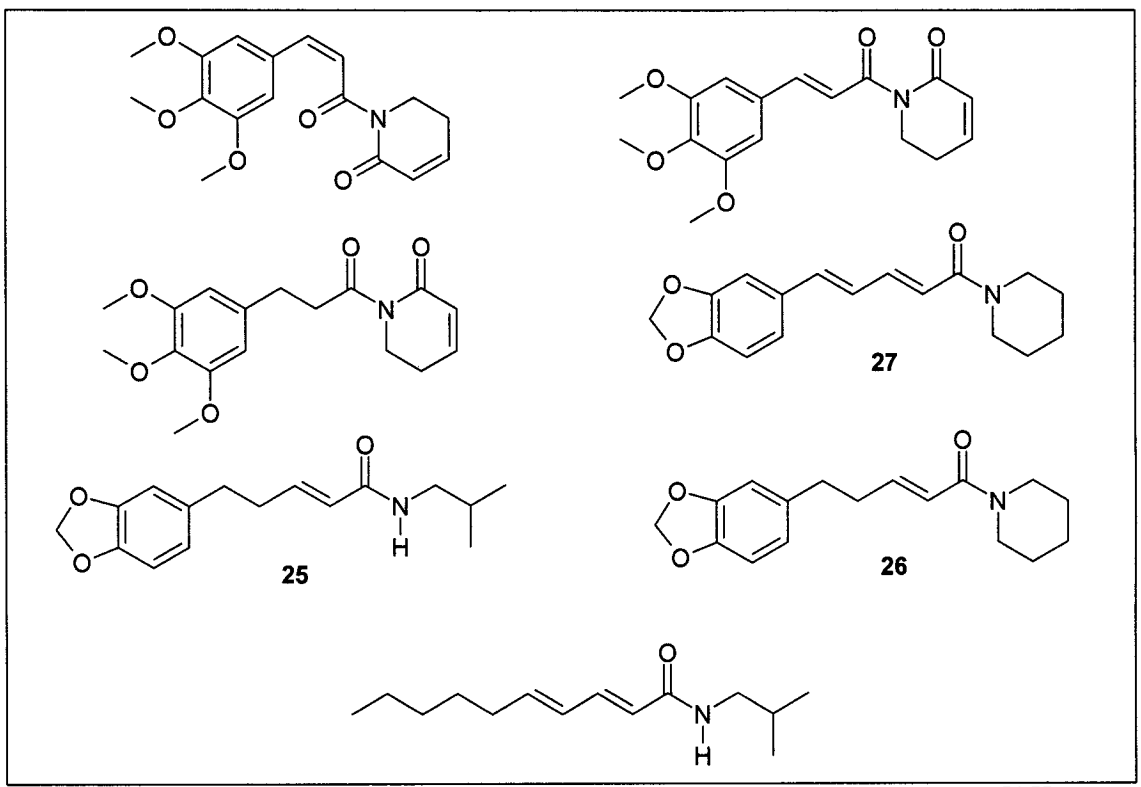


Figure 1.7.3c: Piperamides isolated from the seeds of *P. tuberculatum*.[54,55]

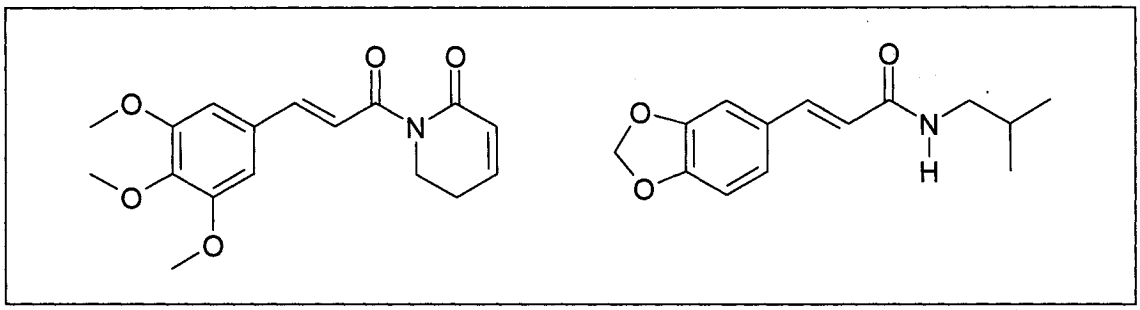


Figure 1.7.3d: Piperamides isolated from the leaves of *P.tuberculatum.*[55]

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Chapter 2

ANTI-ANXIETY ACTIVITY OF THE MARGRAVIACEAE FAMILY AND ITS DEVELOPMENT AS A NOVEL ANTI-ANXIETY THERAPY

2.1. Introduction

This study was initiated by identifying a group of plants belonging to Margraviaceae family which was relatively rare, and essentially unexplored from a phytochemical viewpoint.[1] This provides the distinct possibility of presenting a "new plant" as well as the discovery of novel structures that may have unique and significant biological activities.

A review of the literature revealed a single report which mentioned the presence of flavonoid glycosides in the flowers of one species of the Magraviaceae family (*Noranteaguianensis*).[2] More importantly, some members of this family have been used traditionally by indigenous people in South America to relieve fear-related stress (*see Introduction,* **Section 1.4**). Based on these ethnobotanical reports and later confirmation in conversation with Kekchi healers from Belize, which suggested pharmacological activity, we then became interested in assessing the psychotropic effects of this plant on animal models of fear and anxiety.

The objective was to investigate, and if the ethnobotanical reports were correct, then develop the relatively unknown Margraviaceae family of plants (*S. gilgi* AUBL. and *S. sympetala* AUBL.) as a source of a new anti-anxiety therapy. This would involve extraction, bioassay-guided fractionation, isolation and identification of the active component(s), its synthesis and possible chemical modification in order to produce derivatives with improved activity patterns.

This chapter details the results of this investigation, the exciting discovery of the anti-anxiety activity of the Margraviaceae extracts, the identification of the key active ingredient as well as its development as a novel anti-anxiety therapy.

2.1.1. Natural Product Drug Discovery

The most practical experimental approach utilized in natural product drug discovery is bioassay-guided fractionation.[3] In this endeavour, the starting materials are selected usually by a biologist or botanist (random collection based on information derived from ethnomedical system of medicine or literature surveillance) and extracts are prepared that are suitable for biological evaluation. These extracts are then tested in a bioassay test system, and the plant extract demonstrating a positive response are considered as active leads.

The crude active extracts are fractionated based on solubility in various solvents. Each fraction is monitored in the bioassay test system and the fraction containing the active ingredient(s) is identified. This fraction is then subjected to chromatography, in our hands, flash silica gel chromatography. Eluant fractions are monitored by analytical TLC and fractions containing single spots are combined. Mixtures are re-chromatographed until a number of pure compounds (by TLC) are obtained.

The "pure" compounds are then analyzed by a combination of MS, IR, proton and carbon high field NMR in order to make structure assignments. All the pure compounds are evaluated in the bioassay until one responsible for activity is identified. Based on the accumulated data, the bioactive compound becomes a candidate for more advanced testings and development. This is a multi-disciplinary approach involving chemistry, biology, biochemistry and in the case of anti-anxiety drugs, also psychology. Each discipline contributes to the discovery and development in an obligatory and synergistic manner (**Figure 2.1.1**).

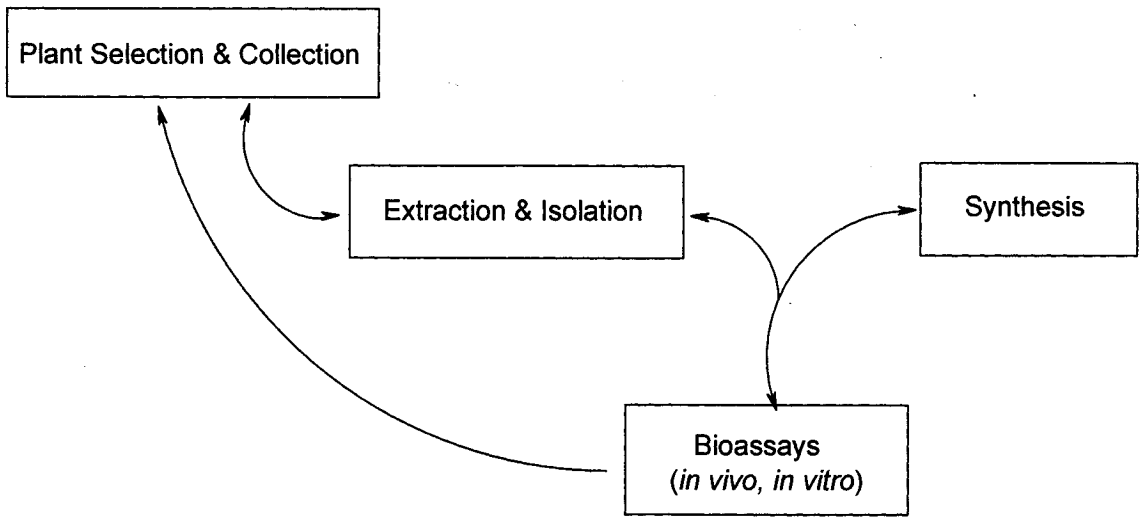


Figure 2.1.1. Inter-relationships in the discovery and development of bioactive natural product.

2.1.2. Bioassay-guided fractionation of Margraviaceae

Various parts of *S.gilgi* and *S.sympetala* were collected at Tortaguero and Horquetas near Eastern Costa Rica. The leaves were stored in 1L Nalgene bottles and covered with 95% ethanol (EtOH) solution in the field for preservation, and during subsequent transport to Ottawa and storage in the fridge. The fruit, small greenish apple-shaped was processed similarly. The woody parts were inspected and the dirt and visible fungus removed. They were then washed with water and air-dried before their eventual transfer to Ottawa.

The leaves and the fruit were processed in Ottawa by chopping into small pieces in a conventional kitchen food processor; and additional 95% EtOH was added to facilitate the extraction process. These mixtures were stored overnight and then filtered. The filter cake was washed with fresh 95% EtOH and the EtOH extracts were evaporated under diminished pressure to yield a dark green almost black gummy substance, that is the crude EtOH extract (SS-CE), which was submitted for anti-anxiety tests in animal models for anxiety. The yield of the SS-

CE based on dry weight was on average 32% from the leaves and 13% from the fruits. The bioassay-guided fractionation of the pshychotropic crude EtOH extract of *S.gilgi* AUBL. leaves by solvent extraction is summarized in the flow diagram (**Figure 2.1.2**).

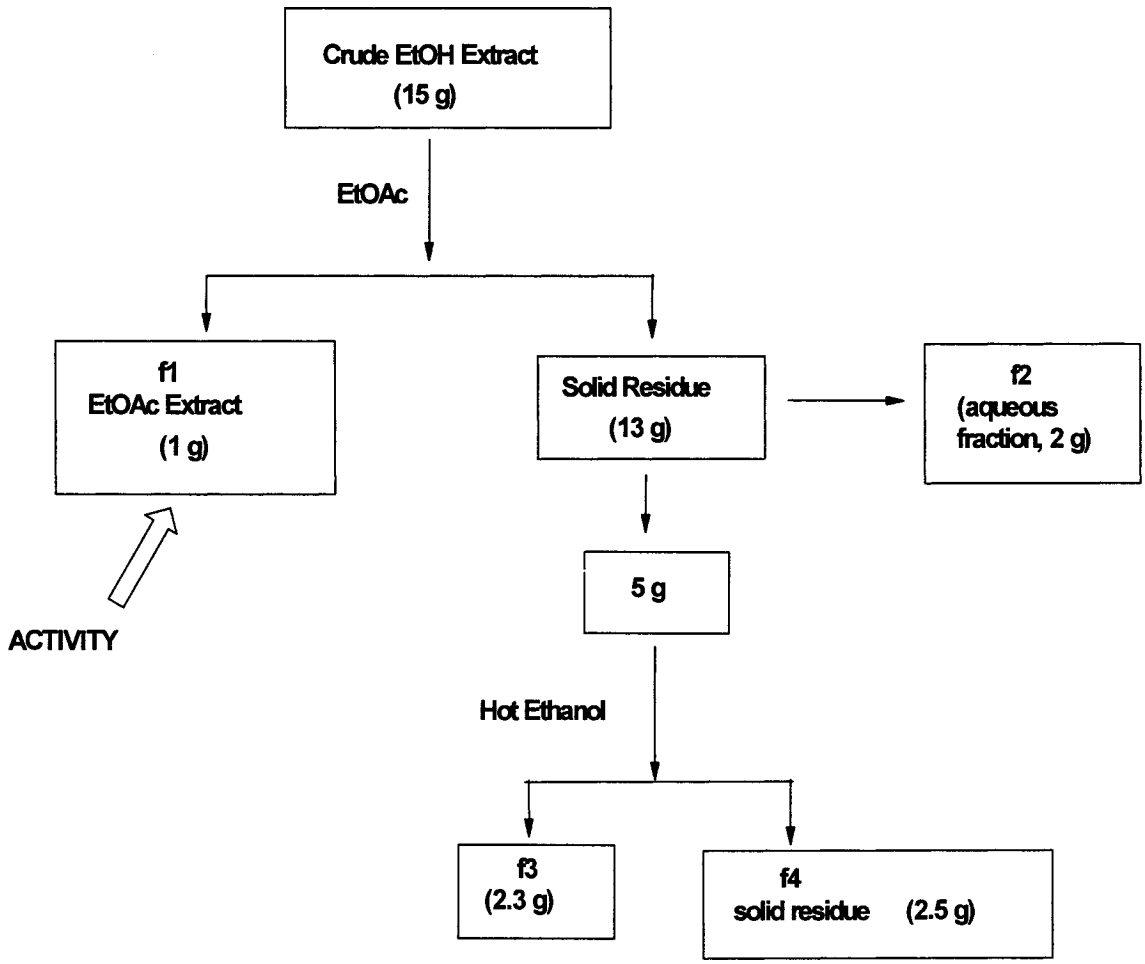


Figure 2.1.2. Bioassay-guided fractionation of the crude EtOH extract from Margraviaceae (*S.gilgi*) leaves.

The crude EtOH extract (15 g) was extracted twice with ethyl acetate (EtOAc) by stirring at room temperature for 4h before filtering, and then once overnight with acetone before it was filtered again. The combined organic

filtrates were concentrated under diminished pressure to afford an EtOAc-soluble extract, fraction 1 (f1), as a dark green material (1.0 g). The remaining EtOAc insoluble material (13.0 g), was a dark brown solid. Part of this solid (2.0 g) was set aside and labelled fraction 2 (f2). Some of this solid (5.0 g) was also further extracted with EtOH by stirring and heating to reflux for five minutes before filtration and concentration under reduced pressure to afford a brown EtOH soluble substance (2.4 g) which was labelled as fraction 3 (f3). The remaining EtOH insoluble fraction (light brown solid, 2.5 g) was designated fraction 4 (f4). Fractions 1-4 were then submitted for *in vivo* bioassays using industry standardized tests for anxiety.

2.1.3. Industry standardized tests for anxiety

The known industry standardized tests (validated industry accepted tests) that are commonly used to assess anti-anxiety activity include the (i) elevated plus maze paradigm (rats and mice), (ii) the acoustic startle and fear potentiated startle paradigms (rats), (iii) punished drinking or Vogel test (rats), (iv) light-dark box (mice) and (v) swim despair test (mouse).[4,5] Some of these tests were used to assess the anxiolytic effects of the crude EtOH plant extracts from Margraviaceae, as well as the various partly purified fractions and eventually the pure compounds.

There are several reasons for proposing several different paradigms. Firstly, humans and animals experience different types of anxiety. For instance, generalized anxiety (which is not cue related) occurs under a variety of situations and is clinically differentiated from anxiety that is specific to a particular environmental condition such as agrophobia (cue related). Since the two forms of anxiety may involve different underlying mechanisms and may require different treatments to alleviate anxiety, it is essential to evaluate effects of treatments in more than one paradigm.

Secondly, other behavioral states may impact on the performance in a particular test paradigms. For instance, if a drug affects locomotor activity or alters the impulsivity of the test subject, the time spent on the open arms of the elevated plus-maze (presumed to reflect anti-anxiety effects) may be interpreted incorrectly. Thus, by having several animal models of anxiety assessment, one can be more certain about the anti-anxiety effects and profile of the test agents.

2.1.3.1. Elevated Plus-Maze Test

The elevated plus-maze (EPM) test relies on the inherent conflict between an innate desire to explore a novel area and the avoidance of its aversive features. Typically, animals spend much more of their time exploring enclosed alleyways (safe zones) and avoid the elevated planks (open vulnerable or anxiety provoking zones). However, when animals are treated with classic anxiolytic drugs such as benzodiazepines (e.g. Valium)[6], they venture out more onto the open arms of the maze.

2.1.3.2. Fear-Potentiated Startle Paradigm

In this test, rats are trained to expect a mild foot-shock when light (a fear cue) is illuminated. Initially, their startle response to a sudden sound (tone 115 dB) was determined in the absence of the light cue. Finally, they are exposed to the same tone but now in the presence of light. Typically, animals show a greater startle response in the presence of light as compared to its absence. However, rats treated with anti-anxiety drugs markedly attenuate fear-potentiated startle response.

2.1.4. Anxiolytic effects of Margraviaceae plant extracts

It was found that the crude EtOH extracts of the leaves of Magraviaceae (SS-CE) demonstrated significant anti-anxiety activity in the elevated plus-mazed test and in the fear potentiated startle paradigm. Subsequent bioassay-directed fractionation of these extracts successively with EtOAc, acetone and hot EtOH yielded an EtOAc-soluble anti-anxiety active fraction, f1, (*see* **Figure 2.1.2**).

When SS-CE and f1 were administered orally to test animals, they both alleviate anxiety in the EPM test (**Figure 2.6.1a**) as well as in the fear potentiated startle paradigm (**Figure 2.6.1b**). The anxiolytic potential of this plant is therefore confirmed in two distinct validated tests for anxiety.

2.2. Discussion of the isolation and identification of plant metabolites from Margraviaceae extracts

In the course of our search for plant extracts as potential anti-anxiety agents, the crude EtOH extracts (SS-CE) from Margraviaceae leaves (collected February 2000) were found to exhibit significant anti-anxiety activity. Subsequent bioassay-directed fractionation by solvent extraction of these extracts yielded an EtOAc-soluble anti-anxiety active fraction (f1), (**Figure 2.1.2**).

The activity guided isolation and purification of f1 by silica gel column chromatography followed by recrystallisation from hexane/CH_2Cl_2 furnished pure betulinic acid (**3**), as the bioactive constituent in 0.01% yield from the dried plant material (**Figure 2.2a**).

Several chemical investigations of the EtOAc and EtOH extracts from the leaves of Margraviaceae (*see* **Section 2.3.5**) led to the isolation of seven known pentacyclic triterpenoids including (**3**), six known flavonoids and three other plant metabolites. A plasticiser (not a metabolite) was also isolated.

Among the seven pentacyclic triterpenoids isolated were two pairs of inseparable isomeric mixtures; (i) α-amyrin (**1a**) and β-amyrin (**1b**), (ii) methyl 2α-hydroxy-ursolic acid (**2c**) and maslinic acid (**2d**) which were isolated as methyl esters, 2α-hydroxy-ursolate (**2a**) and methyl maslinate (**2b**) respectively, after esterification with diazomethane (CH_2N_2). Betulinic acid was isolated both in its free form (**3**) and as methyl ester (**3a**) after CH_2N_2 treatment. Ursolic acid (**5c**) was also isolated as methyl ursolate (**5a**) after methylation with CH_2N_2. The remaining pentacyclic triterpenoid was identified as taraxeryl *trans-p*-hydroxycinnamate (**4**), (**Figure 2.2a**).

The six flavonoids isolated from Margraviaceae were naringenin-4',7-dimethyl ether (**6**), naringenin-7-methyl ether (**7**), an inseparable isomeric mixture of eriodictyol-3', 7-dimethyl ether (**8a**) and eriodictyol-4', 7-dimethyl ether (**8b**), naringenin (**9**) and 3', 5, 5' 7-tetrahydroxyflavanone (**10**), (**Figure 2.2b**). Except for flavanones (**9**) and (**10**), all the other flavanones were isolated after treatment with diazomethane.

The other plant metabolites isolated from Margraviaceae were identified as chondrillaterol (**11**), linolenic acid (**12**) which was isolated both as methyl linolenate (**12a**) and ethyl linolenate (**12b**), and a porphyrin type compound (**13**) whose structure was not completely assigned (**Figure 2.2c**).

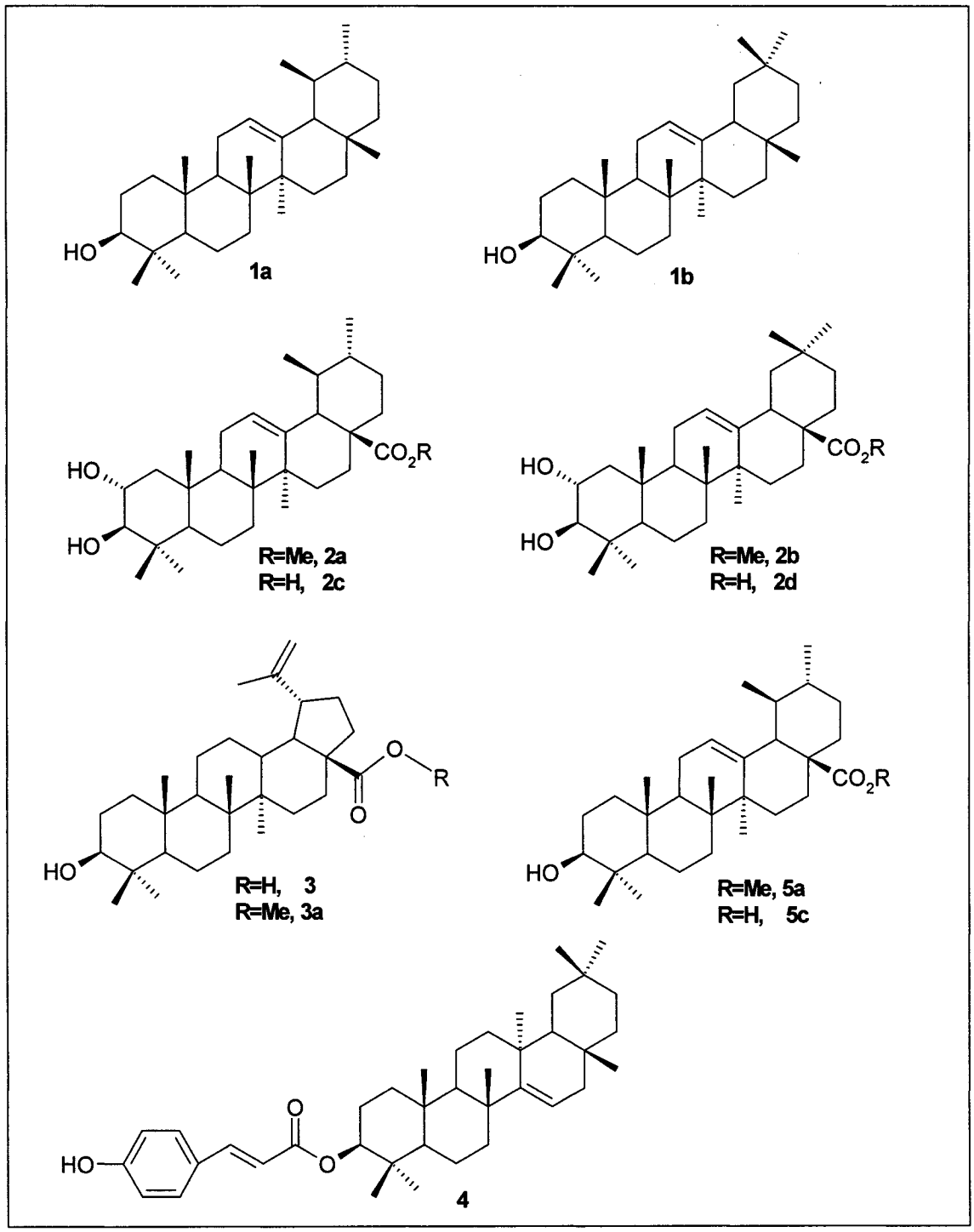


Figure 2.2a: Pentacyclic triterpenoids isolated from Margraviaceae leaf extracts.

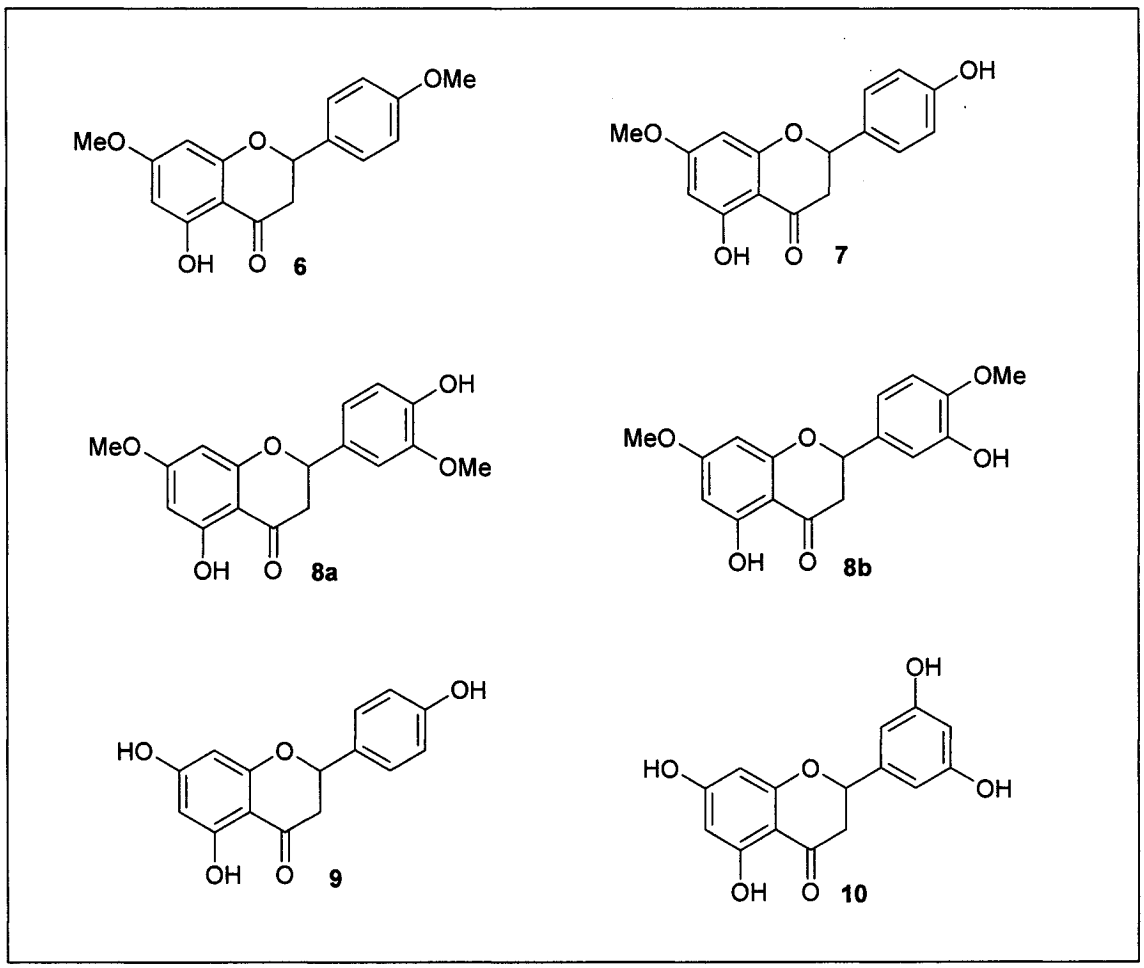


Figure 2.2b: Flavonoids isolated from Margraviaceae leaf extracts.

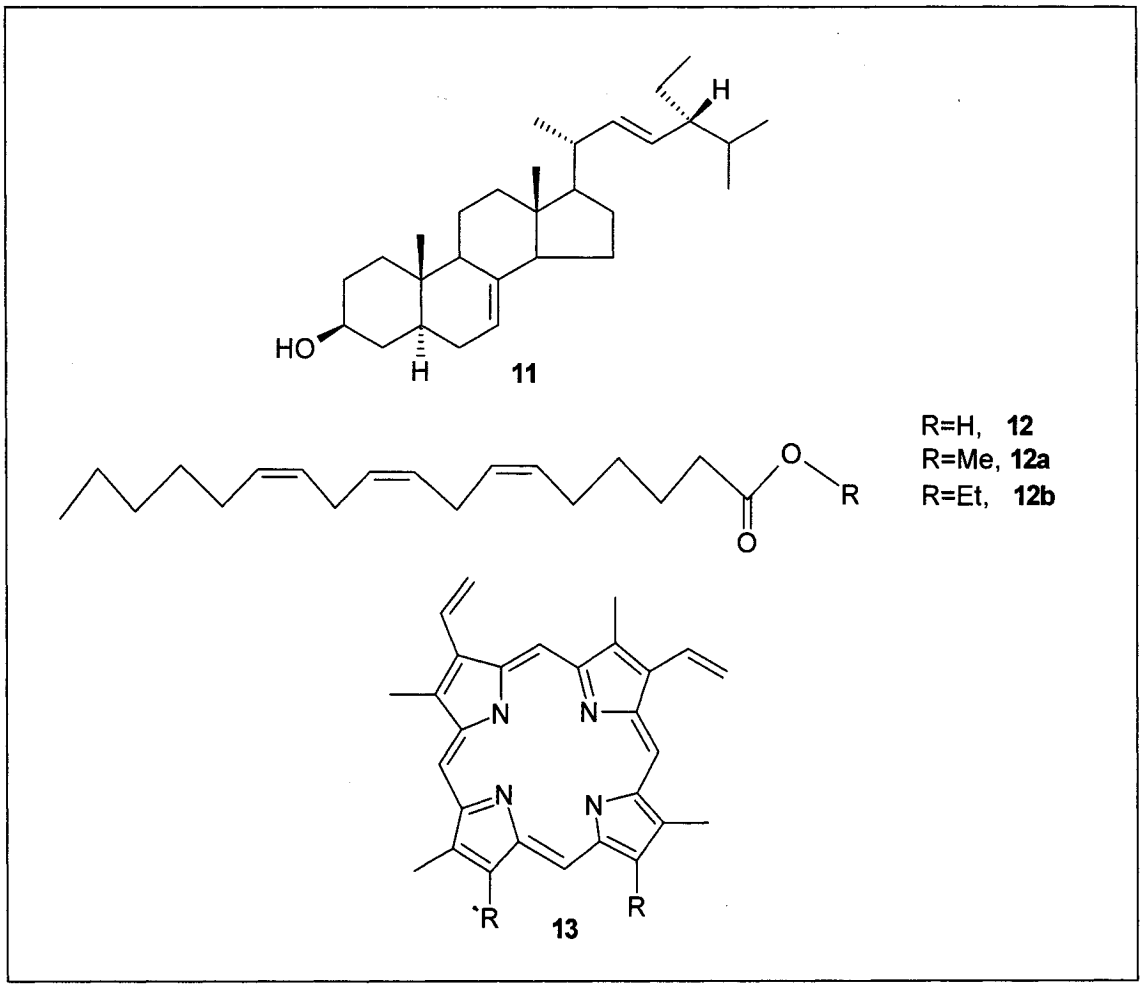


Figure 2.2c: Other metabolites isolated from Margraviaceae plant extracts.

The fruits of Margraviaceae were also subjected to the same extraction and isolation processes. Only compounds (**3**) and (**11**) were isolated from the fruits of Margraviaceae.

The percentage yields for each of these metabolites from the dry weight of Magraviacae plant materials as well as the times of plant collection are summarized in **Table 2.2**. The structural recognition and elucidation of these metabolites were achieved by comparing their spectral data and melting points with those previously reported in the literature as well as to an authentic sample in the case of (**3**).

Table 2.2: % Yields of metabolites from Margraviaceae leaves and fruits[f] based on dry weight obtained at different times of collection.

	% YIELDS				
Metabolites	April 1999	Feb. 2000	May 2002 (normal)	May 2002 (old leaves)	May 2002 (dried leaves)
1a &1b		1.00		0.63	1.10
2a & 2b				0.03	
3	0.09[f]	0.02			
3a				0.01	0.01
4			0.10	0.06	0.07
5a				0.02	
6				0.03	
7				0.06	
8a & 8b				0.02	
9	0.05				
10	0.04				
11	0.09[f]	0.02			
12			0.15		
12a				0.18	0.09
12b			0.02		
13	0.11				

[f]Isolated from the fruits of Margraviaceae.

The amyrins (**1a**) and (**1b**) were found to be the major components in all of the Margraviaceae leaf extracts except for the "normal" leaves collected in May 2002 where they were not isolated at all. In the April 1999 collection, both the amyrins can be seen by ^{1}H NMR, but were not isolated.

Betulinic acid (**3**) was isolated in all of the extracts except for one of the experiments with the May 2002 collections. This may simply be due to a smaller amount of starting extract since (**3**) was isolated from the same collection of old and dried leaves when a larger amount of starting material was employed (*see* **Section 2.3.5**).

Most of the metabolites were isolated from the May 2002 collection (old leaves). Methylation of polar fractions with CH_2N_2 facilitated the isolation of the flavonoids (**6**), (**7**), (**8a**), (**8b**) and the dihydroxy acids (**2a**) and (**2b**).

Variability in the chemical composition of plants (both quality and quantity) occurs as a result of a number of factors such as the availability of nutrients in the soil, storage or drying process and seasonal changes. Warm weather conditions favour the synthesis of certain secondary metabolites while rainy seasons can inhibit their production.[7]

2.2.1. Pentacyclic triterpenoids

Triterpenoids or triterpenes are ubiquitous non-steroidal secondary metabolites of terrestial and marine flora and fauna occurring in the free forms as well as in their ester, ether or glycoside forms. Their presence, even in non-photosynthetic bacteria, has created interest especially in its evolutionary and functional aspects.[8]

Triterpenoids are composed of 30 carbon atoms derived from C_5 isoprene units and may possess acyclic, mono-, di-, tri-, tetra- or pentacyclic carbon skeletons. The pentacyclic triterpenoids appear to be the dominant constituents of this class of natural products, so they have been widely investigated. Despite the remarkable diversity of carbon skeletons that is already known for triterpenoids, new variants and more complex ones continue to emerge.

It is said that the classical era of triterpenoid chemistry ended in 1949 with the structure elucidation of oleanolic acid.[9] The advent of powerful analytical methods and their subsequent development over the years meant that spectroscopic techniques are now routinely employed for structure elucidation of natural products including pentacyclic triterpenoids. As a result, an increasing number of triterpenoids even very complex ones are being isolated and their structures determined.[10]

The medicinal uses of this class of natural products are rather limited. However, considerable research efforts in recent years has revealed their great potential as drugs.[11]

The seven known natural products isolated from Margraviaceae extracts belong to three of the eight major classes of triterpenoids, namely dammarane, euphane, hopane, isoeuphane, lanostane, lupane, oleanane and ursane.[12] Betulinic acid (**3**) belongs to the lupane series of triterpenoids and possess a lupane type carbon skeleton . The α-amyrin (**1a**), methyl ursolate (**5a**), and methyl 2α-hydroxyursolate (**2a**) of the ursane series possess an urs-12-ene carbon skeleton. The β-amyrin (**1b**), methyl maslinate (**2b**) and taraxeryl *trans*-hydroxycinnamate (**4**) all belong to the oleanane series.

It is worth noting that compounds (**1b**) and (**2b**) possess an olean-12-ene carbon skeleton while compound (**4**) possess an olean-14-ene skeleton where the double bond has migrated to the C-14 position. In the presence of acids, olean-14-ene derivatives isomerize to olean-12-ene derivatives.[13]

The methyl esters are not the natural products but derived from the acids by CH_2N_2 treatment.

The structures of the oleanane and ursane series are identical from the A-ring through to the D-ring moiety and differ only at the position of one methyl group (C-29) at ring E. By contrast, in the lupane type triterpenoids such as betulinic acid (**3**), ring E is now a five membered ring with an isopropyl or isopropenyl group attached at C-19 (**Figure 2.2.1**).

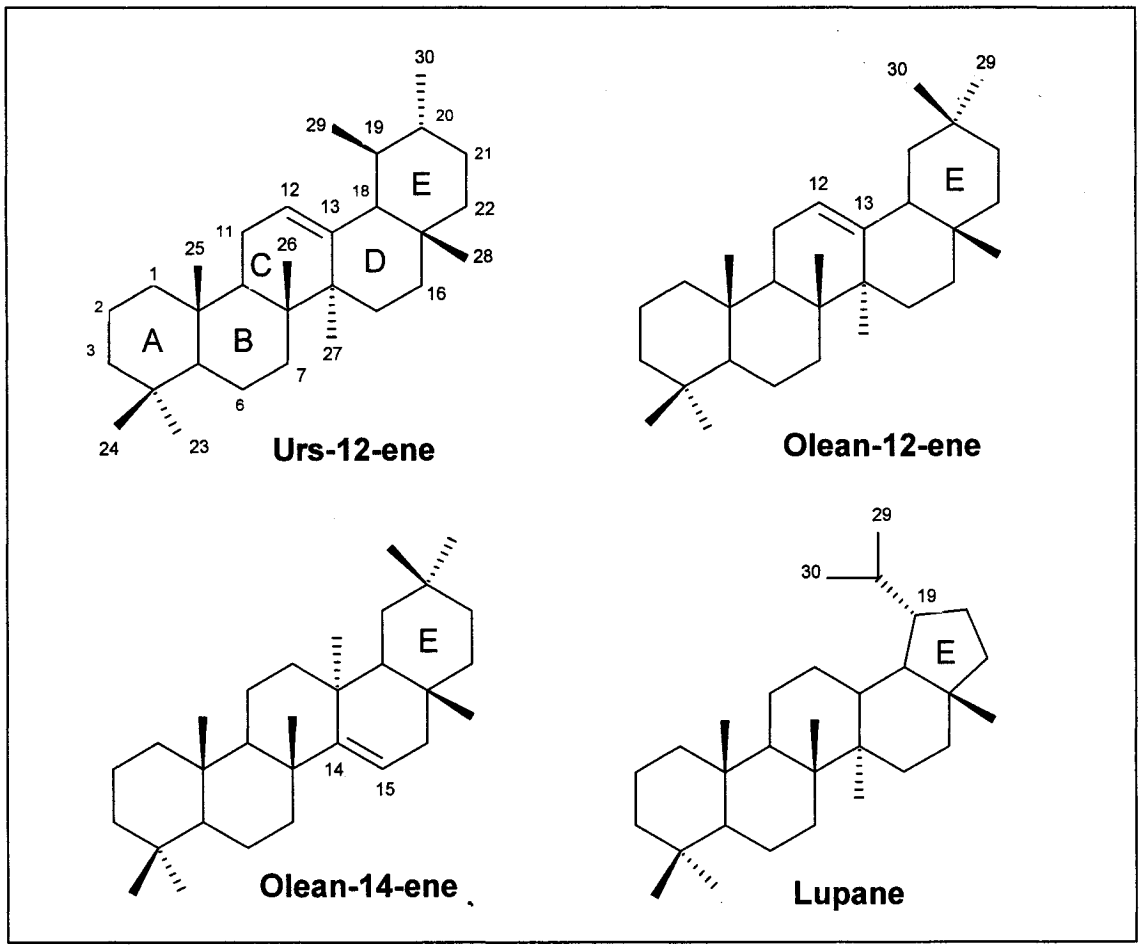
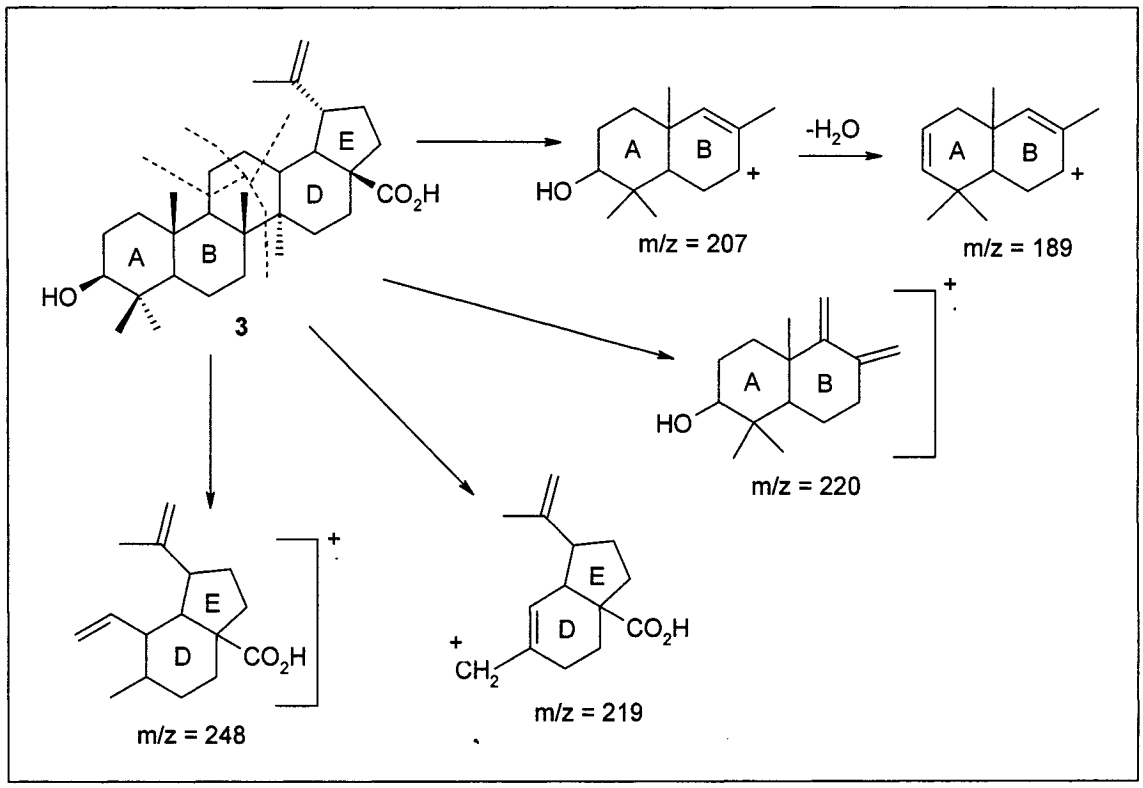


Figure 2.2.1: The different carbon skeletons of the pentacyclic triterpenoids isolated from Margraviaceae plant extracts.

2.2.2. Lupane triterpenoids

The mass spectra of lupane derived triterpenoids such as betulinic acid (**3**) have been documented to exhibit diagnostically valuable fragment ions at m/z 248, 220, 219, 207, 203 and 189 which readily allow their identification. It has been postulated that these ions originated from the cleavage of bonds in ring C (depicted by dotted lines) followed by proton transfer (**Figure 2.2.2**).[14] Virtually all of the lupane derivatives are characterized by an intense peak at m/z 189 irrespective of the nature of substitution in rings A, B, D and E.[15] This intense

peak at m/z 189 can be formed by the loss of water from the fragment ion peak at m/z 207 as depicted in **Scheme 2.2.2**.



Scheme 2.2.2: The postulated cleavage of ring C in lupane derived triterpenoids such as betulinic acid (**3**) and the resulting diagnostic fragment ions.

Lupane type triterpenoids exhibit characteristic ^{13}C resonances for the olefinic carbons of the isopropenyl moiety at δ ~150 (C-20) and δ ~110 (C-29) as well as the for the methine carbon bearing the hydroxyl group (C-3) at δ ~79.[8]

Diagnostic signals were observed for the terminal methylene protons of the isopropenyl moiety (often as broad singlets) at δ ~4.6 (br s, 1H, H-29) and at δ ~4.7 (br s, 1H, H-29'), and for the methine proton bearing the hydroxyl group at C-3 at δ ~ 3.2 either as a multiplet or double doublets (dd, J~10 Hz, J~5 Hz, 1H, H-3α). The vinylic methyl group usually resonatesat δ ~1.7 as a distinct singlet (s, 3H, H-30) while the five tertiary methyl groups normally resonated as five distinguished three-protons singlets at δ 0.74-0.98.

Indeed, these diagnostic features were evident from the spectroscopic data collected for betulinic acid (**3**), its methyl ester (**3a**) and other derivatives of betulinic acid that were synthesized later on in this study *(see* **Section 2.5**).

2.2.2.1. Betulinic acid

Betulinic acid (**3**) was isolated from the EtOAc extracts of Margraviaceae leaves and fruits as a white solid, m.p. 295-297°C (lit.[16] 275-278°C, lit.[17] 283°C, lit.[18] 290-292°C, lit.[19] 290-293°C, lit.[20] 297°C, lit.[21] 311-313°C, lit.[22] 315-318°C, lit.[23] 316-318°C) in 0.01-0.02 % yield of dry weight. This was after repeated silica gel column chromatography eluting with EtOAc-hexane solvent gradient followed by recrystallisation from hexane/CH_2Cl_2. Although the literature m.p. of (**3**) ranges from 272-318°C, the Rf value (0.3 in 30% mixture hexane/EtOAc) was identical with an authentic sample.

The EI mass spectrum of (**3**) displayed the $[M]^+$ at m/z 456 corresponding to the anticipated molecular formula $C_{30}H_{48}O_3$. This was established by HRMS. The fragmentation pattern was typical of a lupane derivative containing a hydroxyl group in ring A or B and a carboxyl group in ring D or E. The major relevant peaks observed at m/z 189 (base peak), 203, 207, 219, 220 and 248 implies the cleavage of ring C (**Scheme 2.2.2**). These peaks matched well with the MS of an authentic sample and literature values[23].

The strong IR absorptions at 3425 and 1687 cm^{-1} confirmed the presence of the hydroxyl and the carboxyl functions respectively.

The most significant signals in the ^1H NMR spectrum of (**3**) were those attributable to the methylene protons of the isopropenyl moiety [δ 4.71 (br s, H-29), δ 4.59 (br s, H-29'), δ 1.67 (s, 3H, H-30)], the methine proton attached to the hydroxyl group at C-3 [δ 3.17 (dd, J=5.2 Hz, J=10.7 Hz, H-3α)], the allylic proton at C-19 [δ 2.99 (ddd, J=5.1 Hz, J=11.2 Hz, J=11.2 Hz, 1H, H-19] and the remaining five tertiary methyl singlets at δ 0.95 (s, 3H, H-27), 0.94 (s, 3H,H-26), 0.91 (s, 3H, H-23), 0.80 (s, 3H, H-25) and at δ 0.73 (s, 3H, H-24). The

assignment of these methyl singlets were made possible by comparison with previously reported data for betulinic acid.[19]

The diagnostic signals in the ^{13}C NMR spectrum of (**3**) were observed for theacid carbonyl group at δ 181.3 (C-28), for the two olefinic carbons at δ 150.9 (C-20) and at δ 110.2 (C-29) and for the methine carbon bearing the hydroxyl group at δ 78.6 (C-3). The ^{13}C NMR data of (**3**) were almost identical with published data for betulinic acid, (**Table 2.2.2.1**).[19]

Betulinic acid (**3**) was first isolated in 1939 by Soliman from the bark of *Cornus florida* L. who gave it the name cornolic acid before it was re-named betulinicacid or betulic acid.[24] The "melaleucin" obtained by Isii and Osima in 1939 is thought to be impure betulinic acid.[24] Barton and Jones suggested that gratioloneisolated from *Gratiola officinalis* by Retzlaff in1902 and by Maurer *et. al*, in 1870 was also identical to (**3**).[24]

In 1948, Bruckner and co-workers showed that a compound which had been extracted from the bark of *Platanus acerifolia* and designated platanolic in 1925, platanin in 1944 and platanol in 1944 was also identical to betulinic acid.[24]

A NAPRALERT search revealed that (**3**) has been found in 91 different families of plants.

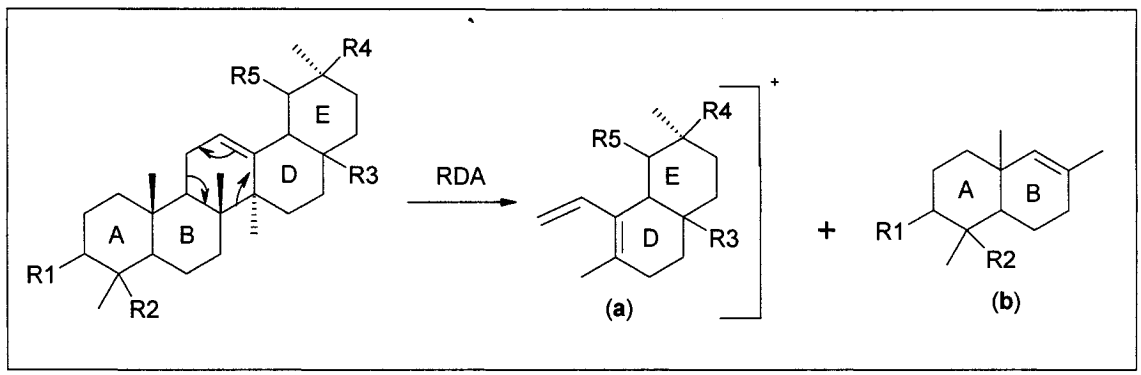
Table 2.2.2.1: ^{1}H and ^{13}C NMR data of betulinic acid (**3**). Comparison of the δ_C of (**3**) with published data for betulinic acid ($\delta_{C'}$).[19]

C	δ_H	δ_C	$\delta_{C'}$
1		38.7	38.7
2		27.3	27.4
3	3.17 (dd, J=5, 11 Hz)	79.0	78.9
4		38.8	38.8
5		55.2	55.3
6		18.2	18.3
7		34.2	34.3
8		40.6	40.7
9		50.4	50.5
10		37.1	37.2
11		20.8	20.8
12		25.4	25.5
13		38.3	38.4
14		42.4	42.4
15		30.5	30.5
16		32.1	32.1
17		56.2	56.3
18		46.8	46.8
19	2.98 (ddd, J=11, 11, 5 Hz)	49.2	49.2
20		150.4	150.3
21		29.6	29.7
22		37.0	37.0
23	0.92 (s)	27.9	27.9
24	0.74 (s)	15.3	15.3
25	0.81 (s)	16.0	16.0
26	0.95 (s)	16.1	16.1
27	0.96 (s)	14.6	14.7
28		180.0	180.5
29	4.72 (s), 4.59 (s,)	109.7	109.7
30	1.67 (s)	19.3	19.3

2.3.3. Olean-12-ene and urs-12-ene triterpenoids

In this work, five out of the seven pentacyclic triterpenoids isolated from Margraviaceae [(**1a**), (**1b**), (**2a**), (**2b**), (**5a**)] possess either an olean-12-ene or an urs-12-ene carbon skeleton. As pointed out earlier, the only difference between these two types carbon skeletons or frameworks lies on the pattern of methyl substitution on ring E where the position of one methyl group has shifted (**Figure 2.2.1**).

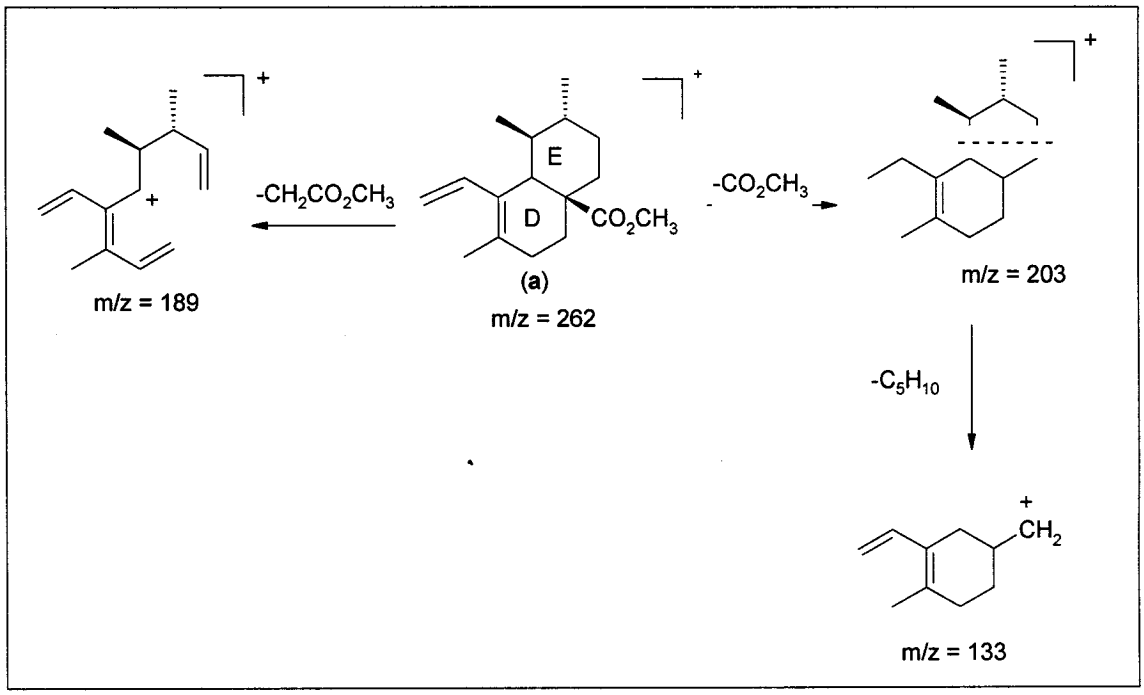
A diagnostic feature of these types of carbon skeletons which readily allows their assignment and identification, comes from their mass spectral fragmentation patterns. The mode of fragmentation of all compounds of this class is controlled by the position of the double bond which undergoes a retro-Diels-Alder (RDA) cleavage of ring C (as indicated by the arrows) and thus, providing fragments (**a**) and (**b**), (**Scheme 2.2.3a**).[14]



Scheme 2.2.3a: Typical RDA cleavage of ring C in olean-12-ene and urs-12-ene pentacyclic triterpenoids.

Typically, the RDA fission leading to species (**a**) can thus be employed as a diagnostic tool for determining the presence of a C-12 and C-13 double bond in the olean-12-ene and urs-12-ene skeletal type triterpenoids. The correctness of this assignment is demonstrated by the fact that substitution in ring A and B does not change the mass of fragment (**a**) while alterations in ring D and E result in the

appropriate mass shifts. Thus, the isomeric mixtures α- amyrin (**1a**) and β-amyrin (**1b**) yielded fragment (**a**) of m/z 218 while the methyl ester of ursolic acid (**5a**) and the isomeric methyl esters (**2a**) and (**2b**) which carry the carbomethoxy group at C-17, exhibit species (**a**) at m/z 262. The commonly observed fragment ions at m/z 203, 189 and 133 due to subsequent fragmentation of species (**a**) are outlined below (**Scheme 2.2.3b**).[14]



Scheme 2.2.3b: Typical EI mass spectral fragmentation of species (**a**) leading to the principal fragment ions observed in urs-12-ene and olean-12-ene type triterpenoids.

Another diagnostic fragmentation product that was readily identified was m/z 189 (207-18). This fragment ion was also previously observed for the lupane type triterpenoids (*see* **Scheme 2.2.2**).[14]

The distinction between the olean-12-ene and the urs-12-ene carbon skeletons is not easy and a variety of chemical and spectroscopic methods have been used for this purpose. However, it appears that the most precise is ^{13}C

NMR spectroscopy where the chemical shifts of the olefinic carbons C-12 and C-13 are particularly diagnostic.[25,26] This method has the advantage that the contamination of a member of one family by the isomer of the other series is generally, immediately detected.

In general, the chemical shift of C-13 appeared at δ ~144-145 in the olean-12- enes and at δ ~140 in the corresponding urs-12-enes. On the other hand, the chemical shift for C-12 in the olean-12-ene is observed at δ ~122 and at δ ~125 in the corresponding urs-12-ene skeleton.

The chemical shifts for C-12 and C-13 of the urs-12-ene [(**1a**), (**2a**), (**5a**)] and olean-12-ene [(**1b**), (**2b**)] triterpenods isolated from Margraviaceae are tabulated in **Table 2.2.3**. These results were in good agreement with the ^{13}C chemical shifts previously reported in the literature for these compounds and are included (in brackets) for comparison.[8,25,27]

Table 2.2.3: Comparing δ_C of olean-12-enes to urs-12-enes with literature values (in brackets).[8,25,27]

Compound	C-12	C-13
1a (urs-12-ene)	124.4 (124.3)	139.6 (139.4)
2a (urs-12-ene)	125.3 (125.3)	138.1 (138.1)
5a (urs-12-ene)	125.5 (125.5)	138.6 (138.5)
1b (olean-12-ene)	121.7 (121.8)	145.2 (145.1)
2b (olean-12-ene)	122.1 (122.0)	143.6 (143.6)

It is evident from **Table 2.2.3**, that C-12 is deshielded by 2-3 ppm and C-13 is shielded by 4-5 ppm in urs-12-ene in comparison to those of corresponding carbons in olean-12-ene. The difference between the two values has been attributed to the presence of the 19β (equatorial) methyl group (C-29) which is in close proximity in the urs-12-enes and thus, steric effects on the C-12 and C-13 chemical shifts operate relative to the olean-12-enes.

The ^{1}H NMR spectra of these olean-12-ene and urs-12-ene triterpenoids also revealed characteristic signals for the proton on a trisubstituted double bond at δ 5.10-5.25 (t, J=3.5-3.7 Hz, H-12). The signal corresponding to the methine proton attached to the hydroxyl at C-3 and C-2 (in the case of compounds **2a** and **2b**) resonated at δ ~ 3.2 and δ ~ 3.6 respectively, while the five methyl singlets resonated in the range of δ ~ 0.69-1.25.

Generally, the ^{1}H NMR spectra of urs-12-ene type compounds such as (**1a**) should exhibit 6 tertiary methyl singlets plus 2 secondary methyl doublets compared to 5 tertiary methyl singlets and 2 secondary doublets each for compounds (**5a**) and (**2a**). On the other hand, the ^{1}H NMR spectra of olean-12-ene type compounds like (**1b**) should show 8 tertiary methyl singlets and only 7 tertiary singlets for (**2b**) with no doublets observed. This spectral pattern was clearly evident in the ^{1}H NMR spectrum of (**5a**) which was isolated as a single pure compound. However, since compounds [(**1a**) and (**1b**), (**2a**) and (**2b**)] were isolated as mixtures, these expected patterns were not as clear-cut due to overlapping signals.

2.2.3.1. Isomeric mixture of α- and β-amyrins

The major component in the EtOAc-soluble active fraction of Magraviaceae leaves was identified to be an isomeric triterpenoid mixture of α-amyrin (**1a**) and β-amyrin (**1b**). This was isolated as a white solid, m.p.179-181°C (lit.[28] 175°C) in 0.6-1.1% of dry weight after silica gel column chromatography followed by recrystallisation from EtOH.

Compounds (**1a**) and (**1b**) are known to occur together in many plant exudates both in the free state and as acetates. It is worth noting that the only difference between the structures of (**1a**) and (**1b**) is in the position of the C-29 methyl group in the E-ring (**Figure 2.2.3.1a**).

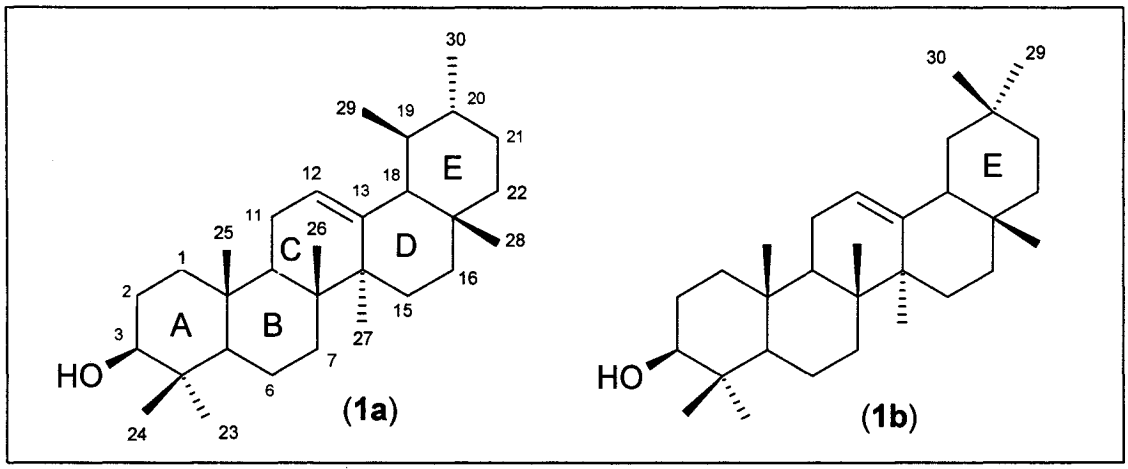


Figure 2.2.3.1a: Comparing the structure of α-amyrin (**1a**) and β-amyrin (**1b**).

The characterization of the individual components of the isomeric mixture of (1a) and (1b) was greatly simplified by the assignment of the olefinic carbons (C 12 and C-13) in their ^{13}C NMR spectrum, since the chemical shifts of the sp^2 carbon atoms are very characteristic for each triterpenoid.[25,26]

As mentioned earlier, ^{13}C NMR spectroscopy is the most precise when it comes to distinguishing the urs-12-ene and olean-12-ene derived triterpenoids and has the advantage that the contamination of one isomer by the other is readily discerned. In this way, it was possible to identify the presence of compounds (**1a**) and (**1b**) by comparison with previously published ^{13}C NMR data for α-and β-amyrin (**Table 2.2.3.1**).[8,25]

Table 2.2.3.1: The ^{13}C NMR data for compounds (**1a**) and (**1b**). Comparison with published data for α- amyrin ($\delta_{C'}$) and β-amyrin ($\delta_{C''}$).[8,25]

C	**1a**	$\delta_{C'}$	**1b**	$\delta_{C''}$
1	38.5	38.6	38.5	38.6
2	27.2	27.1	27.2	27.3
3	79.0	78.8	79.0	78.8
4	38.8	38.6	38.8	38.8
5	55.1	55.1	55.1	55.1
6	18.3	18.2	18.3	18.2
7	32.9	32.8	32.9	32.8
8	39.7	39.9	38.5	38.6
9	47.6	47.5	47.7	47.6
10	36.9	36.9	37.1	37.0
11	23.2	23.2	23.7	23.6
12	124.4	124.3	121.7	121.6
13	139.6	139.4	145.2	145.0
14	42.0	42.0	41.5	41.4
15	28.7	28.6	25.9	26.0
16	26.6	26.5	27.2	27.1
17	33.7	33.6	32.5	32.5
18	59.0	58.9	47.2	47.2
19	39.6	39.5	46.8	46.7
20	39.5	39.5	31.2	31.1
21	31.1	31.1	34.7	34.6
22	41.7	41.6	37.1	37.2
23	28.1	27.9	28.4	28.3
24	15.6	15.6	15.5	15.5
25	15.6	15.6	15.6	15.6
26	16.7	16.8	16.8	16.8
27	23.3	23.3	26.1	26.0
28	28.1	27.9	28.4	28.3
29	17.5	17.3	33.3	33.2
30	21.4	21.3	23.5	23.6

The appearance of signals at δ 139.6 (C-13), 124.4 (C-12) and 79.0 (C-3) ppm indicated the presence of (**1a**) having the basic 3β-hydroxy-urs-12-ene skeleton whereas the signals at δ 145.2 (C-13), 121.7 (C-12) and 79.0 (C-3) ppm

confirmed the presence of the basic 3β-hydroxy-olean-12-ene skeleton in (**1b**). Discernable differences between the chemical shifts of carbons C-18, C-19 and C-20 in ring E of (**1a**) and (**1b**) were also very useful in their identification (**Figure 2.2.3.1c**).

The ^{1}H NMR spectrum of compounds (**1a**) and (**1b**) confirmed the above deductions by displaying diagnostic signals for the olefinic protons at δ 5.10 (t, J=3.5 Hz, H-12) and 5.16 ppm (t, J=3.7 Hz, H-12) for compounds (**1a**) and (**1b**) respectively, and for the hydroxyl bearing methine proton at δ 3.2 (dd, J=10.3 Hz, J=5.7Hz, H-3α), (**Figure 2.2.3.1b**).

. The comparatively large coupling constant (J=10.3) established that the OH group has the 3β-configuration. These data agreed with those reported by Johnson *et. al.*[29]

The methyl signals at δ 0.77-1.11 appeared among a heavily overlapping region. Ideally, eight methyl signals should be observed as singlets for (**1a**) and two of them (H-29 and H-30) as a doublet for (**1b**). Since these compounds were isolated as a mixture these methyl signals were not as clear-cut due to signal overlaps.

The EIMS contained the molecular ion $[M]^+$ at m/z 426 which coincided with the desired molecular formula $C_{30}H_{50}O$; this was confirmed by HRMS. The fragmentation pattern matched the known MS for (**1a**) and (**1b**). The base peak at m/z 218 and the abundant ions at m/z 203, 189 and 133 were attributable to the RDA cleavage of ring C and the subsequent fragmentation of the D/E rings (**Schemes 2.2.3a** and **2.2.3b**).

The broad IR band at 3305 cm^{-1} was consistent with the presence a free OH group.

The isomeric mixture of amyrins (**1a**) and (**1b**) was first extracted from elemi resin and examined by Rose in 1835 who assigned to it the empirical formula $C_{30}H_{66\text{-}68}O$.[28] Hess (1839) and Johnston (1842) even adopted the formula $C_{30}H_{66}O$. Baup (1852) appears to be the first one to use the name "amyrin".[28] In 1874-1875, Fluckiger and Buri considered "amyrin" to be a monohydroxy compound $C_{25}H_{41}OH$, m.p. 177°C that forms a monoacetyl

derivative.[28] In 1876, Stenhouse and Groves isolated "icacin" from the incense tree of British Guiana and gave it the formula $C_{46}H_{76}O$; which was later shown to be a mixture of amyrins.

The first detailed study of the amyrins was carried out by Vesterberg (1887-1890) who examined a specimen of Manila elmi resin, determines its amyrin content to be 16.5% of which 2/3 was the α-amyrin (**1a**). He also realized that these amyrins were isomers and not homologue and also correctly assigned the formula $C_{30}H_{50}O$ to them.

The presence and position of the D-bond in β-amyrin (**1b**) was confirmed by the conversion of oleanolic acid (**5d**) to (**1b**) by Ruzicka *et. al.*, in 1937. The gross structure of α-amyrin (**1a**) was determined by Ruzicka *et. al.*, in 1949. This was confirmed by Corey and Cantrall in 1959 by a 20 steps synthesis of (**1a**) from glycrrhetic acid (a β-amyrin derivative).[30] Several syntheses of β-amyrin (**1b**) have been reported in the literature.[10,29,31,32]

The best source of the amyrins is the Manila elemi resin although both the α- and β-amyrins are very widely distributed in the plant kingdom.[28] In all cases, the β-isomer gives the less soluble and higher melting point derivative. α-Amyrin (**1a**) isolated from the hexane extract of *Achillea ageratum* L. (Asteraceae) has been showen to have high degree of growth inhibition against Hep-2 and McCoy cells.[33]

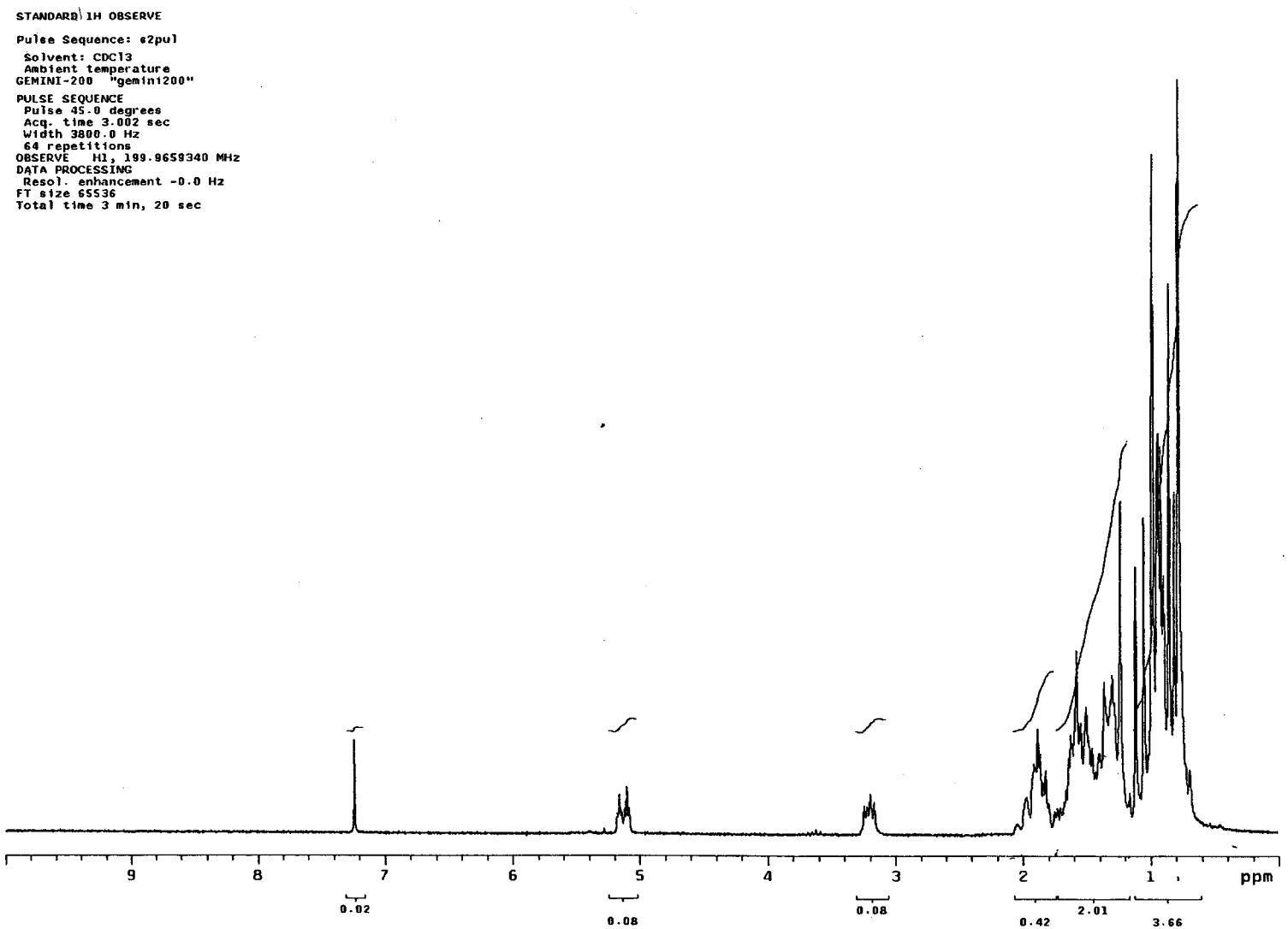


Figure 2.2.3.1b: ^{1}H NMR spectrum of the isomeric mixture of α-amyrin (**1a**) and β-amyrin (**1b**) isolated from Margraviaceae plant extracts.

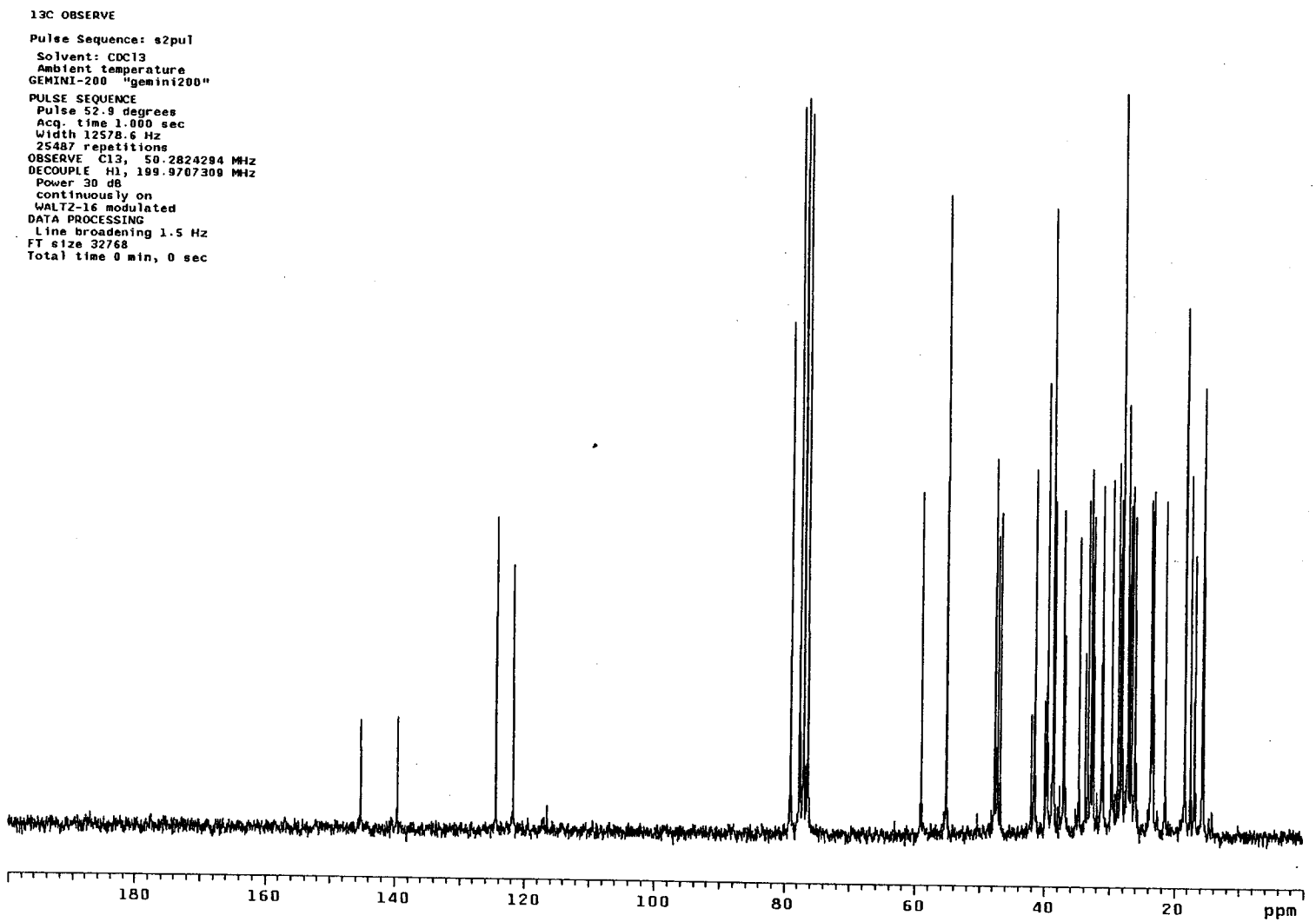


Figure 2.2.3.1c: ^{1}C NMR spectrum of the isomeric mixture of α-amyrin (**1a**) and β-amyrin (**1b**) isolated from Margraviaceae plant extracts.

2.2.3.2. Methyl Ursolate

Ursolic acid (**5c**) was isolated as methyl ursolate (**5a**) from the EtOAc extracts of Magraviaceae leaves as a white solid, m.p. 111-113°C (lit.[34] 111-114°C, lit.[35] 113-114°C, lit.[27] 115-116°C, lit.[36] 166-168°C, lit.[37] 170-172°C) in 0.03% dry weight after methylation with CH_2N_2 followed by repeated Si gel column chromatography.

The identity of (**5a**) was determined by comparing its physical and spectral data with published values for methyl ursolate and its structural isomer methyl oleanolate (**5b**). It is worth noting that compounds (**5a**) and (**5b**) differ only in the position of one methyl group (C-29) in ring E (**Figure 2.2.3.2a**). Moreover, the structure of (**5a**) resembled that of α–amyrin (**1a**) except for an additional CO_2Me group at C-28. Therefore, one would expect their spectral data to be very similar but with appropriate signal changes to account for these differences.

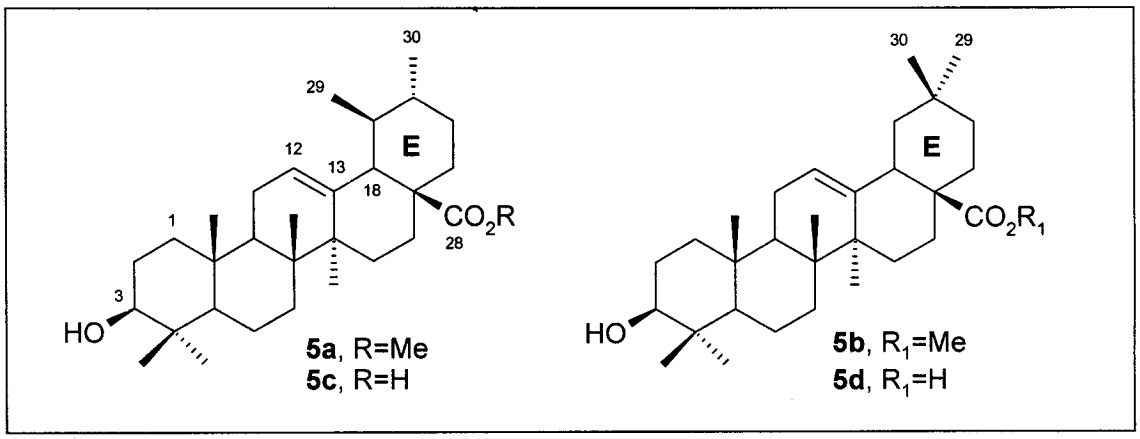


Figure 2.2.3.2a: Urs-12-ene derived triterpenoids (**5a** and **5c**) compared with their structural analogues, the olean-12-enes (**5b** and **5d**).

The ^{13}C NMR was used to establish compound (**5a**) to be an urs-12-ene derived triterpenoid and not its olean-12-ene analogue, methyl oleanolate (**5b**), especially the diagnostic difference in the chemical shifts of the olefinic carbons

(C-12) at δ 125.5 and (C-13) at δ 138.8 for (**5a**) compared to the corresponding resonances at δ 122.1 and δ 143.4 for (**5b**).

Significant differences in the chemical shifts of the carbons in ring E (C-17, C-18, C-19 and C-20) were also observed. The ^{13}C NMR data of (**5a**) were virtually identical with the reported values in the literature for methyl ursolate (**Table 2.2.3.2**).[8,26] The published ^{13}C NMR data for methyl oleanolate (**5b**) are also included in the table for comparison.[8]

In the ^{1}H NMR spectrum of (**5a**), the two doublets at δ 0.91 (d, J=6.4 Hz, H-30) and 0.83 ppm (d, J=6.4 Hz, H-29) produced by the two secondary methyl on ring E were the most useful indicator for the presence of an urs-12-ene skeleton rather than an olean-12-ene derived triterpenoid.

The other important signals were attributed to the presence of a trisubstituted olefinic proton at δ 5.22 (t, J=3.6 Hz, H-12), allylic proton at δ 2.20 (d, J=11.4 Hz, H-18β), a hydroxy bearing methine proton at δ 3.19 (m, 1H, H-3α), a methoxy group at δ 3.58 (s, 3H, CO_2Me), and five tertiary methyl singlets at δ 0.72 (H-26), 0.75 (H-24), 0.89 (H-25), 0.96 (H-23) and at 1.05 ppm (H-27). The ^{1}H NMR data of (**5a**) were comparable with published data for methyl ursolate.[36]

The mass spectrum of (**5a**) contained the $[M]^+$ at m/z of 470 in accordance with its molecular formula $C_{31}H_{50}O_3$. Prominent ions at m/z 262, 203 (base peak), 189 and 133 are due to the RDA cleavage of the C-ring that is characteristic of pentacyclic triterpenoids having a double bond at C-12 and C-13 with CO_2CH_3 and OH groups at C-28 and C-3 respectively (**Schemes 2.2.3a** and **2.2.3b**). These MS data agreed with those reported by Furuya *et. al.*[34] Its IR spectrum exhibited strong absorption bands at 3500 and 1725 cm^{-1} assignable to the hydroxyl and carbonyl groups respectively.

Table 2.2.3.2: The ^{13}C NMR data of compound (**5a**). Comparison with reported literature values for methyl ursolate ($\delta_{C'}$) and methyl oleanolate (**5b**).[8,26]

C	**5a**	$\delta_{C'}$	**5b**
1	38.8	38.8	38.5
2	27.2	27.3	27.1
3	79.0	78.8	78.7
4	38.7	38.8	38.7
5	55.2	55.4	55.2
6	18.3	18.4	18.3
7	32.9	33.0	32.6
8	39.4	39.6	39.3
9	47.5	47.5	47.6
10	36.9	37.0	37.0
11	23.3	23.3	23.4
12	125.5	125.5	122.1
13	138.1	138.0	143.4
14	42.0	42.0	41.6
15	28.1	28.2	27.7
16	24.2	24.3	23.1
17	48.0	48.1	26.6
18	52.8	52.8	41.3
19	39.0	39.1	45.9
20	38.6	38.8	30.6
21	30.6	30.7	33.8
22	36.6	36.7	32.3
23	28.0	28.2	28.1
24	15.4	15.5	15.6
25	15.6	15.7	15.4
26	16.9	16.9	16.8
27	23.6	23.6	26.0
28	178.1	177.7	177.9
29	17.0	16.9	33.1
30	21.2	21.2	23.6
OMe	51.5	51.5	51.3

Ursolic acid (**5c**) originally known as urson, was first isolated from the leaves of bearberry, *Arctostaphylos Uva-ursi*, in 1854 by Trommsdorff who gave it the formula $(C_{10}H_{16}O)n$ while Hlasiwetz proposed the formula $(C_{10}H_{17}O)_n$ in 1855.[24] Gintl (1893) provided the first evidence of a hydroxyl group in ursolic

acid which he considered to be related to sesquiterpenes with a formula of $C_{30}H_{48}O_3$.[24]

In 1918, Dodge noted a close resemblance between (**5c**) and caryophyllin (oleanolic acid) occurring in clove buds.[24] He accepted the formula $C_{30}H_{48}O_3$ but regarded (**5c**) as a hydroxy lactone and not a hydroxy acid. In 1922-1924, Haar performed detailed reaction studies on (**5c**) and showed that it was identical with the alcohol prunol (isolated by Power and Moore in 1910) and the glycol malol (isolated by Sando in 1923).[24] He also considered (**5c**) to be closely related to sesquiterpenes and gave it the formula $C_{31}H_{50}O_3$. Riviere and Pichard (1924-1925) confirmed the identity of malol and (**5c**) and showed that (**5c**) was a hydroxy acid and not a glycol.[24]

The first evidence that (**5c**) was probably a triterpene derivative was obtained by Ruzicka and van Veen in 1929.[24] Jacobs and Flecks provided the first evidence that (**5c**) was an unsaturated acid containing a secondary alcohol group in 1931. These observations and experiments were confirmed and extended by Kuwada and Matsukawa (1933-1938) leading to the conclusion that (**5c**) was a triterpene unsaturated hydroxy acid, containing a secondary alcohol group, having the formula $C_{30}H_{48}O_3$.

More evidence for the structure of (**5c**) was revealed when Goodson found that it could be converted to α-amyrin (**1a**) in 1938. Huzii and Osumi (1939-1940) were the first to propose the pentacyclic structure of (**5c**) but the positions of the double bond, the carboxylic acid group and the methyl groups in the E-ring were incorrectly assigned.

Further progress in the elucidation of the structure of (**5c**) was dependent on the determination of that of α-amyrin (**1a**) since, once this was known, it would only be necessary to find the position of the carboxylic acid. On the basis of the evidence provided by the investigations of Spring, Ruzicka and their collaborators (1943-1953) the structure of (**5c**) was finally identified and correctly assigned.[24]

Compound (**5c**) is widely distributed in the plant kingdom and occurs in great abundance in the waxy coatings of leaves and fruits of apples and pears

and, may act as antifeedant and antimicrobial agents.[38] One mature Bramley apple contains > 50 mg of (**5c**) in its peel.[39]

Ursolic acid (**5c**) has also been isolated from many medicinal plants and a variety of biological activities has been attributed to it including anti-arthritic, anti-ulcer, anti-tumor, anti-HIV, anti-inflammatory activities.[39] The anti-inflammatory activity of (**5c**) prompted Ying *et. al* to investigate its potential as an inhibitor of human leucocyte elastase (HLE) and found that (**5c**) was the most potent of several pentacyclic triterpenoids tested. These authors suggested that (**5c**) interacts with the subsite S_3 of the extended substrate-binding domain in HLE, and that the carboxyl group at C-28 contributes to binding to HLE, since replacement of this group with a hydroxy group, such as in uvaol, the alcohol analogue of (**5c**), reduces potency of inhibition (**Figure 2.2.3.2b**).

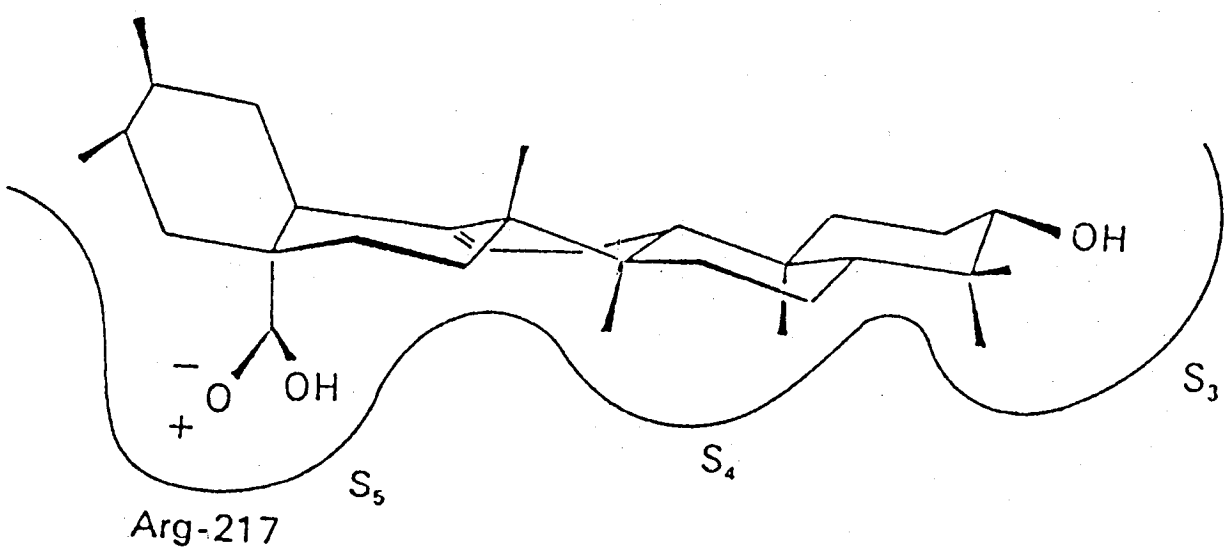


Figure 2.2.3.2b: A diagrammatic representation of the binding of ursolic acid (**5c**) to the extended substrate-binding domain of human leucocyte elastase (HLE).

The anti-inflammatory and anti-ulcer effects of ursolic acid (**5c**) and its structural isomer oleanolic acid (**5d**) *in vitro* and *in vivo*, have been attributed to the inhibition of histamine release as well as lipooxygenase and cyclooxygenase-2 (COX-2) enzyme activity.[40]

Recent findings revealed that synthetic ursolic acid derivatives such as urs-1-en-3-one with various modification to the C-ring increased anti-inflammatory and anti-carcinogenic potency.[41]

Wang and Polya reported that (**5c**) and structurally related triterpenoids are the most potent non-aromatic plant derived inhibitors of cyclic AMP-dependant protein kinase (cAK) yet found.[42] This may be significant in terms of plants defensive mechanisms since a variety of plant defensive metabolites are potent inhibitiors of protein kinases involved in eukaryote signal transduction pathways. These potent triterpenoid inihibitors of cAK have a common structural motif involving polar residues located at opposite ends of a non-polar triterpenoid nucleus and hence, possess an amphiphilic character. A simple model of amphiphile inhibition of cAK was proposed, since a variety of triterpenoids not possessing this structural motif were relatively inactive.

Mallavadhani *et. al.*, recently synthesized long chain (C-12 to C-18) of 3-O-fatty acid esters of (**5c**) and (**5d**) which showed potent antifeedant activity against the agricultural pest tobacco caterpillar larvae (*Spodoptera litura F*).[43]

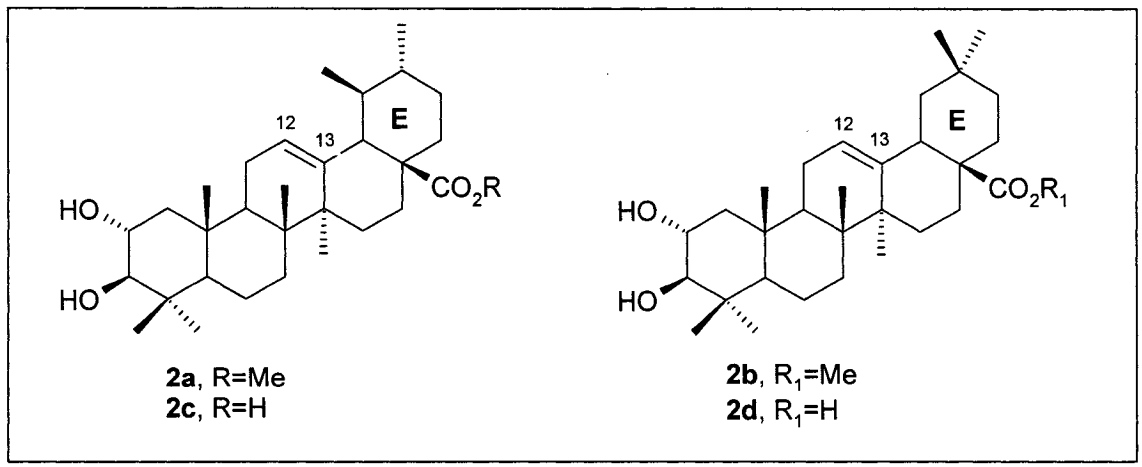
Furthermore, (**5c**) exhibited equipotent anti-HIV activity with oleanolic acid (**5d**) but was slightly toxic.[44] Several synthetic 3-O-acyl ursolic acid and oleanolic acid derivatives have demonstrated relatively potent anti-HIV activity. It has been suggested that the acyl group at C-3 and the structure of the E-ring might play an important role in the anti-HIV potency since these acyl derivatives were generally less potent than the corresponding 3-O-acyl betulinic acid derivatives.

2.2.3.3. Isomeric mixture of methyl 2α-hydroxy-ursolate and methyl maslinate

The isomeric triterpenoid mixture of 2α-hydroxy-ursolic acid (**2c**) and maslinic acid (**2d**) was isolated as methyl esters, methyl 2α-hydroxy-ursolate (**2a**) and methyl maslinate (**2b**) respectively, from the EtOAc extracts of Margraviaceae leaves after treatment with CH_2N_2 followed by repeated Si gel column chromatography and recrystallisation from hexane/CH_2Cl_2.

Compounds (**2a**) and (**2b**) was obtained as a white solid, m.p. 215-218°C, in 0.04% yield (dry weight). The m.p. of (**2a**) and (**2b**) as a mixture has not been reported in the literature. However, the m.p. of the separate pure compounds are as follows; for compound (**2a**), lit.[34] 204-207°C, lit.[45,46] 211-213°C, lit.[27] 211-214°C, lit.[47] 215°C; for (**2b**), lit.[34] 225-227°C, lit.[45] 227-228°C, lit.[48] 228°C, lit.[27] 232-234°C, lit.[49] 249-252°C, lit.[46] 254-260°C.

It should be noted that compounds (**2a**) and (**2b**) have the same structures as methyl ursolate (**5a**) and methyl oleanolate (**5b**) respectively (**Figure 2.2.3.2a**), except for an additional α-hydroxyl group at C-2 (**Figure 2.2.3.3a**).



2a, R=Me
2c, R=H

2b, R_1=Me
2d, R_1=H

Figure 2.2.3.3a: Structures of 2α-hydroxy-ursolic acid (**2c**) and maslinic acid (**2d**) and and their respective methyl esters, (**2a**) and (**2b**).

The EI mass spectrum of (**2a**) and (**2b**) contained a weak $[M]^+$ at m/z 486 corresponding to the anticipated molecular formula $C_{31}H_{50}O_4$. This was confirmed by HRMS. Diagnostic fragment ions (*listed in the Experimental* **Section 2.3**) derived from D/E rings via RDA cleavage of the C ring and their subsequent fragmentations (**Schemes 2.2.3a** and **2.2.3b**) were observed and were also consistent with published data.[34,45] Strong O-H and C=O IR bands appeared at 3723 and 1719 cm^{-1} respectively.

The assignments of the ^{13}C NMR and the ^1H NMR signals of (**2a**) and (**2b**) mixture were performed by reference to the literature data reported for the individual pure compounds of methyl 2α-hydroxyursolate and methyl maslinate respectively,[8,27,45] as well as for their mixture.[37]

Inspection of the ^{13}C NMR data of (**2a**) and (**2b**) showed diagnostic chemical shifts for C-2 at δ 68.9, C-3 at δ 83.8 which were in conformity with an urs-12-ene-2α, 3β-diol or an olean-12-ene-2α-3β-diol skeleton (**Figure 2.2.3.3c**). The resonances at δ 125.3 (C-12) and at δ 138.2 (C-13) confirmed the presence of an urs-12-ene derived triterpenoid while the resonances at δ 122.1 (C-12) and at δ 143.6 (C-13) established the presence of an olean-12-ene derived triterpenoid. The differences in the chemical shifts of C-17, C-18, C-19 and C-20 of ring E further supported the proposed structures for (**2a**) and (**2b**) (**Table 2.2.3.3**).

The ^1H NMR spectrum exhibited characteristic signals for the olefinic protons at δ 5.22 (t, J=3.5 Hz, H-12) and δ 5.21 (t, J=3.5 Hz) for (**2a**) and (**2b**) respectively. Diagnostic signals were also observed for H-2 and H-3 (both attached to hydroxyl bearing carbons C-2 and C-3 respectively) at δ 3.66 (m, H-2) and at δ 2.96-2.97 (d, J=12 Hz, H-3). The coupling constant (J=10-12 Hz) between H-3 and H-2 is comparatively large confirming that the two hydroxyl groups have the 2α- and 3β- configurations. The one-proton signal due to an allylic 18β-hydrogen near δ 2.20 and 2.83 ppm indicated the difference between a methyl urs-12-ene-28-oate the olean-12-ene analogue. The methyl singlets were observed in the range δ 0.69-1.10 ppm (**Figure 2.2.3.3b**).

Table 2.2.3.3: ^{13}C NMR data for compounds (**2a**) and (**2b**). Comparison with data published for methyl 2α-hydroxyursolate ($\delta_{C'}$) and methyl maslinate ($\delta_{C''}$).[8,27,45]

C	**2a**	$\delta_{C'}$	**2b**	$\delta_{C''}$
1	46.8	46.6	46.4	46.3
2	68.9	68.9	68.8	68.9
3	83.8	83.9	83.8	83.9
4	39.2	39.3	39.1	39.1
5	55.4	55.2	55.3	55.2
6	18.4	18.3	18.3	18.3
7	32.9	32.8	32.6	32.5
8	39.6	39.5	39.1	39.0
9	47.5	47.4	47.5	47.5
10	38.3	38.2	38.3	38.2
11	23.4	23.4	23.5	23.6
12	125.3	125.3	122.0	122.1
13	138.2	138.1	143.8	143.6
14	42.1	42.0	41.7	41.6
15	28.0	27.9	27.6	27.6
16	24.3	24.1	23.1	23.0
17	48.1	48.0	46.6	46.5
18	52.8	52.7	41.3	41.2
19	39.1	39.1	45.8	45.8
20	38.9	38.8	30.7	30.7
21	30.7	30.7	33.8	33.8
22	36.6	36.6	32.3	32.3
23	28.7	28.6	28.6	28.6
24	16.8	16.9	16.8	16.9
25	16.7	16.7	16.8	16.9
26	17.0	17.0	16.8	16.9
27	23.6	23.6	26.0	25.9
28	178.2	178.3	178.0	178.1
29	19.9	16.9	33.1	33.1
30	21.2	21.2	23.5	23.6
OMe	51.5	51.5	51.5	51.5

Potent inhibitory activity against HIV-protease has been attributed to masilinic acid (**5d**).[50]

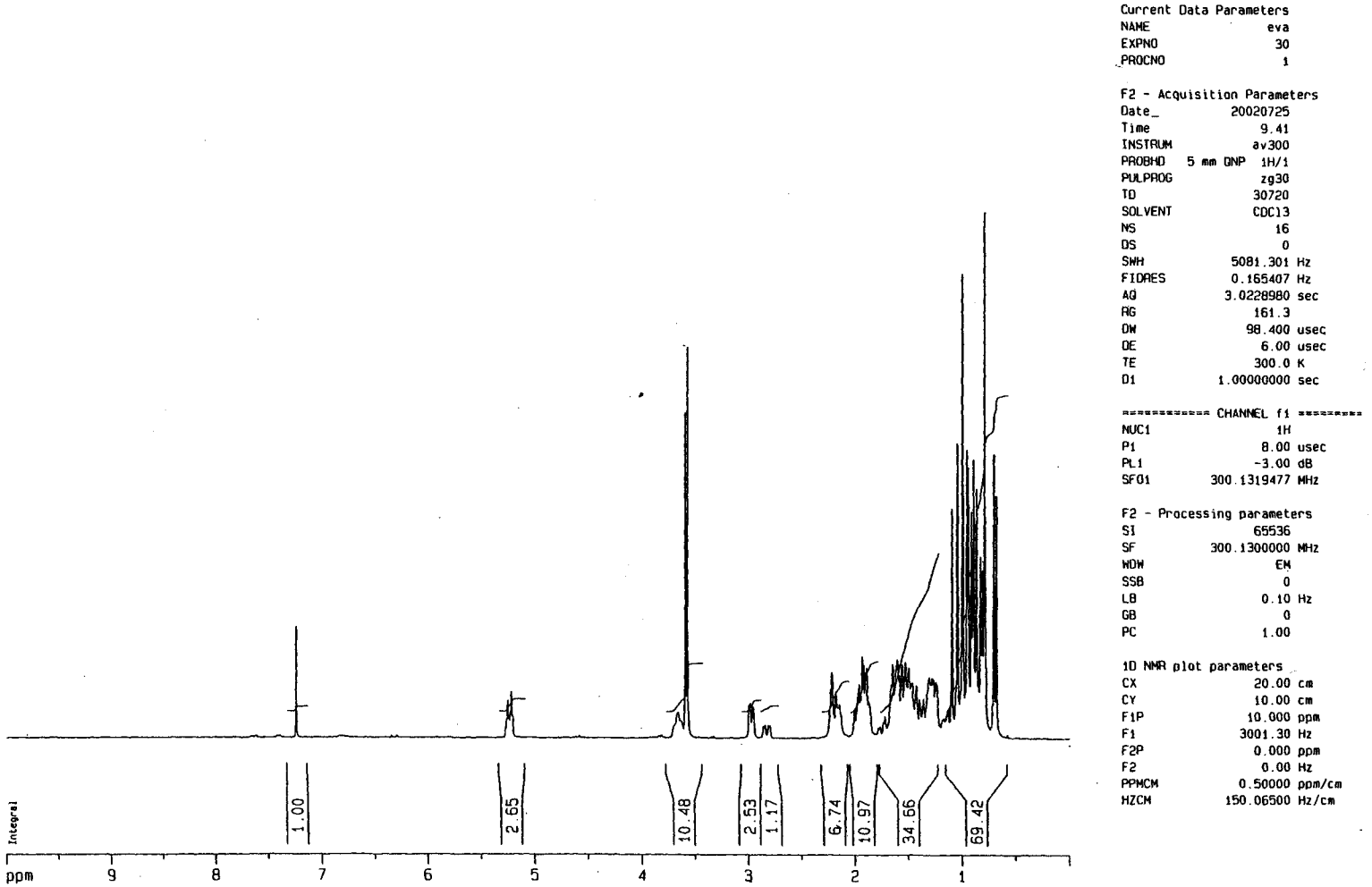


Figure 2.2.3.3b: ^{1}H NMR spectrum of the isomeric mixture of 2α-hydroxy-ursolic acid (**2a**) and maslinic acid (**2b**) isolated from Margraviaceae plant extracts.

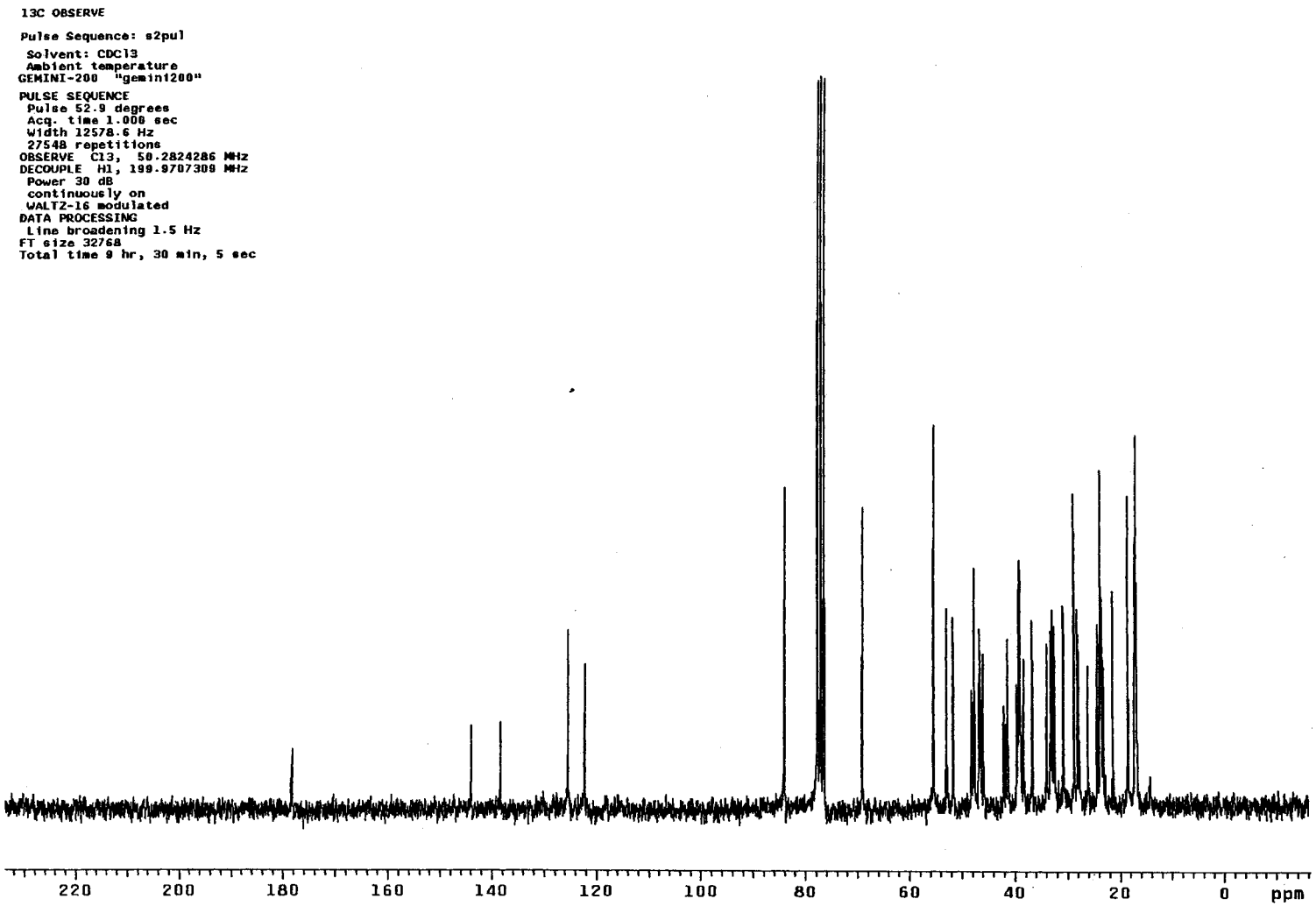
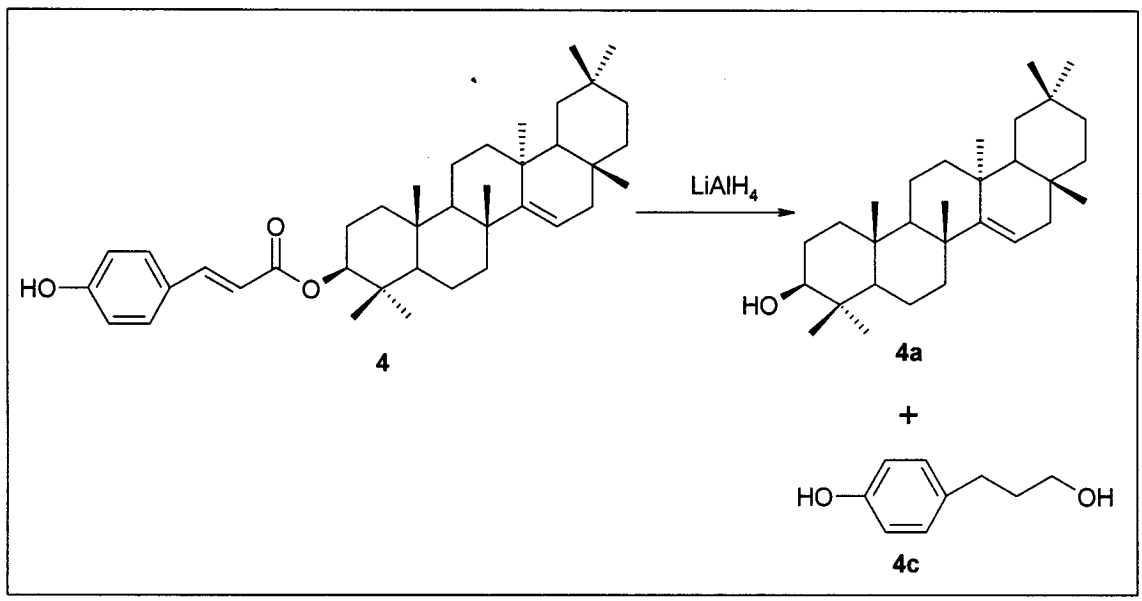


Figure 2.2.3.3c: ^{13}C NMR spectrum of the isomeric mixture of 2α-hydroxy-ursolic acid (**2a**) and maslinic acid (**2b**) isolated from Margraviaceae plant extracts.

2.2.4. Olean-14-ene Triterpenoids

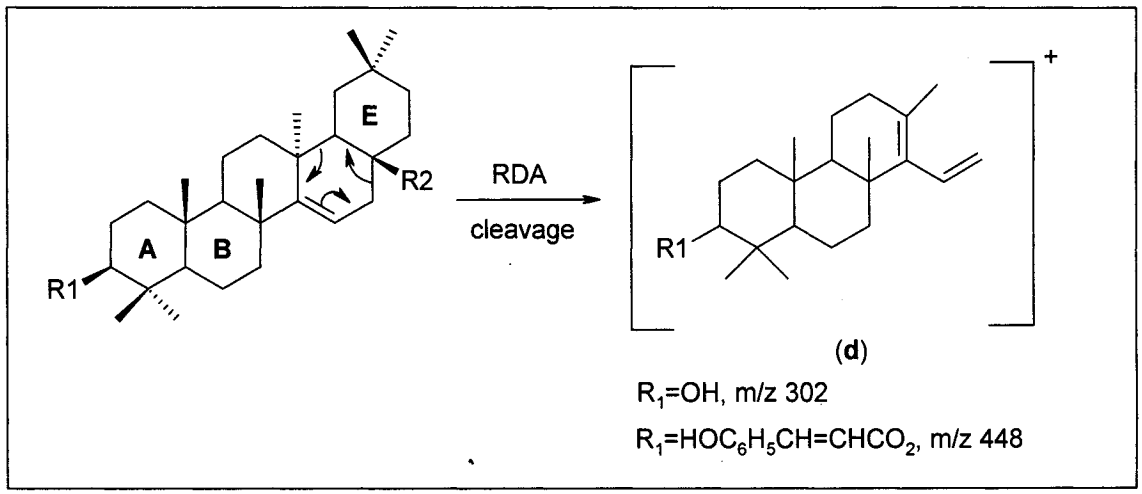
The isolate taraxeryl trans-p-hydroxycinnamate (**4**) belongs to a class of triterpenoids known as taraxeranes or D-friedooleananes which possess an olean-14-ene or taraxer-14-ene carbon skeleton.[8]

The structure of (**4**) was established primarily by analysis and comparison with previoulsy reported data for taraxeryl *trans-p*-hydroxycinnamate[54] and by $LiAlH_4$ reduction of (**4**) in order to liberate the olean-14-ene triterpenoid moiety (**4a**), known as taraxerol or alnulin (**Scheme 2.2.4a**).[51] The other reduction product was 4-hydroxyphenyl-propanol (**4c**). It is interesting to note that the carbonyl group and the double bond of the *p*-hydroxycinnamate moiety were both reduced by $LiAlH_4$ in this reaction, and that the allylic alcohol was not observed at all. This phenomenon is known in the literature.[52]



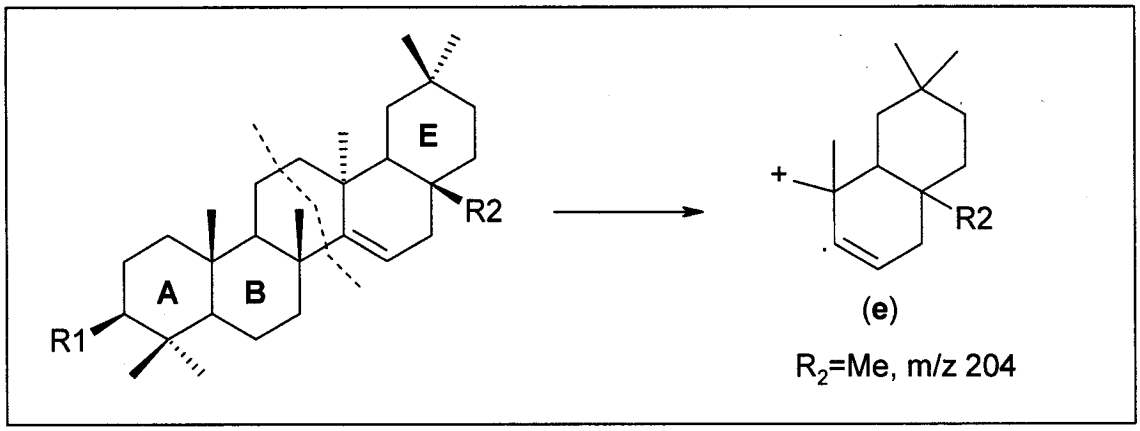
Scheme 2.2.4a: $LiAlH_4$ reduction of compound (**4**) in order to liberate the triterpenoid moiety, taraxerol or alnulin (**4a**).

Compounds (**4**) and (**4a**) undergo retro-Diels-Alder (RDA) fragmentation similar to that previously observed for the urs-12-ene and olean-12-ene derived triterpenoids (**Scheme 2.2.3a**) except, that cleavage of ring D occurs rather than ring C (**Scheme 2.2.4b**).[14] This cleavage process results in the formation of a characteristic fragment ion (**d**) at m/z 302 and at m/z 448 when the substituent R_1 is a hydroxyl and a *p*-hydroxycinnamate group respectively.



Scheme 2.2.4b: Typical RDA cleavage of the D-ring in an olean-14-ene type triterpenoid.

An alternative mode of cleavage that is also characteristic of olean-14-ene derived skeletons involves the fission of the C-ring (indicated by the dotted lines) thereby, producing an abundant fragment ion (**e**) at m/z 204 derived from rings D and E if the substituent R_2 is a methyl group as in the case of compound (**4**) and (**4a**), (**Scheme 2.2.4c**).[14,53]



Scheme 2.2.4c: Typical fission reaction of the C-ring in an olean-14-ene type triterpenoid.

The olefinic carbons of olean-14-ene derivatives exhibited characteristic resonances at approximately 158.1 and 117.0 ppm for the olefinic carbons, C-14 and C-15 respectively.[8] The ^{13}C NMR data of compounds (**4**) and (**4a**) are presented in **Table 2.2.4a** together with the published data for taraxerol ($\delta_{C'}$).[53]

The ABX pattern centred at $\delta \sim 5.5$ typical of the vinylic proton at C-15 in these types of compounds were observed in the ^1H NMR spectra of both (**4**) and (**4a**).[13] The diagnostic ^1H NMR resonances used in the identification of compounds (**4**) and (**4a**) are summarised in **Table 2.2.4b**.

The ^{13}C and ^1H NMR chemical shifts of (**4**) have not been reported in the literature and their assignments were accomplished largely by comparison with those of its acetate[54] and *cis* isomer (taraxeryl *cis*-*p*-hydroxycinnamate).[55]

Table 2.2.4a: ^{13}C NMR data for compounds (**4**) and (**4a**). Comparison with published data for taraxerol ($\delta_{C'}$).[53]

C	**4**	**4a**	$\delta_{C'}$
1	37.5	38.0	38.1
2	23.6	27.1	27.3
3	81.0	79.0	79.2
4	38.0	39.0	39.1
5	55.6	55.5	55.7
6	18.7	18.9	19.0
7	33.1	35.1	35.3
8	39.0	38.9	38.9
9	48.7	48.7	48.9
10	37.4	37.7	37.9
11	17.5	17.5	17.7
12	36.6	35.8	35.9
13	37.9	37.5	37.9
14	158.0	158.0	158.1
15	116.9	116.9	117.0
16	33.7	36.6	36.9
17	35.8	38.0	38.1
18	49.1	49.2	49.4
19	41.2	41.3	41.4
20	28.8	28.8	29.0
21	35.1	33.7	33.9
22	37.7	33.0	33.2
23	28.0	28.0	28.1
24	16.7	15.4	15.6
25	15.5	15.4	15.6
26	29.9	29.8	30.1
27	25.9	25.9	26.0
28	29.8	29.8	30.1
29	33.3	33.3	33.5
30	21.3	21.3	21.5
p-hydroxy-cinnamate ester			
C-1'	167.3		
C-2'	127.4		
C-3'	144.0		
C-4'	116.3		
C-5' and C-9'	130.0		
C-6' and C-8'	115.8		
C-7'	157.6		

Table 2.2.4b: Diagnostic ^{1}H NMR signals for compounds (**4**) and (**4a**). Comparison with published data for alnulin or taraxerol ($\delta_{H'}$).[53,54]

H	4	4a	$\delta_{H'}$
H-3α	4.57, t J=8.0 Hz	3.17, dd J=10.3, 6.3 Hz	3.19, dd J=8.0, 5.0 Hz
H-15	5.52, dd J=8.3, 3.2 Hz	5.51, dd J=8.2, 3.3 Hz	5.52, dd J=11.2, 4 Hz
H-23	0.93	0.93	0.96
H-24	0.93	0.90	0.94
H-25	0.80	0.78	0.82
H-26	1.08	1.06	1.10
H-27	0.96	0.95	0.99
H-28	0.88	0.80	0.83
H-29	0.89	0.88	0.92
H-30	0.89	0.88	0.94
H-2'	6.28, d J=16.0 Hz		
H-3'	7.58, d J=16.0 Hz		
H-5'/9'	7.49, d J=8.6 Hz		
H-6'/8'	6.89, d J=8.7 Hz		

2.2.4.1. Taraxerol *trans-p*-hydroxycinnamate

Taraxeryl *trans-p*-hydroxycinnamate (**4**) also known as careoborin was isolated from the EtOAc and EtOH extracts of Margraviaceae leaves in 0.06-0.1% dry weight, as a white solid, m.p. 273-275°C (lit.[54] 257-258°C) after repeated silica gel column chromatography followed by recrystallisation from MeOH/$CHCl_3$.

Compound (**4**) was first isolated from the leaves of *Carea arborea* in 1981 as pale yellow globules.[54] It has also been isolated together with its *cis* isomer as a mixture from *Bernardia laurentii*.[56]

The molecular formula of (**4**), $C_{39}H_{56}O_3$, was established on the basis of the $[M]^+$ at m/z 572 and HRMS. The fragment ion peaks at m/z 448 and at m/z 204 would be formed by a RDA cleavage of ring D that is characteristic of olean-14-ene skeleta (**Scheme 2.2.4b**) and, as a result of the fission reaction of ring C (**Scheme 2.2.4c**) respectively. The base peak at m/z 147 confirmed the presence of the *p*-hydroxycinnamate moiety. The presence of a free OH group and the C=O functions were apparent by the strong IR bands at 3500 and 1701 cm^{-1} respectively.

Diagnostic signals were observed for the D-ring vinylic proton at δ 5.51 (dd, J=8.3 Hz, J=3.2 Hz, 1H, H-15) and for the methine proton bearing the cinnamoyl moiety at δ 4.57 (t, J=8.0 Hz, H-3α). The two symmetrical doublets integrating for 2H each at δ 6.82 (d, J=8.7 Hz, 2H, H-6'/H-8') and 7.49 (d, J=8.6 Hz, 2H, H-5'/H-9') confirmed the presence of a *p*-disubstituted aromatic ring. The pair of one proton doublets at δ 6.28 and 7.58 (J=16 Hz) is characteristic of *trans* olefinic protons of the cinnamoyl moiety. The chemical shifts of the eight tertiary methyl groups were comparable with those reported for the *cis* isomer of (**4**), (**Table 2.2.4b**).[55]

The double bond at the C-14 and C-15 positions was readily recognized from the ^{13}C NMR spectrum of (**4**) since these carbons resonated at δ 158.0 and 117.0 ppm respectively. Significant signals were observed for C-3 (bearing the cinnamate ester group) at δ 81.0, for the ester carbonyl at δ 167.3 and, for the aromatic carbons of the *p*-hydroxy-cinnamate ester moiety (**Table 2.2.4a**).

2.2.4.2. Taraxerol

Taraxerol (**4a**) also commonly known as alnulin, was isolated as a pure white solid, m.p. 277-278°C (lit.[57] 273-275°C, lit.[54] 277-278°C, lit.[58] 277-280°C, lit.[53] 278-279°C, lit.[59] 282-283°C, lit.[60] 282-285°C, lit.[61] 287-289°C), in 71% yield after silica gel column chromatography.

The EI mass spectrum of (**4a**) displayed a $[M]^+$ at m/z 426 corresponding to the desired molecular formula $C_{30}H_{50}O$; which was established by HRMS. The fragmentation pattern was typical of olean-14-ene derivatives (**Schemes 2.2.4c**, and **2.2.4b**) and also in good agreement with MS data previously published by Deshmane and Dev for taraxerol.[57]

The comparison of the ^1H NMR data of (**4a**) with published data[54,59] for taraxerol ($\delta_{C'}$) revealed great similarity (**Table 2.2.4b**), (*see* **Figure 2.2.4.2a**). The olefinic carbons C-15, C-14 and C-3 (bonded to OH) resonated at δ 116.9, 158.0 and 79.0 ppm respectively, and were the most diagnostically valuable (**Table 2.2.4a**), (*see* **Figure 2.2.4.2b**).

Compound (**4a**) was first isolated as tiliadin from the bark of lime in 1900 by Braeutigam who gave it the molecular formula $C_{21}H_{32}O_2$, m.p. 228-229°C.[28] In 1912, Power and Browning isolated (**4a**) from dandelion and assigned to it the formula $C_{29}H_{48}O$.[28] Zellner *et. al.*, isolated (**4a**) as alnulin ($C_{35}H_{60}O$, m.p.261°C) from the bark of *Alnus incana* L. in 1924, but he quickly revised his formula to $C_{30}H_{50}O$.[28] In 1926, Zellner isolated tiliadin from the bark of Tilia platyphyllos ($C_{28}H_{48}O$, m.p. 275°C).[28]

Taraxerol (**4a**) was first obtained pure by Gerloff in 1936, from the bark of *Tilia cordata* and he also determine its correct composition ($C_{30}H_{50}O$, m.p. 276°C).[28] In 1941, Takeda isolated (**4a**) as skimmiol from *Skimmia japonica* and suggested that it was probably taraxerol.[28] Koller *et. al.,* (1950) were the first to point out that taraxerol was identical to alnulin and tiliadin which was confirmed by Brooks in 1953.[28] The final confirmation of the structure of (**4a**) was provided by the partial synthesis of taraxeryl acetate from β-amyrin (**1b**) in 1955 by Beaton and his colleagues.[62]

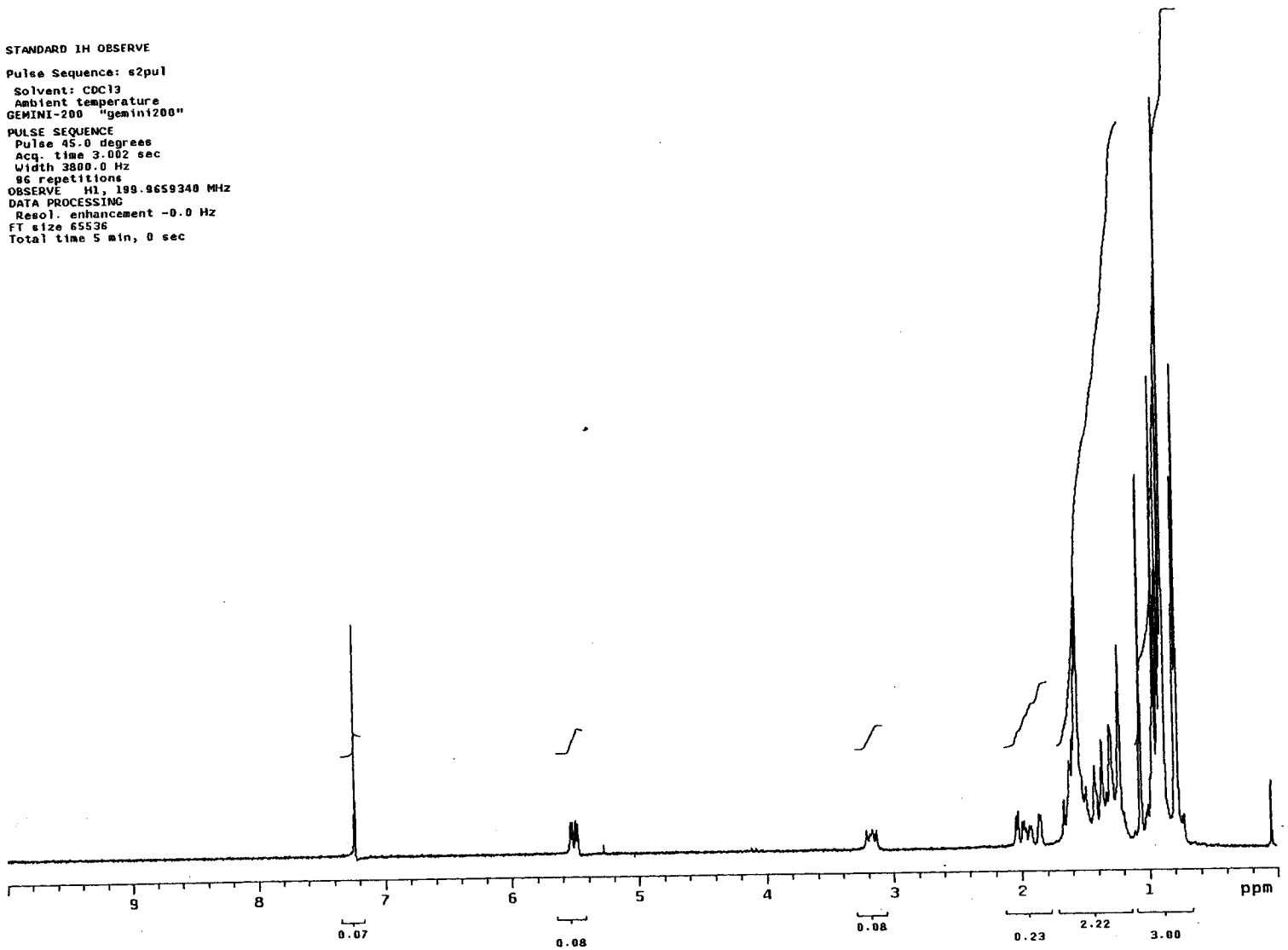


Figure 2.2.4.2a: ^{1}H NMR spectrum of taraxerol or alnulin (**4a**).

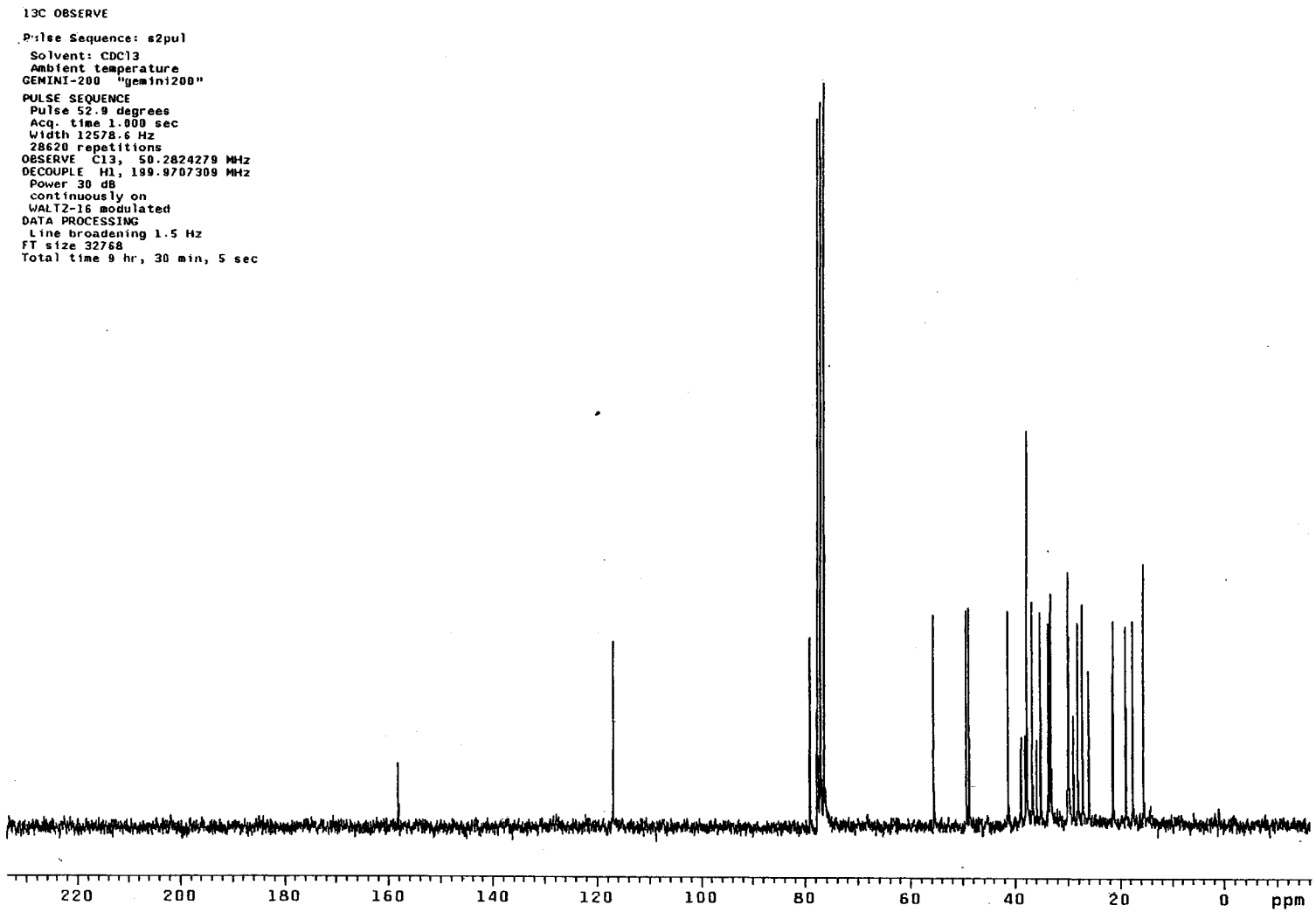


Figure 2.2.4.2b: ^{13}C NMR spectrum of taraxerol or alnulin (**4a**)

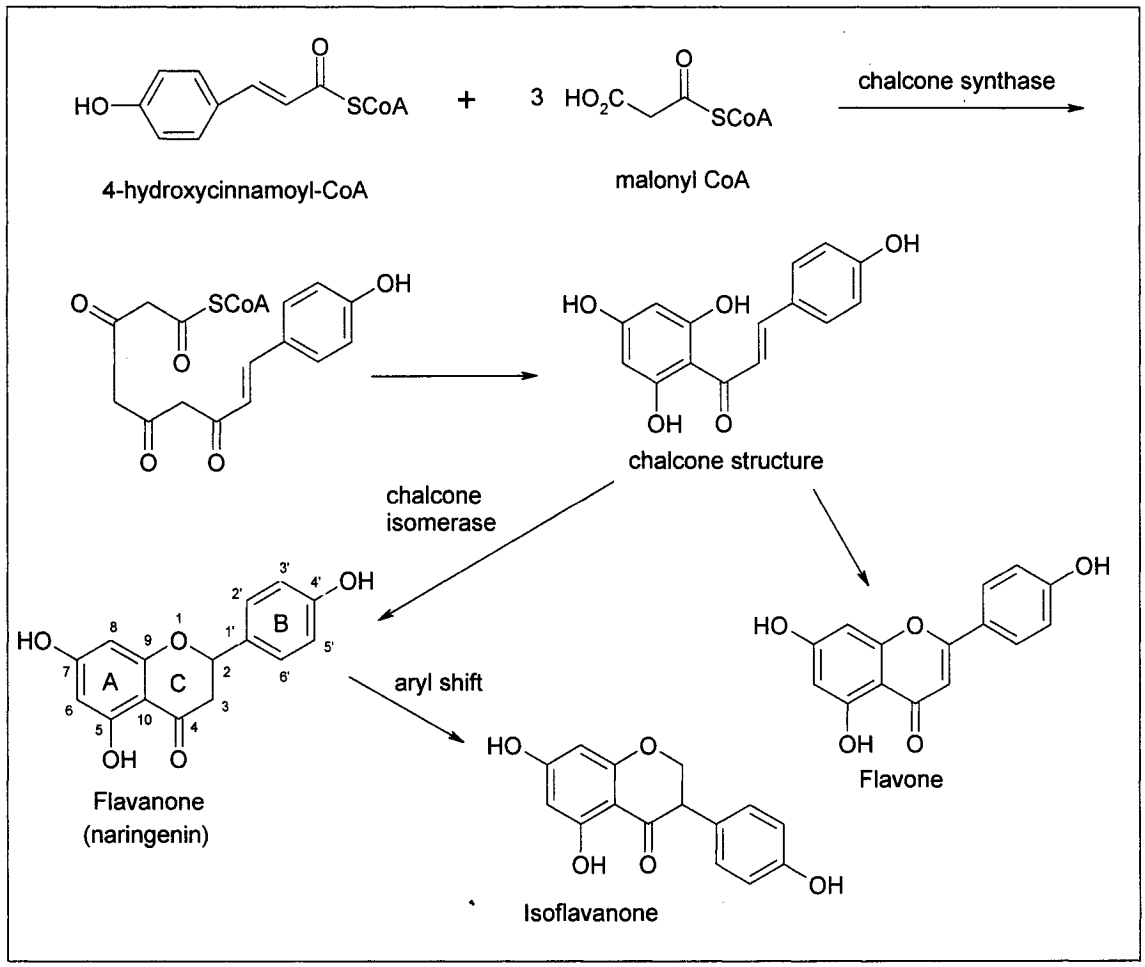
2.2.5. Flavonoids

Flavonoids are the largest group of naturally occurring phenol derivatives and are present in substantial amount in plants (0.5-1.5%).[63] They occur both in the free state (flavonoid aglycones), as glycosides, and as dimeric compounds or biflavanoids. It is estimated that 4000-5000 of these compounds are now known with ~500 flavonoid aglycones comprising of the seven main classes of flavonoids viz., flavones, isoflavones, flavanones, flavonols, dihydroflavonols, chalcones and dihydrochalcones.[64]

The chalcones although not strictly flavonoids are biosynthetically related, showing a mixed biogenesis from shikimates and polyketides.[65] All contain fifteen carbon atoms in their basic nucleus and these are arranged in a C_6-C_3-C_6 configuration (C_6 represents an aromatic ring).[66]

The accepted biosynthetic pathway to these large families of compounds is shown in **Scheme 2.2.5a**.[65] Cytochrome P450-dependant mono-oxygenase and 2-oxoglutarate-dependant dioxygenase (2-ODDs) have been reported to be invloved in flavonoid synthesis.[67].

The magnificent colors of autumn leaves are due largely to flavonoids.[68] In particular, flavones and their close relatives are usually yellow (Latin: *flavus* yellow) and form the largest group of heterocycles found in higher plants. They also contribute to the color and flavour in the processing of many food (vegetables, fruits, herbs) and drinks (tea, wine).[63]



Scheme 2.2.5a: Biosynthesis of common flavonoid aglycones.

The therapeutic usefulness of flavonoids have recently been recognized. Various biological activities of flavonoids have been reported including anti-inflammatory, cystostatic, antiviral, antimutagenic and *in vivo* anticancer activity.[69] Manthey and Guthrie reported the antiproliferative activities of citrus flavonoids against six common human cancer lines (lung, prostate, colon, melanoma and both the estrogen receptor positive (ER+) and (ES-) breast cancer.[70] Recently, Lin *et. al.*, reported biflavonoids as novel antituberculosis agents.[71]

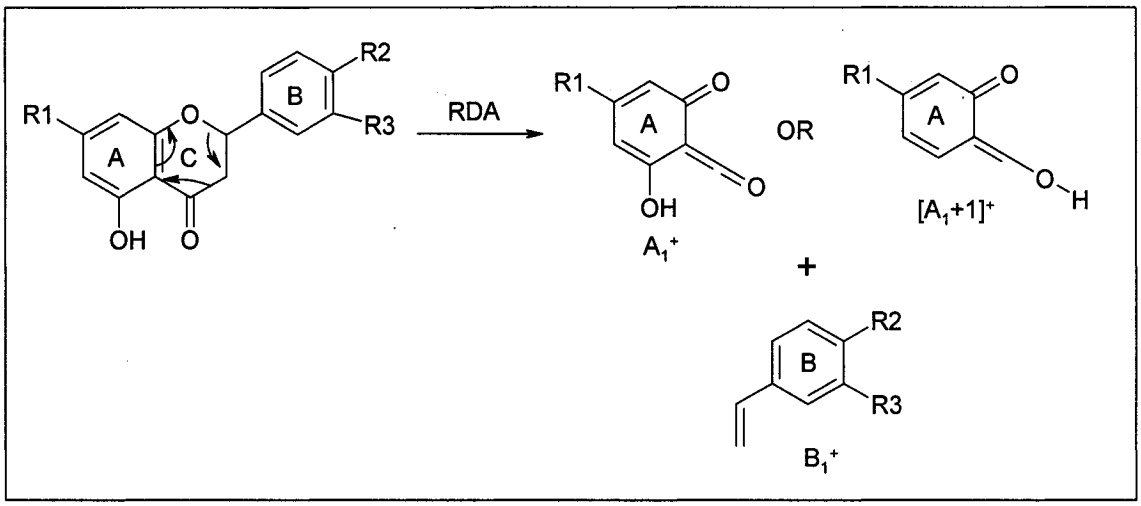
It appears that the biological effects of these flavonoids is due to their interaction with DNA and regulatory enzymes.[69] Their antioxidant activity (involving efficiency in capturing free radicals) is highly influenced by the

presence of hydroxyl and methoxy group on the aromatic rings.[63,69] The ortho-dihydroxy structure (catecholic moiety) in the B ring of some flavonoids has been shown to be the active center for metal chelation, scavenging and antioxidant activity thereby, preventing mammalian cytotoxicity caused by reactive oxygen species.

The beneficial actions of citrus flavonoids have been attributable to their protection of the microvascular endothelium during oxidative stress especially in cancer and inflammation cases.[70] Much of the anti-inflammatory activities of citrus flavonoids arise from their antioxidant properties as well as their abilities to block key steps in arachidonic acid metabolism. The strong synergistic effect between citrus flavonoids and vitamin C is now well documented. It is likely that multiple mechanisms will be found for the several classes of flavonoids, thus holding promise as potentially useful anticancer agents.

The various classes of flavonoids exhibit specific, diagnostic and generally predictable fragmentation patterns in their EI mass spectra.[72] Most flavonoid aglycones yield intense peaks for the molecular ion [M^+] which is often the base peak. Generally, sufficient information from their fragmentation patterns can be obtained to determine their molecular weight, the elemental formula, the substitution patterns in the A and B rings as well as the class of flavonoid. The most significant fragmentation in terms of flavonoid identification involves the typical retro-Diels Alder (RDA) cleavage of ring C producing characteristic fragment ions from the A and B rings and their subsequent fragmentations (**Scheme 2.2.5b**).[73]

Mabry and Markham have developed a systematic nomenclature to define the primary A and B rings fragments (designated as A_1, A_2 ... and B_1, B_2 etc.,) generated by EI-MS of flavonoid aglycones (**Figure 2.2.5a**).[72,74]



Scheme 2.2.5b: Typical RDA cleavage of a flavonoid aglycone (flavanone).

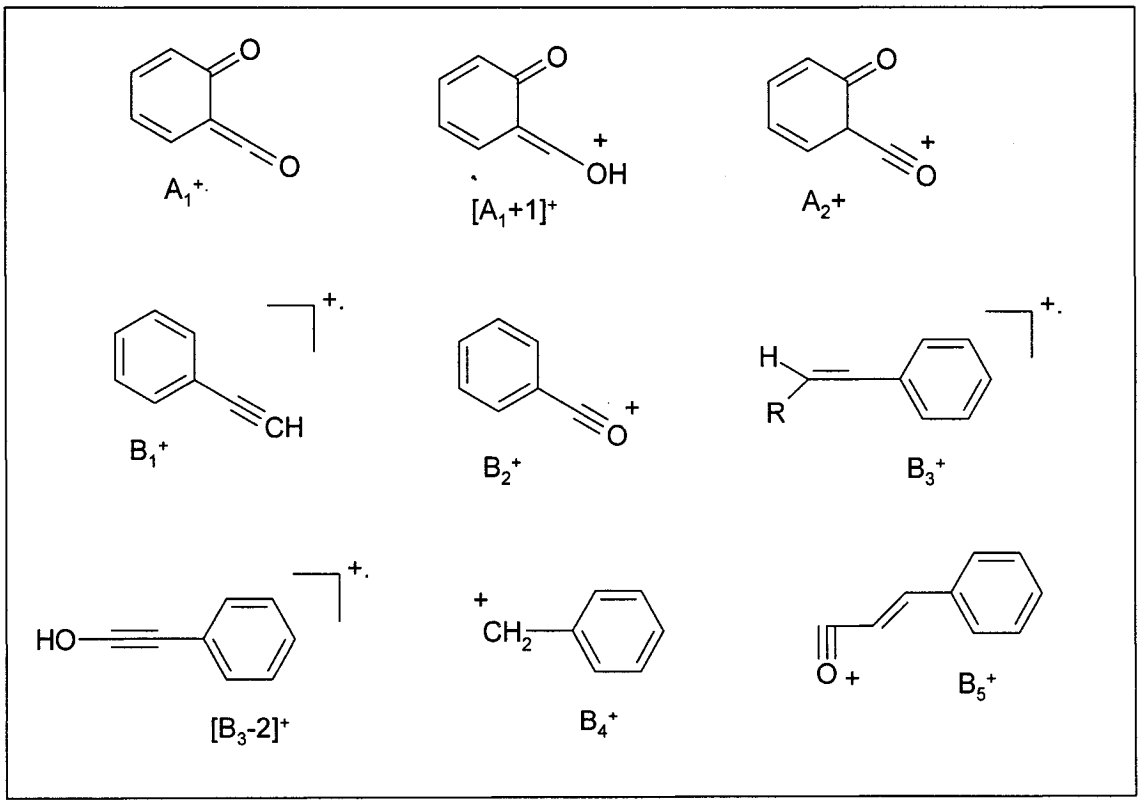


Figure 2.2.5a: Systematic nomenclature to defining the primary EI mass spectral A and B ring fragments of flavonoid aglycones.

A comprehensive amount of information about the MS of flavonoid aglycones has been published by Marbry and Markham (1975), Mabry and Ulubelen (1980), Markham (1988) Grayer (1989) as well as Hendin and Phillips (1992) which can be utilized in their differentiation and greatly simplify their identification.[72]

The unequivocal identification of the flavonoids isolated in this work was based on the comparison of their spectroscopic data and physical properties with published data. The spectroscopic data suggested the presence of a flavanone skeleton in all of the six flavonoids (**6**, **7**, **8a**, **8b**, **9**, **10**) isolated from Margraviaceae leaf extracts (**Figure 2.2b**).

2.2.6. Flavanones

During the early stages of this project (April-May 1999), a collection of Magraviaceae leaves from Costa Rica was extracted as outlined in **Figure 2.3.5e** to give an acetone extract. Naringenin (**9**) and 3',5,5',7'-tetrahydroxyflavanone (**10**) were isolated from this extract in 0.04-0.05% yields of dry weight. These two flavanones were not 100% pure by ^{1}H NMR after repeated silica gel column chromatography, but were pure enough to be characterised.

Naringenin (**9**) is one of the most important and fairly common flavanones in nature, whereas flavanone (**10**) bearing the 3',5'-dihydroxylation pattern, is very rare.[73]

In a latter collection also made in Costa Rica in May 2002, the di- and mono- methyl ether of naringenin namely naringenin 4',7-dimethyl ether (**6**) and naringenin 7-methyl ether (**7**) as well as the two isomeric dimethyl ether forms of the flavanone eriodictyol or naringenin-3'4'-dihydroxyflavanone, (**8a**) and (**8b**) were isolated from an EtOAc extract of Margraviaceae leaves in 0.02-0.06% yield after treatment with CH_2N_2 followed by repeated column chromatography. Flavanones (**8a**) and (**8b**) were isolated as an inseparable mixture (**Figure 2.3.5b**).

Since flavanones (**6**) and (**7**) were isolated after methylation with CH_2N_2, it is possible that they are both present in Margraviaceae as naringenin (**9**). Similarly, eriodictyol 3', 7-dimethyl ether (**8a**) and eriodictyol 4'-7-dimethyl ether (**8b**) could be present in Margraviaceae as eriodictyol. Eriodictyol like naringenin is widely distributed in higher plants and both are parent compounds of several natural flavanones.[73]

All natural flavanones and 3-hydroxyflavanone exist in the thermodynamically favoured conformation with the 2 or 2,3 substituents equatorial and the (2S)-configuration.[75] This was in view of the high value of the coupling constant (J~13 Hz) between protons in the C-2 and C-3 positions originating from diaxial interactions (H_{2a}-H_{3a}), compared to a much smaller coupling constant (J~3 Hz) between the axial-equatorial protons (H_{2a}-H_{3e}) in a stable half-chair conformation (**Figure 2.2.6**).

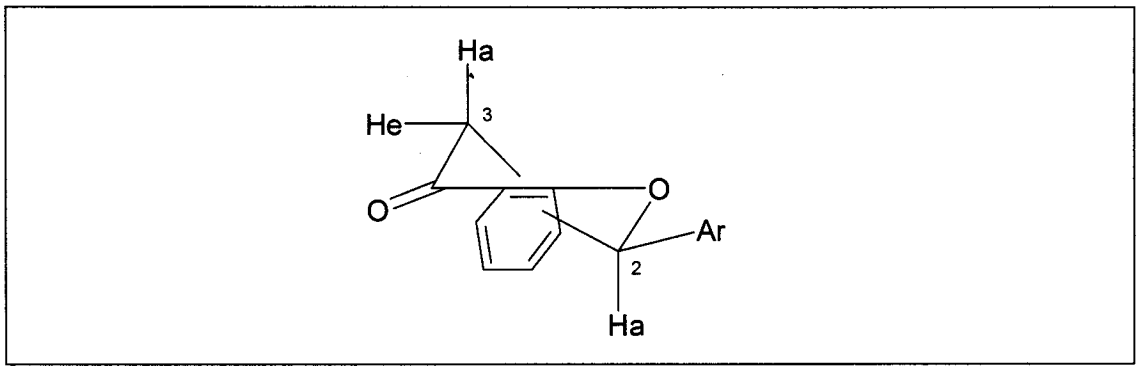


Figure 2.2.6: Half-chair conformation of flavanones which results in a large coupling constant ($J_{2a,3a}$).

Two distinct and diagnostic spectral features of these flavanones (**6**, **7**, **8a**, **8b**, **9**, **10**) that made their identification relatively straightforward. Firstly, in all of their ^{1}H NMR spectra, the appearance of an ABX pattern at δ ~5.31 (dd, J~3.0 Hz, J~13 Hz, H-2_{ax}), at δ ~3.05 (dd,J~13 Hz, J~17 Hz, H-3_{ax}) and δ ~2.80 (dd, J~3.0 Hz, J~17 Hz, H-3_{eq}) together with the hydrogen-bonded (chelated) proton

signal at δ ~12.0 (s, OH at C-5) proved the presence of a flavanone nucleus in all of these compounds.

Secondly, in the EI-MS spectra of these compounds, the presence of fragment ions characteristic of RDA fragmentation of a flavanone skeleton was readily detected (**Scheme 2.2.5b**). Additionally, the significant A and B ring fragment ions classified according to the standard nomenclature system of flavanones was very helpful in the elucidation of their structural detail (**Scheme 2.2.5c**).

A general method for the synthesis of flavanones involves the condensation of an appropriate hydroxyacetophenone with a suitable substituted benzaldehyde in the presence of an alkaline to give the chalcone which is then cyclized to a flavanone.[76,77]

2.2.6.1. Naringenin- 4',7-dimethyl ether

Naringenin- 4',7-dimethylether (**6**), was isolated as a white solid, m.p.115-116°C (lit.[78] 115.5-115.9°C, lit.[79] 114-115°C) in 0.03% yield of dry weight after treatment with diazomethane followed by repeated silica gel column chromatography.

The EI mass spectrum of (**6**) exhibited a $[M]^+$ ion peak at m/z 300 that was in accordance with its molecular formula $C_{17}H_{16}O_5$. This was confirmed by HRMS. The mass fragment peaks at m/z 166 and m/z 134 (base peak) are typical of the RDA cleavage of ring C of a flavanone (**Scheme 2.2.5b**). This also indicated the location of one methoxy group on each of the rings A and B. The peaks at m/z 167, 121 and 193 are consistent with further fragmentation of a flavanone following the RDA process. The MS data of (**6**) also matched well with reported data.[78,79] Its IR spectrum showed strong bands at 3010 and 1637 cm^{-1} due to the H-bonded OH (C-5) chelated to the conjugated carbonyl group (C-4) respectively.

The ^1H NMR spectrum recorded for (**6**) displayed the ABX pattern typical of a flavanone nucleus as a three one-proton double doublets at δ 5.35 (dd,

J=3.1 Hz, J=12.9 Hz, H-2_{ax}), at δ 3.09 (dd, J=12.9 Hz, J=17.2 Hz, H-3_{ax}) and at δ 2.76 (dd, J=3.2 Hz, J=15.7 Hz, H-3_{eq}). The phenolic singlet at δ 12.01 is consistent with the presence of the chelated hydroxyl group at C-5. The two proton *meta*-coupled doublet at δ 6.05 (d, J=2.3 Hz, H-8) and δ 6.02 (d, J=2.3 Hz, H-6) indicated a 5,7 disubstitution pattern in ring A. The two symmetrical doublets at δ 6.93 (dd, J=2.1 Hz, J=6.7 Hz, H-3'/5') and 7.36 (dd, J=1.8 Hz, J=6.7 Hz H-2'/6') suggested the presence of a *p*-substituted B-ring (AA'BB' pattern). The two singlets at δ 3.78 (s, 3H) and at δ 3.81 (s, 3H) confirmed the presence of two methoxy groups at C-7 and C-4' positions respectively. The ^{13}C NMR spectrum of (**6**) was assigned by comparison with known literature data for 5-hydroxy-7,4'-dimethoxy flavanone which were in excellent agreement, (**Table 2.2.6.1**).[79,80]

Table 2.2.6.1. : ^{13}C NMR data of flavanone (**6**). Comparison with literature data reported for naringenin 4', 7-dimethyl ether (δ_C') and (δ_C").[79,80]

C	6	δ_C'	δ_C"
2	79.0	78.9	79.0
3	43.2	43.2	43.2
4	196.0	196.0	196.0
5	164.1	164.1	164.2
6	95.1	95.0	95.1
7	168.0	168.0	168.0
8	94.2	94.2	94.2
9	162.9	162.9	162.9
10	103.1	103.1	103.2
1'	130.3	130.3	130.6
2'	127.7	127.7	127.7
3'	114.2	114.2	114.3
4'	160.0	160.0	160.1
5'	114.2	114.2	114.3
6'	127.7	127.7	127.7
7-OMe	55.7	55.7	55.6
4'-OMe	55.4	55.7	55.3

Flavanone (**6**) was first reported to be naturally occurring in the bud excretions of *Betula ermani* by Wollenweber and Egger in 1971.[73] Lam and Wrang reported that flavanone (**6**) was present in higher amounts in the leaves of *Dahlia tenuicaulis* collected in autumn than in leaves collected in winter or the spring.[78]

2.2.6.2. Naringenin- 7-methyl ether

Naringenin- 7-methyl ether (**7**), or sakuranetin, was isolated from the polar fraction of a diazomethane treated EtOAc extract of Margraviaceae as an impure green solid, so the m.p. was not determined (lit.[81] 151-153°C, lit.[82] 152°C) after repeated silica gel column chromatography. A green pigment appeared to run together with (**7**) on TLC which was difficult to separate. The structure of (**7**) was verified by acetylation with acetic anhydride and pyridine to afford 5,4'-diacetyl-7-methoxyflavanone (**7a**).

The EI mass spectrum of (**7**) displayed a molecular ion, $[M]^+$ at m/z 286 in agreement with its calculated formula $C_{16}H_{14}O_5$. This was established by HRMS. The protonated ion at m/z 167 (base peak) and the ion at m/z 120 are typical of a RDA cleavage (**Scheme 2.2.5b**). These ions confirmed that the methoxy group is attached to ring A at C-7 and the hydroxyl group is attached to ring B at C-4'. Its IR spectrum showed two strong bands at 3387 and 1641 cm^{-1} attributable to the hydroxyl and the chelated carbonyl groups respectively.

The ^1H NMR spectrum of (**7**) was quite similar to that of flavanone (**6**) except for the disappearance of the C-4' methoxy signal at δ 3.81 (s, 3H). One three-proton singlet was observed at δ 3.78, assignable to the methoxy substituent at C-7. The remaining signals were almost superimposable with that of flavanone (**6**).

Acetylation of (**7**) gave its diacetate (**7a**), whose MS revealed a $[M]^+$ at m/z 370 according to its molecular formula $C_{20}H_{18}O_7$, and was confirmed by HRMS. The ^1H NMR signals due to these two additional acetoxy groups appeared as two singlets at δ 2.30 (s, 3H) and 2.37 (s, 3H). This resulted in the disappearance of

the one proton singlet normally observed at δ 12.01 due to the chelated OH group at C-5.

The ^{13}C NMR data for (**7**) was consistent with published data for sakuranetin.[81] These data were also compared with those of its isomer isosakuranetin (**Table 2.2.6.2**). The most significant difference is observed for the resonances of C-7 and C-4'. That is, C-7 resonated at δ 168.0 and 164.9 ppm whereas C-4' resonated at δ 156.2 and 160.0 ppm for sakuranetin and isosakuranetin respectively.

Table 2.2.6.2: ^{13}C NMR data for flavanone (**7**). Comparison with published data for sakuranetin (δ**c'**) and isosakuranetin (δ**c"**).[81]

C	7	δc'	δc"
2	79.0	80.2	78.9
3	43.1	43.2	43.1
4	196.1	196.0	196.1
5	164.1	164.2	164.3
6	95.1	95.1	96.7
7	168.0	168.0	164.9
8	94.2	94.4	95.5
9	162.9	162.9	163.2
10	103.1	103.1	103.3
1'	130.4	130.5	130.3
2'	128.0	128.0	127.7
3'	115.7	115.6	114.2
4'	156.2	156.2	160.0
5'	115.6	115.6	114.2
6'	128.0	128.0	127.7
7-OMe	55.7	55.7	
4'-OMe			55.4

Sakuranetin (**7**) takes its name from the Japanese word sakura, for cherry, and is commonly found in *Prunus* where it was first isolated by Japanese in 1908. Isosakuranetin (**9b**) is also a natural product which was first isolated also

from *Prunus* in 1957.[83] Isosakuranetin has known cytotoxic, fungicide and allelopathic properties.[84]

A very brief treatment of naringenin with CH_2N_2 or alkylation with methyl sulfate and sodium hydrogen carbonate yielded sakuranetin. This is because the C-7 hydroxyl group is the most acidic in polyhydroxyflavanones and the 5-hydroxyl group the least acidic.[85]

2.2.6.3. Isomeric mixture of eriodictyol- 3',7 -dimethyl ether and eriodictyol- 4',7-dimethyl ether

The isomeric mixture of eriodictyol-3',7-dimethyl ether (**8a**) and eriodictyol-4',7-dimethyl ether (**8b**) were isolated after repeated silica gel column chromatography followed by washing with hexane, from the polar fraction of a diazomethane treated EtOAc extract of Margraviaceae leaves. Compounds (**8a**) and (**8b**) were obtained as a pale yellow gum in 0.02% yield of dry weight. The m. p. of this mixture has not been recorded in the literature. However the m. p. of the separate pure compounds are known; for (**8a**), lit.[86] 148-149°C, lit.[81,87] 148-150°C, lit.[88] 149°C, lit.[89] 149-150°C; for compound (**8b**), lit.[81] 165-168°C.

The EI mass spectrum of (**8a**) and (**8b**) displayed a $[M]^+$ at m/z 316 in agreement with its molecular composition $C_{17}H_{16}O_6$; which was confirmed by HRMS. The diagnostic fragment ions m/z 166, protonated ion m/z 167 and m/z 150 are ascribable to the RDA cleavage of ring C which placed one methoxy substituent on ring A and the other methoxy and one hydroxyl substituent on ring B (**Scheme 2.2.5b**). This fragmentation pattern agreed with literature patterns.[87,89]

The ^{1}H NMR spectrum of flavanone (**8a**) was almost superimposable over that of (**8b**). The characteristic signals included the ABX system at δ 5.32 (dd, J=2.8 Hz, J=12.8 Hz, H-2_{ax}) for **8a**, at δ 5.30 (dd, J=12.6, J=3.3 Hz, H-2_{ax}) for **8b**, and at δ 3.06 (dd, J=12.8 Hz, J=17.2 Hz, H-3_{eq}), at δ 2.76 (dd, J=3.2 Hz, J=17.2 Hz, H-3_{ax}) along with the H-bonded proton signal at δ 12.0 (OH at C-5). These

signals proved the presence of a flavanone skeleton. The two meta-coupled aromatic protons at δ 6.05 (d, J=2.4 Hz) and 6.02 ppm (d, J=2.4 Hz) proved the presence of a 5,7-disubstitution pattern on the A ring. In ring B, H-2' appeared as a meta-coupled doublet (J=2.3 Hz) at δ 7.02 for (**8b**). The methoxy singlets were observed at δ 3.79 [7-OMe groups for both (**8a**) and (**8b**)], at δ 3.92 [3'-OMe for (**8a**)] and at δ 3.90 [4'-OMe for (**8b**]. The remaining aromatic signals appeared as a two-proton multiplet at δ 6.83-6.95. These data matched well with those in the literature.[87,89]

The resonances in the ^{13}C NMR spectrum of (**8a**) and (**8b**) were in good agreement with published data for their individual pure compounds (**Table 2.2.6.3**).[81] The most diagnostic carbons were observed for C-2' and C-5'. Thus, C-2' resonated at δ 108.7 and 112.6 ppm for compounds (**8a**) and (**8b**) respectively while C-5' resonated at δ 114.5 and 110.6 ppm for compounds (**8a**) and (**8b**) respectively.

Flavanones (**8a**) and (**8b**) are both natural products. Flavanone (**8a**) was first isolated by Geismann from the bark of *Meliocope sarcococca* in 1958, while (**8a**) was first isolated by Christiansen and Boll from the bark of peach (*Prunus persica*) in 1966.[90]

Table 2.2.6.3 : ^{13}C NMR data of flavanones (**8a**) and (**8b**). Comparison with published data for eriodictyol-3'7-dimethyl ether (δ**c'**) and eriodictyol-4',7-dimethyl ether (δ**c"**).[81]

C	8a	δc'	8b	δc"
2	79.3	79.4	79.0	79.0
3	43.4	43.4	43.2	43.2
4	196.0	196.0	196.0	196.0
5	164.1	164.1	164.1	164.1
6	95.1	95.1	95.1	95.1
7	168.0	168.0	168.0	168.0
8	94.2	94.3	94.2	94.2
9	162.8	162.8	162.8	162.8
10	103.1	103.1	103.1	103.1
1'	130.2	130.2	131.5	131.5
2'	108.7	108.7	112.6	112.6
3'	146.7	146.7	145.9	145.9
4'	146.2	146.2	147.0	147.0
5'	114.5	114.5	110.6	110.6
6'	119.6	119.6	118.2	118.2
7-OMe	55.7	55.7	55.7	55.7
3'-OMe	56.0	56.1		
4'-OMe			56.0	56.0

2.2.6.4. Naringenin

Naringenin (**9**) was isolated as a pale yellow solid in 0.05% yield of dry weight from an acetone extract of Margraviaceae leaves (**Figure 2.3.5e**). However, it was still impure by ^1H NMR after repeated silica gel column chromatography and its m.p. was not determined. The literature melting points are as follows; lit.[83] 248°C, lit.[81] 248-250°C, lit.[91] 250-251°C, lit.[60] 251°C, lit.[92] 252-253°C.

The EI mass spectrum of (**9**) revealed a $[M]^+$ at m/z 272 (also the base peak) which matched its molecular formula, $C_{15}H_{12}O_5$. Diagnostic mass fragments at m/z 153 ($[A+1]^+$), 152 (A_1^+), 120 (B_3+) and 107 (B_4^+), follow the

typical RDA fragmentation of the C-ring and its subsequent fragmentations, (**Schemes 2.2.5b** and **2.2.5c**), and were in conformity with published data.[72,93]

The ^{1}H NMR data of naringenin (**9**) recorded in the experimental section coincided with published data,[81] as well as with an authentic sample recently isolated in our laboratory by Matieu Lalonde from *Prunus serotina* Ehrh (black berry).[94]

Naringenin (**9**) occurs in its free state, as glycosides and ethers in plants like *Acacia*, *Artemisia* L. and the woods of *Prunus*.[81,85] Power and Tutin were the first to study naringenin in 1907 but thought they were dealing with a chalcone. In 1928, Asahina and co-workers established that naringenin was a flavanone. This was supported by work of Geismann (1946), Narasimhachari *et. al.*, (1949), Hasegawa and Shirato (1953) and its synthesis from *p*-hydroxy-cinnamic acid and phloroglucinol by Molho and Chandenson in 1959.[85]

Compound (**9**) is one of the compounds that is responsible for the increase drug uptake by grapefruit juice. It is an inhibitor of the cytochrome P450 enzyme (CYP3A4) that is responsible for first pass metabolism of drugs in small intestine.[94] Its presence in black berry is medically relevant since it is used as a hydroalcoholic tincture for treatment of coughs in naturopathic medicine.

In chemical ecology (woods), (**9**) may act as a synergist for other compounds in the defense of black berry against predation because of its ability to inhibit metabolism. Naringenin derivatives are well documented as bitter principles in grapefruit and have been extensively researched by the citrus industry.[95]

2.2.6.5. 3',5,5',7-Tetrahydroxyflavanone

3',5,5',7-Tetrahydroxyflavanone (**10**) was isolated as a yellow solid from an acetone fraction of Margraviaceae leaves in 0.04% yield of dry weight. Its m.p. was not determined due to lack of material (lit.[76] 186-188°C, lit.[77] 208-209°C). Although flavanone (**10**) was still impure after repeated chromatography, diagnostic features were clearly evident in its ^{1}H NMR and MS spectra.

The EIMS of (**10**) showed intense peaks at m/z 288 ($[M]^+$), 152 (A^+), 153 ($[A+1]^+$), 136 (B_3^+), 123 (B_4^+), 57 and 43 (base peak) typical of a flavanone skeleton of the postulated structure (Schemes **2.2.5b** and **2.2.5c**). The $[M]^+$ at m/z 288 was an accurate match for its molecular formula $C_{15}H_{12}O_6$ which was confirmed by HRMS. The fragmentation pattern also agreed with previously reported MS data for 3',5,5',7-tetrahydroxyflavanone.[77]

The ^1H NMR spectrum of (**10**) exhibited characteristic signals similar to that of (**9**) especially for the protons in rings A and C. The protons in the B ring which now contains two hydroxyl groups give rise to a one proton singlet at δ 7.04 (s, H-4') and a two proton singlet at δ 6.89 (s, H-2'/H-6'). The ^1H NMR data of (**10**) were compatible with literature data.[76,77]

Flavanone (**10**) was first reported in the literature in 1994, where it was wrongly assigned as the structure of the natural product Huazhongilexone isolated from the leaves of the Chinese *Ilex centrochinensis*.[77] However, in 1996, this assignment was corrected by Uffe *et. al.*, who synthesized the racemic compound (**10**).[77] Two fairly low yielded syntheses of (**10**) are known in the literature.[76,77]

Compound (**10**) has recenlty been isolated from a plant, *Pseudotsuga sinensis Dode*.[96]

2.2.7. Chondrillasterol and a Porphyrin type compound

The other important metabolites isolated from Margraviaceae leaf extracts were chondrillaterol (**11**) and a porphyrin type compound (**13**) whose structure was not fully assigned (**Figure 2.2c**).

Chondrillasterol (**11**) was isolated as a white solid, m.p. 164-165°C (lit.[97] 154-157, lit.[98] 163-165°C) both from the leaves (0.09%) and the fruits (0.02%) of Magraviaceae after silica gel column chromatography followed by washing with hexane.

The EIMS spectrum of (**11**) contained a $[M]^+$ peak at m/z 412 coinciding with its molecular formula $C_{29}H_{48}O$; which was further established by HRMS. A broad O-H stretch was observed at 3400 cm^{-1} with no C=O absorption.

The ^1H NMR spectrum of (**11**) exhibited olefinic protons at δ 5.14 (m, 2H, H-22 and H-23) and at 5.02 (dd, J=8.7 Hz, J=14.4 Hz, H-7). A one proton multiplet at δ 3.57 typical of a proton involved in a CH-OH function was assignable to H-3α. The methyl signals were observed at δ 0.53 (s, 3H, H-18), 0.79 (m, H-19, H-26, H-29), 0.84 (d, J=6.3 Hz, H-27) and1.01 (d, J=6.6 Hz, H-21). The methyl groups were assigned by comparison with published data for the acetate of (**11**) and related sterols.[98-100]

The ^{13}C NMR resonances of (**11**) were assigned by comparison with those reported by Junkuszew *et. al.*, for chondrillasterol, (**Table 2.2.7**).[101] The most significant signals were observed at δ 117.4 (C-7), 139.5 (C-8), 138.0 (C-22), 129.4 (C-23) for the olefinic protons and at δ 71.0 for C-3 bearing the hydroxyl group.

Metabolite (**13**) was a dark green solid which was isolated in 0.1% yield from a $CHCl_3$ extract of Margraviaceae leaves after repeated column chromatography, followed by recrystallisation from hexane (*see* **Section 2.3.5**).

The structure of (**13**) was partially determined to be a porphyrin derivative after comparison of its ^1H NMR spectrum with similar structures previously described in the literature.[102-105] Diagnostic signals were observed for the *meso* protons (methine) at δ 9.41 (double doublet-like) and for the AMX system at δ 7.96 (dd, J=17.8 Hz, J=11.5 Hz, H_X), at δ 6.27 (d, J=17.8 Hz, H_M) and at δ 6.15 (d, J=11.6 Hz, H_A) [*see* **Figure 2.2.7a** for the ^1H NMR of (**13**)]. The ^1H NMR spectra of other related porphyrin structures are also given in **Figure 2.2.7b** for comparison.[102]

Table 2.2.7: ^{13}C NMR data for compound (**11**). Comparison with published data for chondrillasterol (δ**c'**).[101]

C	11	δc'
1	37.1	37.1
2	31.4	31.4
3	70.9	71.0
4	37.9	37.9
5	40.2	40.2
6	29.6	29.6
7	117.4	117.4
8	139.5	139.5
9	49.9	49.4
10	34.1	34.1
11	21.5	21.5
12	38.4	38.4
13	43.2	43.2
14	55.1	55.0
15	22.9	22.9
16	28.4	28.4
17	55.8	55.8
18	12.9	12.9
19	12.0	12.1
20	40.7	40.7
21	21.2	21.2
22	138.1	138.0
23	129.3	129.4
24	51.2	51.2
25	37.1	37.1
26	21.3	21.3
27	18.9	18.9
28	25.3	25.3
29	12.1	12.1

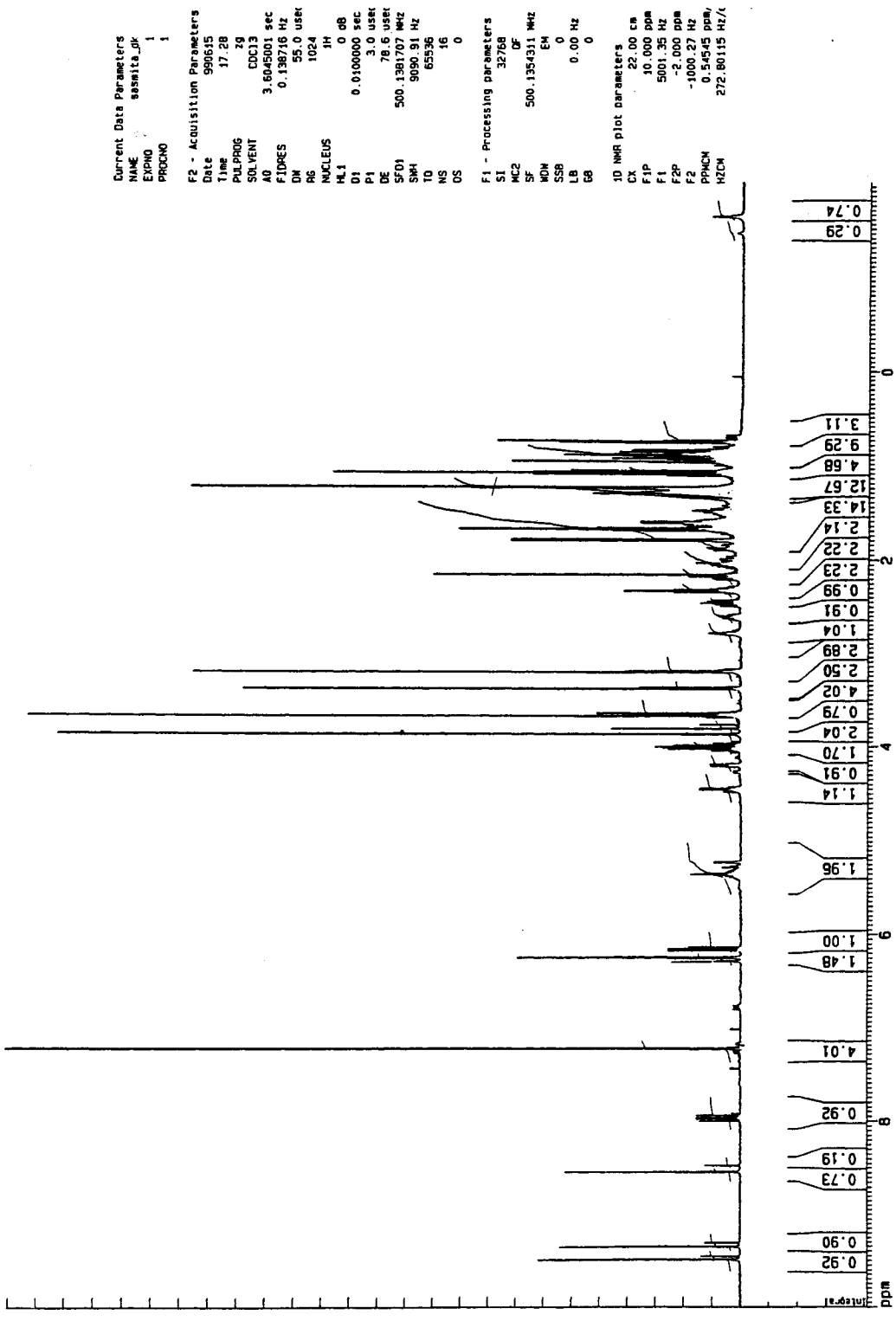


Figure 2.2.7a: ^{1}H NMR spectrum of a porphyrin type compound (**13**) isolated from Margraviaceae plant extracts.

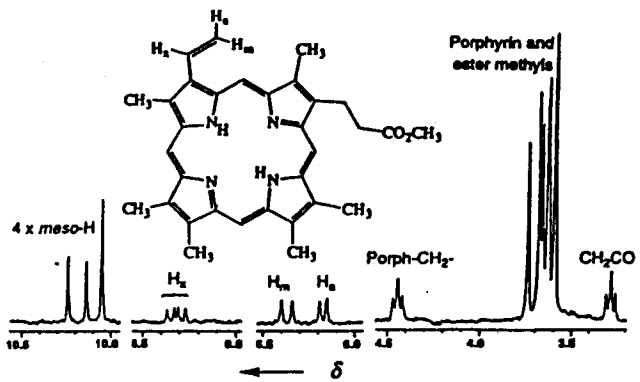
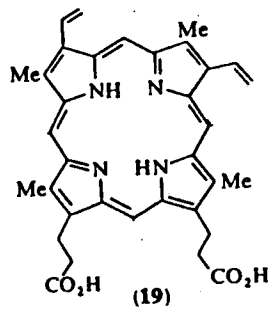


Figure 4. 300 MHz proton NMR spectrum of the esterified monovinylporphyrin product from incubations of **3b** with CRH.



(19)

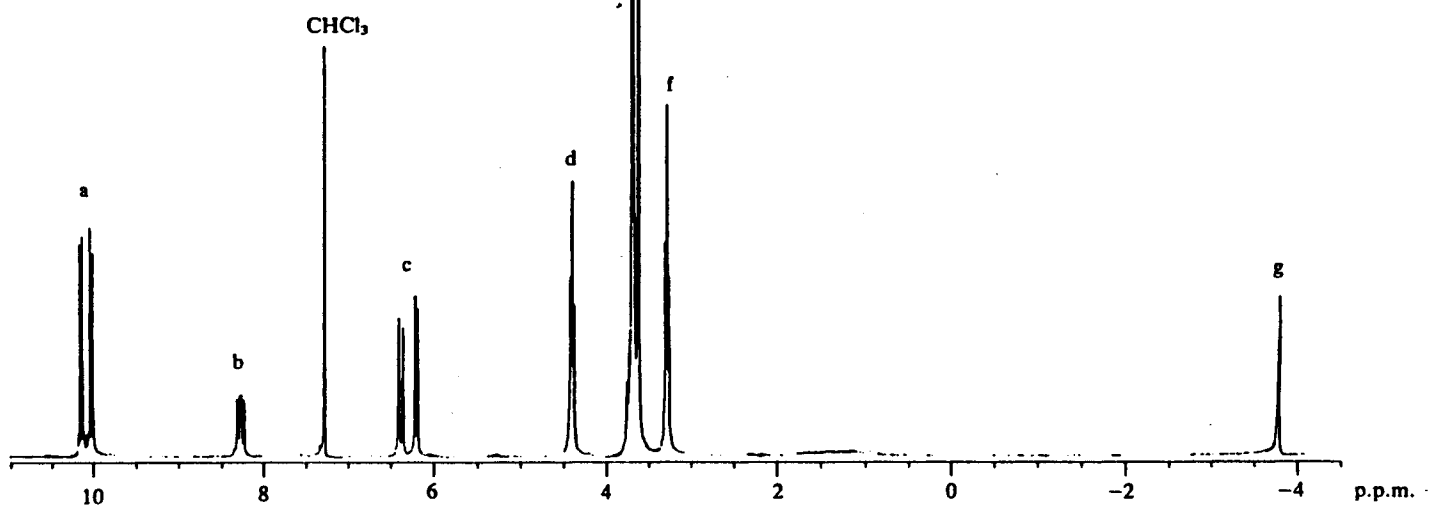


Figure 1 360 MHz Proton NMR spectrum, in $CDCl_3$, of protoporphyrin-IX (19) dimethyl ester. Assignments: a, methine protons; b, vinyl CH; c, vinyl CH_2; d, propionate CH_2CH_2CO; e, nuclear methyls and methyl esters; f, propionate CH_2CH_2CO; g, NH

Figure 2.2.7b: ^{1}H NMR of porphyrin type structures from the literature.[102]

2.3. Experimental : Part 1

GENERAL: Melting points were determined using a Thomas Hoover Capillary melting point apparatus and are uncorrected. The IR spectra were recorded in chloroform solution or neat employing a Bomem-Michelson MB-100 FT/IR spectrophotometer. Mass spectra were obtained by means of a VG 7070E spectrometer. ^{1}H and ^{13}C spectra were recorded on a Bruker AMX-500, Bruker AMX-300 and Varian Gemini XL-200 spectrometers with shifts reported in ppm. The multiplicities of the NMR signals were reported using the following abbreviations, singly or in combinations: singlets (s), broad singlet (br s), doublet (d), triplet (t), quartet (q) and multiplet (m).

Most of the solvents used in the reactions were distilled prior to use. THF was dried by distillation from a benzophenone ketyl. Dichloromethane was dried by distillation from calcium hydride. DMF and DMSO were distilled and stored over activated molecular sieves. Thin layer chromatography (TLC) were carried out using Kieselgel 60 F254 precoated 0.25 mm plates. Visualization of the spots was facilitated by UV irradiation followed by charring of a TLC which has been dipped in a molybdate solution. The molybdate solution was prepared by dissolving ammonium molybdate (2.5 g) and cerric sulphate hydrate (1.0 g) in a solution of sulphuric acid (10 mL) in distilled water (90 mL). Silica gel 270-400 Mesh was used for flash chromatography.

2.3.1. Plant Materials

A supply of Margraviaceae leaves, *Souroubea gilgi* [voucher No. UO19108] and *Souroubea sympetala* [voucher No. UO19151] were collected at Tortaguero and Horquetas, Eastern Costa Rica in May 1997, April 1999, February 2000 and May 2002 by Professors Arnason and Durst. The leaves, fruit, twigs etc., were immediately immersed in 95% ethanol (EtOH) in 1L Nalgene bottles in the field for preservation, during its transportation to Ottawa

and subsequent storage in a refrigerator. During May 2002, fresh leaves were also dried at 50°C and placed in well sealed platic bags before transport to Ottawa.

2.3.2. Preparation of EtOH extracts from Magraviaceae leaves

The leaves of *S. gilgi* (collected May 1997) and the 95% EtOH were blended in a conventional kitchen blender and additional 95% EtOH was added when required to facilitate the blending process. The blended material was left to stand for 1 day at room temperature (22°C) and then filtered. The insoluble dried fibre weighed 104.3 g. The filtrate was concentrated under diminished pressure and freeze dried for 2 days to afford a dark green gum of the EtOH extract (32.0 g). The crude EtOH extract was submitted for *in vivo* bioassays with the appropriate animal (rats and mice) test model systems, described in **Sections 2.1.3** and **2.6**. The crude EtOH plant extract was shown to be anxiolytic.

2.3.3. Preparation of test samples

Test samples (50 vials) were also prepared in order to represent the total crude plant material by mixing 50 mg of the brown solid residue from the aqueous fraction (f2) with 10 mg of the green EtOAc fraction (f1) already dissolved in 0.5 mL EtOH. Another set of test samples (100 vials) were also prepared to represent the active EtOAc fraction by dissolving 20 mg of the EtOAc extract in 1 mL of EtOH.

2.3.4. Bioassay-guided fractionation of Magraviaceae extracts

The bioassay-guided fractionation by solvent extraction of the pshychotropic plant material by solvent extraction is summarized in the flow diagram (**Figure 2.1.2**). The crude EtOH extract (15.0 g) prepared as in **Section 2.3.2** was extracted twice with ethyl acetate (EtOAc) by rapidly stirring with

EtOAc (2 x 50 mL) at room temperature for 4h before filtering. The remaining solid was stirred overnight (15h) with acetone (100 mL) and the mixture was again filtered. The combined organic extracts were concentrated *in vacuo* to afford 1.0 g of green gum, labelled the EtOAc extract or fraction 1 (f1). The remaining residue was a brown solid (13.0 g). 2.0 g of this fraction was removed and labelled fraction 2 (f2). Another 5.0 g of it was re-extracted by refluxing with hot EtOH (40 mL) to afford 2.4 g of a light brown EtOH soluble extract, fraction 3 (f3). The light brown EtOH insoluble material weighed 2.5 g; it was designated fraction 4, (f4).

Fractions 1-4 were submitted for *in vivo* bioassays using industry standardized tests for anxiety described in **Sections 2.1.3** and **2.6**. The active fraction was found to be fraction 1 (f1).

2.3.5. Isolation of plant metabolites from Margraviaceae

Several Margraviaceae plant extracts were prepared and purified in order to permit the isolation of plant metabolites and with the ultimate goal of identifying the biologically active principle(s).

In the first extraction experiment, *S.gilgi* leaves (collected February 2000) were extracted as in **Section 2.3.2** to afford an EtOH extract (40.1 g). The residue of dry leaf fibre weighed 178.2 g. The EtOH extract which was stirred rapidly with EtOAc (3 x 150 mL) at room temperature for 4h then filtered. This procedure was repeated twice; once for 4h and then once overnight (15h). The combined EtOAc extracts were concentrated under reduced pressure to furnish a dark green EtOAc extract (8.0 g). Part of the this EtOAc extract was used to prepare test samples for bioassays (**Section 2.3.3**), and the remaining EtOAc material (5.7 g) was chromatographed over silica gel eluting with hexane-EtOAc mixture of increasing polarity to afford two major fractions. **Note**: Fractions are reported in order of increasing polarity.

Fraction 1 (1.3 g), was a white solid of an inseparable mixture of α- amyrin (**1a**) and β-amyrin (**1b**). Fraction 2 (560 mg) was re-chromatographed over silica

gel (hexane-EtOAc solvent gradient) yielding two subfractions. Subfraction 1 (40 mg) after it was washed with hexane (5 x 1 mL) yielded chondrillasterol (**11**) as a white solid (26 mg). Subfraction 2 (60 mg) was again chromatographed on silica gel eluting with hexane-EtOAc solvent gradient to afford a pale yellow solid (35 mg) which after recrystallisation from hexane/CH_2Cl_2 furnished pure betulinic acid (**3**) as a white solid (25 mg), **Figure 2.3.5a**.

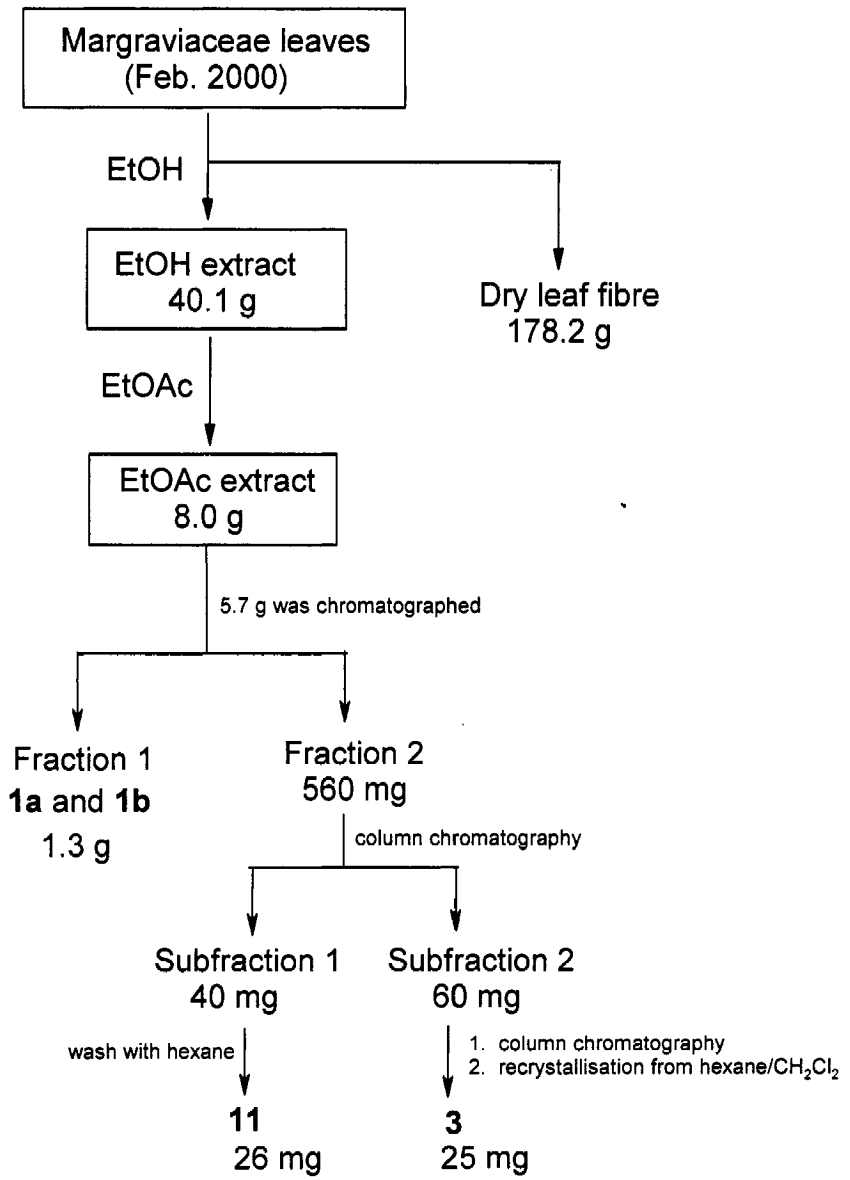


Figure 2.3.5a: First extraction experiment with Magraviaceae leaves.

In a second extraction experiment, the old leaves of *S.gilgi* plant (collected May 2002) was extracted as described in **Section 2.3.2** yielding an EtOH extract (29.7 g) and 99.0 g of dry fibre. The EtOH was stirred with EtOAc (3 x 500 mL) at room temperature before filtering (twice for 4h and once overnight). The combined EtOAc extracts were concentrated *in vacuo* to yield a dark green EtOAc extract (5.4 g) which was subjected to silica gel chromatography (hexane EtOAc gradient system) to afford 5 main fractions (F1-F5), (**Figure 2.3.5b**).

Fraction 1 (710 mg), the non-polar materials was a dark orange liquid which was very difficult to purify. By ^{1}H NMR it appeared to be a mixture of fatty acids and triglycrides and was not investigated further. Fraction 2 (800 mg) was re-purified by Si gel chromatography eluting with hexane-EtOAc gradient solvent system followed by recrystallisation from EtOH to afford 620 mg of an inseparable mixtures of amyrins (**1a**) and (**1b**). Fraction 3 (100 mg) was re-chromatographed over silica gel eluting with hexane-EtOAc gradient to afford a pale yellow solid which after washing with hexane yielded taraxeryl *trans*-p-hydroxycinnamate (**4**) as a white solid (62 mg).

Fraction 4 (900 mg) was treated with CH_2N_2 followed by silica gel column chromatography eluting with hexane-EtOAc gradient to afford 5 main subfractions: subfraction 1 was a white solid (180 mg) of pure methyl linolenic acid (**13**); subfraction 2 was a fairly pure white solid (30 mg) of naringenin-4',7-dimethyl ether (**6**); subfraction 3 was an impure white solid (10 mg) of betulinic acid methyl ester (**3a**); subfraction 4 was a pure white solid (24 mg) of methyl ursolate (**5a**), and subfraction 5 was an impure green solid (60 mg) of naringenin-7-methyl ether (**7**). Further attempts to purifiy subfraction 5 by washing with hexane (5 x 1 mL) and by silica gel column chromatography. The structure of (**7**) was verified by acetylation of 20 mg of (**7**) with acetic anhydride and pyridine in $CHCl_3$ to afford 5,4'-diacetyl-4-methoxyflavanone (**7a**).

Fraction 5 (350 mg) was also treated with CH_2N_2 and chromatographed over silica gel eluting with hexane-EtOAc mixtures of increasing polarity to obtain 2 main subfractions. Subfraction 1 was washed with hexane to obtain an inseparable mixture of eriodictyol-3',7-dimethyl ether (**8a**) and eriodictyol-4',7-

dimethyl ether (**8b**) as a yellow gum (22 mg). Subfraction 2 was re-purified using silica gel chromatography (hexane-EtOAc gradient) furnishing an inseparable isomeric mixture of methyl-2α-hydroxyursolate (**2a**) methyl maslinate (**2b**) as a white solid (33 mg), which was further recrystallised from hexane/CH_2Cl_2.

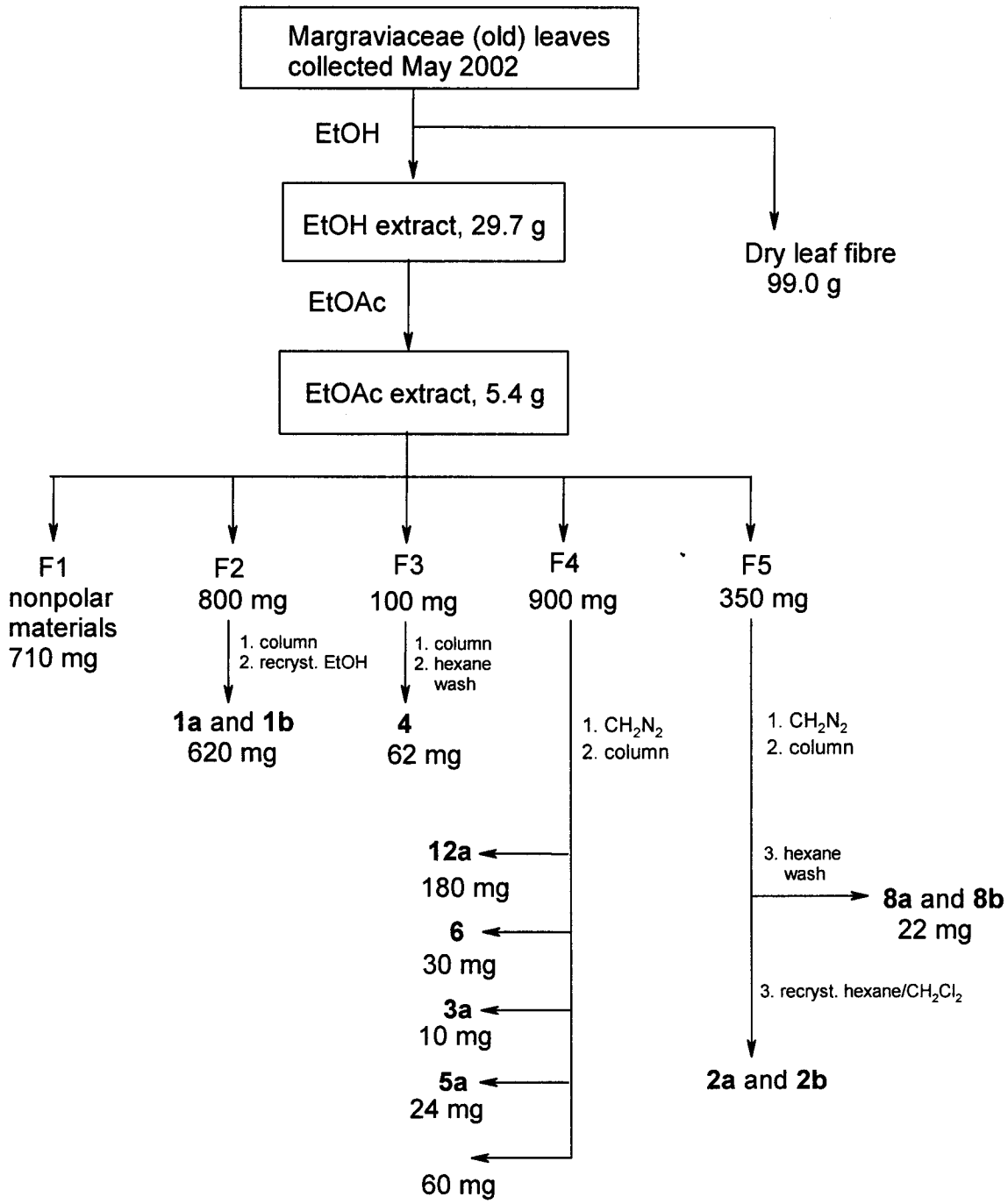


Figure 2.3.5b: Second extraction experiment with Magraviaceae leaves

In the third experiment, dried ground leaves (150 g) of *S.gilgi* (collected May 2002) were soaked in 95% EtOH (1.8 L) at room temperature overnight, before the mixture was filtered and concentrated *in vacuo* to afford an EtOH extract (9.00 g) and the dried leaf fibre (137.7 g).

The crude EtOH extract (9.00 g) was subjected to silica gel column chromatography with gradient elution (hexane-EtOAc) to furnish 4 main fractions (F1-F4).

Fraction 1 (970 mg) was a dark orange liquid which was again difficult to purify. By ^1H NMR it also appeared to be a mixture of fatty acids and triglycerides as observed previously. Fraction 2 (1.5 g) was an inseparable mixture of amyrins, compounds (**1a**) and (**1b**).

Fraction 3 (620 mg) was re-chromatographed to yield after re-crystallization from MeOH-$CHCl_3$ 100 mg of taraxeryl *trans*-p-hydroxycinnamate (**4**). In order to verify the structure of (**4**), it was reduced with $LiAlH_4$ yielding taraxerol or alnulin (**4a**) and 4-hydroxyphenyl-propanol (**4c**), (**Scheme 2.2.4a**).

Fraction 4 (1.7 g) was treated with CH_2N_2, rechromatographed on a silica gel column eluting with a gradient of hexane-EtOAc mixtures to furnish 130 mg of methyl linolenate (**12a**) and 15 mg of betulinic acid methyl ester (**3a**), (**Figure 2.3.5c**).

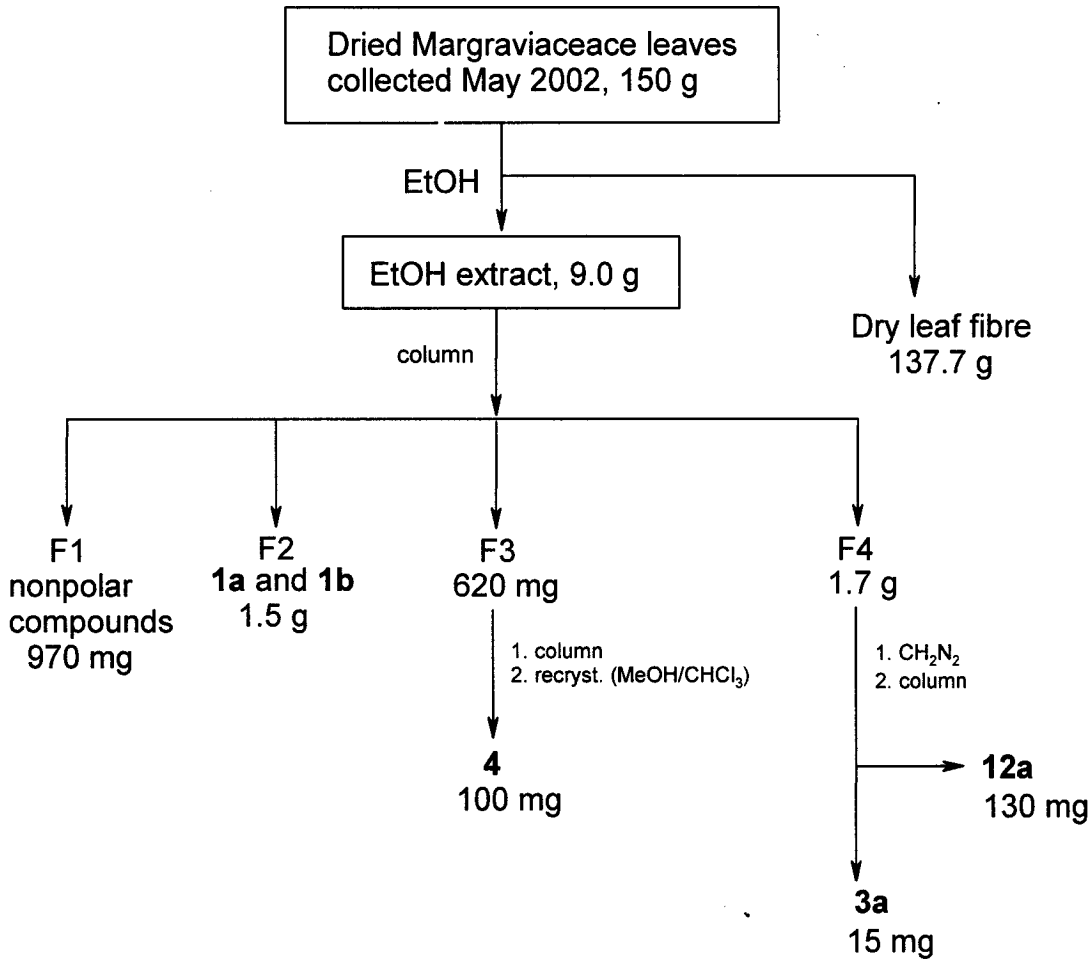


Figure 2.3.5c: Third extraction experiment with Margraviaceaea leaves

In a fourth experiment, *S.gilgi* leaves (normal) were extracted as in **Section 2.3.2**. yielding an EtOH extract (24.9 g) and the dry leaf fibre (77.8 g). Part of the EtOH extract (14.9 g) was extracted thrice by stirring with EtOAc (3 x 500 mL) at room temperature, twice for 4h and once overnight, filtering after each stirring. The combined EtOAc extracts were concentrated *in vacuo* affording an EtOAc extract (2.5 g) which was subjected to silica gel column chromatography eluting with hexane-EtOAc gradient to give 4 main fractions (F1-F4).

Fraction 1, the non-polar fraction was a dark orange liquid (400 mg) which was hard to purify as observed before. However, after repeated silica gel column chromatography using hexane-EtOAc gradient as eluant, ethyl linolenate (**12b**) was isolated as a pale yellow liquid (20 mg). By ^{1}H NMR, this fraction consists

mainly of a mixture of fatty acids and triglycerides similar to those observed in the previous extractions.

Fraction 2 (560 mg) was re-chromatographed over silica gel eluting with hexane-EtOAc gradient to afford plasticiser as a white solid (482.2 mg). Fraction 3 was a white solid (80 mg) consisting mainly of compound (**4**) by ^{1}H NMR and was not purified further. Fraction 4 was a white solid (120 mg) that was identified to be linolenic acid (**12**), **Figure 2.3.5d**.

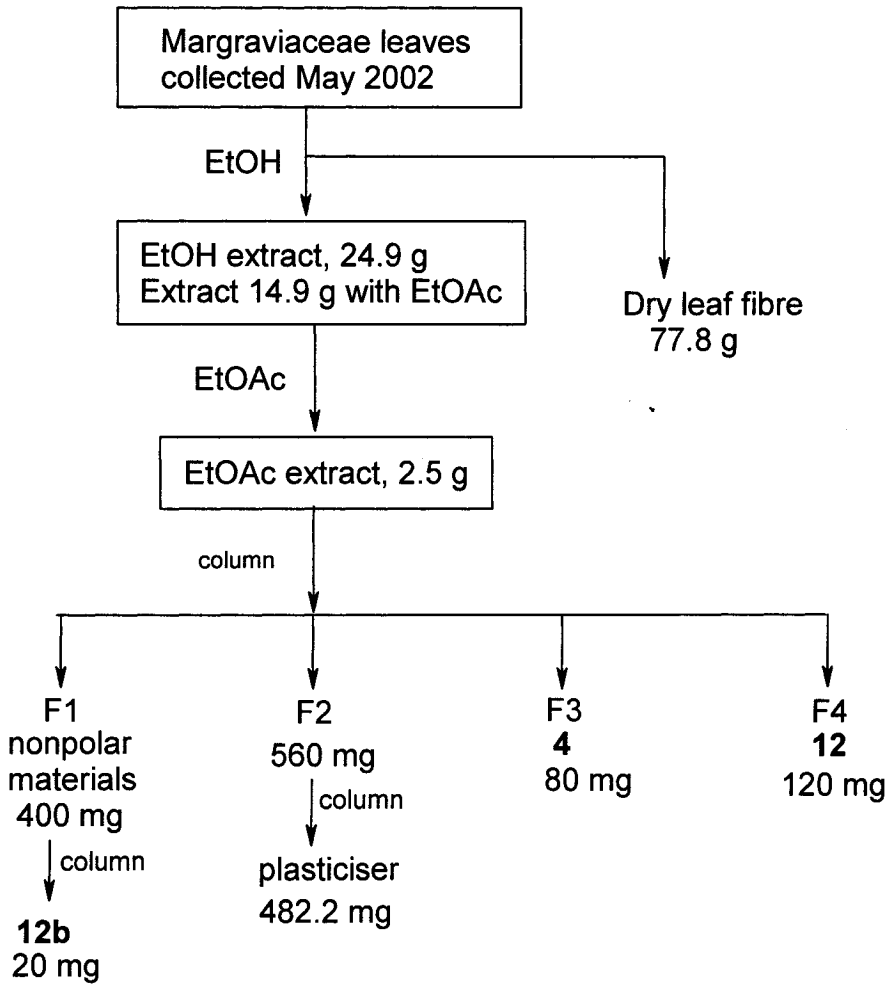


Figure 2.3.5d: Fourth extraction experiment with Magraviaceae leaves.

In a fifth extraction, *S. sympetala* leaves (collected April 1999) were extracted as in **Section 2.3.2** to afford an EtOH extract (71.3 g) and dry fibre (157.6). Part of this EtOH extract (12.7 g) was extracted successively with hexane, chloroform ($CHCl_3$), acetone and methanol (MeOH) by stirring with each solvent twice (2 x 50 mL) for 4h at room temperature before it was filtered and concentrated *in vacuo* affording a hexane extract (1.1 g), $CHCl_3$ extract (130 mg), acetone extract (360 mg) and a MeOH extract (8.6 g).

The chloroform extract was chromatographed over silica gel eluting with hexane EtOAc gradient to obtain a porphyrin type compound (**13**) as a dark green solid (30 mg) and whose structure was not fully assigned. The acetone extract was also subjected to silica gel column chromatography using hexane-EtOAc gradient as eluant to afford 15 mg of naringenin (**9**) and 10 mg of 3',5,5'7-tetrahydroxyflavanone (**10**) both as pale yellow solids. Both compounds (**9**) and (**10**) were not 100% pure by ^1H NMR but were not purified further due to lack of material (**Figure 2.3.5e**).

The ripe fruits of *S.gilgi* (collected April 1999) were processed in a similar way to the *S. gilgi* leaves (**Section 2.3.2**) in a sixth extraction to afford an EtOH extract (8.6 g) and the dried fruit fibre (21.2 g). The EtOH extract (8.6 g) was extracted thrice by stirring with EtOAc (3 x 50 mL) at room temperature twice for 4h and then once overnight, filtering each time. The combined EtOAc extracts were evaporated to dryness *in vacuo* yielding an EtOAc extract (370 mg) which was purified by silica gel chromatography using hexane-EtOAc gradient as eluants furnishing 20 mg of betulinic acid (**3**) and 22 mg of chondrillasterol (**11**) after washing with hexane (**Figure 2.3.5f**).

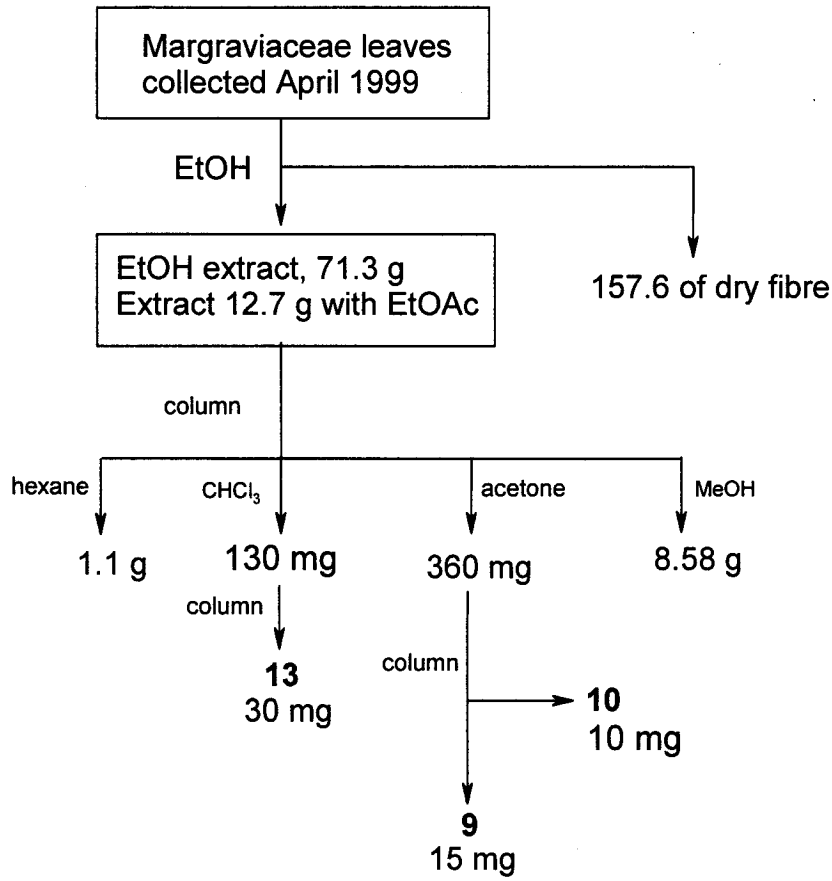


Figure 2.3.5e : Fifth extraction experiment with Magraviaceae leaves.

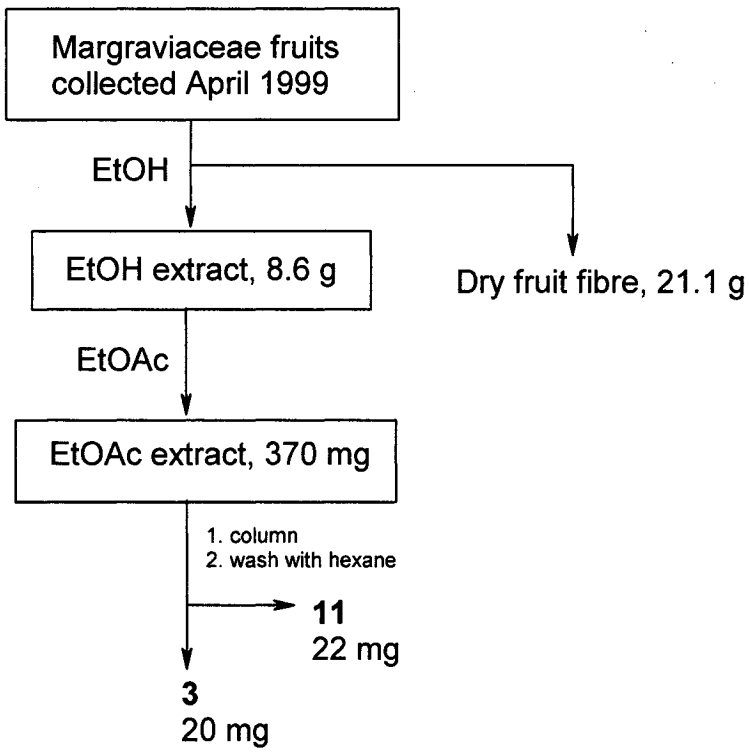
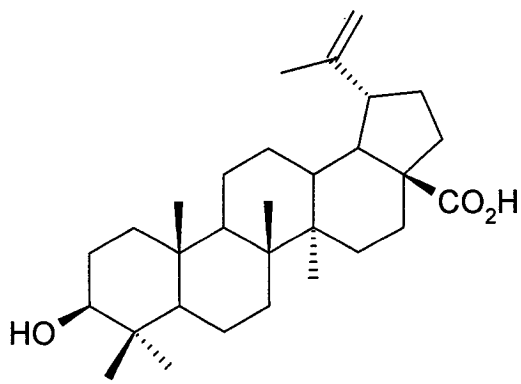


Figure 2.3.5f : Sixth extraction experiment with Magraviaceae fruits.

2.3.6. Pentacyclic Triterpenoids

2.3.6.1. Betulinic acid

Other names: 3β-Hydroxy-lup-20(29)-en-28-oic acid, betulic acid.



3

m.p. 295-297°C (lit.[16] 275-278°C, lit.[17] 283°C, lit.[18] 290-292°C, lit.[19] 290-293°C, lit.[20] 297°C, lit.[21] 311-313°C, lit.[22] 315-318°C, lit.[23] 316-318°C).

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 4.72 (s, H-29), 4.59 (s, H-29'), 3.17 (dd, J=11.4 Hz, J=4.7 Hz, 1H, H-3α), 2.98 (ddd, J=11.4 Hz, J=5.4 Hz, H-19), 1.67 (s, 3H, H-30), 0.96 (s, 3H, H-27), 0.95 (s, 3H, H-26), 0.92 (s, 3H, H-23), 0.81 (s, 3H, H-25), 0.74 (s, 3H, H-24).

^{13}C NMR (500 MHz, $CDCl_3$): δ (ppm) 179.9 (C=O), 150.4 (C-20), 109.7 (C-29), 79.0 (C-3), 56.3, 55.4, 50.6, 49.3, 46.9, 42.5, 40.7, 38.9, 38.8, 38.4, 37.2, 37.0, 34.4, 32.2, 30.6, 29.7, 27.9, 27.4, 25.5, 20.9, 19.4, 18.3, 16.1, 16.0, 15.3, 14.7.

IR υ_{max} ($CHCl_3$, cm^{-1}): 3425 (OH), 2940, 2870, 1687 (C=O), 1645,1453, 1372, 1232, 1191,1029, 999, 882.

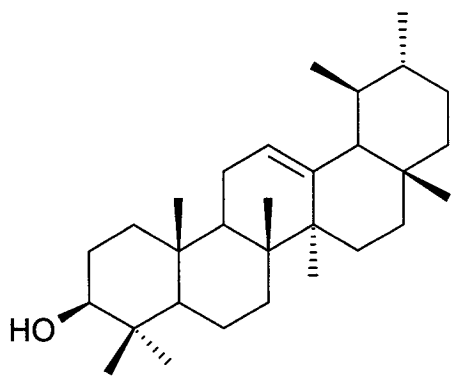
MS (EI) *m/z* (rel. int.): 456 $[M]^+$ (26), 438 $[M-H_2O]^+$ (13), 411 $[M-CO_2H]^+$ (7), 248 (27), 220 (20), 207 [fragment (**c**)]$^+$ (57), 203 (43), 189 $[207-H_2O]^+$ (100), 135 (51), 121 (42), 107 (38), 95 (50), 81 (52), 69 (64), 57 (37), 55 (56), 43 (78), 41 (48).

HRMS: *Calculated* for $C_{30}H_{48}O$, 456.36036, *found* 456.35902.

2.3.6.2. Isomeric mixture of α- and β-amyrins

α-Amyrin (1a)

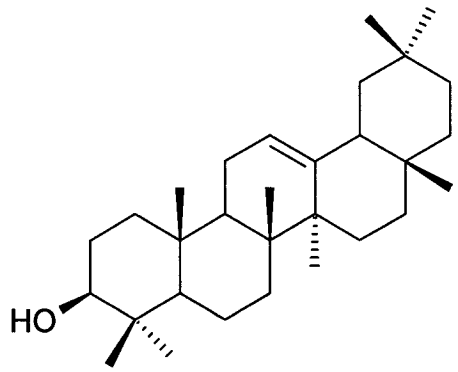
Other names: 3β-Hydroxyurs-12-ene, urs-12-en-3β-ol, viminalol, α-amirin, α-amyrine.



1a

β-Amyrin (1b)

Other names: 3β-Hydroxyolean-12-ene, olean-12-en-3β-ol, β-amirin, β-amyrine, β-amyrenol



1b

m.p. 179-181°C (lit.[28] 175°C)

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 5.16 (t, J=3.7 Hz, 1H, H-12β), 5.10 (t, J=3.5 Hz, 1H, H-12α), 3.20 (dd, J=10.3 Hz, J=5.7 Hz, 1H, H-3α), 1.23 (s, 3H, H-27α), 1.11 (s, 3H, H-27β), 1.05 (s, 3H, H-25β), 0.98 (br s, 9H), 0.96 (d, J=6.4 Hz, 3H, H-30α), 0.93, (d, J=6.0 Hz, 3H, H-29α), 0.92 (s, 3H, H-29β), 0.85 (s, 6H), 0.81 (s, 3H, H-26β), 0.77 (br s, 12H).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 145.2 (C-13 for **1b**), 139.6 (C-13 for **1a**), 124.4 (C-12 for **1a**), 121.7 (C-12 for **1b**), 79.0 (C-3), 59.0, 55.1, 47.7, 47.6, 47.2, 46.8, 42.0, 41.7, 41.5, 39.7, 39.6, 39.5, 38.8, 38.5, 37.1, 36.9, 36.8, 34.7, 33.7, 33.3, 32.9, 32.6, 32.5, 31.2, 31.1, 28.7, 28.4, 28.1, 27.2, 26.6, 26.1, 25.9, 23.7, 23.5, 23.3, 23.2, 21.4, 18.3, 17.5, 16.8, 16.7, 15.7, 15.6, 15.5.

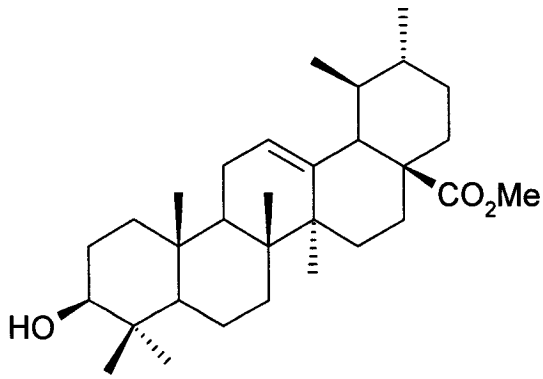
IR υ_{max} ($CHCl_3$, cm^{-1}): 3305 (OH), 2967, 2946, 2922, 2870, 2855, 1655, 1463, 1389, 1307, 1246, 1190, 1035, 996,, 925, 827.

MS (EI) *m/z* (rel. int.): 426 $[M]^+$ (7), 408 $[M-H_2O]^+$ (1), 219 (44), 218 [retro Diels Alder (RDA) fragment (**a**)]$^+$ (100), 207 [fragment (**c**)]$^+$ (26), 203 [218 - CH_3]$^+$ (54), 189 [207 - H_2O] (34), 135 (14), 109 (24).

HRMS: *Calculated* for $C_{30}H_{50}O$, 426.38617, *found* 426.38399.

2.3.6.3. Methyl Ursolate

Other names: Ursolic ester methyl ester, 3β-hydroxy-urs-12-en-28-oic acid methyl ester



5a

m.p. 111-113°C (lit.[34] 111-114°C, lit.[35] 113-114°C, lit.[27] 115-116°C, lit.[36] 166-168°C, lit.[37] 170-172°C)

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 5.22 (t, J=3.6 Hz, 1H, H-12), 3.58 (s, 3H, OMe), 3.19 (m, 1H, H-3α), 2.20 (d, J=11.4 Hz, H-18), 1.05 (s, 3H, H-27), 0.96 (s, 3H, H-23), 0.91 (d, J=6.4 Hz, 3H, H-30), 0.89 (s, 3H, H-25), 0.83 (d, J=6.4 Hz, 3H, H-29), 0.75 (s, 3H, H-24), 0.72 (s, 3H, H-26).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 178.1 (C=O), 138.1 (C-13), 125.5 (C-12), 79.0 (C-3), 55.2, 52.8, 51.5, 48.0, 47.5, 42.0, 39.4, 39.0, 38.8, 38.7, 38.6, 36.9, 36.6, 32.9, 30.6, 28.1, 28.0, 27.2, 24.2, 23.6, 23.3, 21.2, 18.3, 17.0, 16.9, 15.6, 15.4

IR υ_{max} ($CHCl_3$, cm^{-1}): 3505 (OH), 2928, 2871, 2361, 2253, 1725 (C=O), 1514, 1457, 1378, 1307, 1233, 1168, 1113, 1093, 1031, 997, 950, 913, 733, 662.

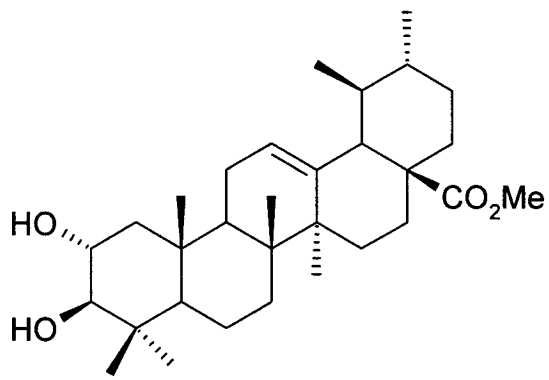
MS (EI) *m/z* (rel. int.): 470 $[M]^+$ (3), 452 $[M\text{-}H_2O]^+$ (3), 262 [RDA fragment (**a**)]$^+$ (96), 207 [fragment (**c**)] $^+$ (22), 203 $[262\text{-}CO_2CH_3]^+$ (100), 189 $[207\text{-}H_2O]^+$ or $[203\text{-}C_5H_{10}]^+$ (26), 133 (49), 119 (20), 69 (15), 55 (18), 41 (17).

HRMS: *Calculated* for $C_{31}H_{50}O_3$, 470.37599, *found* 470.37508

2.3.6.4. Isomeric mixture of methyl 2α-hydroxy-ursolate and methyl maslinate.

Methyl 2α-hydroxy-ursolate (2a)

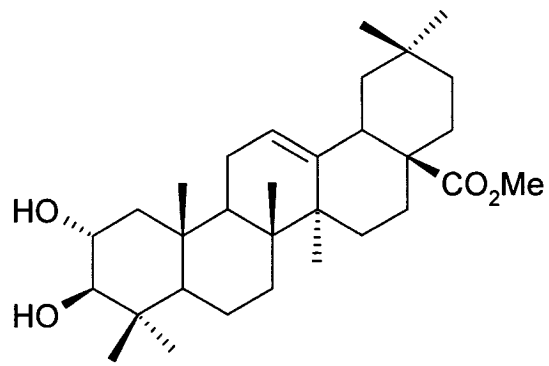
Other names: Ursolic acid methyl ester, 2α-hydroxyursolic acid methyl ester, 2α, 3β-dihydroxy-urs-12-en-28-oic acid methyl ester.



2a

Methyl maslinate (2b)

Other names : Maslinic acid methyl ester, 2α-hydroxyoleanolic acid methyl ester, 2α, 3β-dihydroxy-olean-12-en-28-oic acid methyl ester.



2b

m.p. 215-218°C

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 5.26 (t, J=3.5 Hz, 1H, H-12, olean-), 5.22 (t, J=3.5 Hz, 1H, H-12urs-), 3.66 (m, 1H, H-2β), 3.60 (s, 3H, OMe, olean-), 3.58 (s, 3H, OMe, urs-), 2.97, (dd, J=9.0 Hz, J=2.1 Hz, H-3α), 2.84 (dd, J=13.6 Hz, J=4.0 Hz H-18, olean-), 1.10 (s, 3H), 1.05 (s, 3H), 1.00 (s, 6H), 0.96 (s, 3H), 0.95 (s, 3H), 0.91 (d, J=6.7 Hz, 3H), 0.87 (s, 3H), 0.86 (s, 3H), 0.82 (d, J=6.7 Hz, 3H), 0.80 (s, 6H), 0.71 (s, 3H), 0.69 (s, 3H).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 178.3 (C=O), 178.1 (C=O), 143.8 (C-13 for **2b**), 138.2 (C-13 for **2a**), 125.3 (C-12 for **2a**), 122.1 (C-12 for **2b**), 83.9 (C-3), 68.9 (C-2), 55.2, 52.7, 51.5, 48.0, 47.5, 47.4, 46.6, 46.5, 46.3, 45.8, 42.0, 41.6, 41.2, 39.5, 39.3, 39.1, 39.0, 38.8, 38.2, 36.6, 33.8, 33.1, 32.8, 32.5, 32.3, 30.7, 28.6, 27.9, 27.6, 25.9, 24.1, 23.6, 23.4, 23.0, 21.2, 18.3, 17.0, 16.9, 16.7, 16.5.

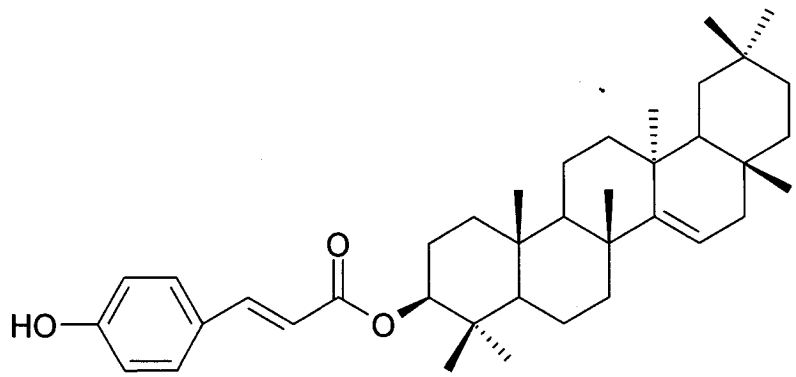
IR υ_{max} ($CHCl_3$, cm^{-1}): 3424 (OH), 2928, 2860, 1719 (C=O), 1655, 1638, 1508, 1459, 1378, 1264, 1165, 1049, 1033, 998, 921, 863, 825, 757, 664.

MS (EI) *m/z* (rel. int.): 486 $[M]^+$ (1), 468 $[M-H_2O]^+$ (1), 262 [RDA fragment (**a**)]$^+$ (68), 249 (11), 233 (3), 223 [fragment (**c**)]$^+$ (5), 204 (15), 203 [262 $-CO_2Me]^+$ (100), 202 (19), 189 (22), 133 (32).

HRMS: *Calculated* for $C_{31}H_{50}O_4$, 486.37091, *found* 486.36963

2.3.6.5. Taraxeryl *trans*-p-hydroxycinnamate

Other names : Careaborin, D-friedoolean-14-en-3-ol, 27-Norolean-14-en-3-ol, (2E)-3-(4-hydroxyphenyl)-2-propenoate.



4

m.p. 273-275°C (lit.[54] 257-258°C)

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 7.58 (d, J=16.0 Hz, 1H, H-3'), 7.49 (d, J=8.6 Hz, 2H, H-5'/9'), 6.82 (d, J=8.7 Hz, 2H, H-6'/8'), 6.28 (d, J=16.0 Hz, 1H, H-2'), 5.51 (dd, J=6.8 Hz, J=3.1 Hz, 1H, H-15), 4.57 (t, J=8.0 Hz, 1H, H-3α), 1.08 (s, 3H, H-26), 0.96 (s, 3H, H-27), 0.93 (s, 6H, H-23/H-24), 0.89 (s, 6H, H-29/H-30), 0.88 (s, 3H, H-28), 0.80 (s, 3H, H-25).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 167.3 (C=O), 158.0 (C-14), 157.6 (C-7'), 144.0 (C-3'), 130.0 (C-5'/C-9'), 127.4 (C-2'), 116.9 (C-15), 116.3 (C-4'), 115.8 (C-6'/C-8'), 81.0 (C-3α), 55.6, 49.1, 48.7, 41.2, 39.0, 38.0, 37.7, 37.5, 37.4, 36.6, 35.8, 35.1, 33.7, 33.3, 33.1, 29.9, 29.8, 28.8, 28.0, 25.9, 23.6, 21.3, 18.7, 17.5, 16.7, 15.5.

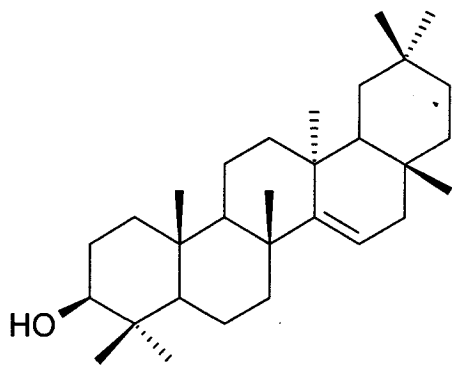
IR υ_{max} ($CHCl_3$, cm^{-1}): 3630 (OH), 2934, 2361, 2342, 1701 (C=O), 1655, 1560, 1542, 1474, 1168, 826, 669.

MS (EI) *m/z* (rel. int.): 572 $[M]^+$ (5), 448 [RDA fragment (d)]$^+$ (13), 269 (20), 204 [fragment (**e**)]$^+$ (39), 189 (16), 164 (14), 147 (100), 135 (15), 133 (15), 107 (14), 95 (15), 91 (17), 69 (16), 55 (14), 41 (14).

HRMS: *Calculated* for $C_{39}H_{56}O_3$, 572.42295, *found* 572.42421.

2.3.6.6. Taraxerol

Other names: Alnulin, taraxer-14-en-3β-ol, taraxen-3β-ol, 3-picenol, tiliadin, skimmiol, 27-norolean-14-en-3-ol, D-friedoolean-14-en-3β-ol.



4a

To a stirred solution of taraxeryl *trans*-p-hydroxycinnamate (**4**), (47 mg, 8.21 x 10^{-5} mol) in THF (10 mL) under nitrogen at 0°C, was added $LiAlH_4$ (15.6 mg, 4.11 x 10^{-4} mol) and the mixture was left to stir at r.t. (22°C) for 3h. The reaction mixture was quenched with saturated NH_4Cl solution (1 mL), water (10 mL), and the organic solvent was removed *in vacuo* before it was extracted with EtOAc (3 x 10 mL). The combined EtOAc extract was dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography eluting with hexane-EtOAc gradient solvent

system to afford (**4a**) as a white solid, (25 mg, 71%). The other reduction product was 4-hydroxyphenyl-propanol (**4c**), a pale brown liquid (10 mg).

m.p. 277-278°C (lit.[56] 273-275°C, lit.[54] 277-278°C, lit.[57] 277-280°C, lit.[53] 278-279°C, lit.[58] 282-283°C, lit.[59] 282-285°C, lit.[60] 287-289°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 5.51 (dd, J=8.2 Hz, J=3.3 Hz, 1H, H-15), 3.17 (dd, J=10.3 Hz, J=6.3 Hz, 1H, H-3α), 1.06 (s, 3H, H-26), 0.95 (s, 3H, H-27), 0.93 (s, 3H, H-23), 0.90 (s, 3H, H-29), 0.88 (s, 6H, H-25/H-30), 0.80 (s, 3H, H-28), 0.78 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 158.0 (C-14), 116.9 (C-15), 79.0 (C-3), 55.5, 49.2, 48.7, 41.3, 38.9, 38.7, 38.0, 37.7, 37.5, 36.6, 35.8, 35.1, 33.7, 33.3, 33.0, 29.9, 29.8, 29.7, 28.8, 28.0, 27.1, 25.9, 21.3, 18.7, 17.5, 15.4.

IR υ_{max} ($CHCl_3$, cm^{-1}): 3627, 2931, 2360, 2342, 1472, 1384, 1056, 1037. 999, 863, 669, 642.

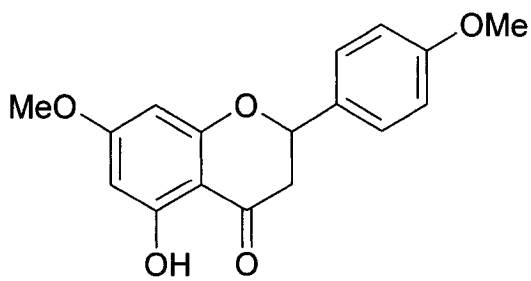
MS (EI) *m/z* (rel. int.): 426 $[M]^+$ (16), 411 (15), 302 [RDA fragment (**d**)]$^+$ (53), 287 (46), 205 (28), 204 [fragment (**e**)]$^+$ (100), 203 (14), 189 (23), 135 (55), 133 (40), 121 (36), 107 (35), 95 (42), 81 (37), 69 (59), 43 (39), 41 (64).

HRMS: *Calculated* for $C_{30}H_{50}O$, 426.3867, *found* 426.38583.

2.3.7. Flavanones

2.3.7.1. Naringenin-4', 7-dimethyl ether

Other names: Narigenin-4', 7-dimethyl ether, 5-hydroxy-4',7-dimethoxyflavanone



6

m.p. 115-116°C (lit.[77] 115.5-115.9°C, lit.[78] 114-115°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 12.01 (s, 1H, OH-5), 7.36 (dd, J= 6.7 Hz, J=1.8 Hz, 2H, H-2'/6'), 6.93 (dd, J=6.7 Hz, J=2.1 Hz, 2H, H-3'/5'), 6.05 (d, J=2.3 Hz, 1H, H-6), 6.02 (d, J=2.3 Hz, H-8), 5.35 (dd, J=12.9 Hz, J=3.1 Hz, 1H, H-2_{ax}), 3.81 (s, 3H, OMe-7), 3.78 (s, 3H, OMe-4'), 3.09 (dd, J=17.2 Hz, J=12.9 Hz, 1H, H-3_{ax}), 2.76 (dd, J=17.3 Hz, J=3.2 Hz, H-3_{eq}).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 196.1 (C=O), 168.0 (C-7), 164.1 (C-5), 162.9 (C-9), 160.0 (C-4'), 130.3 (C-1'), 127.7 (C-2', C-6'), 114.2 (C-3', C-5'),103.1 (C-10), 95.1 (C-6), 94.2 (C-8), 79.0 (C-2), 55.7 (OMe-7), 55.4 (OMe-4'), 43.2 (C-3).

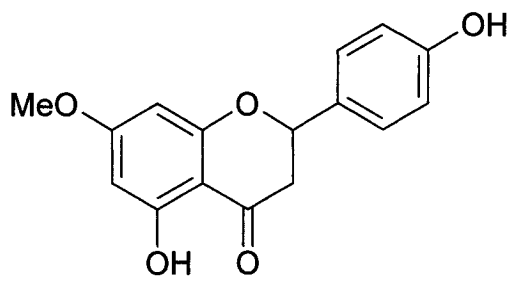
IR υ_{max} ($CHCl_3$, cm^{-1}): 3010 (OH), 2975, 2941, 2913, 2361, 1637 (C=O), 1578, 1491, 1447, 1306, 1278, 1210, 1176, 1114, 1071, 1030, 989, 832, 745, 572.

MS (EI) *m/z* (rel. int.): 300 $[M]^+$ (70), 299 (42), 193 (17), 166 [RDA fragment $A_1]^+$ (19.8), 134 [RDA fragment $B_1]^+$ (100), 121 (56), 91 (15).

HRMS: *Calculated* for $C_{17}H_{16}O_5$, 300.09978, *found* 300.09904.

2.3.7.2. Naringenin-7-methyl ether

Other names: Sakuranetin, naringenin-7-methyl ether, 4',5-dihydroxy-7-methoxyflavanone.



7

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 12.01 (s, 1H, OH-5), 7.29 (dd, J= 8.7 Hz, J=2.5 Hz, 2H, H-2'/6'), 6.85 (dd, J=6.6 Hz, J=2.0 Hz, 2H, H-3'/5'), 6.05 (d, J=2.3 Hz, 1H, H-6), 6.02 (d, J=2.3 Hz, H-8), 5.32 (dd, J=13.0 Hz, J=3.0 Hz, 1H, H-2_{ax}), 3.78 (s, 3H, OMe-7), 3.07 (dd, J=17.2 Hz, J=12.9 Hz, 1H, H-3_{ax}), 2.75 (dd, J=17.2 Hz, J=3.1 Hz, H-3_{eq}).

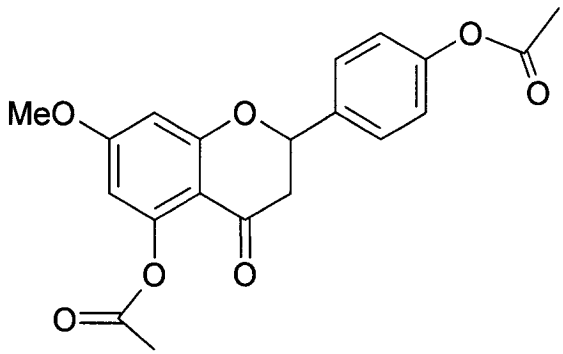
^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 196.1 (C=O), 168.0 (C-7), 164.1 (C-5), 162.9 (C-9), 156.2 (C-4'), 130.4 (C-1'), 128.0 (C-2'/C-6'), 115.6 (C-3'/C-5'), 103.1 (C-10), 95.1 (C-6), 94.2 (C-8), 79.0 (C-2), 55.7 (OMe-7), 43.2 (C-3).

IR υ_{max} ($CHCl_3$, cm^{-1}): 3387 (OH), 2934, 2360, 2342, 1641 (C=O), 1574, 1519, 1445, 1306, 1205, 1073, 1031, 961, 885, 834, 742, 667, 571.

MS (EI) *m/z* (rel. int.): 286 $[M]^+$ (89), 285 (50), 193 (29), 167 [RDA fragment A_1 + 1]$^+$ (100), 166 [RDA fragment $A_1]^+$ (32), 138 (18), 120 [RDA fragment $B_1]^+$ (40).

HRMS: *Calculated* for $C_{16}H_{14}O_5$, 286.08413, *found* 286.08461

5,4-Diacetoxy-7-methoxyflavanone



7a

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 7.45 (dd, J= 7.5 Hz, J=1.7 Hz, 2H, H-2'/6'), 7.13 (dd, J=6.7 Hz, J=2.0 Hz, 2H, H-3'/5'), 6.40 (d, J=2.4 Hz, 1H, H-6), 6.27 (d, J=2.4 Hz, H-8), 5.44 (dd, J=13.4 Hz, J=3.0 Hz, 1H, H-2_{ax}), 3.81 (s, 3H, OMe-7), 2.98 (dd, J=16.8 Hz, J=13.3 Hz, 1H, H-3_{ax}), 2.71 (dd, J=16.7 Hz, J=3.0 Hz, 1H, H-3_{eq}).

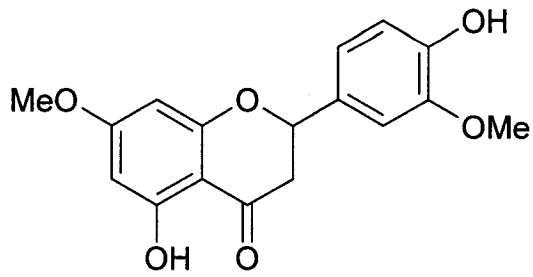
MS (EI) *m/z* (rel. int.): 370 $[M]^+$ (3.9), 329 (20), 328 (100), 327 $[M\text{-}CH_3CO]^+$ (18), 286 (52), 285 (49), 193 (30), 167 (52), 166 (53), 120 (38), 57 (32), 55 (28), 43 (32), 41 (27).

HRMS: *Calculated* for $C_{20}H_{18}O_7$, 370.10526, *found* 370.10815.

2.3.7.3. Isomeric mixture of Eriodictyol-3', 7-dimethyl ether and eriodictyol-4', 7-dimethyl ether

Eriodictyol-3', 7-dimethyl ether (8a)

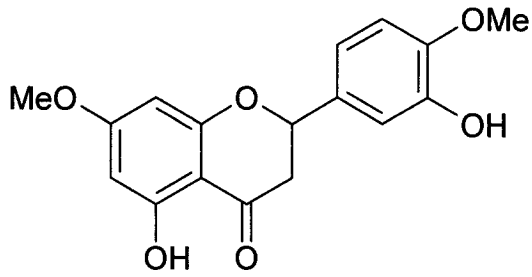
Other names: Homoeriodictyol, 4',5-dihydroxy- 3,7'-dimethoxyflavanone.



8a

Eriodictyol-4', 7-dimethyl ether (8b)

Other names: 5,3'-Dihydroxy-7,4' dimethoxyflavanone.



8b

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 12.00 (s, 1H, OH-5), 7.02 (d, J=2.3 Hz, H-2'), 6.83-6.95 (m, 2H, H-5'/6'), 6.05 (d, J=2.4 Hz, 1H, H-6), 6.02 (d, J=2.3 Hz, H-8), 5.32 (dd, J=12.8 Hz, J=2.8 Hz, 1H, H-2_{ax}) 5.30 (dd, J=12.6 Hz, J=3.3 Hz, 1H, H-2_{ax}), 3.92 (s, 3H, OMe-4'), 3.90 (s, 3H, OMe-4'), 3.79 (s, 3H, OMe-7), 3.06 (dd, J=17.2 Hz, J=12.8 Hz, 1H, H-3_{ax}), 2.75 (dd, J=17.2 Hz, J=3.2 Hz, H-3_{eq}).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 196.1 (C=O), 168.0, 164.1(C-5), 162.8(C-9), 147.0 (C-4'), 146.7 (C-3'), 146.2 (C-4'), 145.9 (C-3'), 131.5 (C-1'), 130.2 (C-1'), 119.6 (C-6'), 118.2 (C-6'), 114.5 (C-5'), 112.6 (C-2'), 110.6 (C-5'), 108.7 (C-2'), 103.1 (C-10), 95.1 (C-6), 94.2 (C-8), 70.3 (C-2), 79.0 (C-2), 55.7 (OMe-7), 56.0 (OMe-4'/OMe-3'), 43.4 (C-3), 43.2 (C-3).

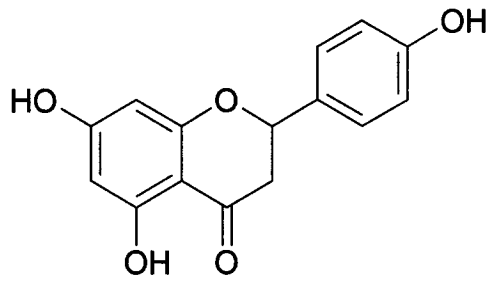
IR υ_{max} ($CHCl_3$, cm^{-1}): 3438 (OH), 2923, 2846, 2247, 1642 (C=O), 1635 (C=O), 1571, 1365, 1262, 1146, 1076, 959, 908, 805, 734, 643, 605.

MS (EI): 316 $[M]^+$ (75), 315 (27), 262 (35), 203 (50), 193 (27), 167 [RDA fragment A_1 + 1]$^+$ (74), 150 [RDA fragment B_1]$^+$ (41), 137 (69), 133 (31), 44 (55), 29 (100).

HRMS: *Calculated* for $C_{17}H_{16}O_6$, 316.09469, *found* 316.09461

2.3.7.4. Naringenin

Other names: 4',5,7-Trihydroxyflavanone, 5,7-dihyroxy-2-(4-hydroxyphenyl) chroman-4-one.



9

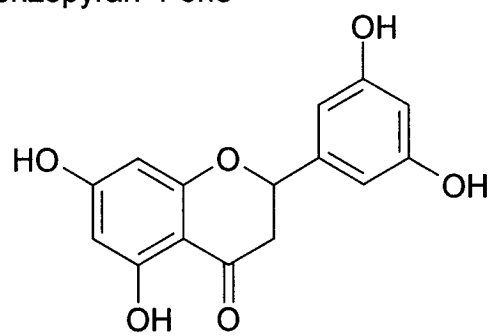
^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 12.19 (s, 1H, OH-5), 7.38 (d, J= 8.0 Hz, 2H, H-2'/6'), 6.89 (d, J=8.0 Hz, 2H, H-3'/5'), 5.92 (s, 2H, H-6/H-8), 5.43 (dd, J=12.0 Hz, J=3.2 Hz, 1H, H-2_{ax}), 3.17 (dd, J=17.0 Hz, J=12.0 Hz, 1H, H-3_{ax}), 2.71 (dd, J=17.0 Hz, J=3.0 Hz, H-3_{eq}).

IR υ_{max} ($CHCl_3$, cm^{-1}): 3419 (OH), 2086, 1642 (C=O), 1455, 1294, 1313, 1236, 1120, 1092, 963, 830, 749, 715.

MS (EI) *m/z* (rel. int.): 272 $[M]^+$ (100), 271 (47), 179 (31), 166 (23), 153 [RDA fragment A_1 + 1]$^+$ (99), 124 (16), 120 [RDA fragment B_1]$^+$ (77), 44 (25).

2.3.7.5. 3', 5, 5', 7-Tetrahydroxyflavanone

Other names: 4H-1-Benzopyran-4-one



10

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 12.17 (s, 1H, OH-5), 7.04 (s, 2H, H-4'), 6.89 (d, J=8.0 Hz, 2H, H-2'/6'), 5.93 (s, 2H, H-6/H-8), 5.38 (dd, J=12.0 Hz, J=3.2 Hz, 1H, H-2_{ax}), 3.15 (dd, J=17.0 Hz, J=12.0 Hz, 1H, H-3_{ax}), 2.74 (dd, J=17.04 Hz, J=3.0 Hz, H-3_{eq}).

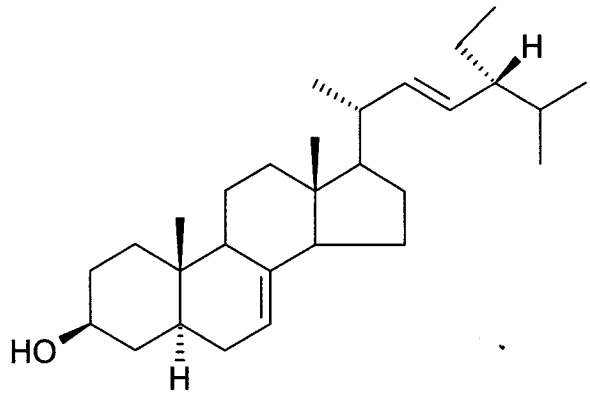
IR υ_{max} ($CHCl_3$, cm^{-1}): 3357 (OH), 2926, 2858, 2356, 1639 (C=O), 1627, 1600, 1596, 1443, 1340, 1268, 1219, 856, 818, 727, 657.

MS (EI) *m/z* (rel. int.): 288 $[M]^+$ (74), 287 (34), 179 (51), 166 (52), 153 [RDA fragment A_1 + 1]$^+$ (87), 152 [RDA fragment A_1]$^+$ (21), 151 (30) 137 (31), 136 [RDA fragment B_1]$^+$ (55), 135 (31), 123 (41), 121 (39), 111 (29), 109 (35), 97 (53), 95 (39), 91 (34), 85 (41), 83 (47), 81 (42), 71 (54), 69 (56), 67 (32), 57 (81), 55 (63), 43 (100), 41 (53).

HRMS: *Calculated* for $C_{15}H_{12}O_6$, 288.06342, *found* 288.06249.

2.3.7.8. Chondrillasterol

Other names: 3β-Hydroxy-24-ethyl-5α-cholesta-7, 22-diene.



11

m.p. 164-165°C (lit.[96] 154-157, lit.[97] 163-165°C)

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 5.14 (m, 2H, H-22/H-23), 5.02 (dd, J=14.4 Hz, J=8.7 Hz, H-7), 3.57 (m, H-3α), 1.01 (d, J=6.6 Hz, Me, H-21), 0.84 (d, J=6.3 Hz, Me, H-27), 0.79 (m, 3xMe, H-19, H-26, H-29), 0.53 (s, Me, H-18).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 139.5 (C-8), 138.1 (C-22), 129.4 (C-23), 117.4 (C-7), 71.0 (C-3), 55.8, 55.0, 51.2, 49.4, 43.2, 40.7, 40.2, 37.9, 37.1, 34.1, 31.4, 28.4, 25.3, 22.9, 21.5, 21.3, 18.9, 12.9, 12.1.

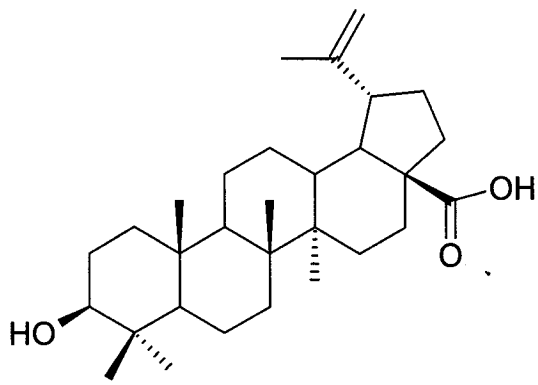
IR υ_{max} ($CHCl_3$, cm^{-1}): 3400 (OH), 2937, 2364, 1560, 1457, 1381, 1039, 829, 796, 668.

MS (EI) *m/z* (rel. int.): 412 $[M]^+$ (34), 394 $[M-H_2O]^+$ (4), 273 (33), 272 (26), 271 (100), 269 (28), 255 (45), 147 (31), 107 (45), 95 (37), 83 (56), 81 (70), 69 (41), 55 (60), 43 (29).

HRMS: *Calculated* for $C_{29}H_{48}O_1$, 412.37052, *found* 412.36900.

2.4. The bioactive constituent from Margraviaceae

In the course of our search for the bioactive constituent from the leaves of Margraviaceae family, it was found that the crude EtOH extract (SS-CE) demonstrated significant anti-anxiety activity. Subsequent bioassay-directed fractionation by solvent extraction of this extract successively with EtOAc and hot EtOH showed that the activity resided solely in the EtOAc soluble fraction, fraction 1 (f1), (*see* **Figure 2.1.2**). Further bioassay-guided chromatography of the active fraction (f1), resulted in the isolation of betulinic acid (**3**) as the bioactive anti-anxiety agent (*see* **Section 2.6**).



Betulinic acid (3)

2.4.1. Betulinic acid: A bioactive anti-anxiety agent

It was demonstrated unambiguously in two different standard industry accepted anxiety assays that betulinic acid (**3**) was responsible for the anti-anxiety activity of Margraviaceae extracts. When (**3**) was administered orally to rats (1 mg/kg), it produced a statistically significant anti-anxiety effect both in the fear potentiated (**Figure 2.6.2a**) and the EPM tests for anxiety (**Figure 2.6.2b**). These examples illustrate that (**3**) is a major biologically active constituent of Margraviaceae. When a pure sample of (**3**) was used at 0.5-1.0 mg/kg, the

activity of (**3**) is comparable to that of the gold standard anti-anxiety compounds, benzodiazepines (Valium).[6] The anti-anxiety activity of (**3**) is observed for at least 4h after oral administration. No other changes in behaviour were observed.

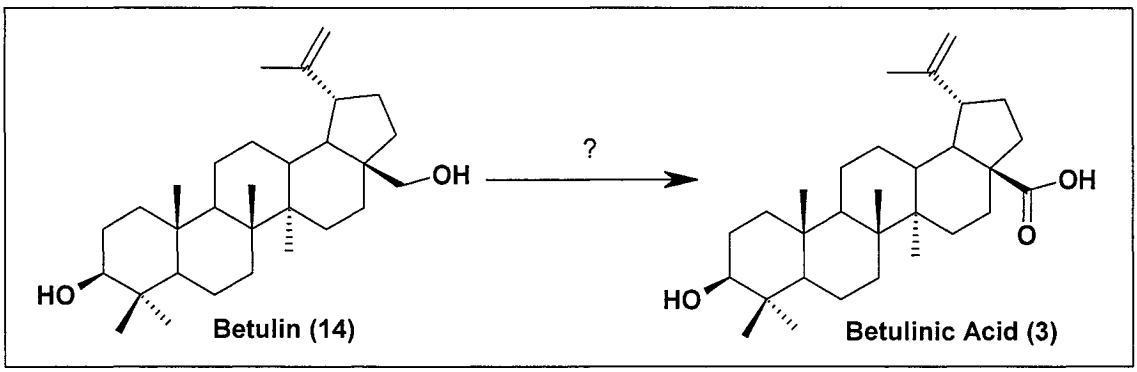
The effect of betulinic acid (**3**) on CD-1 strain of mice was also tested (**Figure 2.6.2c**). It was found that (**3**) was equally effective as anti-anxiety agent in mice. These data demonstrated that the anxiolytic effect of betulinic acid (SS-01) is not restricted to rats, as it can be observed in mice as well.

2.4.2. Synthesis of Betulinic Acid

An important aspect of this project was to synthesize betulinic acid (**3**) in large scale not only for biological evaluation but also, for the production of a series of potentially important derivatives that may have anti-anxiety activity superior to (**3**). The structural complexity of (**3**), possessing five fused rings and eight chiral centers, presents a formidable total synthetic challenge to any organic chemist. Fortunately, another natural product betulin (**14**), the major extractive component of white-barked birch trees (comprising typically 10-15% of the outer bark), can easily be converted into (**3**).[106,107]

The ready availability and the unlimited supply of (**14**) from local collections (Calabogie area, Ontario), made (**14**) an extremely attractive starting material for the synthesis of (**3**). Indeed, this was the general synthetic strategy that was envisioned and employed in the synthesis of (**3**) and, subsequently, many of its derivatives (**Scheme 2.4.2**).

Birch trees are widespread in the northern latitudes of the world. The levels of (**14**) is known to increase with age and the outer bark contains about four times as much betulin as the inner bark (**Figure 2.4.2**).[108]



Scheme 2.4.2: The general synthetic strategy employed in the synthesis of betulinic acid (**3**) from betulin (**14**).

Figure 2.4.2: Picture of white-barked birch trees (*Betula* species).

2.4.2.1. Betulin

Betulin (**14**) having the molecular formula $C_{30}H_{50}O_2$, is one of the first natural products obtained from plants (*Betula alba*) dating back to 1788. It was assigned the name betulin by Mason in 1831.[28] It was nearly a century after the original isolation of (**14**) that any attempt was made to determine its chemical composition. Hausmann assigned to it the formula $C_{36}H_{60}O_3$ in 1876 while Traubenberg (1917) suggested the formula $C_{24}H_{40}O$ and a double bond located in a cyclic system. In 1922, Schulze and Pieroh proposed that (**14**) was a dihydric alcohol with the formula $C_{32}H_{52}O_2$ or $C_{33}H_{54}O_2$ while Dischendorfe adopted the formula $C_{30}H_{48}O_2$ or $C_{30}H_{50}O_2$ in the same year and later $C_{30}H_{52}O_2$.[28]

In 1926, Dishendorfer and Grillmayer regarded (**14**) as $C_{30}H_{50}O_2$ plus or minus 2H. Meanwhile, Vesterberg and Vesterberg favoured $C_{30}H_{50}O_2$ which was finally confirmed by Ruzicka and coworkers in 1932. The absolute configuration of compound (**14**) was assigned by application of the Horeau method and diastereomeric reaction products detected by GC. Two reviews of (**14**) are known in the literature, the latest one appeared in 1989.[106]

Birch bark containing 10-14% betulin has been used as an antiseptic, and in folk medicine to treat skin diseases, malaria and gout. Feeding deterrent activity, anti-inflammatory, allelopathic and insecticidal activities have been attributed to betulin and betulin-containing herbs.[106] Various betulin derivatives such as 3,28-Di-O-(3'3',-dimethylglutaryl)-betulin exhibited significant anti-HIV potency.[109]

In our laboratory, betulin (**14**) was extracted from white birch bark by cutting the bark into small rectangular pieces before it was ground to powder using a Wiley Mill. The powdered bark was then immersed in EtOAc for 24h at room temperature. The mixture was filtered, washed with extra solvents and the filtrate was concentrated *in vacuo* to yield a dark brown crude extract in 15-29% yield. Compound (**14**) was recrystallized in 12-19% dry weight (**Table 2.4.2.1**).

Table 2.4.2.1: Effect of different solvents on the extraction and recrystallization of betulin (**14**) from birch bark powder.

Extraction Solvent	Birch bark powder (g)	Crude extracts of (**14**), (g)	Amount of (**14**) recrystallized (g)	% of (**14**) recrystallized
(i). EtOAc	138.1	40.1	26.6 ($MeOH/CHCl_3$)	19
(ii). CH_2Cl_2	138.1	38.6	21.6 (Isopropanol/H_2O)	16
(iii).Acetone/water	138.1	55.6	16.3 (Isopropanol)	12

In an effort to compare the effects of different solvents in the extraction of (**14**) from birch bark, EtOAc, CH_2Cl_2 and acetone/water (Giner's procedure)[110] were employed in the extraction of 138.1 g of dried birch bark powder. The Giner's procedure involved extracting the birch bark powder twice with boiling acetone. The combined acetone extract was concentrated before water was added followed by cooling in ice (**Table 2.4.2.1**).

There appeared to be no significant difference between the EtOAc and CH_2Cl_2 extractions. The Giner's procedure yielded both a better quantity of crude yield and quality of (**14**) as indicated by a pale yellow solid compared to a slightly darker yellow or brown crude residue obtained from the EtOAc and CH_2Cl_2 extractions, although the % yield of recrystallized (**14**) was lower.

A variety of solvents (MeOH, $MeOH/CHCl_3$, $EtOH/H_2O$, EtOH/acetone and isopropanol) were used for the recrystallisation of crude betulin. The best solvents for recrystallizing (**14**) appeared to be $MeOH/CHCl_3$, isopropanol and isopropanol/H_2O.

It should be noted that the purification of (**14**) by recrystallization was insufficient since the solid remained pale yellow (after it was recrystallized thrice from 10% CH_3Cl/MeOH or isopropanol) compared to pure compound which is a

white solid, m.p. 248-249°C (lit.[60] 248-251°C, lit.[111] 254-256°C, lit.[112] 255-256°C, lit.[113] 257°C). Therefore, silica gel column chromatography was necessary to remove this pale yellow pigment (impurity). This observation was in accordance with those previously reported by Hayek *et. al*, in 1989.[106]

However, recrystallised betulin (**14**) appeared pure by ^{1}H NMR. This material was used for large scale synthesis (5-20 g) of betulinic acid (**3**), and the brown impurity was removed later on in the synthesis by silica gel column chromatography.

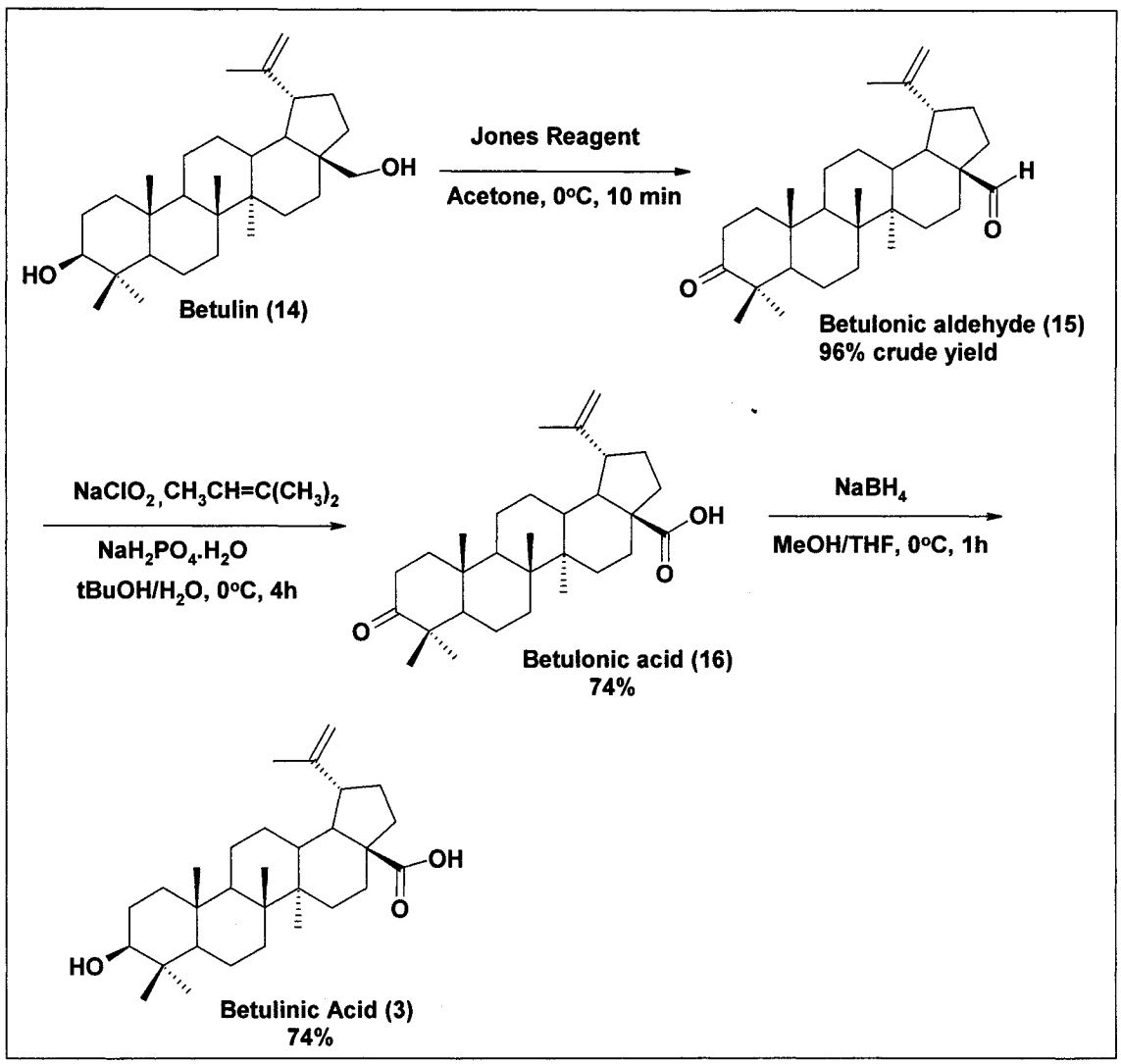
The identification of (**14**) was made through comparison of its melting point and spectral data with those in the literature. The EIMS of (**14**) displayed a $[M]^+$ at m/z 442 which was in accordance with its molecular formula, $C_{30}H_{50}O_2$, This was confirmed by HRMS. The fragmentation pattern listed in the experimental section was characteristic of the lupane type triterpenoid. The IR spectrum of (**14**) revealed a broad hydroxyl absorption at 3352 cm^{-1} with no carbonyl absorption.

Both the ^{1}H and ^{13}C NMR data of (**14**) were in excellent agreement with literature data.[114,115] The ^{1}H NMR spectrum of (**14**) revealed a methylene moiety at δ 4.65 (d, J=2.0 Hz, H-29), 4.55 (d, J=1.8 Hz, H-29'). The hydroxymethylene unit (CH_2OH) was obvious from the AB system at δ 3.77 (dd, J=10.9 Hz, J=1.4 Hz, H-28) and at δ 3.30 (d, J=10.8 Hz, H-28'). The hydroxyl group at C-3 and its β-orientation was supported by the double doublet at 3.16 (dd, J=10.9 Hz, J=5.1 Hz, H-3α). The allylic proton resonated at δ 2.36 (ddd, J=10.6 Hz, J=10.6 Hz, J=5.8 Hz, H-19). The vinylic methyl group appeared at 1.65 (s, 3H), while the five tertiary methyl singlets were observed at 0.99 (s, H-26), 0.95 (s, H-27), 0.94 (s, H-23), 0.79 (s, H-25), and at δ 0.74 (H-24).

The ^{13}C NMR of (**14**) displayed diagnostic signals for the olefinic carbons at δ 150.3 (C-20) and 109.6 (C-29) as well as for the carbons bearing the secondary and primary hydroxyl functions at δ 79.0 (C-3) and 60.5 (C-28) respectively.

2.4.2.2. Synthesis of betulinic acid from betulin

It was anticipated that a large scale synthesis of betulinic acid (**3**) from betulin (**14**) could be performed through a simple synthetic approach. Indeed, a 3-step conversion of (**14**) to (**3**) involving a Jones' oxidation, $NaClO_2$ oxidation and $NaBH_4$ reduction followed by recrystallization of (**3**) from MeOH appeared to be the most efficient method (**Scheme 2.4.2.2**). This is a slight modification of Pezzuto's relay synthesis.[116]



Scheme 2.4.2.2: Synthesis of betulinic acid (**3**) from betulin (**14**).

Crude betulin (recrystallised three times from $MeOH/CHCl_3$ or isopropanol/H_2O) was converted to betulonic aldehyde (**15**) by Jones' oxidation (CrO_3/H_2SO_4/acetone/0°C) in a 5-10 minutes reaction (>95% crude yield). Compound (**15**) was then subjected to $NaClO_2$ oxidation in the presence of 2,3-dimethyl-2-butene and sodium dihydrogen phosphate in $tBuOH/H_2O$ to afford betulonic acid (**16**) in 74 % isolated yield.

The $NaClO_2$ oxidation of (**15**) to (**16**) was initially carried out in the presence of sulfamic acid (H_2NSO_3) in water and THF. However, this reaction condition resulted in the formation of several additional complex compounds which were not identified. One of these compounds appeared just below (**16**) and (**3**) on TLC which made the purification of (**16**) and the subsequent recrystallisation of pure betulinic acid (**3**) from MeOH very difficult. This complication was eliminated when $NaClO_2$ was used with 2,3-dimethyl-2-butene and sodium dihydrogen phosphate in $tBuOH/H_2O$.

It was critical that (**3**) thus obtained could be purified by recrystallisation because of its poor solubility (in almost every solvent), which made its purification by column chromatography very difficult especially when dealing with more than 1 g of material. The reduction of (**16**) to (**3**) was conveniently carried out with $NaBH_4$ in methanol (MeOH) in quantitative yield.

Pezzuto *et. al* reported a 95:5 ratio of β to α isomers of (**3**).[116] However, in our hands, recrystallization of the crude product from $MeOH/CHCl_3$ afforded pure betulinic acid (**3**) as white needles in 70-75% yield. The 500 MHz NMR showed no evidence of contamination by the α-isomer.

Compound (**15**) was purified as a white solid, m.p.163-165°C (lit.[28] 165-166°C). Other authors reported betulonic aldehyde as a colorless gum or colorless oil.[117] The EIMS of (**15**) showed a $[M]^+$ at m/z 438 that matched with its molecular formula $C_{30}H_{46}O_2$. This was confirmed by HRMS. The fragmentation pattern was typical of a lupane derived triterpenoid of the proposed structure (**Scheme 2.2.2**) and was also comparable with literature values.[117] Its IR spectrum contained major absorption bands at 1703 (C=O) and at 3378 cm^{-1} (OH).

The ^{1}H and ^{13}C NMR shift assignments of (**15**) were accomplished through comparison with published data for betulonic aldehyde.[109,117] The ^{1}H NMR spectrum of (**15**) resembled that of (**14**) except for the disappearance of the secondary alcohol signal at δ 3.16 and the primary alcohol (CH_2OH, AB system) signals at δ 3.77 and 3.30 ppm observed for (**14**). These signals are now replaced by an aldehyde signal at δ 9.64 (d, J=1.7 Hz, H-28) and signals for protons adjacent to the carbonyl group (C-3) at δ 2.46 (m, 1H, H-2) and at δ 2.36 (m, 1H, H-2) respectively.

The ^{13}C NMR exhibited diagnostic signals at δ 217.9 ascribed to the keto functionality at C-3, at δ 206.4 attributable to the aldehydic carbon at C-28 and at δ 149.6 (C-20) and 110.1 (C-29) due to the olefinic carbons.

Betulonic aldehyde (**15**) has been previously synthesized by PDC oxidation[109] and PCC oxidation (87%) of betulin.[109,117] It has also formed from Oppenauer oxidation of betulin using benzoquinone as hydrogen acceptor, from heating (**14**) in with copper bronze at 300°C and by mild oxidation of betulin with chromic acid in aqueous acetic at low temperature.[28]

Compound (**15**) has recently been identified as a new natural product from yellow birch bark (*B. alleghaniensis*) in bark samples from three different locations in Quebec, Canada.[118] It has been reported to inhibit B162F2 cell proliferation by induction of apoptosis (cellular suicide in cancer cells without affecting healthy cells).[119]

Betulonic acid (**16**) was isolated pure as a white solid, m. p. 247-249 (lit.[120] 236-238°C, lit.[116] 247-249 °C, lit.[28] 253°C, lit.[111] 261-264°C), after silica gel column chromatography (EtOAc-hexane solvent gradient). Its EIMS revealed a $[M]^+$ at m/z 454 that agreed with its molecular formula $C_{30}H_{46}O_3$, and was etablished by HRMS. The fragment losses were characteristic of a lupane derived triterpenoid (**Scheme 2.2.2**) and was in agreement with those previously reported in the literature.[111] Its IR spectrum showed carbonyl absorptions at 1696 (acid) and 1642 cm^{-1} (ketone) and a broad hydroxyl band at 3300 cm^{-1}.

The complete assignment of the proton and carbon NMR shifts of (**16**) was made by comparison with reported spectroscopic data for betulonic

acid.[111,121] The ^{1}H NMR spectrum of (**16**) was similar to that of (**15**) except for the disappearance of the aldehydic proton at δ 9.64. The protons adjacent to the carbonyl group at C-3 appeared as a two proton multiplet at δ 2.46 (m, 2H, H-2).

The ^{13}C NMR spectrum of (**16**) exhibited two carbonyl functionalities at δ 218.4 (keto group at C-3) and at δ 182.5 (acid group at C-28). The olefinic carbons resonated as usual at δ 150.3 (C-20) and 109.8 (C-29).

Compound (**16**) has been isolated from *Orthopterygium huancuy* and from the stem bark of *Evodia meliafolia* as a natural product.[120,122] Incubation of (**3**) with resting cell suspensions of phenobarbital-induced *Baccillus megaterium* ATCC 14581 resulted in microbial transformation of (**3**) to (**16**).[123] Microbial transformation of (**16**) by the filamentous fungus *Chaetomium longirostre* yielded oxygenated derivatives with more potent anti-tumor promoting effects.[124]

It has been shown that (**16**) has allelopathic activity and selectivity over germination and growth of monocotyledons species with average inhibition of (-) 50% on the germination of *Hordeum vulgare* and average stimulation of 30% on the germination of *Allium cepa*.[125]

Dihydrobetulonic acid has been reported to be the most active compound when cytotoxicity evaluation was performed against human melanoma cells.[126] Moreover, several oximes and amines derivative of (**16**) have been evaluated against cultured human melanoma.

The physical and spectroscopic data of betulinic acid (**3**) synthesized in this work were in excellent agreement with those previously observed for an authentic sample, and that isolated from the Margraviaceae plant extracts (*see* **Sections 2.2.2.1.** and **2.3.6.1**).

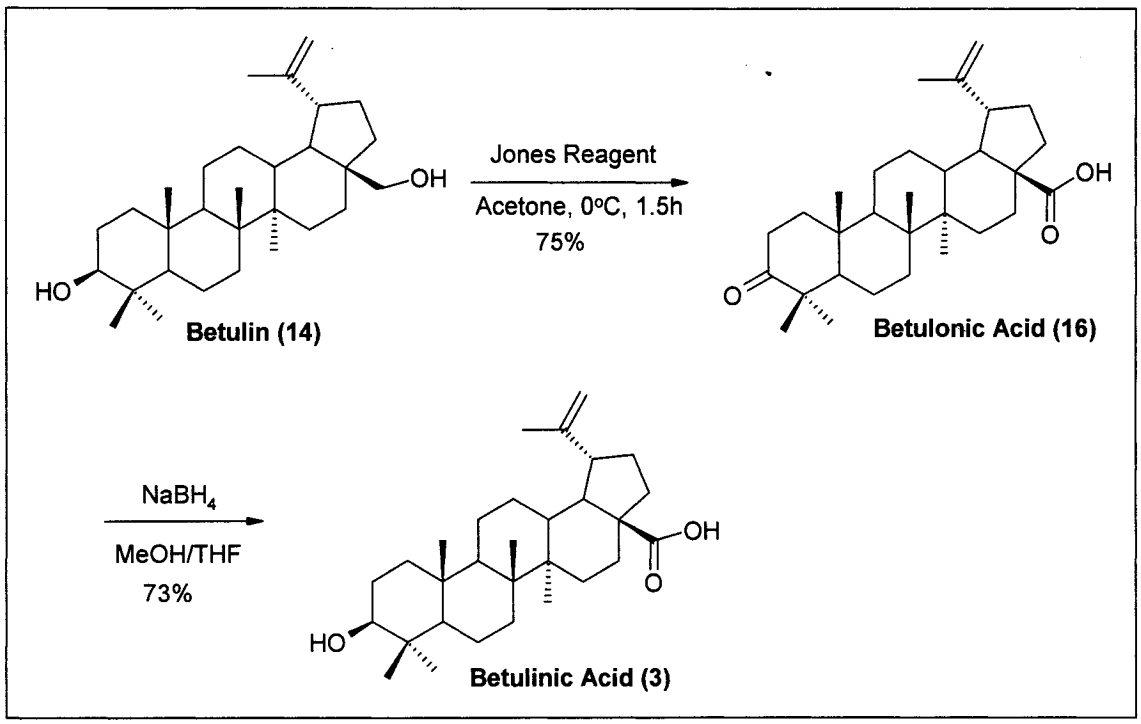
2.4.2.3. Previous syntheses of betulinic acid

Betulinic acid (**3**) was first synthesized as 3-acetyl betulinic acid (**14c**) by Ruzicka *et. al.*, in1938 in low overall yield.[127] In this synthesis, the two hydroxyl groups of betulin (**14**) were acetylated to betulin 3,28-diacetate (**14e**) and, by using a slight excess of 0.1N alkali over that required for the saponification of one

group, a monoacetate of (**14c**) was obtained. The monoacetate of betulin was oxidized with chromic acid to the corresponding 3-acetyl-28-aldehyde which was then further oxidized to 3-acetyl betulinic acid (**14d**).

More recently (1997), Pezzuto and his colleagues reported two syntheses of (**3**) from (**14**).[116] Firstly, in a two steps synthesis, crude betulin (recrystallized thrice from MeOH/$CHCl_3$) was subjected to Jones' oxidation for 1.5h to afford betulonic acid (**16**) in 75% yield after column chromatography.

Compound (**16**) was reduced with $NaBH_4$ in MeOH/THF to (**3**) in quantitative yields as a 5:95 mixture of α- and β- isomers and was recrystallized from MeOH to afford 75% β-isomer as white needles (**Scheme 2.4.2.3a**). This synthesis by Pezzuto *et. al.*, is a two step version of the synthetic protocol employed in our own synthesis of betulinic acid (**Scheme 2.4.2.2**). However, in our laboratory, when crude betulin was oxidized with the Jones' reagent, (**16**) was obtained in very low yield (20-30%).



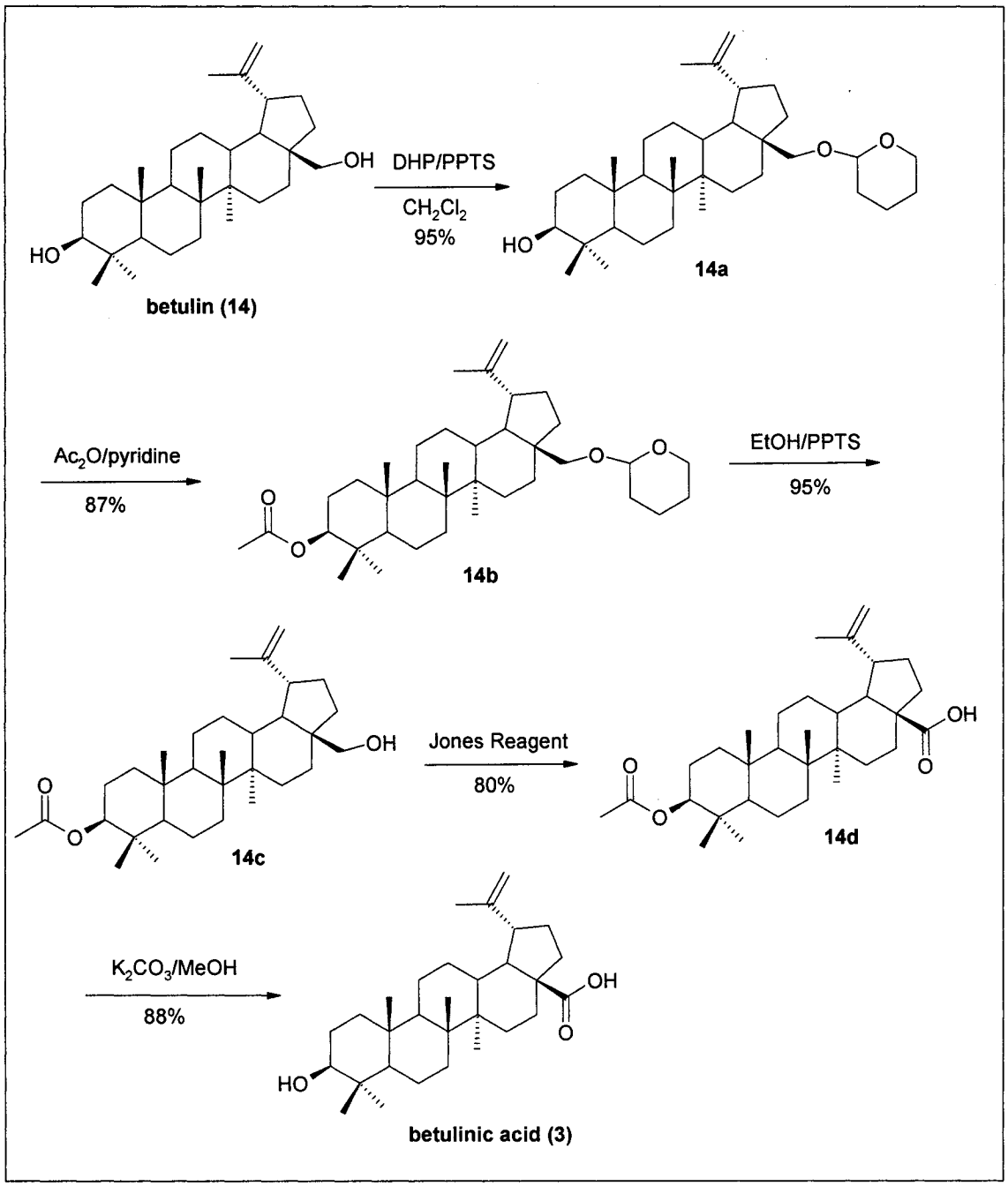
Scheme 2.4.2.3a: The first Pezzuto synthesis of betulinic acid (**3**) from betulin (**14**).[116]

In a second five steps synthesis of (**3**) from (**14**), the primary alcohol of betulin was monoprotected as a THP ether (DHP/CH_2Cl_2/PPTS, 95%) and the secondary alcohol was acetylated (Ac_2O/pyridine) to afford (**14b**) in 87% yield. The THP protecting group was removed (MeOH/PPTS, 95%) and the acetylated betulin was subjected to Jones' oxidation to furnish the carboxylic acid acetate (**14d**). The acetyl group was removed (K_2CO_3/MeOH) to give the of β-isomer betulinic acid (**3**). (**Scheme 2.4.2.3b**). The overall yield according to Pezzuto *et. al.*, is 55%.[116]

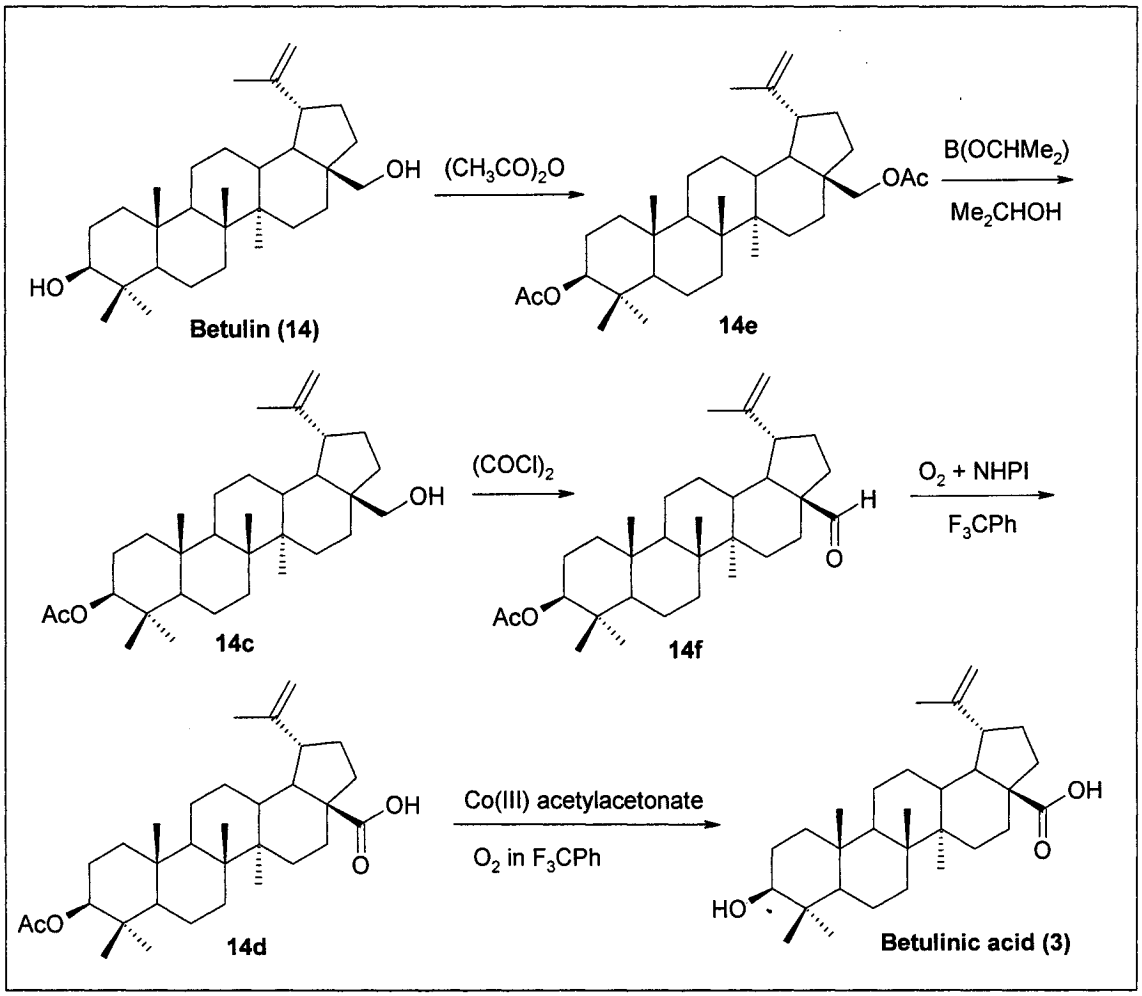
These authors concluded that the conversion of (**14**) to (**3**) involving Jones' oxidation, $NaBH_4$ reduction (**Scheme 2.4.2.3a**) followed by recrystallization from MeOH is the most efficient for large scale synthesis (30 g), despite the slightly lower yield.

Last year, Roshchin and his coworkers patented an improved method for the synthesis of (**3**) which involves oxidation of (**14**) to (**16**) using PCC complexed with acetic anhydride in DMF (2.5 or 3 : 1).[128] The $NaBH_4$ reduction of (**16**) yielded the α/β isomer of (**3**), (5 : 95). The natural isomer (β) was recrystallized from C2 and C-4 alcohol.

Another method for manufacturing (**3**) from (**14**) was documented by Krasutsky for patent application.[129] In this method, (**14**) was diacetylated to betulin 3,28-diacetate, then regioselectively mono-deacetylated using $B(OCHMe)_2$ in Me_2CHOH to afford betulin 3-acetate (**14c**). Betulin-3-acetate is oxidized with oxalyl chloride in CH_2Cl_2 to the corresponding betulinaldehyde 3-acetate (**14f**), which was further oxidized to betulinic acid 3-acetate (**14d**) using O_2 and N-hydroxyphthalimide (NHPI) in F_3CPh. Subsequent deacetylation with cobalt (III) acetylacetonate and O_2 in F_3CHPh furnished betulinic acid (**3**), (**Scheme 2.4.2.3c**).



Scheme 2.4.2.3b: The second Pezzuto synthesis of betulinic acid (**3**) from betulin (**14**).[116]



Scheme 2.4.2.3c: Synthesis of betulinic acid (**3**) from betulin (**14**) submitted for patent application.[129]

It is hard to see an advantage of this method (**Scheme 2.4.2.3c**) relative to ours or Pezzuto's short synthesis except for avoiding the use of chromium (Cr) as the oxidizing agent. Should betulinic acid (**3**) be eventually used as a human medicine, it is expected that oxidants other than Cr^{6+} be employed or data would need to be supplied to show that Cr residue meets safety levels.

2.4.2.4. Biosynthesis of betulinic acid and related pentacyclic triterpenoids

Betulinic acid (**3**) and pentacyclic triterpenoids are biosynthetically derived from the cyclization of (3S)-2,3-oxidosqualene or squalene oxide (**Scheme 2.4.2.4**).[65,130]

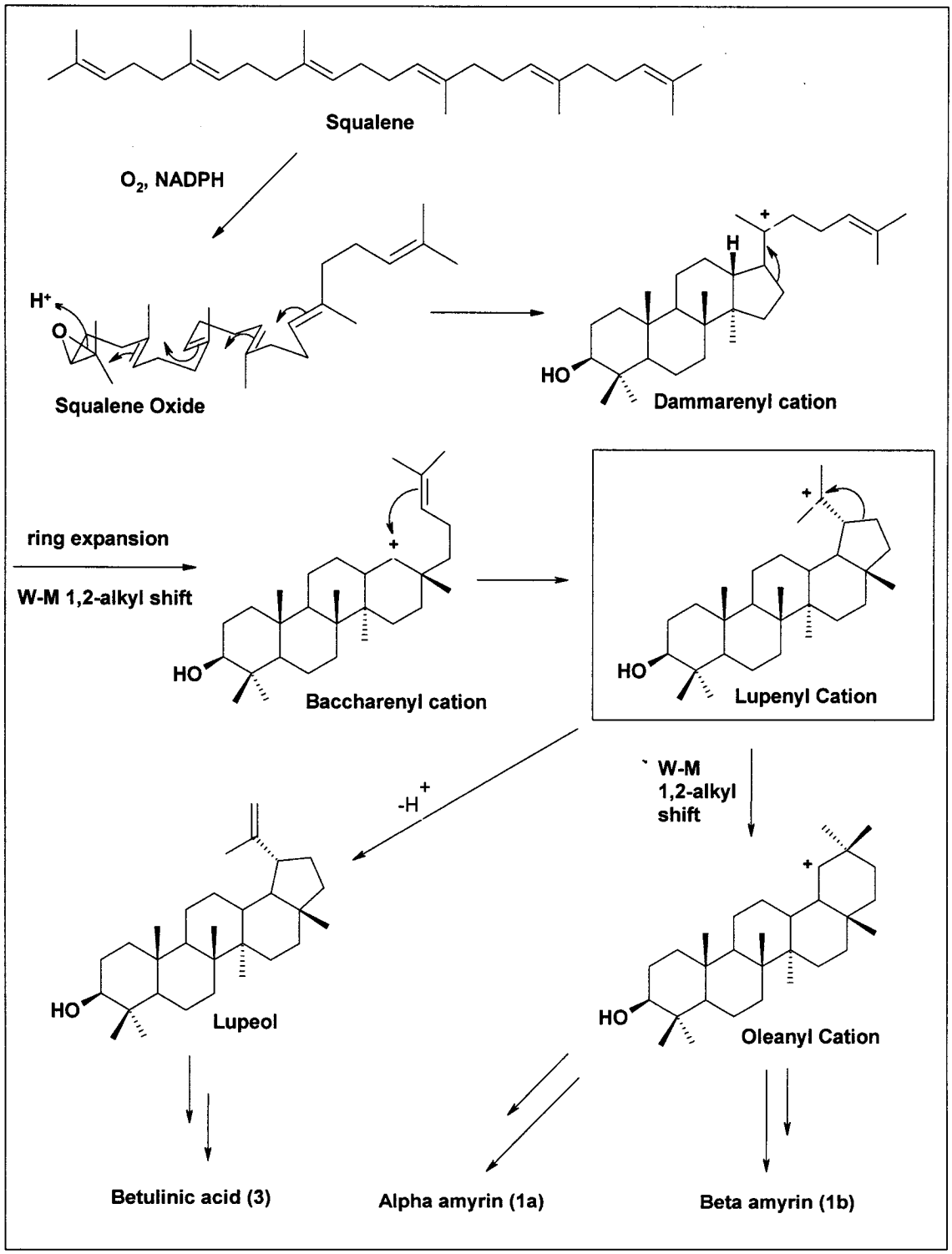
The cyclization mechanisms leading to lupeol and the amyrins have the early part in common. The reaction is initiated by an acid catalysed oxirane ring opening followed by cation-π cyclization via the pre-chair-chair-chair-boat conformation yielding the tetracyclic dammarenyl cation.

Ring expansion to baccharenyl cation and fifth ring closure give the tertiary lupenyl cation where branching of pathways begins. Thus, proton abstraction from one of the gem-dimethyl groups give lupeol which is eventually oxidized to (**3**) while ring expansion at the expense of a tertiary cation (for a secondary cation) give the olenyl cation.

The olenyl cation undergoes several Wagner-Meerwein rearrangements (W-M) and in accordance with the "biogenetic isoprene rule" proposed by Ruzika *et. al* to afford both the α- and the β-amyrins.[27] This rule postulates three 1,2-hydride shifts in α-amyrin (**1a**) formation (one from C-13 to C-18, one from C-18 to C-19 and one from C-19 to C-20) and two 1,2-hydride shifts in the β-amyrin (**1b**) formation (one from C-13 to C-18 and one from C-18 to C-19).

Finally, elimination of 12-pro-R or 12-pro-S hydrogen forms the 12(13) double bond and an inversion of stereochemistry occurs at C-18.

Seo *et. al.*, have demonstrated that both (**1a**) and (**1b**) can be further oxidized to their corresponding acids and hydroxylated compounds like 2α-hydroxyursolic acid (**2c**) and maslinic acid (**2d**) respectively.[27]



Scheme 2.4.2.4: Biosynthesis of betulinic acid (**3**) and related pentacyclic triterpenoids isolated from Margraviaceae plant extracts.

2.5. Derivatization of betulinic acid

The C-3 hydroxyl, C-17 carboxylic acid and the C-20 exo-methylene moiety in betulinic acid (**3**) can easily be modified (**Figure 2.5**). It was envisioned that simple chemical modifications at these positions could provide important, and even novel derivatives of betulinic acid with potent anti-anxiety activity superior to betulinic acid. Such entities with superior properties can potentially be developed as an anti-anxiety therapeutic agent (single entity drug).

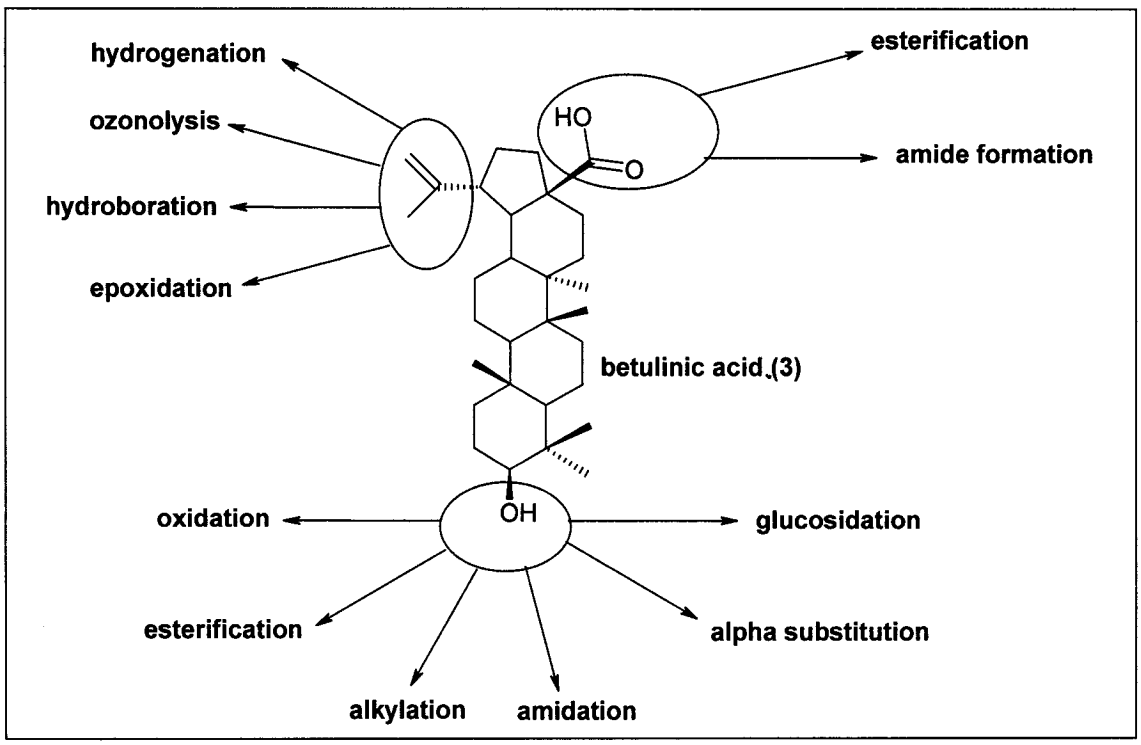


Figure 2.5: Derivatization of betulinic acid (3).

A series of betulinic acid derivatives were then synthesized. The carboxylic acid function of betulinic acid was modified by esterification and amide formation. Hydrogenation, ozonolysis, hydroboration and epoxidation should be easily carried out on the terminal double bond. The 3β-OH group can be esterified, alkylated, oxidized, α-substitued and, or glycosylated.

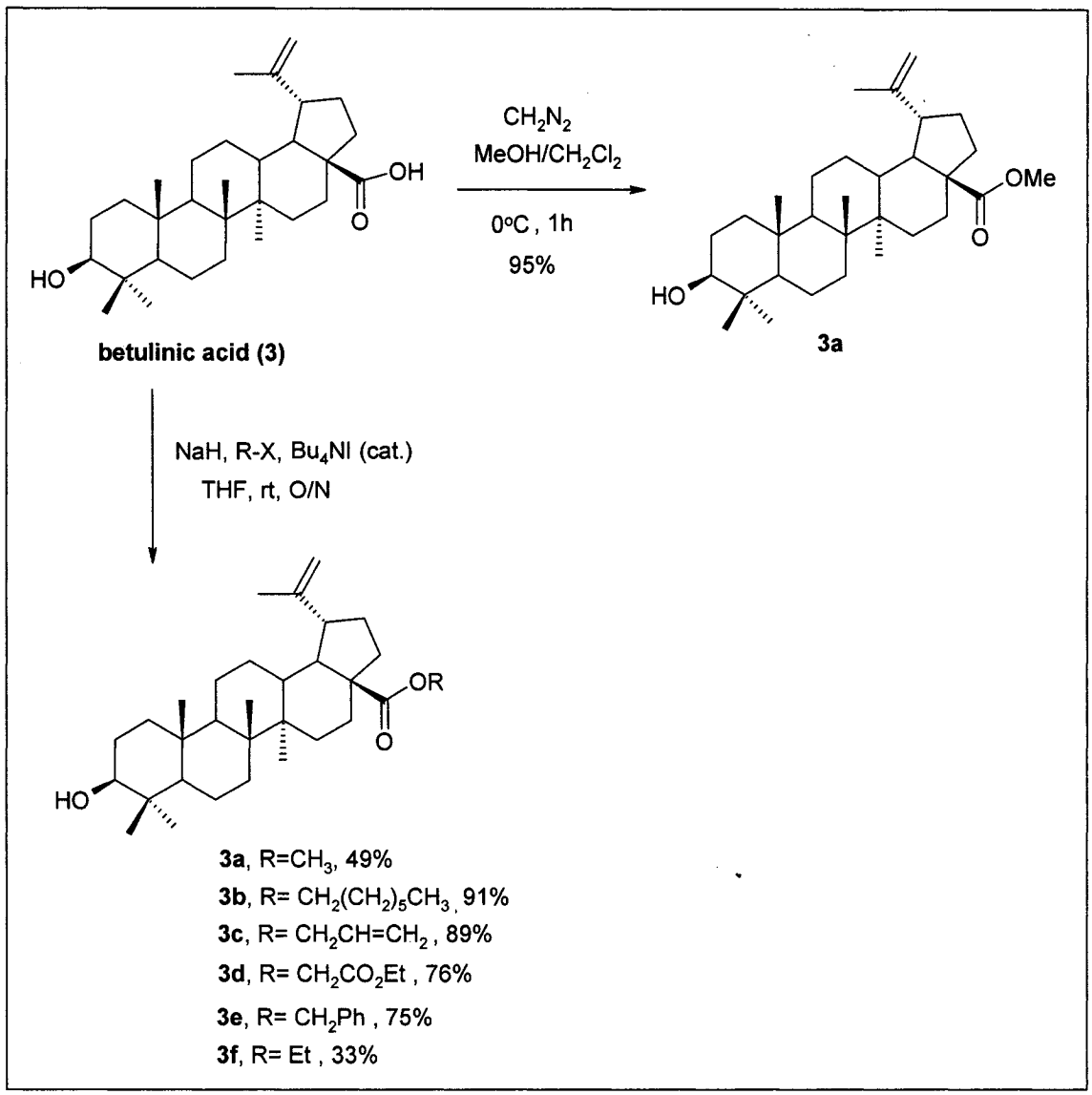
2.5.1. Esterification of betulinic acid

The classic esterification procedures ($MeOH/H^+$, $SOCl_2/MeOH$) failed to esterify betulinic acid (**3**), and resulted in recovered starting materials. This is presumably due to the hindered tertiary nature of the β-positioned carboxylic acid group (neopentenyl system). This observation is consistent with a recent report by Snyder and Deng who synthesized the methyl ester of an analog of betulinic acid in 87% yield by nucleophilic substitution by the carboxylate anion (MeI/CsF) after failed attempts with the classic esterification method.[131] This phenomenon has also been noted in older literature.[24]

In our laboratory, the methyl ester of betulinic acid (**3a**) was prepared by treating (**3**) with diazomethane to furnish (**3a**) in excellent yield (**Scheme 2.5.1**). Surprisingly, in several attempts to prepare the 3-O-methyl ether derivative of betulinic acid, with (KOH, Me_2SO_4, MeOH), (KOH, DMSO, CH_3I), (NaH, Me_2SO_4, THF) and (KOH, DMSO, Me_2SO_4 heated to 100°C) we obtained instead the methyl ester of betulinic acid (**3a**) in moderate yields (40-49%), and with no traces of the 3-O-methyl ether derivative of betulinic acid being formed. Moreover, when (**3**) was treated with silver I oxide and methyl iodide in $CHCl_3$, (**3a**) was obtained in 58% yield with recovered starting material.

It is interesting to note that, the 3-O-methyl ether derivative of betulinic acid has been previously prepared in 97% yield under vigorous conditions (NaH, CH_3I, THF), refluxing for about 41 hours. This is the only single report of the 3-O-methyl ether derivative of betulinic acid (3β-methoxylup-20(29)-en-28-oic acid) known in the literature.[132]

The general utility of the esterification conditions (NaH, R-X, Bu_4NI, THF), was demonstrated through the synthesis of several novel ester derivatives of betulinic acid (**3b-d**), and the known esters (**3a**), (**3e**) and (**3f**) in fairly good yields, except in the case of (**3f**), (**Scheme 2.5.1a**).

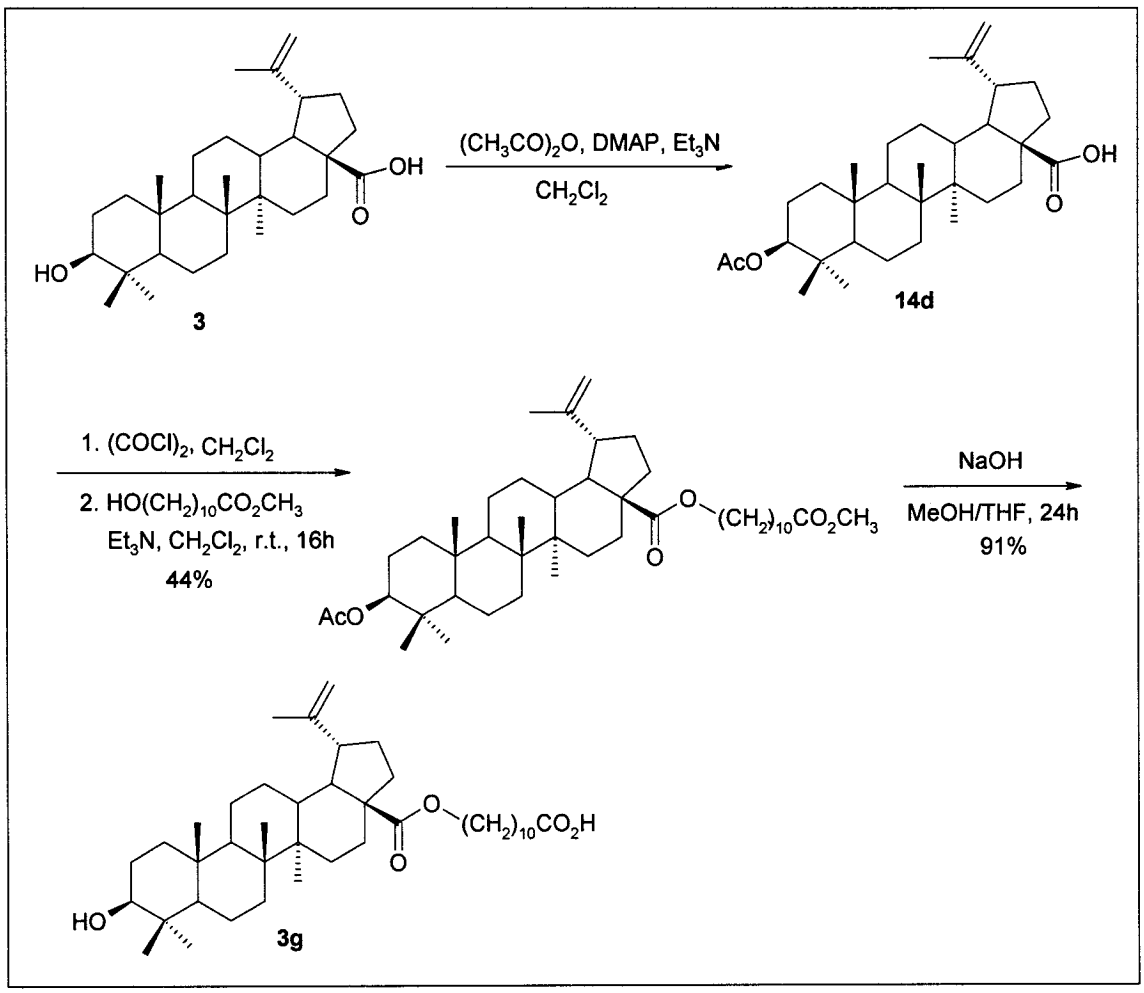


Scheme 2.5.1a: Preparation of the ester derivatives of betulinic acid (**3**).

A survey of the literature revealed several esterification procedures that have been employed in the esterification of betulinic acid and dihydrobetulinic acid. For instance, in 1994, betulinic acid 28-O-carboxymethylmethylate was synthesized by reacting betulinic acid with methyl chloroacetate (NaH, DMF) overnight but in low yield (33.5%).[19]

In 1996, 11-[3β-Hydroxylup-20(29)-en-28-oyl]oxy]undecanoic acid (**3g**) was obtained via a 4-steps synthesis from betulinic acid.[132] Crude acid chloride of 3β-acetyl betulinic acid (prepared from 3β-acetyl betulinic acid (**14d**) and oxalyl

chloride) was treated with methyl 11-hydroxyundecanoate (CH_2Cl_2, Et_3N, rt, 16h to furnish the desired ester (**3g**) after deprotection with aqueous NaOH in MeOH/THF (**Scheme 2.5.1b**). In 1997, benzyl betulinate (**3e**) was obtained in 94% yield after refluxing betulinic acid, benzyl bromide and K_2CO_3 in anhydrous acetone for 2h.[133]



Scheme 2.5.1b: A literature synthesis of an ester derivative of betulinic acid, (**3g**).[132]

The ^{1}H and ^{13}C NMR shift assignments of the synthetic ester derivatives of betulinic acid (**3a-3f**) were largely accomplished through comparison of their spectra with published values for betulinic acid (**3**) and its methyl ester derivative or methyl betulinate (**3a**). The ^{1}H and ^{13}C NMR resonances of the esters (**3a-3f**) mimicked those observed for (**3**) closely, but additional resonances appeared due to the new ester functionalities introduced at C-28.

The key NMR resonances observed for these esters are summarised in **Tables 2.5.1a** and **2.5.1b**.

The IR spectra of esters (**3a-3f**) contained carbonyl and hydroxyl stretching frequencies at 1693-1762 and at 3405-3536 cm^{-1} respectively.

The fragmentation patterns in the MS of (**3a-3f**) with ion peaks at m/z 262, 248, 233, 220, 219, 207 and 189 are typical of analogs of betulinic acid (**Scheme 2.2.2**).

Table 2.5.1a: The key ^{1}C NMR resonances of esters **3a-3f** (δ, ppm, $CDCl_3$).

Carbon	3a	3b	3c	3d	3e	3f
C-3	79.0	78.9	78.9	78.9	78.9	78.9
C-20	150.5	150.7	150.6	150.5	150.5	150.7
C-29	109.6	109.5	109.6	109.6	109.5	109.5
C-28	176.7	176.6	175.7	175.4	175.7	176.1
CO_2Me	51.3					
CO_2CH_2		64.0	64.6	61.3	65.7	59.8
CO_2CH_2				60.2		
CH=			132.5			
CH_2=			118.1			
Ph					136.4	
					128.4	
					128.2	
					128.0	

Table 2.5.1b: The key ^{1}H NMR resonances of esters **3a-3f** (δ, ppm, $CDCl_3$).

Proton	**3a**	**3b**	**3c**	**3d**	**3e**	**3f**
H-3α	3.15, dd J=10.3, 5.5 Hz	3.16, dd J=9.9, 5.5 Hz	3.15, dd J=10.2, 5.5 Hz	3.15,dd J=10.4, 5.4 Hz	3.15, dd J=10.3, 5.2 Hz	3.16,dd J=10.3, 5.4 Hz
H-19	2.97, ddd J=10.8, 5.9 Hz	3.00, ddd J=10.8, 4.8 Hz	3.00, dd J=11.1, 3.9 Hz	2.95, dd J=10.8, 4.5 Hz	3.00, ddd J=11.0, 4.9 Hz	3.00,ddd J=11.0, 4.9 Hz
H-29	4.70, d J=2.2 Hz	4.70, d J=1.6	4.70, br s	4.70, d J=1.6	4.70, br s	4.70, br s
H-29'	4.56, dd J=2.2, 1.4 Hz	4.56, br s	4.54, br s	4.57, br s	4.57, br s	4.57, br s
C-30-Me	1.65, s	1.66, s	1.65, s	1.66, s	1.68, s	1.65, s
C-27-Me	0.93, s	0.94, s	0.93, s	0.94, s	0.93, s	0.94, s
C-26-Me	0.93, s	0.89, s	0.93, s	0.93, s	0.92, s	0.94, s
C-25-Me	0.79, s	0.79, s	0.79, s	0.79, s	0.73, s	0.79, s
C-24-Me	0.72, s	0.73, s	0.72, s	0.72, s	0.72, s	0.73, s
C-23-Me	0.88, s	0.86, s	0.88, s	0.90, s	0.77, s	0.89, s
CO_2Me	3.63, s					
CO_2CH_2		4.04, m	4.54, br s	4.57, m		4.11, m
$CH_X=CH_AH_B$			5.90, m			
$CH_X=CH_AH_B$			5.31, dd J=17.2, 1.4			
$CH_X=CH_AH_B$			5.20, dd J=10.3, J=1.4			
$CO_2CH_2CO_2Et$				4.19, q J=7.2 Hz		
CH_2Ph					5.14, d J=12.3 Hz	
CH_2Ph					5.06, d J=12.3 Hz	
Ph					7.32, br s	

2.5.1.1. Methyl betulinate

Betulinic acid was best methylated with ethereal diazomethane (CH_2Cl_2/MeOH, 0°C, 1h) to afford betulinic acid methyl ester or methyl betulinate (**3a**) as a white solid in 95% yield with a melting point of 220-221°C (lit.[111] 221-222°C, lit.[134] 222-224°C from MeOH and 224-225°C from benzene, lit.[23] 223-224C, lit.[21,24,135] 224-225°C).

The EIMS spectrum of (**3a**) displayed a weak $[M]^+$ peak at m/z 470 corresponding to the molecular formula $C_{31}H_{50}O_3$ which was confirmed by HRMS. The fragmentation pattern was almost identical with previously reported MS data for (**3a**).[136] The IR spectrum of (**3a**) displayed strong OH and C=O absorptions at 3405 and 1726 cm^{-1}, respectively.

The key signals in the ^1H NMR spectrum of (**3a**), were observed for the methylenic protons at δ 4.70 (d, J=2.2 Hz, H-29) and at δ 4.56 (dd, J=2.2 Hz, J=1.4 Hz, H-29'); for the methoxy protons at δ 3.63 (s, 3H); for the methine proton attached to the hydroxyl group at δ 3.15 (dd, J=10.3 Hz, J=5.5 Hz, H-3α) and for the allylic proton at δ 2.97 (ddd, J=10.8 Hz, J=10.8 Hz, J=5.9 Hz, H-19). The vinylic methyl group resonated at δ 1.65 (s, H-30) while the five tertiary methyl groups resonated at δ 0.93 (s, 6H, H-26/H-27), 0.88 (3H, H-23), 0.79 (3H, H-25) and at δ 0.72 (, 3H, H-24), (**Figure 2.5.1.1a**).

The key resonances in the ^{13}C NMR spectrum of (**3a**), corresponded to the ester carbonyl at δ 176.7 (C-28); the olefinic carbons at δ 150.6 (C-20) and at δ 109.6 (C-29); the hydroxyl bearing carbon at δ 79.0 (C-3) and the methoxy carbon at δ 51.3 (**Figure 2.5.1.1b**). Both the carbon and the proton chemical shifts were in excellent agreement with literature values.[114,135,136]

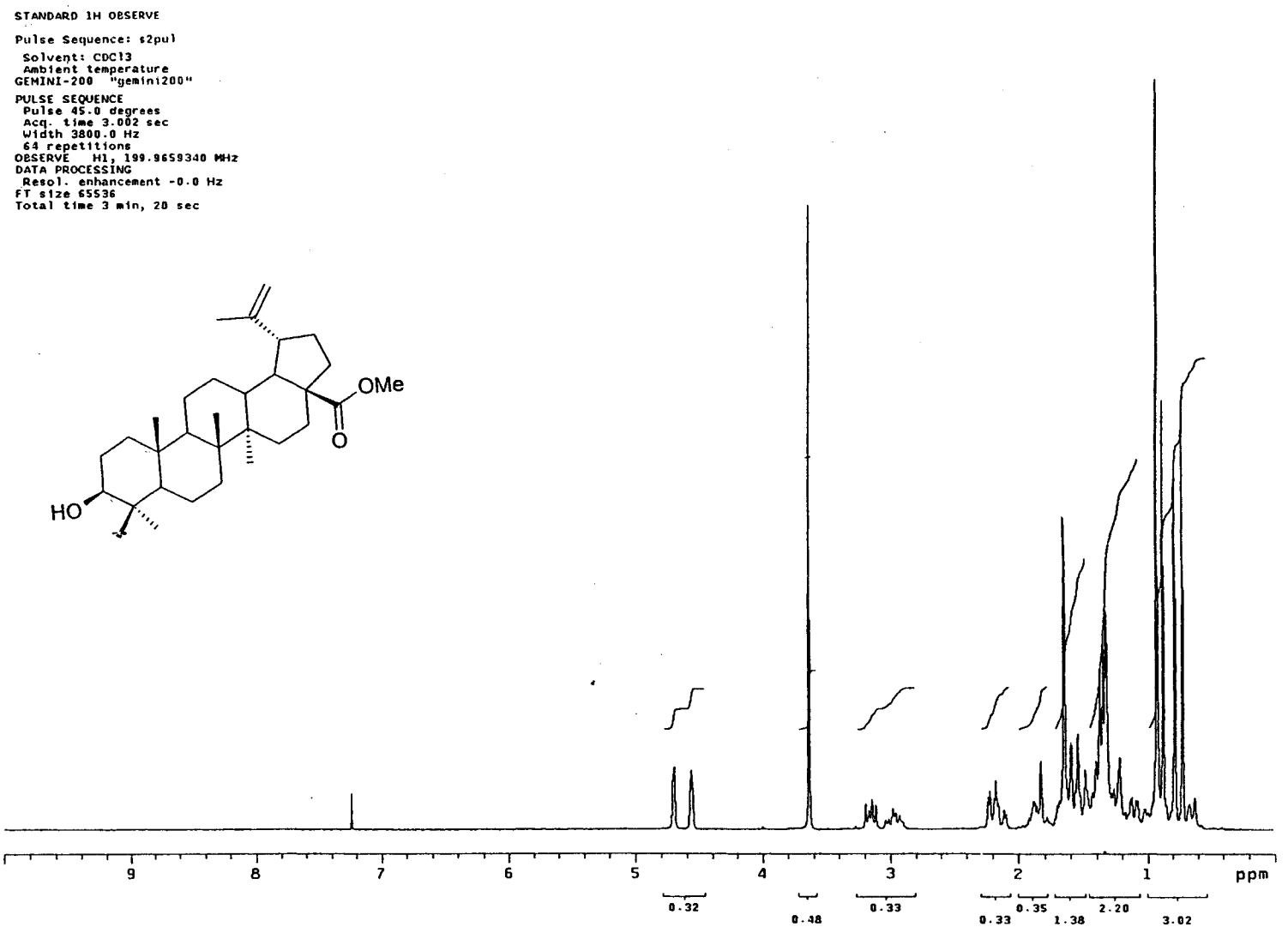


Figure 2.5.1.1a: ^{1}H NMR spectrum of methyl betulinate or betulinic acid methyl ester (**3a**).

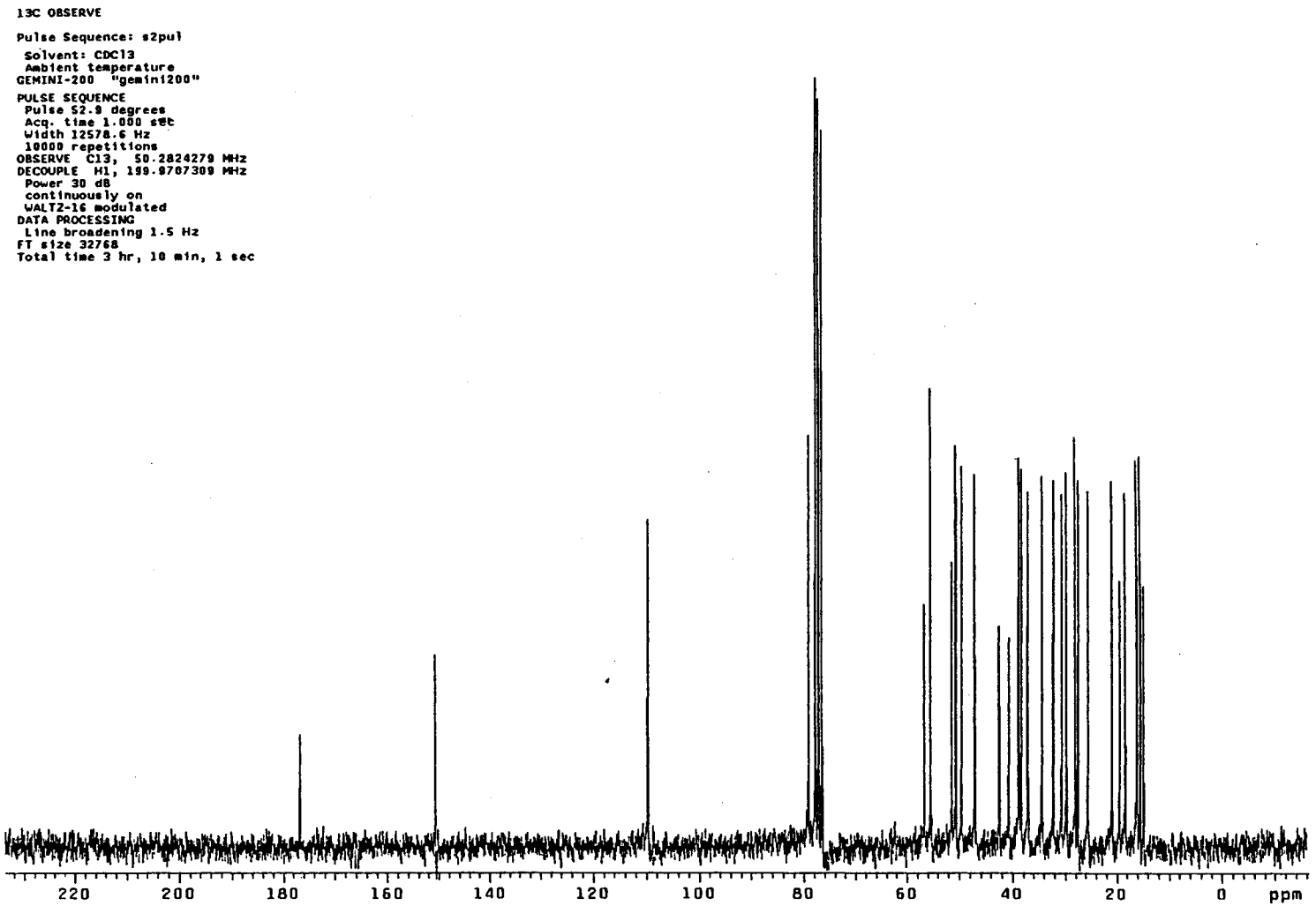


Figure 2.5.1.1b: ^{13}C NMR spectrum of methyl betulinate or betulinic acid methyl ester (**3a**).

2.5.1.2. Heptyl betulinate

The reaction of 1-bromoheptane with betulinic acid in the presence of sodium hydride (NaH) with catalytic amounts of tetrabutylammonium iodide (10% Bu_4NI) in THF at room temperature overnight, resulted in the formation of heptyl betulinate (**3b**) as a white fluffy solid, m.p. 54-56°C, in 91% yield. Ester (**3b**) is a new compound.

The EI mass spectrum of (**3b**) exhibited a $[M]^+$ ion peak at m/z 554 which agreed with the predicted molecular formula $C_{37}H_{62}O_3$; this was confirmed by HRMS. The fragmentation pattern was consistent with a betulinic acid derivative of the proposed structure.

The ^1H NMR spectrum of (**3b**), was characterized by the disappearance of the methoxy singlet at δ 3.63 observed for (**3a**) and the appearance of a new signal at δ 4.04 (m, 2H) ascribed to the CO_2CH_2 part of the heptyl ester moiety. The remaining protons of the ester moiety appeared at the heavily overlapping region of the spectrum at δ 1.9-0.7 and was not easily identified (**Figure 2.5.1.2a**).

The ^{13}C NMR data of (**3b**) records five new resonances at δ 64.0 for CO_2CH_2 part of the ester moiety and at δ 31.8, 28.9, 28.7 and 26.1 ppm for the remaining heptyl portion of the ester moiety.

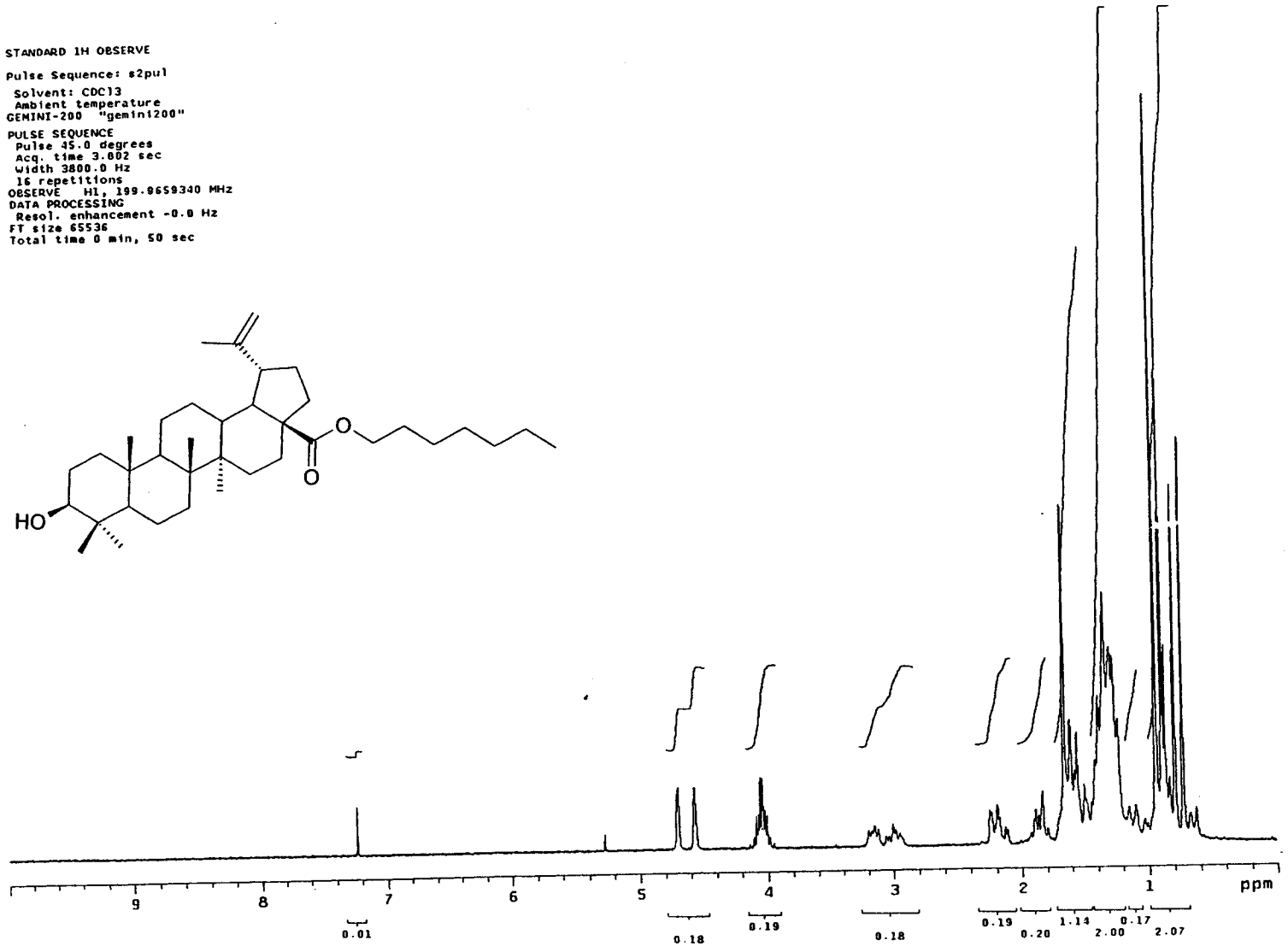


Figure 2.5.1.2a: ^{1}H NMR spectrum of heptyl betulinate (**3b**).

2.5.1.3. Allyl betulinate

The esterification of betulinic acid with allyl bromide using the same procedure described above, furnished allyl betulinate (**3c**) as a white fluffy solid, m.p. 65-68°C in 89% yield after silica gel column chromatography. Ester (**3c**) has not been reported in the literature.

Inspection of the EIMS of (**3c**) revealed a $[M]^+$ at m/z 496 which was compatible with the anticipated molecular formula $C_{33}H_{52}O_3$; and confirmed by HRMS. The fragment losses recorded in the experimental section were consistent with a lupane derived triterpernoid of the postulated structure.

The ^1H NMR spectrum of (**3c**) exhibited a new broad three proton singlet at δ 4.54 (br s, 3H) assigned to one of the protons of the methylene system (H-29), which apparently resonated together with the two protons of the CO_2-CH_2-C=C ester moiety. The other new signals were centred at δ 5.31 (dd, J=17.2, J=1.4 Hz, H_A), at δ 5.20 (dd, J=10.3 Hz, J=1.4 Hz, H_B) and at δ 5.90 (m, H_x) assignable to the ABX system of the allyl system of the ester moiety was (**Figure 2.5.1.3a**).

In the ^{13}C NMR spectrum of (**3c**), three new signals appeared at δ 132.5, 118.1 and at δ 64.6 attributable to the respective olefinic carbons and the CO_2CH_2 part of the allyl ester moiety.

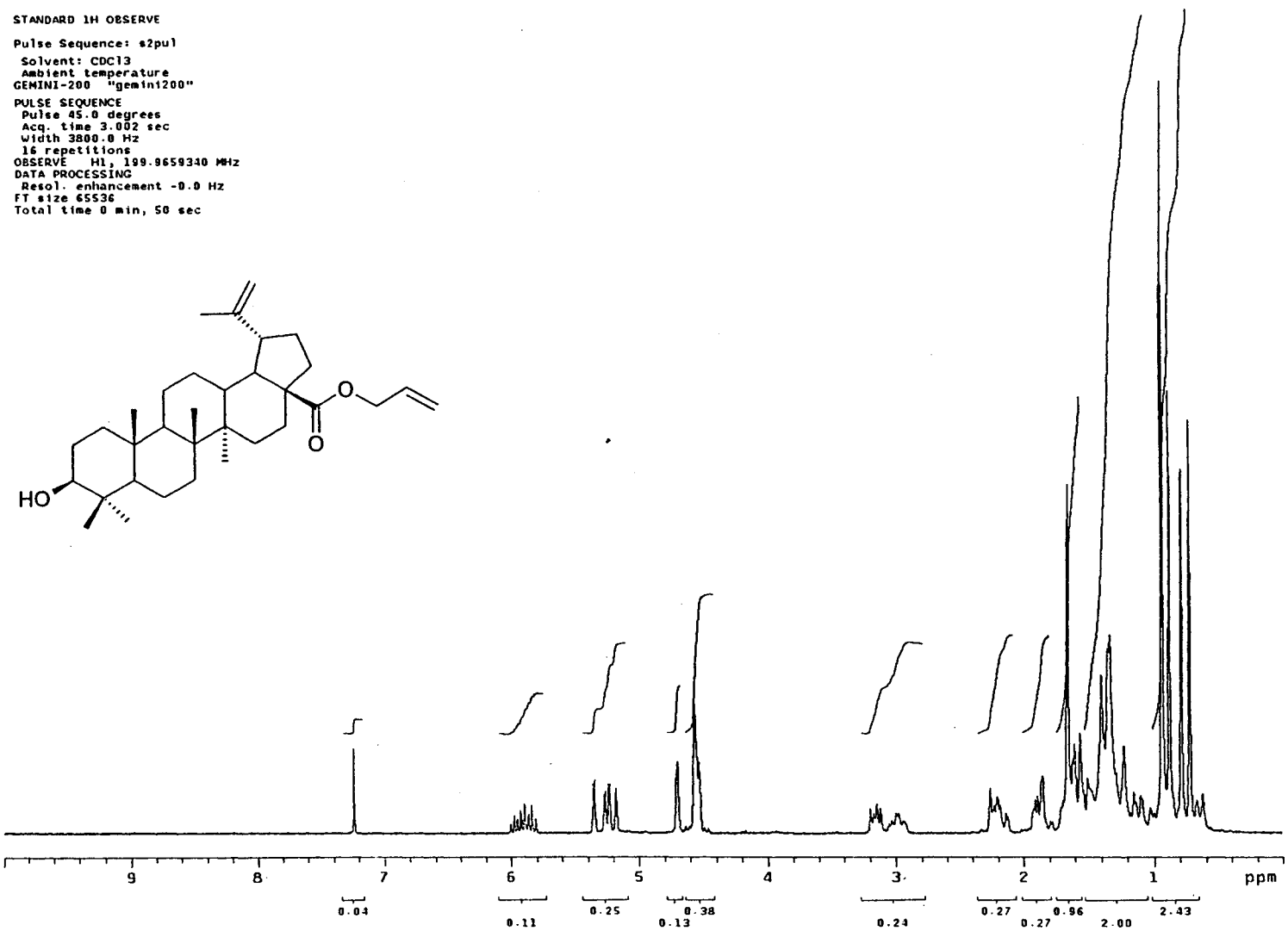


Figure 2.5.1.3a: ^{1}H NMR spectrum of allyl betulinate (**3c**).

2.5.1.4. Ethyl acetoxy betulinate

The reaction of betulinic acid with ethyl bromoacetate under the same reaction conditions described above resulted in the formation of ethyl acetoxy betulinate (**3d**) as a white fluffy solid, m.p. 67-69°C, in 76% yield, after silica gel column chromatography. Ester (**3d**) is a new compound.

The $[M]^+$ at *m/z* of 542 in the EIMS of (**3d**) agreed with its molecular formula $C_{34}H_{54}O_5$; which was further establisehd by HRMS. The fragmentation pattern listed in the experimental section was characteristic of a betulinic acid derivative of the postulated structure.

Significant signals in the ^1H NMR spectrum of (**3d**) included a broad singlet integrating to three protons at δ 4.57 (br s, 3H) attributable to one of the methylenic protons (H-29) signal being merged together with the signals for the two protons of the CO_2-CH_2-CO_2 part of the ester moiety. A new quartet at δ 4.19 (q, J=7.2 Hz, 2H) was attributed to the CH_2 portion of the ethyl group (**Figure 2.5.1.4a**).

The ^1C NMR spectrum of (**3d**) recorded new resonances for the ethyl acetoxy ester moiety at δ 168.0 (C=O), 61.3, 60.2 and at δ 14.1.

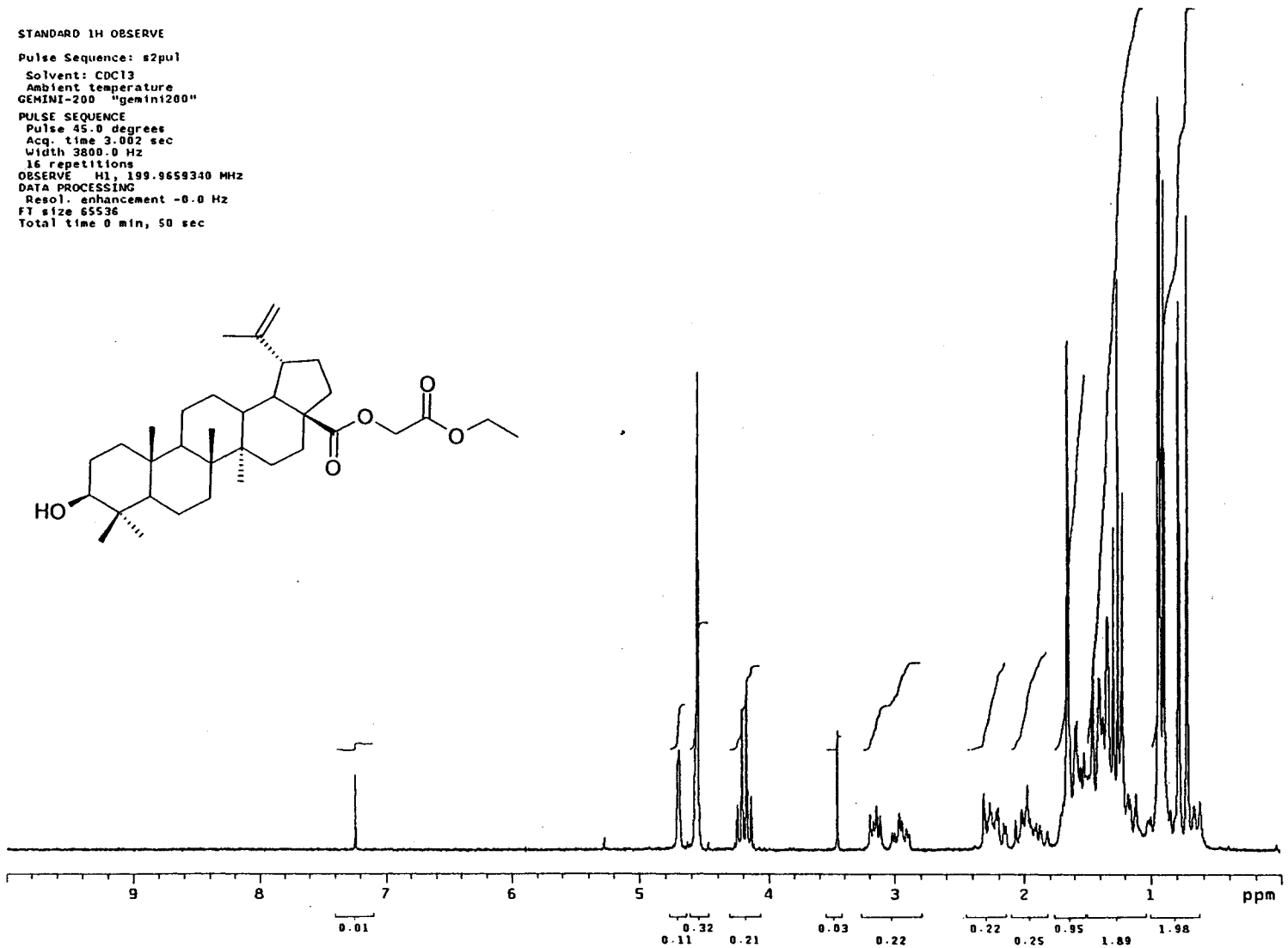


Figure 2.5.1.4a: ^{1}H NMR spectrum of ethyl acetoxy betulinate (**3d**).

3.5.1.5. Benzyl betulinate

Using the same procedure outlined above, betulinic acid was esterified with benzyl bromide furnishing benzyl betulinate (**3e**) as a white solid, m.p.187-189°C (lit.[133] 184-185°C) in 75% yield after silica gel column chromatography.

The molecular ion peak $[M]^+$ at m/z 546 of the EIMS of (**3e**) was consistent withthe desired molecular formula $C_{37}H_{54}O_3$; this was confirmed by HRMS. The mode of fragmentation recorded in the experimental section is typical of a betulinic acid derivative of the proposed structure. Resonances in the ^1H NMR spectrum of (**3e**) agreed with published data (**Table 2.5.1b**).[133] The ^{13}C NMR data of (**3e**) have not been previously reported in the literature. The key signals at δ 65.7,136.4, 128.4, 128.2 and at δ 128.0 in the ^{13}C NMR spectrum of (**3e**) confirmed the presence of the benzyl moiety (**Table 2.5.1a**).

2.5.1.6. Ethyl betulinate

The esterification of betulinic acid with ethyl iodide under the same reaction conditions described above proceeded with limited success. After stirring the reaction mixture for 2 days at room temperature, ethyl betulinate (**3f**) was isolated as a white solid, m.p.197-198°C (lit.[23] 200-201°C), in 33% yield (with recovered starting material) after silica gel column chromatography. Robertson and his colleagues have prepared (**3f**) by treating betulinic acid (**3**) with excess diazoethane in chloroform.[23]

The EIMS of (**3f**) exhibited the $[M]^+$ at m/z 484 which was in conformity with its molecular formula $C_{32}H_{52}O_3$; this was verified by HRMS. The fragment ion losses listed in the experimental section were consistent with the postulated structure. The ^1H NMR spectrum of (**3f**) revealed a new multiplet at δ 4.11 integrating to two protons ascribed to the CH_2 portion of the ester moiety (**Table 2.5.1b**). The new resonances at δ 59.8 in the ^{13}C NMR of (**3f**) confirmed the presence of the ethyl ester moiety (**Table 2.5.1a**).

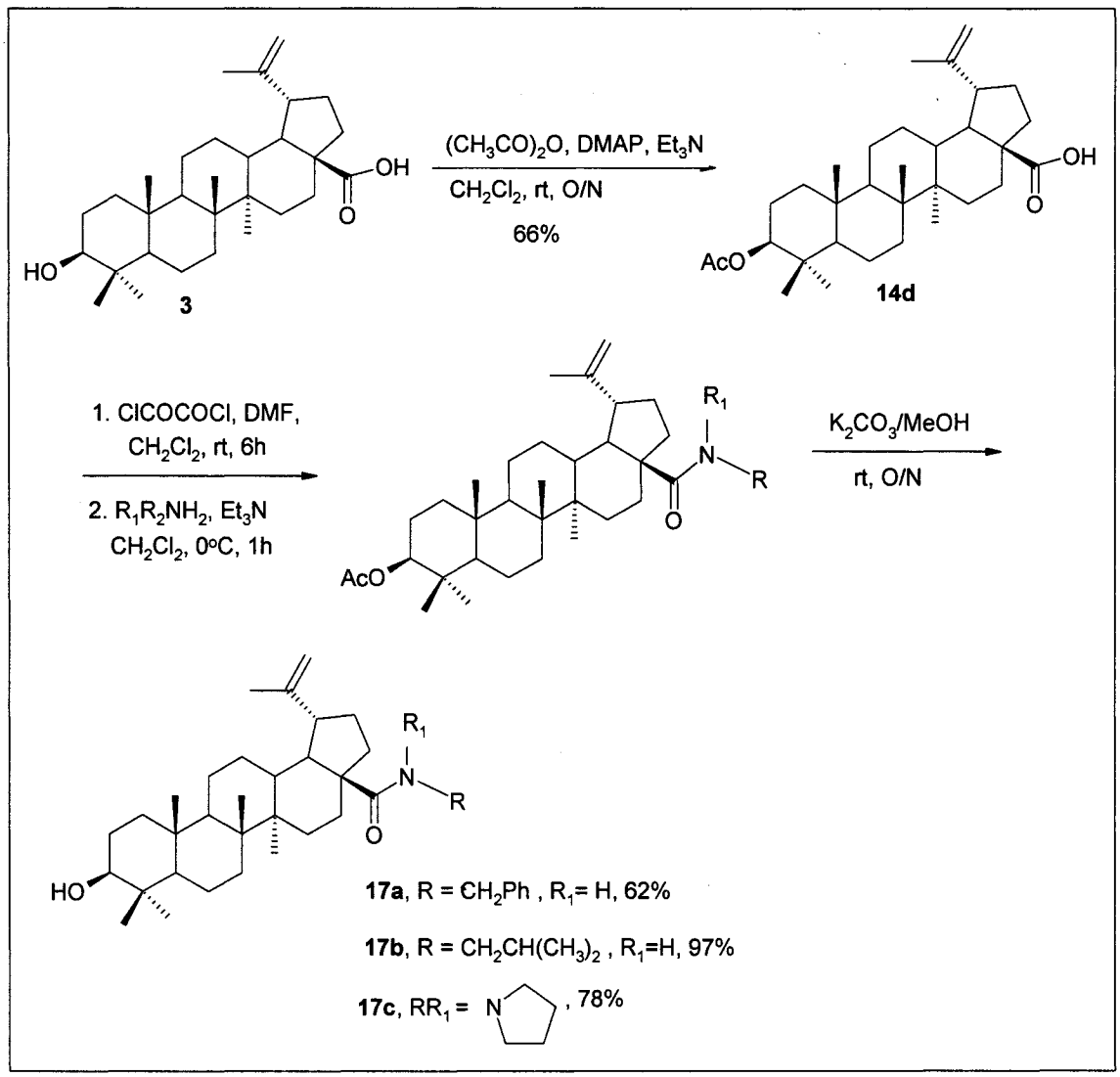
2.5.2. Preparation of betulinic acid amides

Several novel amides of betulinic acid (**17a-17c**) were synthesized via a 4-step synthesis in quite good yields. 3β-acetyl betulinic acid (**14d**) [which was prepared by reacting betulinic acid with acetic anhydride (DMAP, Et_3N,CH_2Cl_2)], was treated with oxalyl chloride and a few drops of DMF in CH_2Cl_2 to afford 3β-acetoxylup-20(29)-en-28-oyl chloride, which was then coupled with the appropriate amine in the presence of Et_3N in CH_2Cl_2 at 0°C, furnishing the corresponding amide, after aqueous work up and cleavage of the acetate protecting group with K_2CO_3/MeOH (**Scheme 2.5.2**).

Compound (**14d**) was purified as a white solid, m.p. 275-277°C (lit.[137] 258°C, lit.[16] 269-271C, lit.[19]) in 66% yield after silica gel column chromatography. The ^1H and ^{13}C NMR data of (**14d**) agreed with published data.[19,137]

Amides (**17a-17c**) are all new compounds. The ^1H NMR shifts of these amides were assigned by comparison with literature data of similar amide derivatives of betulinic acid (**3**) recently reported in the literature.[138,139]

Analysis of the ^1H and ^{13}C NMR shifts of (**17a-17c**) revealed diagnostic features attributable to (**3**) but with new signals arising from the newly introduced amide functionalities at C-28 (**Tables 2.5.2a** and **2.5.2b**).



Scheme 2.5.2: Synthesis of amide derivatives of betulinic acid (**17a-17c**).

Tables 2.5.2a : The key ^{1}H NMR signals for amides (**17a-17c**) (δ, ppm $CDCl_3$).

Proton	17a	17b	17c
CONH	5.82, t J=5.7 Hz	5.61, t J=5.8 Hz	-
H-3α	3.16, m	3.12, m	3.09, m
H-13	2.47, dt J=12.2, J=3.2 Hz	2.46, dt J=12.2, J=3.2 Hz	2.46, dt J=12.1, J=3.4 Hz
H-19	3.16, m	3.12, m	3.09, m
H-29	4.72, d J=2.1 Hz	4.71, d J=2.2 Hz	4.69, d J=2.2 Hz
H-29'	4.58, d J=2.1 Hz	4.56, d J=2.3 Hz	4.54, d J=2.3 Hz
C-30-Me	1.67, s	1.65	1.65
C-27-Me	0.94, s	0.94, s	0.93, s
C-26-Me	0.94, s	0.94, s	0.93, s
C-25-Me	0.80, s	0.80, s	0.79, s
C-24-Me	0.74, s	0.74, s	0.72, s
C-23-Me	0.89, s	0.89, s	0.91, s
N-CH_2Ph	4.47, dd J=14.7, 5.8 Hz		
N-CH_2Ph	4.35, dd J=14.0, 5.6 Hz		
Ph	7.29, m		
N-CH_2CH		3.12, m	
N-CH_2CH		2.98, m	
H_2C-N-CH_2			3.40, m

Tables 2.5.2b: The key ^{13}C NMR signals for amides **17a-17c** (δ, ppm $CDCl_3$).

Carbon	17a	17b	17c
CONH	175.9	175.9	173.6
C-3	79.0	79.0	78.9
C-20	151.0	151.9	151.6
C-29	109.3	109.2	108.9
Ph	139.2		
	128.7		
	127.8		
	127.3		
CH_2Ph	43.3		
$CH_2CH(CH_3)_2$		46.6	
		29.4	
		20.2	
$N(CH_2CH_2)_2$			46.3
			25.6

2.5.2.1. Betulinic acid benzyl amide

When the acid chloride of 3β-acetyl betulinic acid was coupled with benzylamine in the presence of Et_3N in CH_2Cl_2 at 0°C, N-[3β-hydroxylup-20(29)-en-28-oyl]-benzylamine (**17a**) was obtained after aqueous work up and cleavage of the acetate protecting group with K_2CO_3/MeOH (**Scheme 2.5.2**). Compound (**17a**) was isolated in 62% yield as a white solid, m.p. 239-242°C, after silica gel column chromatography (hexane-EtOAc solvent gradient).

The EIMS of (**17a**) yielded a $[M]^+$ at m/z 545 which agreed with the anticipated molecular formula $C_{37}H_{55}NO_2$; this was confirmed by HRMS. The fragmentation pattern reported in the experimental section is typical of a betulinic acid derivative of the postulated structure. The strong IR bands at 3448 and 1638 cm^{-1} indicated the presence of OH and CONH functionalities.

In the ^{1}H NMR spectrum of **17a** (**Figure 2.5.2.1a**), a new one proton triplet with a coupling constant of 5.7 Hz at δ 5.82 characteristic of the CONH moiety, couples with the CH_2 protons of the benzyl moiety at δ 4.47 (dd, J=14.7 Hz, J=5.8 Hz, 1H) and at δ 4.35 (dd, J=14.0 Hz, J=5.6 Hz, 1H). This was also readily determined from the COSY spectrum (**Figure 2.5.2.1b**). The phenyl group resonated at δ 7.29 (m, 5H). A distinctive signal due to H-13 was observed at δ 2.47 (dt, J=12.2 Hz, J=3.2 Hz). As usual, the isopropenyl moiety resonated at δ 4.72 (d, J=2.0 Hz, H-29), δ 4.58 (d, J=2.1 Hz, H-29') and at δ 1.67 (s, 3H, H-30). The two protons multiplet at δ 3.16 was assignable to H-3α and H-19 while the five singlets at δ 0.94 (s, 6H, H-27/H-26), 0.89 (s, 3H, H-23), 0.80 (s, 3H, H-25), and at δ 0.74 (s, 3H, H-24) accounted for the to the five tertiary methyl groups (**Table 2.5.2a**).

The ^{13}C NMR spectrum of **17a** (**Figure 2.5.2.1c**), contained diagnostic resonances at δ 175.9 (C-28), 151.0 (C-20), 109.3 (C-29) and at δ 79.0 (C-3) attributable to the CONH moiety, the two olefinic carbons, and the alcohol bearing carbon respectively. The aromatic carbons were evident from the signals at δ 139.2, 128.7, 127.8 and at δ 127.3 (**Table 2.5.2b**). Carbon resonances at δ 46.7, 43.3 and 37.8 were assigned to C-19, CH_2 carbon of the benzyl moiety and C-13 respectively using the HMQC spectrum (**Figure 2.5.2.1d**) and the DEPT 135 spectrum (**Figure 2.5.2.1e**).

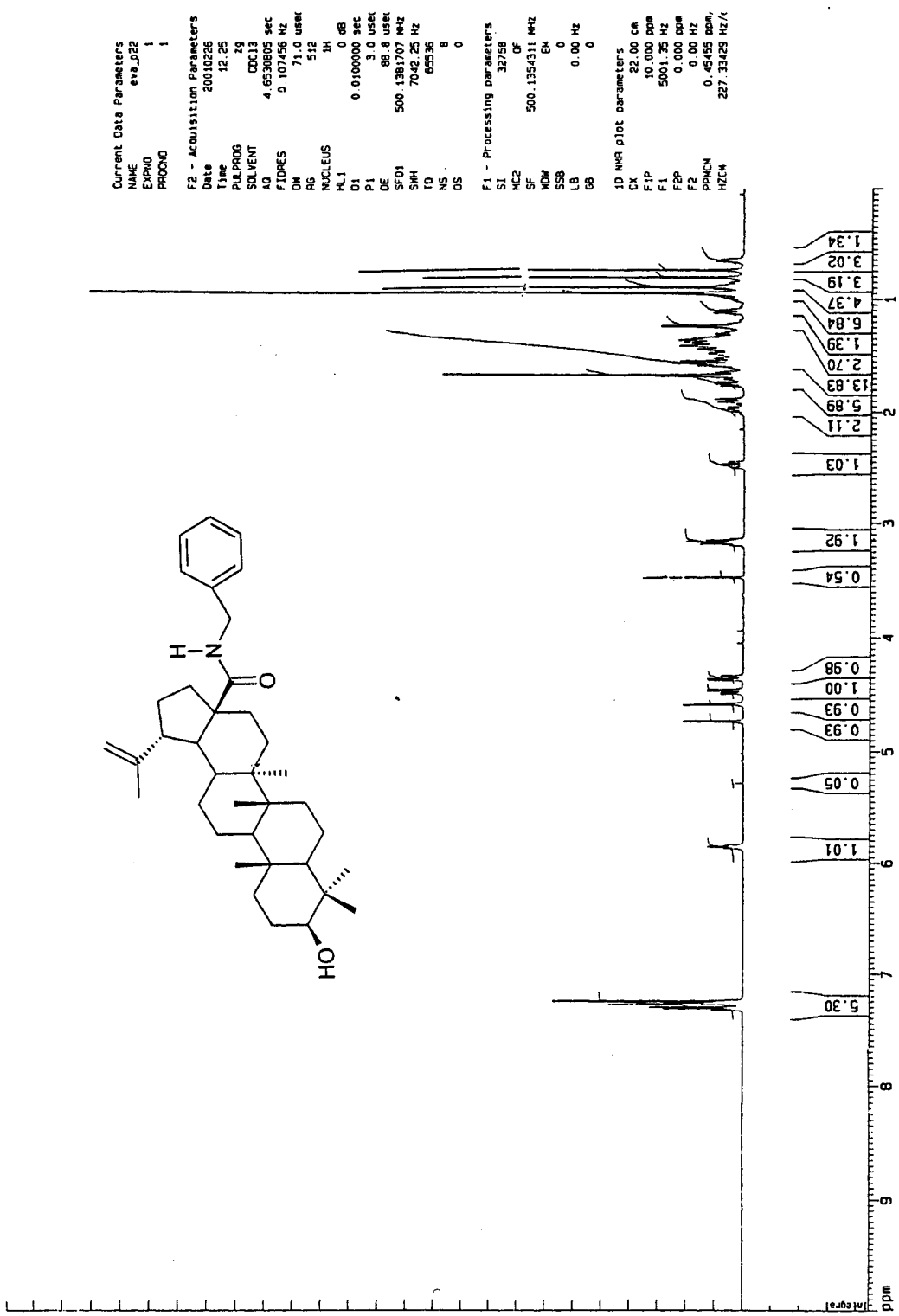


Figure 2.5.2.1a: ^{1}H NMR spectrum of betulinic acid benzyl amide (**17a**).

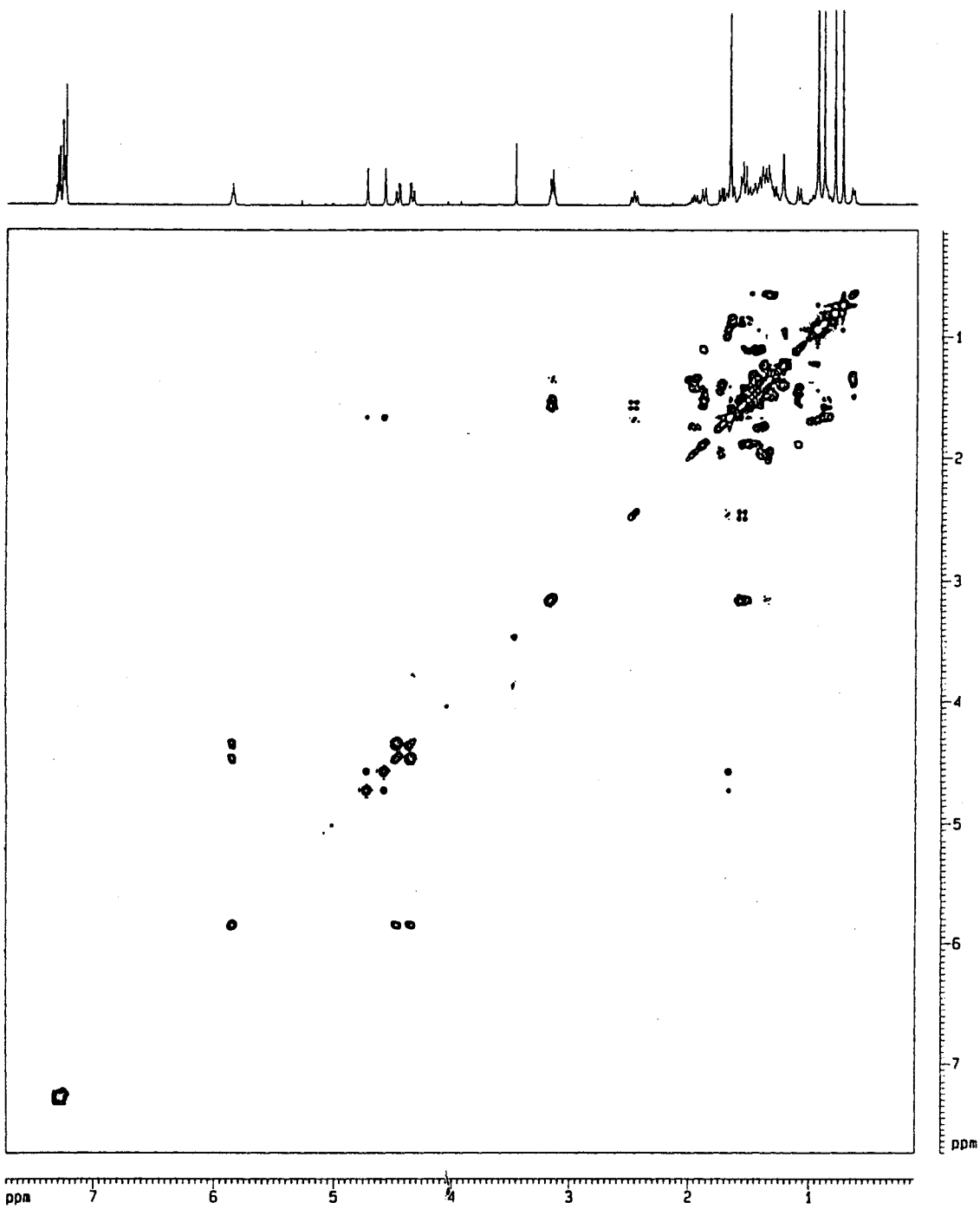


Figure 2.5.2.1b: COSY spectrum of betulinic acid benzyl amide (**17a**).

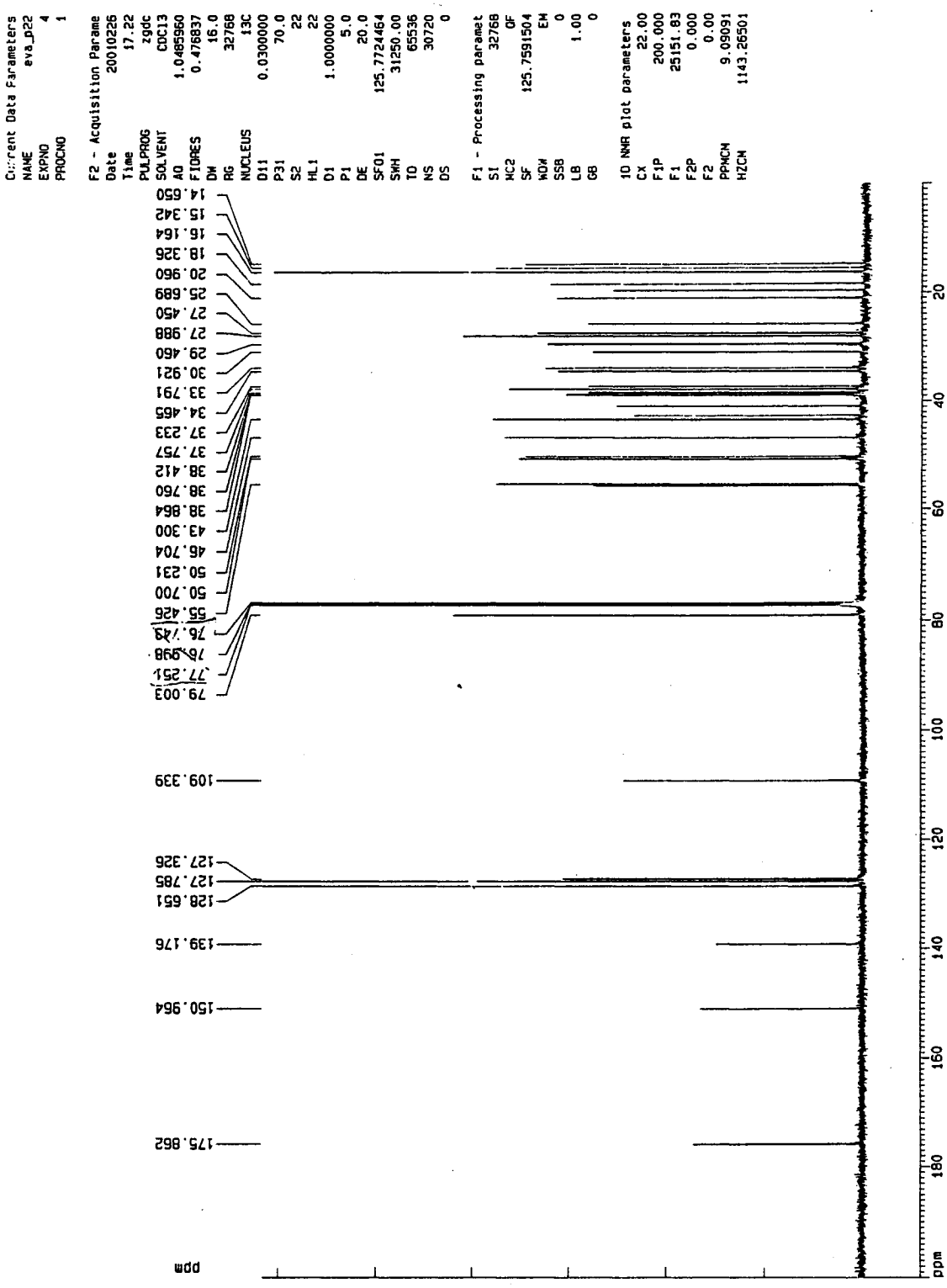


Figure 2.5.2.1c: ^{13}C NMR spectrum of betulinic acid benzyl amide (**17a**).

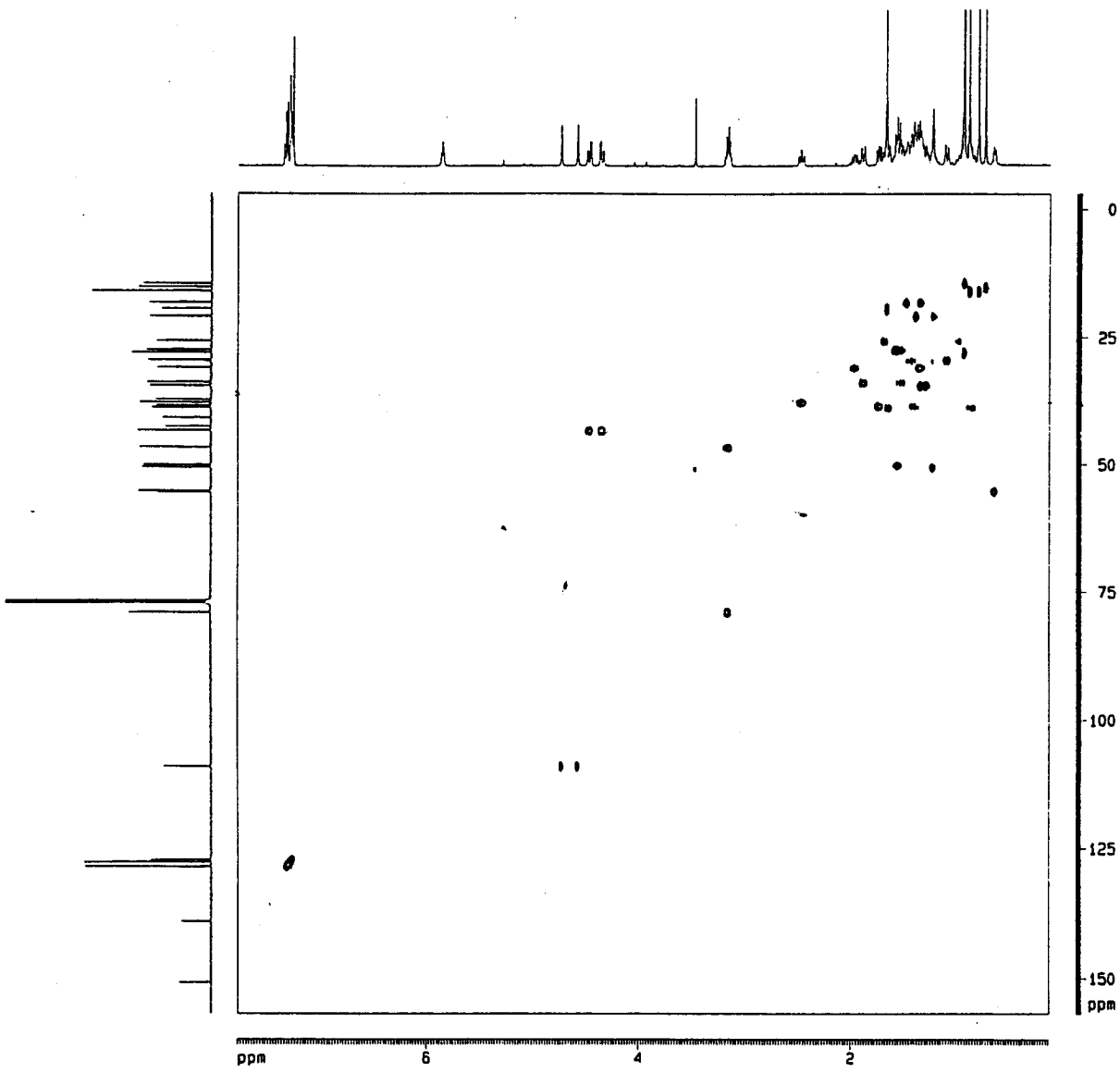


Figure 2.5.2.1d: HMQC spectrum of betulinic acid benzyl amide (**17a**).

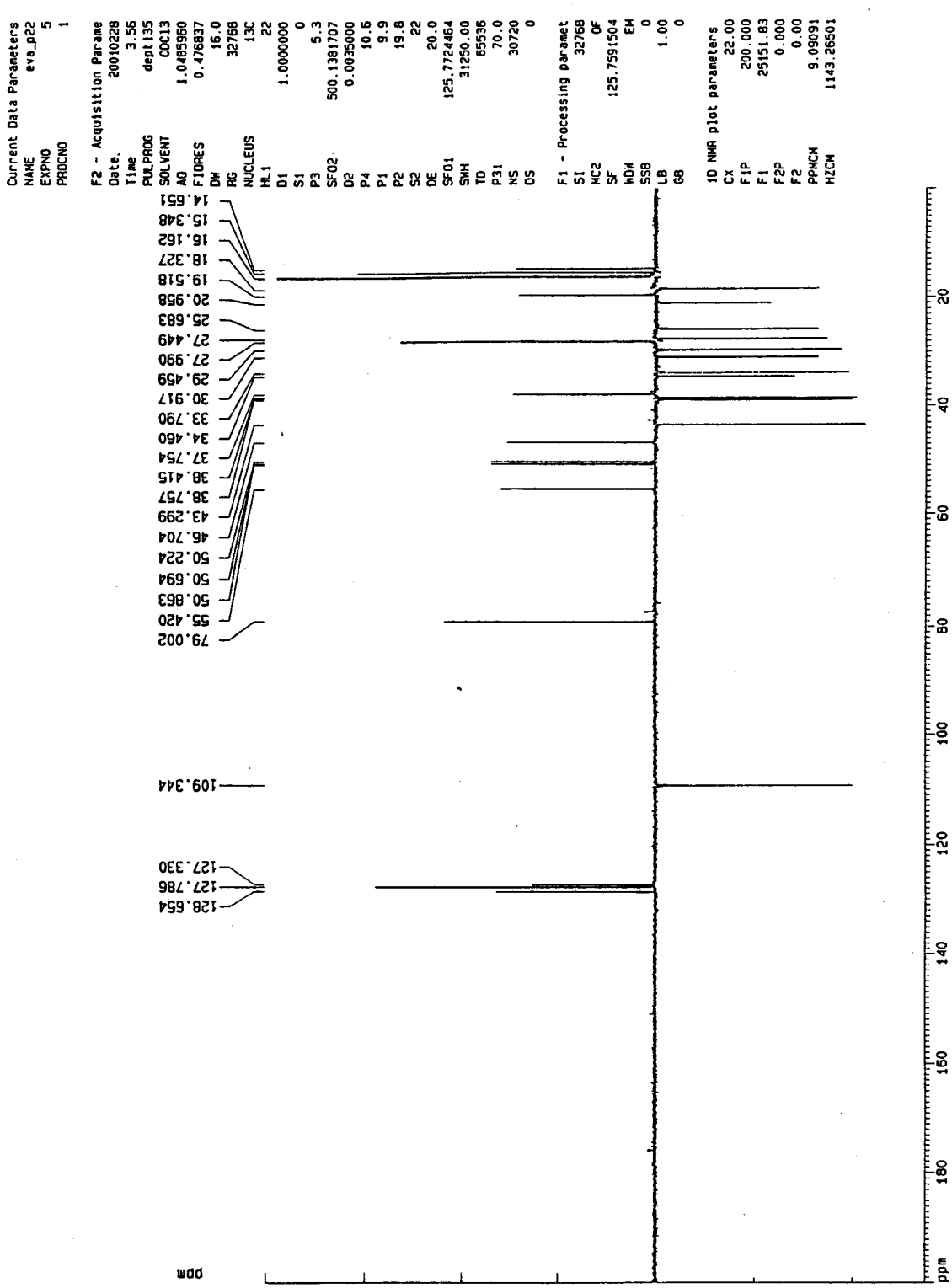


Figure 2.5.2.1e: DEPT spectrum of betulinic acid benzyl amide (**17a**).

2.5.2.2. Betulinic acid isobutyl amide

The reaction of 3β-acetoxylup-20(29)-en-28-oyl chloride with isobutylamine under the same reactions procedure described above furnished N-[3β-hydroxylup-20(29)-en-28-oyl]-isobutylamine (**17b**) as a white solid, m.p. 216-217°C, in 97% yield after silica gel column chromatography.

The EIMS of (**17b**) revealed a $[M]^+$ at m/z 511 (also the base peak) corresponding to the calculated molecular formula $C_{34}H_{57}NO_2$; which was established by HRMS. The fragmentation pattern reported in the experimental section was consistent with a betulinic acid derivative of the proposed structure. The strong IR absorptions at 3448 and 1638 cm^{-1} proved the presence of the OH and the CONH moieties respectively.

The ^1H NMR spectrum of **17b** (**Figure 2.5.2.2a**), displayed a simlar triplet to that observed for (**17a**) at δ 5.61 (t, J=5.8 Hz) characteristic of the CONH moiety. The overlapping signal at δ 3.12 (m, 3H) was attributableto H-3α, H-19 and one of the CH_2 protons of the isoproply moiety. The other proton of the CH_2 portion of the isopropyl group appeared as a multiplet at δ 2.98.

The ^{13}C NMR spectrum of (**17b**) revealed new resonances at δ 46.6, 29.4 and at δ 20.2 (two equivalent carbons) attributable to the isopropyl moiety. Using the HMQC spectrum and the DEPT 135 spectrum, C-19, NCH_2 , and C-13 were assigned to the signals at δ 46.7, 46.6 and 37.7 ppm respectively.

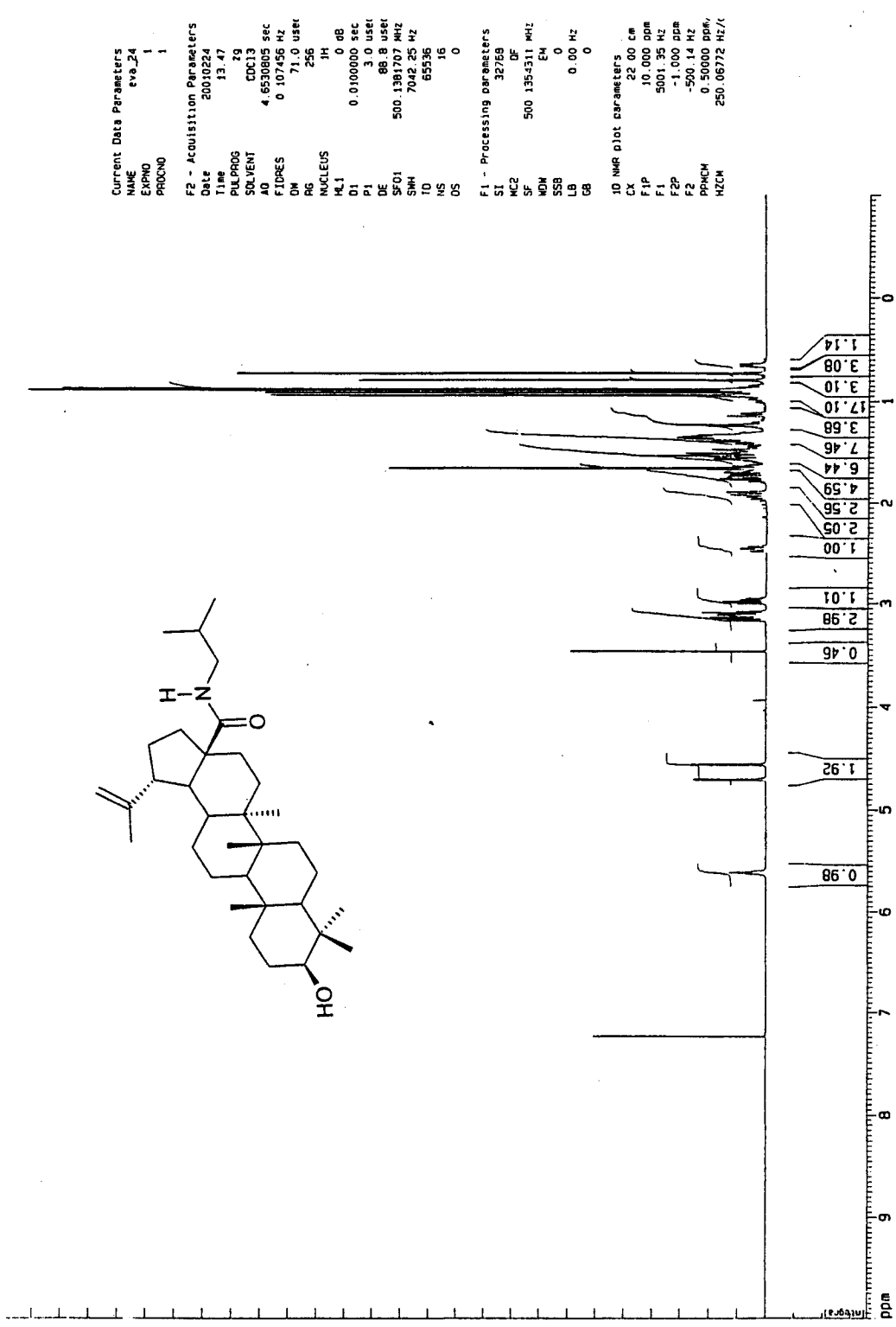


Figure 2.5.2.2a: ^{1}H NMR spectrum of betulinic acid isobutyl amide (**17b**).

2.5.2.3. Betulinic acid pyrrolidenyl amide

Betulinic acid pyrrolidenyl amide (**17c**) was obtained when 3β-acetoxylup-20(29)-en-28-oyl chloride was coupled with pyrrolidine under the same procedure outlined above (**Scheme 2.5.2**).Compound (**17c**) was isolated as a white solid, m.p. 223-226°C, in 78 % yield after silica gel column chromatography.

The EIMS of (**17c**) contained the $[M]^+$ at m/z 509 agreeing with its molecular formula $C_{34}H_{57}NO_2$; this was confirmed by HRMS. The mass spectral ions listed in the experimental section were consistent with a betulinic acid derivative of the assigned structure. The IR spectrum of (**17c**) exhibited CONH and OH absorptions at 1637 and 3424 cm^{-1} respectively.

The ^1H NMR spectrum of **17c** (**Figure 2.5.2.3a**) revealed the disappearance of the one proton triplet at δ 5.6-5.8 characterisitic of the NH proton of the CONH moiety that were observed for (**17a**) and (**17b**). The new multiplet at δ 3.40 (m, 4H) supported the presence of the two CH_2 groups adjacent to nitrogen in the pyrrolidine moiety. The H-3α and the H-19 protons displayed overlapping signals at δ 3.09 (m, 2H), **Table 2.5.2a**.

The ^{13}C NMR spectrum of (**17c**) exhibited similar resonance patterns to that of (**17b**). The resonances at δ 46.3 and 25.6 ppm in the ^{13}C NMR spectrum of (**17c**) were assigned to the two equivalent carbons of the pyrrolidine moiety, with the downfield shift assigned to the carbons adjacent to nitrogen.

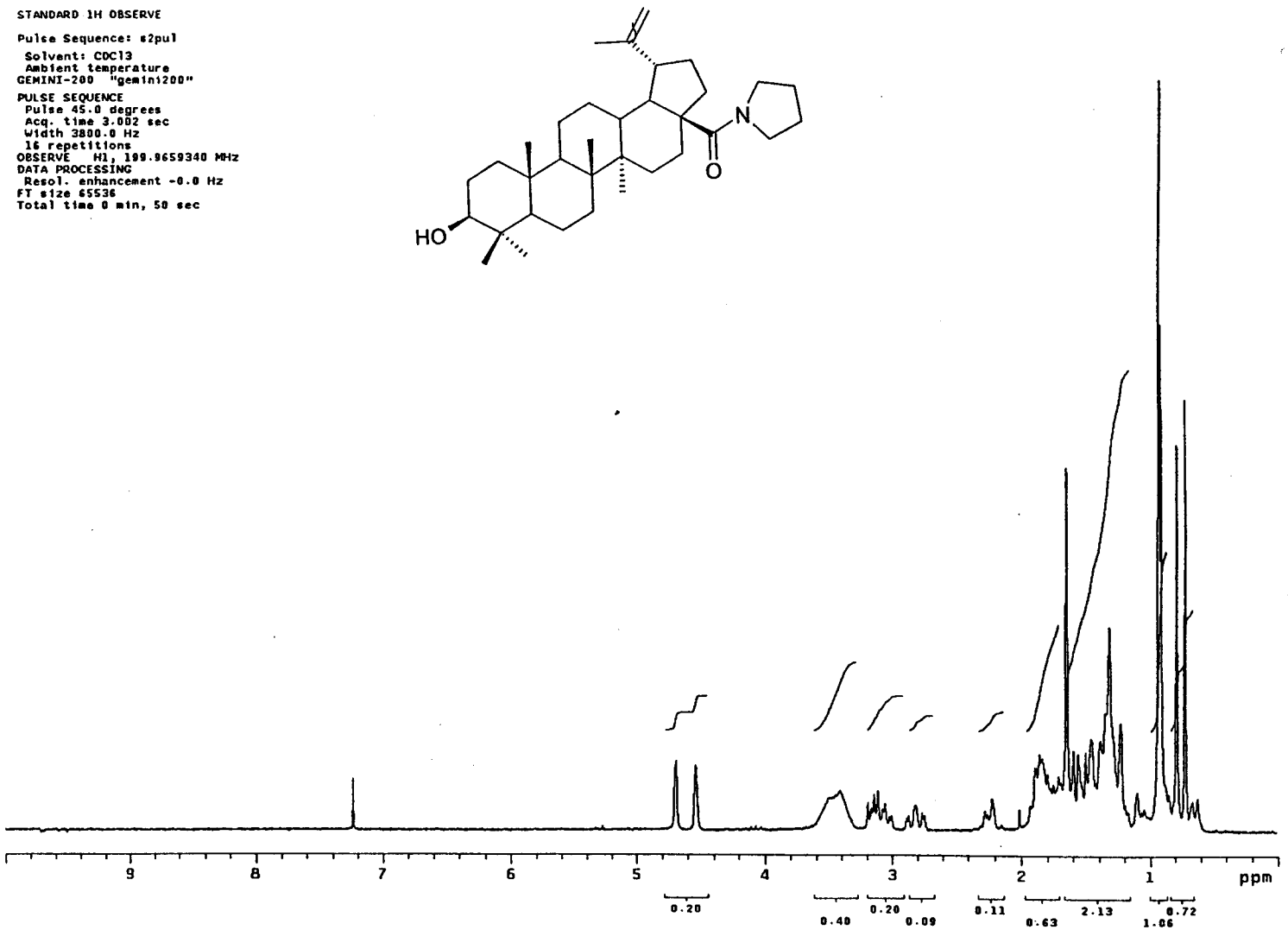
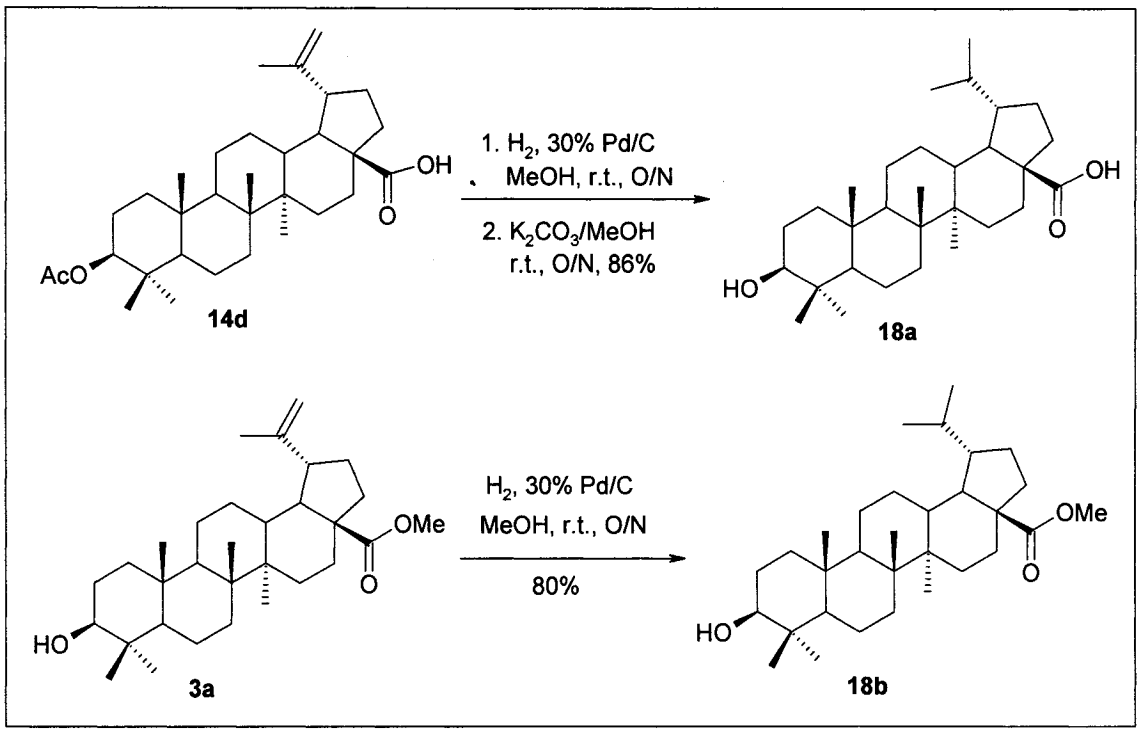


Figure 2.5.2.3a: ^{1}H NMR spectrum of betulinic acid pyrrolidenyl amide (**17c**).

2.5.3. Hydrogenation of betulinic acid

The reduction of the double bond between C-20 and C-29 of betulinic acid was accomplished via hydrogenation of 3β-acetyl betulinic acid (**14d**) using 30% Pd/carbon in MeOH at room temperature overnight, followed by deprotection of the acetate group at C-3 with K_2CO_3 in MeOH to afford dihydrobetulinic acid (**18a**) in 86% yield. Compound (**14d**) was employed in this reaction since it was more soluble in MeOH than betulinic acid (**3**).

Methyl betulinate (**3a**) was also successfully hydrogenated under the same reaction conditions to afford methyl dihydrobetulinate (**18b**) in 80% yield (**Scheme 2.5.3.1**).



Scheme 2.5.3.1: Hydrogenation of betulinic acid derivatives (**14d**) and (**3a**) to their respective dihydrobetulinic acid derivatives (**18a**) and (**18b**).

2.5.3.1. Dihydrobetulinic acid

Dihydrobetulinic acid (**18a**) was isolated as a white solid, m.p. 297-299°C (lit.[19] >300°C, lit.[21] 311-315°C) in 86% yield, after silica gel column chromatography (**Scheme 2.5.3.1**). In the ^{1}H NMR spectrum of (**18a**), the olefinic protons (H-29 and H-29') normally observed at δ 4.72 and δ 4.59 for betulinic acid and betulinic acid derivatives were absent. The spectroscopic data collected for (**18a**) were in excellent agreement with published data.[19]

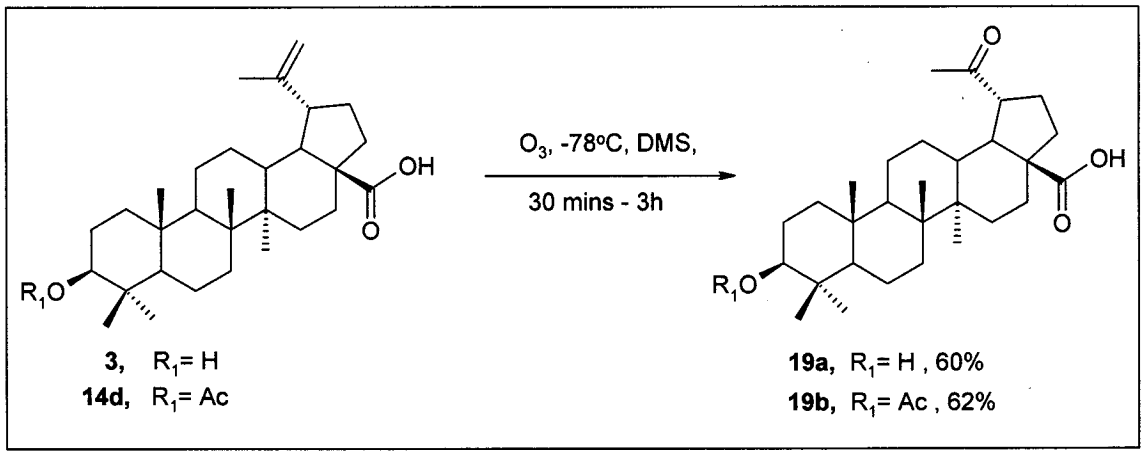
Compound (**18a**) has been reported to be slightly more potent than betulinic acid (**3**) in inhibiting HIV replication in H-9 lymphocytes.[19] Derivatives of (**18a**) have been synthesized and evaluated for their inhibitory effects on HIV-1 replication in acutely infected H-9 cells.[133]

2.5.3.2. Methyl dihydrobetulinate

Methyl dihydro betulinate (**18b**) was obtained as a white solid, m.p.236-238°C (lit.[23] 238-240°C), after silica gel column chromatography (**Scheme 2.5.3.1**). The $[M]^+$ at m/z 472 observed in the EIMS spectrum of (**18b**) corresponded to the molecular formula $C_{31}H_{52}O_3$ which was confirmed by HRMS. As for compound (**18a**), the olefinic protons (H-29 and H-29') were absent in the ^{1}H NMR spectrum of (**18b**). The remaining resonances was almost identical to those of (**18a**) except for methoxy signal at δ 3.63 (s).

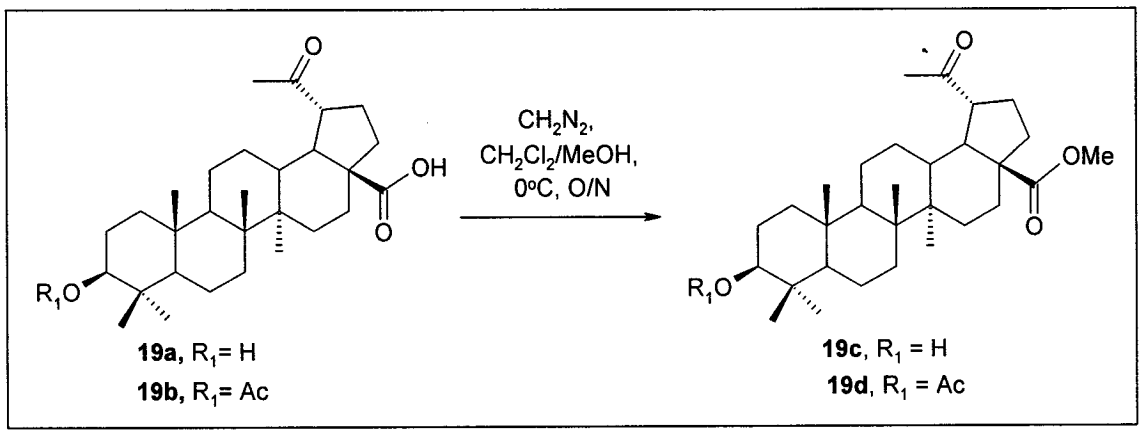
2.5.4. Ozonolysis of betulinic acid and its derivatives

The double bond in the C-20 position of betulinic acid (**3**) and 3β-acetyl betulinic acid (**14d**) were readily converted to their respective ketone moieties (**19a**) and (**19b**) by ozonolysis (O_3/DMS) at -78°C (**Scheme 2.5.4a**).



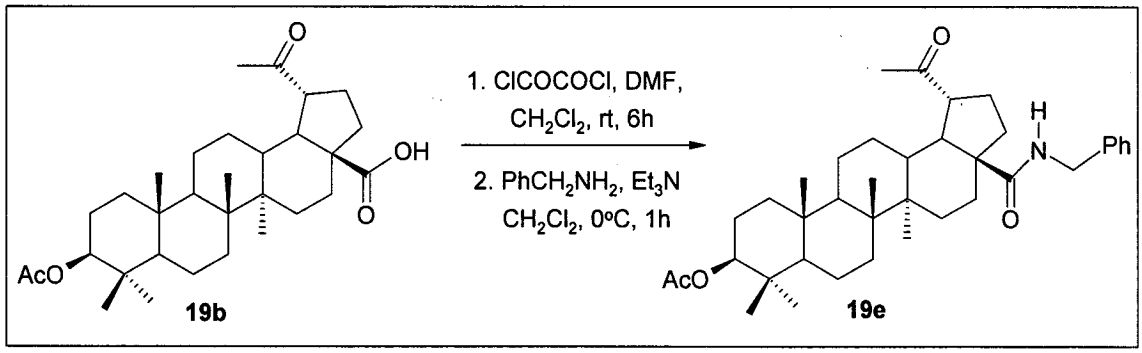
Scheme 2.5.4a: Ozonolysis of betulinic acid (**3**) and 3β-acetyl betulinic acid (**14d**) to their respective ketone moieties (**19a**) and (**19b**).

The ozonized products (**19a** and **19b**) were further derivatized by methylation with CH_2N_2 to their respective methyl esters (**19c**) and (**19d**) in good yields (**Scheme 2.5.4b**).



Scheme 2.5.4b: Methylation of ozonized products (**19a**) and (**19b**) to their corresponding methyl esters (**19c**) and (**19d**).

Moreover, derivatization of (**19b**) to benzyl amide (**19e**) was also carried out successfully (**Scheme 2.5.4c**) using the procedure previously described (*see* **Section 2.5.2**) for the synthesis of the amide derivatives of betulinic acid.



Scheme 2.5.4c: Further derivatization of the ozonized product (**19b**) to its benzyl amide (**19e**).

2.5.4.1. Platanic acid

Ozonolysis (O_3/DMS) of betulinic acid (**3**) at -78°C furnished platanic acid (**19a**) as a white solid (60%), m.p. 279-282°C (lit.[19] 279-282°C) in 60% yield after silica gel column chromatography (**Scheme 2.5.4a**). Compound (**19a**) has been previously prepared in 75% yield by ozonolysis of (**3**) in dioxane/H_2O using OsO_4/$NaIO_4$.[126]

The EIMS of (**19a**) displayed the $[M]^+$ at m/z 458 corresponding to the molecular formula $C_{29}H_{46}O_4$ which was further established by HRMS. Its IR spectrum indicated the presence of an OH and C=O groups by the strong bands at 3466 and 1701 cm^{-1} respectively.

The ^1H NMR spectrum of (**19a**) revealed a disappearance of the methylenic protons H-29 and H-29' at δ 4.72 and at δ 4.59 respectively, and the appearance of a three proton singlet at δ 2.15 characteristic of a methyl group adjacent to a carbonyl group (C-20). An overlapping two proton multiplet at δ 3.2 was assignable to H-3α and H-19. The five tertiary methyl signals resonated at δ 0.98 (s, 3H, H-27), 0.94 (s, 3H, H-26), 0.89 (s, 3H, H-23), 0.80 (s, 3H, H-25) and at δ 0.73 (s, 3H, H-24).

Diagnostic signals in the ^{13}C NMR spectrum corresponded to the ketone and carboxylic acid moiety as well as the carbon bearing the hydroxyl function which appeared at δ 212.1 (C-20), 181.3 (C-28), and at δ 78.9 (C-3) respectively.

Both the ^{1}H NMR and ^{13}C NMR data of (**19a**) were in good agreement with those reported by Fujioka and his coworkers.[19]

Platanic acid (**19a**) is a natural product which was first isolated almost simultaneously by Thomas and Muller in 1961, from the bark of *P. occidentalis* and by Aplin *et. al.,* 1963, from the bark of *Platanus x hybrida* Brot.[134] Compound (**19a**) was first reported together with betulinic acid (**3**) as anti-HIV principles from the leaves of *S. claviflorum* in 1994.[19]

2.5.4.2. Methyl platanate

Treatment of platanic acid (**19a**) with ethereal diazomethane furnished methyl platanate (**19c**) as a white solid, m.p. 250-251°C (lit.[134] 250-251°C), (**Scheme 2.5.4b**). Compound (**19c**) has been previously prepared by ozonolysis (O_3/N_2) of methyl betulinate (**3a**).[134]

The ^{1}H NMR spectrum of (**19c**) was almost superimposable with that of (**19a**) except for the appearance of the methoxy protons at δ 3.65 (s, 3H, OMe). The ^{13}C NMR spectrum disclosed diagnostic signals at δ 212.4 (C-20) for the ketone moiety, at δ 176.5 (C-28) due to the ester moiety, and at δ 78.8 (C-3) for the alcoholic carbon.

2.5.4.3. 3β-Acetyl platanic acid

The ozonolysis of 3β-acetyl betulinic acid (**14d**) using O_3/DMS at -78°C afforded 3β-acetyl platanic acid (**19b**) in 62% yield as a white solid, m.p. 252-255°C (lit.[140] 252-255°C, lit.[132] >250°C), after silica gel column chromatography (**Scheme 2.5.4a**).

The ^{1}H NMR spectrum of (**19b**) resembled that of (**19a**) except that H-3 now resonated at δ 4.44 (m, 1H, H-3α) no longer overlapped with H-19 at δ 3.23

(m, 1H, H-19). The acetyl and acetate protons were observed at δ 2.16 (s, 3H, CH_3CO) and at δ 2.02 (s, 3H, OAc).

The ^{13}C NMR spectrum displayed diagnostic resonances for three carbonyl functions at δ 212.2 (CH_3CO), 182.3 (CO_2H), 171.1 (OAc) and for the carbon bearing the acetate function at δ 80.8 (C-3).

2.5.4.4. 3β-Acetyl methyl platanate

When 3β-acetyl platanic acid (**19b**) was treated with ethereal diazomethane at 0°C, methyl 3β-acetyl platanate (**19d**) was obtained in 78% yield as a white solid m.p. 204-206°C (lit.[134] 204-206°C, lit.[140] 205-207°C, lit.[134] 206-207°C) after silica gel column chromatography (**Scheme 2.5.4b**).

Compound (**19d**) has been previously prepared by acetylation of methyl platanate (**19c**).[134] The ^1H and the ^{13}C NMR spectra of (**19d**) resembled that of (**19b**) except for the new methoxy signals at δ 3.64 and at δ 51.4 respectively.

2.5.4.5. N-[3β-Acetoxy-nor-20-oxolupan-28-oyl]-benzylamine

Further derivatization of 3β-acetoxy platanic acid (**19b**) was performed by reacting it with oxalyl chloride and five drops of DMF in CH_2Cl_2 to afford the corresponding acid chloride, which was then coupled with benzylamine in the presence of Et_3N in CH_2Cl_2 furnishing N-[3β-acetoxy-nor-20-oxolupan-28-oyl]-benzylamine (**19e**) as a white solid, m.p. 280-283°C, in 51% yield after silica gel column chromatography (**Scheme 2.5.4c**).

Compound (**19e**) is a new compound. The $[M]^+$ at m/z 589 in the EIMS of (**19e**) was compatible with its molecular formula $C_{38}H_{55}O_4$; and was established by HRMS. The mode of fragmentation was typical of a lupane derivative of the proposed structure. Its IR spectrum contained strong absorptions at 3376 (N-H), 1733 (CH_3CO_2), 1713 (CONH) and at 1647 cm^{-1} (CH_3CO).

The ^{1}H NMR spectrum of (**19e**) in **Figure 2.5.4.5a** displayed diagnostic signals for the phenyl group at δ 7.32 (m. 5H, Ph); for the CONH moiety at δ 5.91 (t, 5.8 Hz, CONH), which couples with the CH_2 portion of the benzyl group as depicted by the COSY spectrum (**Figure 2.5.4.5b**); for the benzyl protons at δ 4.58 (dd, J=14.8 Hz, J=5.9 Hz, CONHCH-Ph), and at δ 4.32 (dd, J=14.7 Hz, J=5.6 Hz, CONHCH'-Ph); for the methine proton bearing the acetate moiety at δ 4.44 (dd, J=11.5 Hz, J=4.8 Hz, H-3α); for the methine proton adjacent to the acetyl moiety at δ 3.46 (dt, J=11.3, J=4.3 Hz, H-19); for the methine group at C-13 at δ 2.24 (dt, J=12.0 Hz, J=4.2 Hz, H-13); for the acetyl and acetate groups at δ 2.15 (s, CH_3CO) and at δ 2.01 (s, CH_3CO_2); and the five tertiary methyl singlets at δ 0.96 (s, 3H, H-27), 0.86 (s, 3H, H-26), 0.82 (s, 3H, H-23), 0.81 (s, 3H, H-25), 0.80 (s, 3H, H-24).

Diagnostic signals in the ^{13}C NMR spectrum of (**19e**) corresponded to three carbonyl functions at δ 212.9 (CH_3CO), 175.7 (CONH) and at δ 170.9 (OAc) (**Figure 2.5.4.5c**). The aromatic protons resonated at δ 139.0, 128.7, 127.8, 127.4, whereas the carbon bearing the acetate moiety (C-3) resonated at δ 80.9. Analysis of the HMQC spectrum (**Figure 2.5.4.5d**) and the DEPT 135 spectra (**Figure 2.5.4.5e**) allowed the assignment of C-13, C-19 and CH_2Ph to the signals at δ 50.4 and 43.3 ppm respectively.

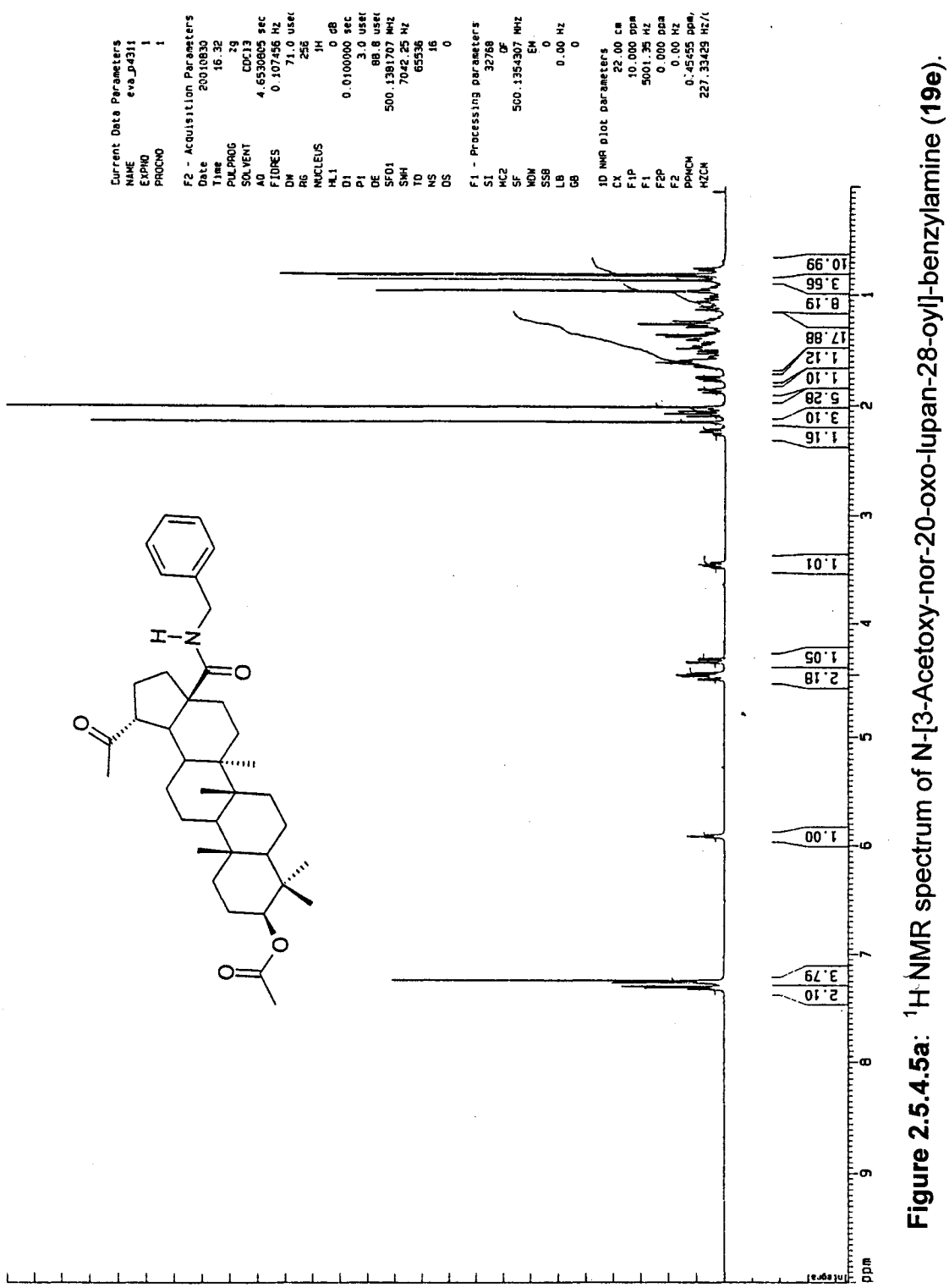


Figure 2.5.4.5a: ^{1}H NMR spectrum of N-[3-Acetoxy-nor-20-oxo-lupan-28-oyl]-benzylamine (**19e**).

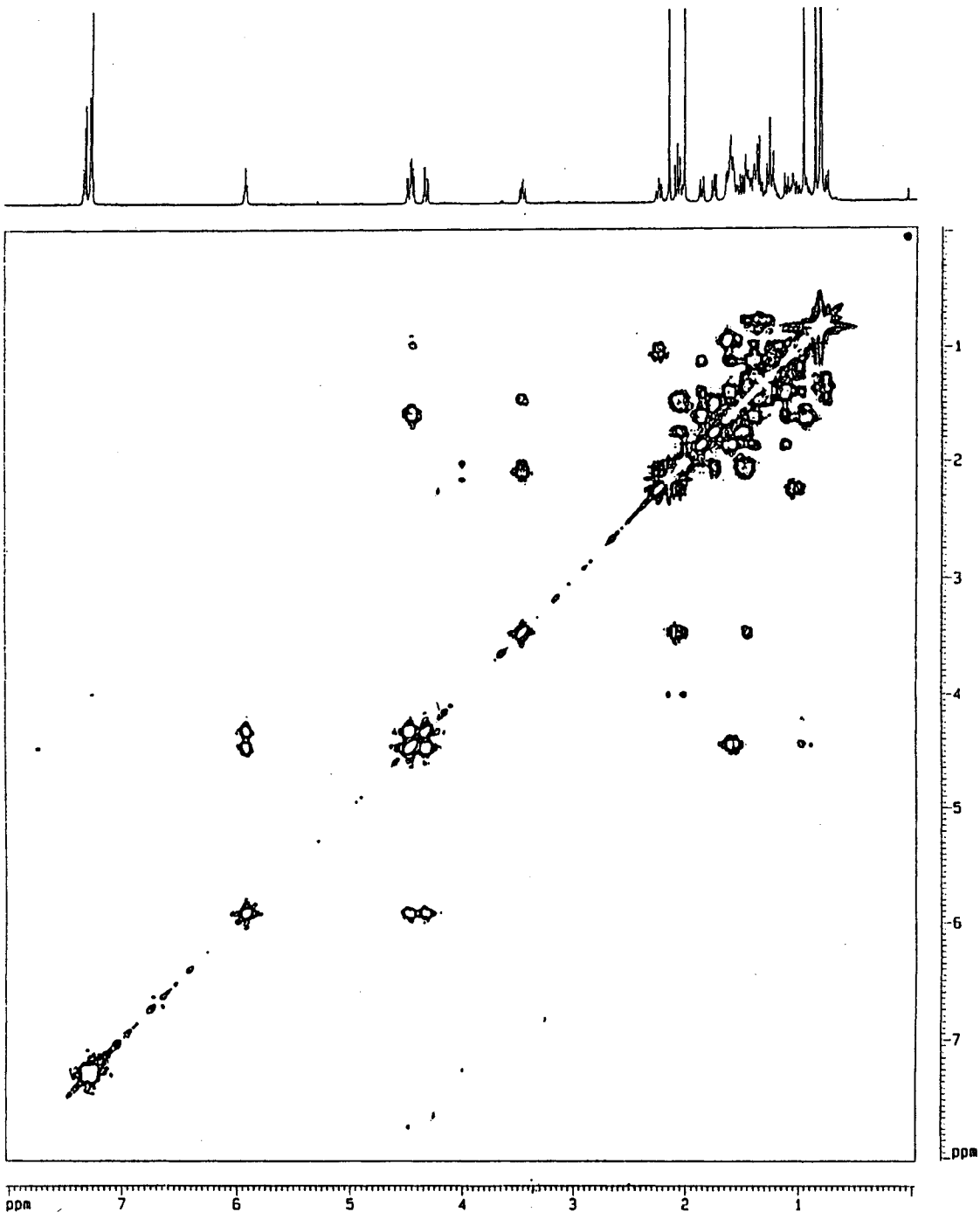


Figure 2.5.4.5b: COSY spectrum of N-[3β-acetoxy-nor-20-oxolupan-28-oyl]-benzylamine (**19e**).

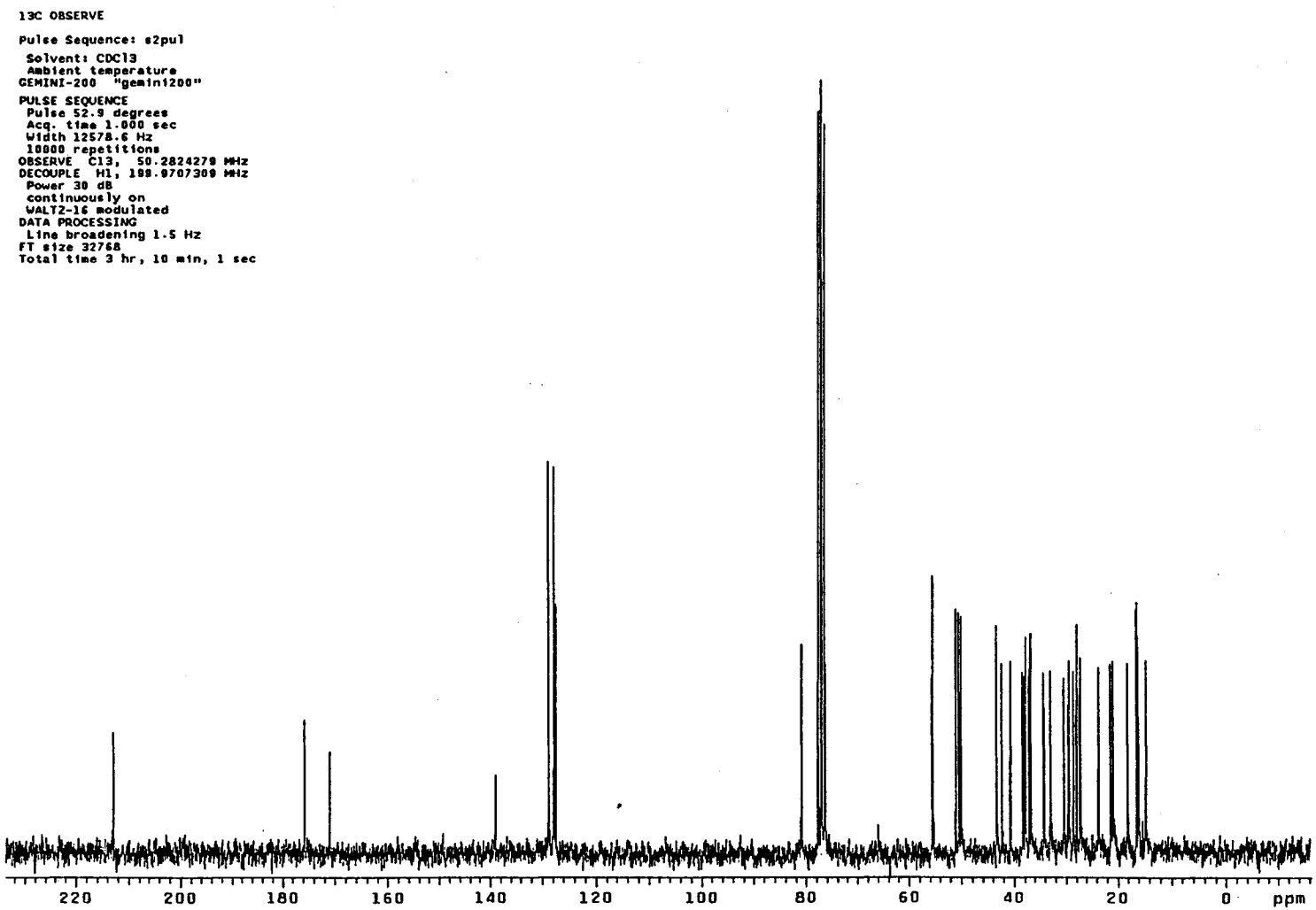


Figure 2.5.4.5c: ^{13}C NMR spectrum of N-[3β-Acetoxy-nor-20-oxo-lupan-28-oyl]-benzylamine (**19e**).

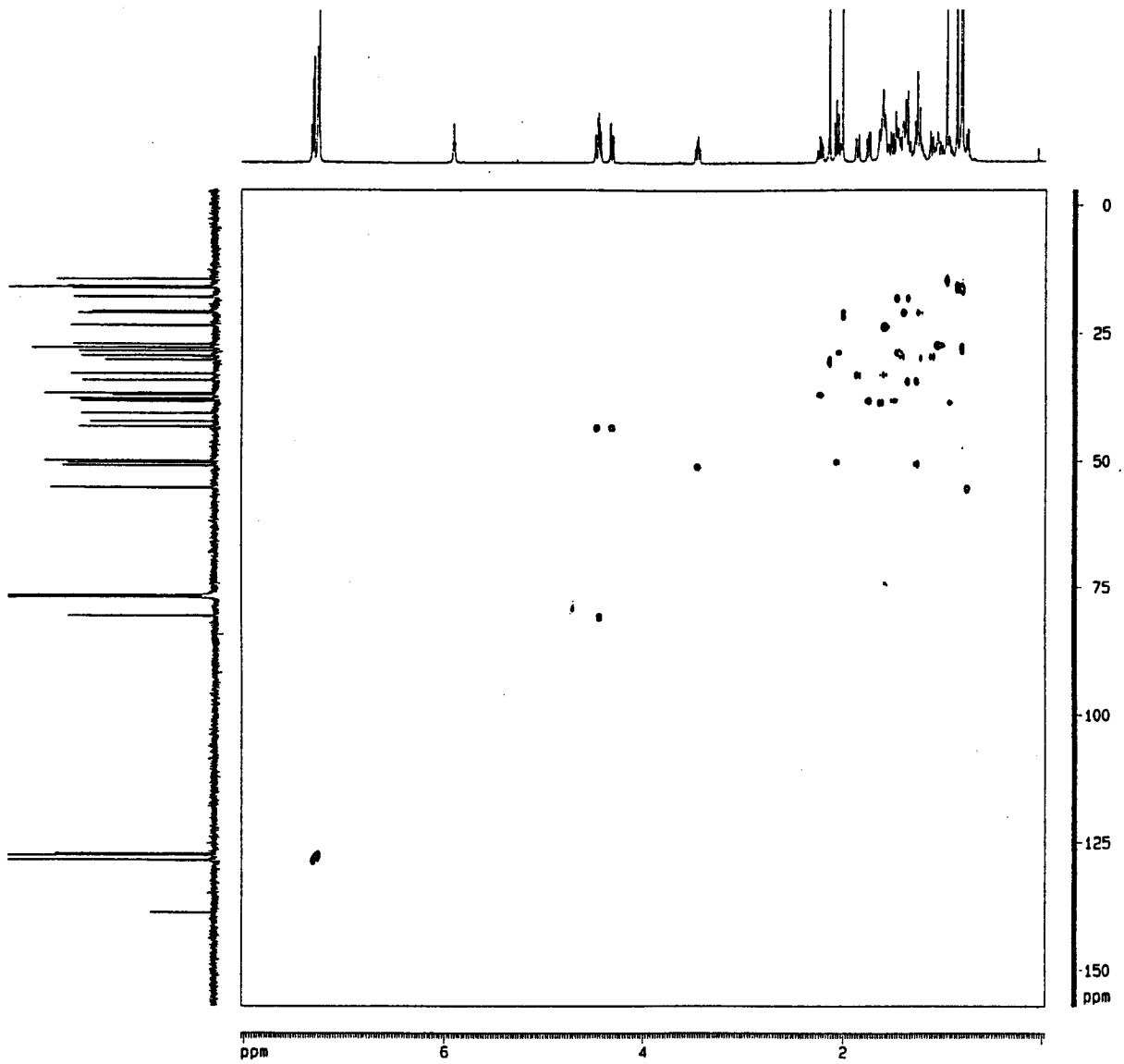


Figure 2.5.4.5d: HMQC spectrum of N-[3β-acetoxy-nor-20-oxolupan-28-oyl]-benzylamine (**19e**).

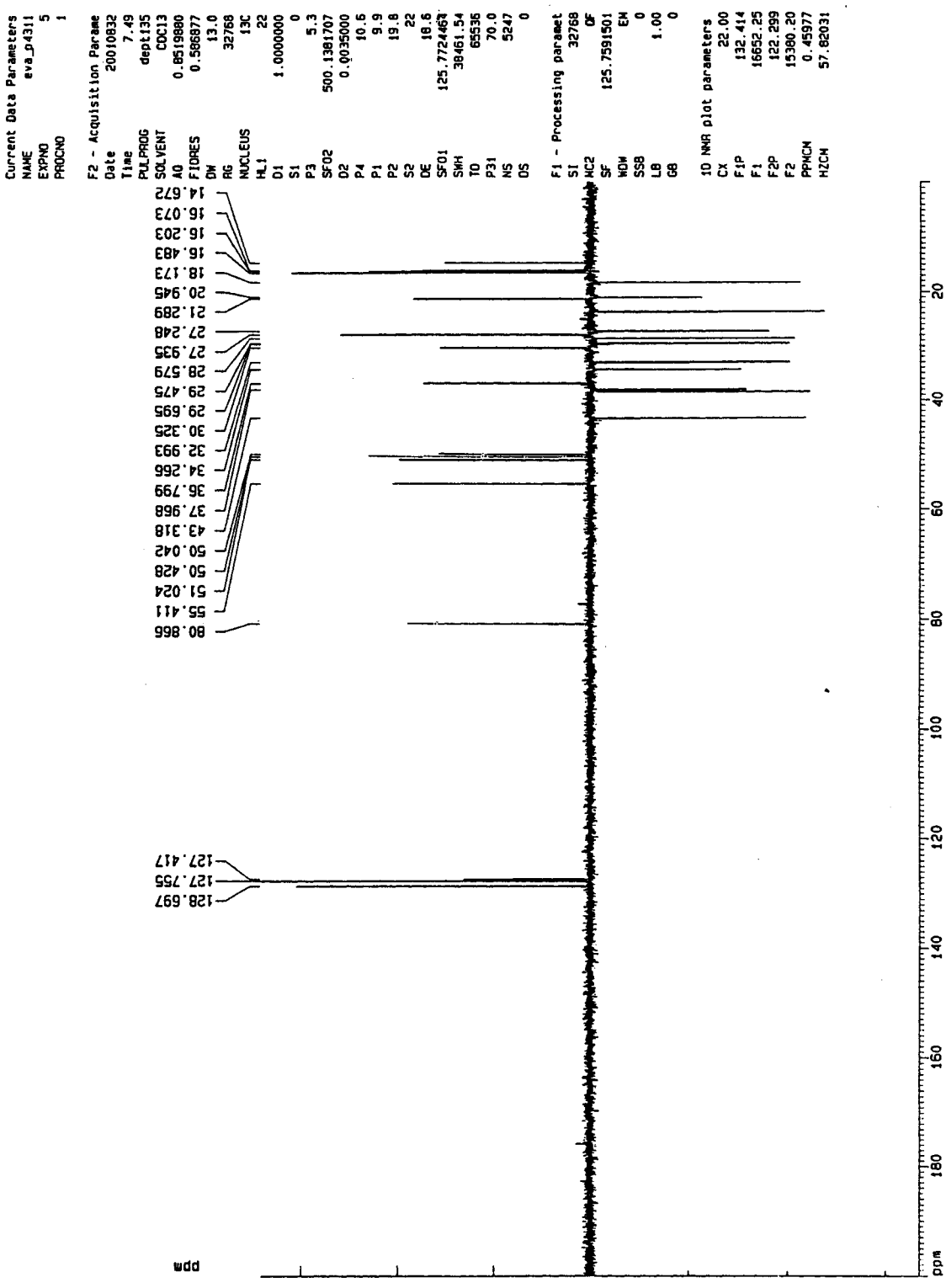
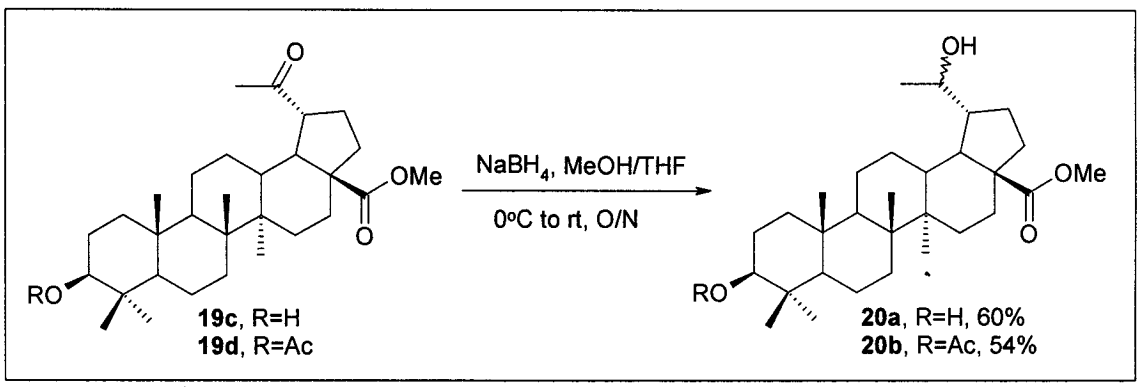


Figure 2.5.4.5e: DEPT 135 spectrum of N-[3-Acetoxy-nor-20-oxo-lupan-28-oyl]-benzylamine (**19e**).

2.5.5. Reduction of ozonized products of methyl betulinate

The 20-keto moiety of the ozonolysis products methyl platanate (**19c**) and methyl 3β-acetyl platanate (**19d**) were further reduced with $NaBH_4$ (MeOH/THF, 0°C) to their corresponding secondary alcohols methyl 3β, 20- dihydroxy-lupan-28-oate (**20a**) and methyl 3β-acetoxy, 20-hydroxy-lupan-28-oate (**20b**) respectively in reasonable yields (**Scheme 2.5.5**). The TLC for both these reactions showed two new close spots expected from the formation of two stereoisomers, but only the major stereoisomer was isolated pure and characterized in each case. The stereochemistry at C-20 was not defined.



Scheme 2.5.5: Reduction of ozonized products (**19c**) and (**19d**) to their respective secondary alcohols (**20a**) and (**20b**).

The crude ^1H NMR spectrum of (**20a**) showed a one proton quartet at δ 3.84 (J=6.3 Hz) and a one proton multiplet at δ 4.01 due to the methine proton bearing the hydroxyl group at C-20. Similarly, in the case of (**20b**), the one proton quartet at δ 3.88 (J=6.4 Hz) and the one proton multiplet at δ 4.01 confirmed the presence of the two stereoisomers.

Attempt to reduce the 20-keto group in platanic acid (**19a**) with $NaBH_4$ under the same reaction conditions was unsuccessful (only produced starting materials). However, recently, Kim *et. al.*, reported successful reduction of (**19a**) with $NaBH_4$ by refluxing in THF to afford the corresponding secondary alcohol, 20-hydroxy platanic acid (20-hydroxy-lupan-28-oic acid) in 80% yield.[126]

2.5.5.1. Methyl 3β, 20-dihydroxy-lup-28-oate

Methyl 20-dihydroxy-lup-28-oate (**20a**) was isolated as a white solid, m.p. 194-196°C in 60 % yield after silica gel column chromatography (**Scheme 2.5.5**). Compound (**20a**) is a new compound. Its EIMS showed the $[M]^+$ at m/z 474 corresponding to the desired molecular formula $C_{30}H_{50}O_4$, which was further established by HRMS. The IR spectrum indicated the presence of carbonyl and hydroxyl absorptions at 1714 and 3407 cm^{-1} respectively.

The ^1H NMR spectrum of (**20a**) exhibited diagnostic signals for the two methine protons bearing the secondary alcohol groups at δ 3.84 (q, 1H, J=6.3 Hz, H-20) and at δ 3.17 (dd, 1H, J=10.4 Hz, J=5.6 Hz, H-3α). The methoxy protons resonated at δ 3.63 (s, 3H) and the five tertiary methyl protons resonated at δ 0.94 (s, 6H, H-27, and H-26), 0.88 (s, 3H, H-23), 0.80 (s, 3H, H-25) and at δ 0.73 (s, 3H, H-24).

In the ^{13}C NMR spectrum of (**20a**), the most significant signals were observed for one carbonyl group at δ 176.9 (C-28) and for the two alcoholic carbons at δ 78.9 (C-3) and at δ 68.9 (C-20).

2.5.5.2. Methyl 3β-acetyl, 20-hydroxy-lup-28-oate

Methyl 3β-acetyl, 20-hydroxy-lup-28-oate (**20b**) was isolated as a white solid, m.p. 256-259°C, in 54% yield after silica gel colum chromatography (**Scheme 2.5.5**). Compound (**20b**) has not been reported in the literature. Its EIMS revealed a $[M]^+$ at m/z 516 was in agreement with its molecular formula $C_{32}H_{52}O_5$; this was verified by HRMS. The IR bands at 1720 and 3538 cm^{-1} established the presence of the OH and C=O functions respectively.

The ^1H NMR spectrum of (**20b**) showed great resemblance to that of (**20a**) except for the methine proton at C-3 now bearing the acetoxy moiety appeared at δ 4.45 (dd, 1H, J=10.8, J=5.5 Hz, H-3α), (**Figure 2.5.5.2**). The acetoxy protons appeared at δ 2.02 (s, CH_3-CO_2). The methine proton bearing the secondary

alcohol function at C-20 was observed as a quartet at δ 3.88 (q, J=6.4 Hz, H-20) while the methoxy singlet resonated at δ 3.63 (s, 3H). The five tertiary methyl protons were evident from the singlets observed at δ (s, H-27), 0.88 (s, H-26), 0.83 (s, H-23), 0.82 (s, H-25) and at δ 0.81 (s, H-24).

The ^{13}C NMR spectrum of (**20b**) exhibited as diagnostic resonances two carbonyl resonances at δ 176.9 and δ 171.1 due to the methyl ester and the acetate moiety respectively, the methine carbon bearing the acetate moiety (C-3) at δ 81.0 and the alcoholic carbon (C-20) at δ 68.9.

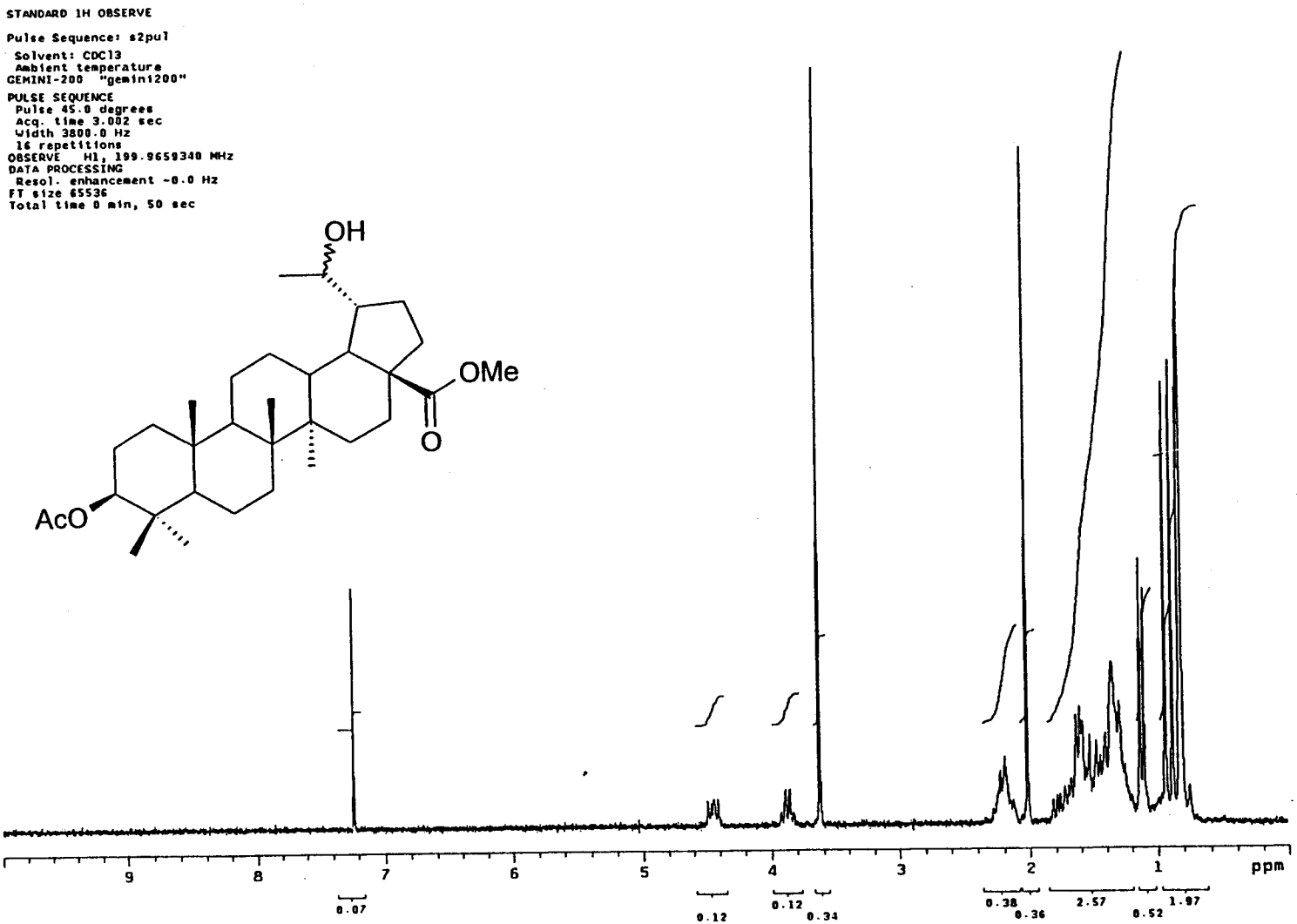
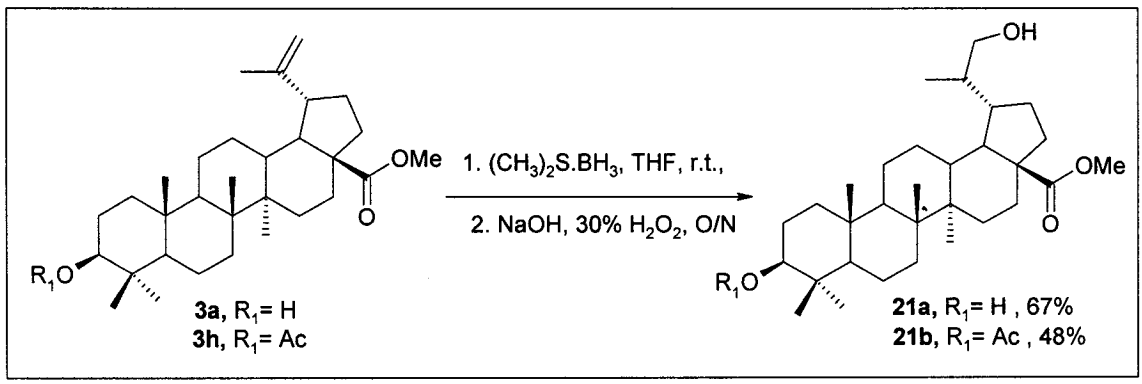


Figure 2.5.5.2: ^{1}H NMR spectrum of Methyl 3β-acetyl-20-hydroxy-lup-28-oate (**20b**).

2.5.6. Hydroboration of betulinic acid derivatives

The double bonds in betulinic esters (**3a**) and (**3h**) were converted to their corresponding primary alcohols by subjecting these compounds to hydroboration conditions, [$(CH_3)_2S.BH_3$, THF for 7h followed by NaOH and 30% w/w H_2O_2]. This gave their respective primary alcohol derivatives (**21a**) and (**21b**) in fairly good yields (**Scheme 2.5.6**).

Recently, Dinda and his coworkers reported the hydroboration of (**3a**) by bubbling diborane gas [prepared from $NaBH_4$ and BF3-etherate in diglyme] through a solution of (**3a**) in THF for 2h, before the dropwise addition of NaOH and 30% H_2O_2 to afford (**21a**) in 97% yield.[141]



3a, R_1= H
3h, R_1= Ac

21a, R_1= H , 67%
21b, R_1= Ac , 48%

Scheme 2.5.6: Hydroboration of betulinic acid derivatives (**3a**) and (**3h**) to their respective primary alcohol derivatives (**21a**) and (**21b**).

2.5.6.1. Methyl 3β, 29-dihydroxy-lup-28-oate

The hydroboration of the methyl ester of betulinic acid (**3a**) resulted in the formation of methyl 3β, 29-dihydroxy-lup-28-oate (**21a**) in 67% yield which was isolated as a white solid m.p. 218-220°C (lit.[141] 212-214°C) after silica gel column chromatography (**Scheme 2.5.6**).

In the EIMS spectrum of (**21a**), the $[M]^+$ appeared at m/z 488 coinciding with the expected molecular formula $C_{31}H_{52}O_4$; this was verified by HRMS. The mass spectral data were in line with those in the literature.[141]

The ^1H NMR spectrum of (**21a**) showed the disappearance of the signals due to the isopropenyl moiety in betulinic acid methyl ester and the apprearance of new signals due to the newly introduced isopropanol moiety. Resonances attributed to the CH_2 part of the isopropanol moiety appeared as two double doublets at δ 3.76 (dd, J=10.4 Hz, J=4.6 Hz, H-29) and at δ 3.39 (dd, J=10.5 Hz, J=8.1 Hz, H-29'). The methoxy and the methine (bearing the hydroxyl group at C-3) protons appeared at δ 3.62 (s, OMe) and at δ 3.17 (dd, J=10.1 Hz, J=5.6 Hz, H-3α) respectively. These chemical shifts agreed reported values for (**24a**).[141]

Diagnostic signals in the ^{13}C NMR spectrum (**21a**) were observed for the carboxyl functionality at δ 176.6 (C-28), and for the two alcoholic carbons at δ 78.9 (C-3), and at δ 64.2 (C-29). The ^{13}C NMR data of (**21a**) have not been reported in the literature.

2.5.6.2. Methyl 3β-acetyl, 29-hydroxy-lup-28-oate

The hydroboration of methyl 3β-acetyl betulonate (**3h**) furnished methyl 3β-acetoxy, 29-hydroxy-lup-28-oate (**21b**) in 48% yield, as a white solid, m.p. 234°C-235°C, after silica gel column chromatography (**Scheme 2.5.6**). This compound has not been reported in the literature.

In the EIMS spectrum of (**21b**), the $[M]^+$ at m/z 530 and the HRMS established the anticipated molecular formula $C_{33}H_{54}O_5$. The fragmentation pattern was characteristic of a lupane type triterpenoid of the postulated structure.

The ^1H NMR spectrum of (**21b**) was similar to that of (**21a**) except that the H-3α bearing the acetate group appeared at δ 4.45 (dd, J=10.8 Hz, J=5.6 Hz), (*see* **Figure 2.5.6.2a**). The methoxy and the acetoxy protons resonated at δ 3.62

(s, 3H, OMe) and at δ 2.02 (s, 3H, CH_3CO_2) respectively. The resonances for the CH_2 part of the isopropanol moiety which coupled to each other, were observed at δ 3.76 (dd, J=10.4 Hz, J=4.6 Hz, H-29) and at δ 3.40 (dd, J=10.4 Hz, J=8.0 Hz, H-29'), (**Figure 2.5.6.2b**). The methyl group at C-30 appeared as a new doublet at δ 0.94 (d, J=6.9 Hz) while the remaining five tertiary methyl singlets resonated as usual at δ ~ 0.94-0.73.

The ^{13}C NMR spectrum of (**21b**) contained diagnostic signals for the carbonyl functionalities at δ 176.7 (CO_2Me) and at δ 171.0 (OAc), and for the two alcoholic carbons at δ 80.9 (C-3), and at δ 64.3 (C-29), (**Figure 2.5.6.2c**). The DEPT 135 spectra of (**21b**) confirmed these assignments (**Figure 2.5.6.2d**).

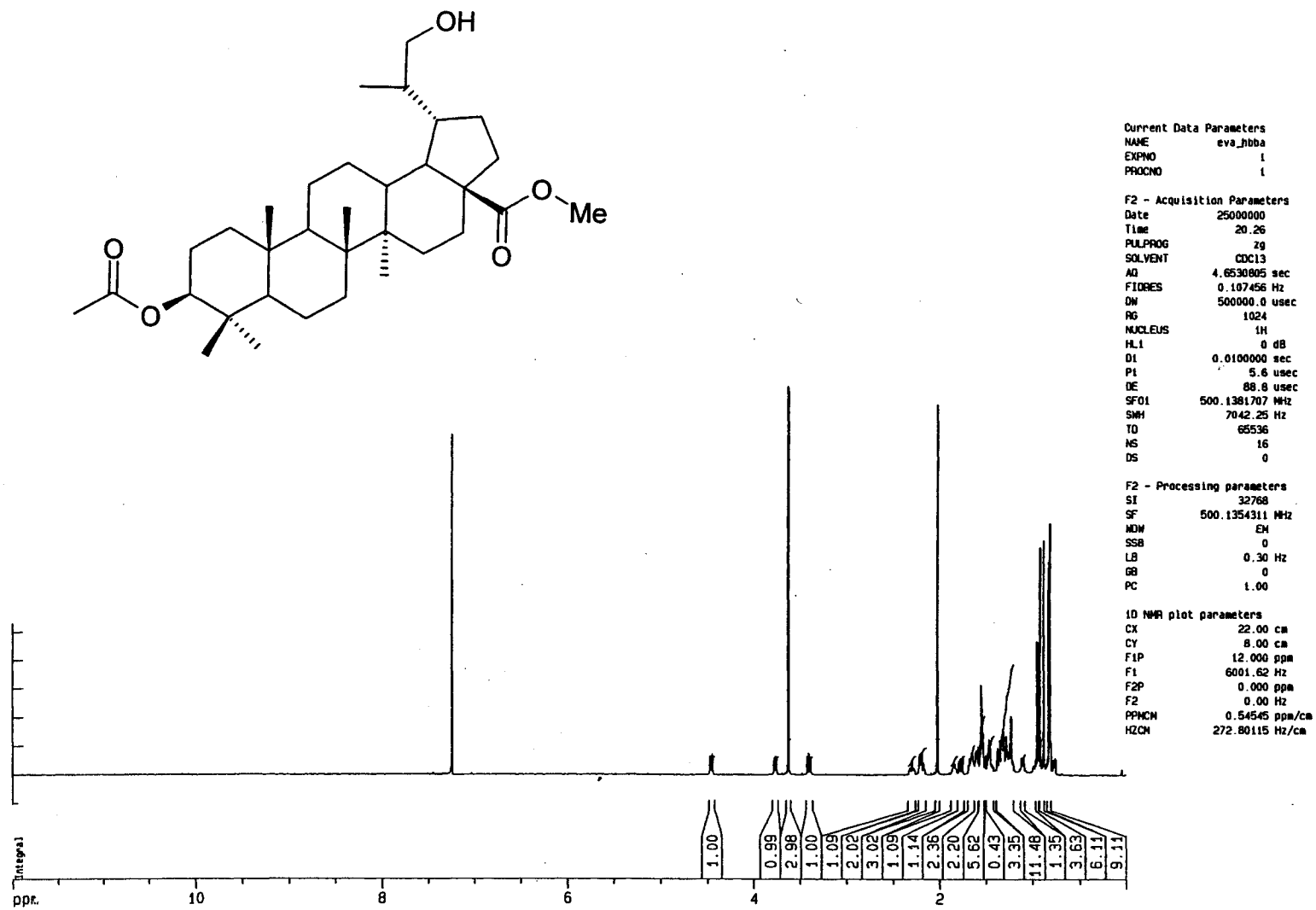


Figure 2.5.6.2a: ^{1}H NMR spectrum of Methyl 3β-acetyl, 29-hydroxy-lup-28-oate (**21b**).

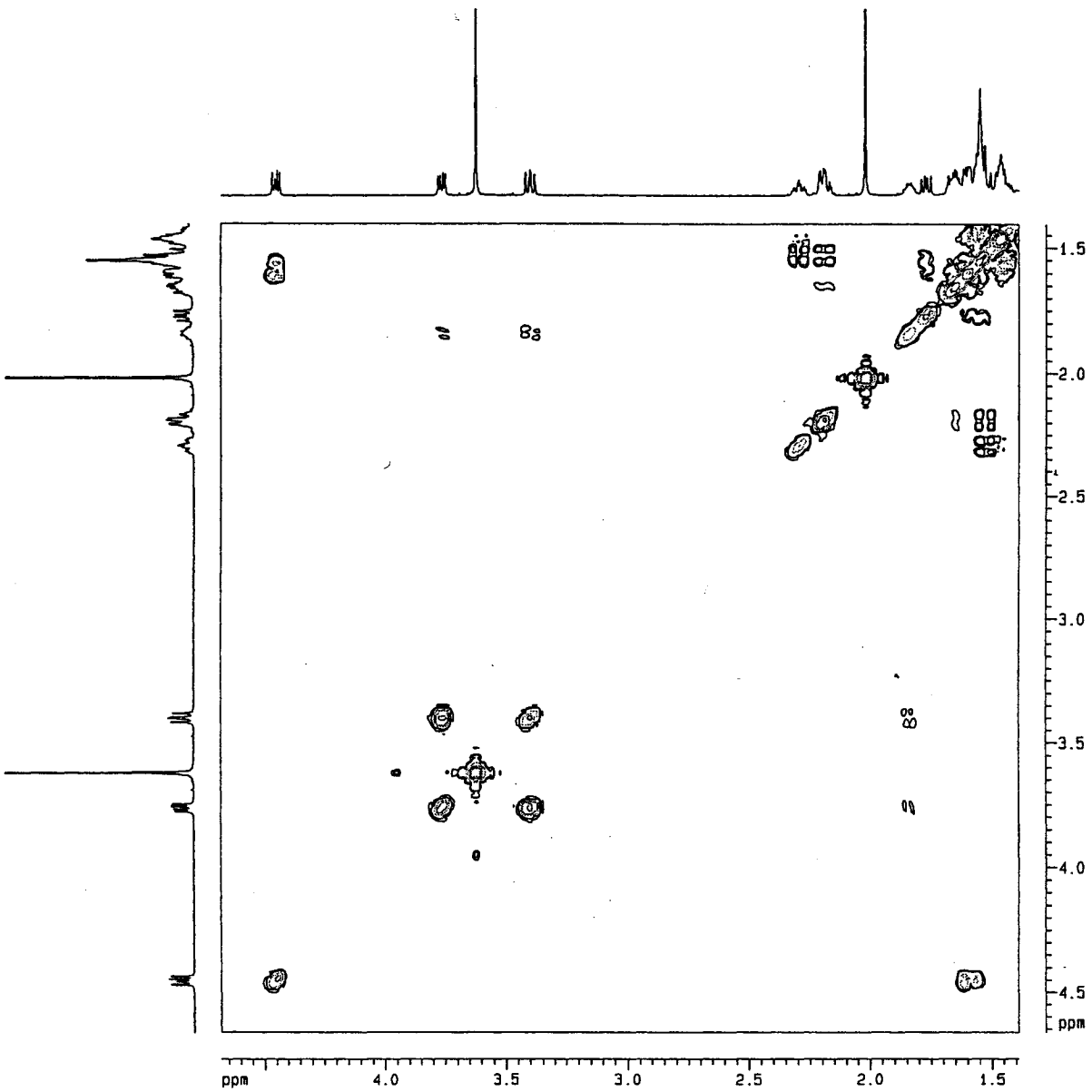


Figure 2.5.6.2b: COSY spectrum of Methyl 3β-acetyl, 29-hydroxy-lup-28-oate (**21b**).

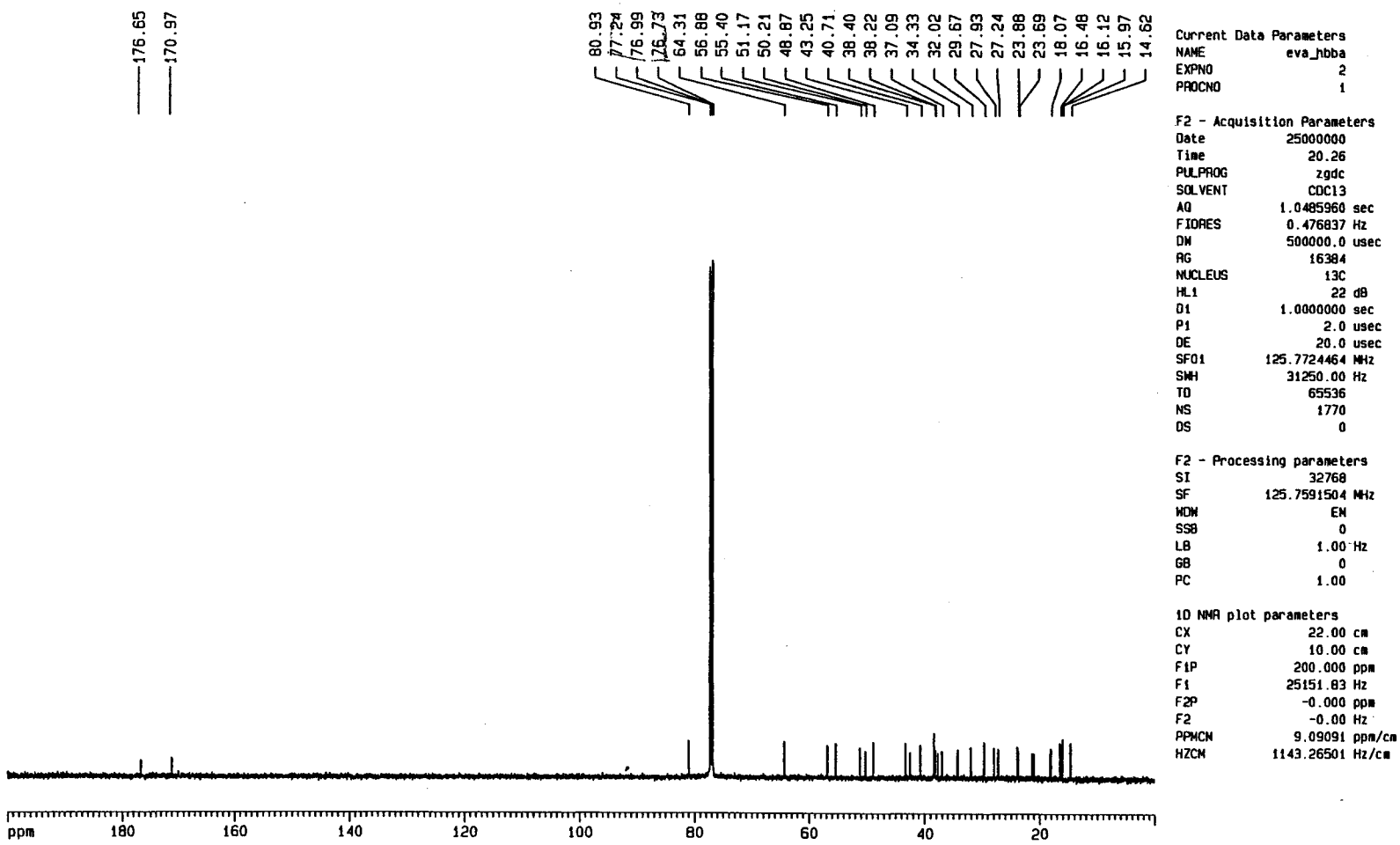


Figure 2.5.6.2c: ^{13}C NMR spectrum of Methyl 3β-acetyl, 29-hydroxy-lup-28-oate (**21b**).

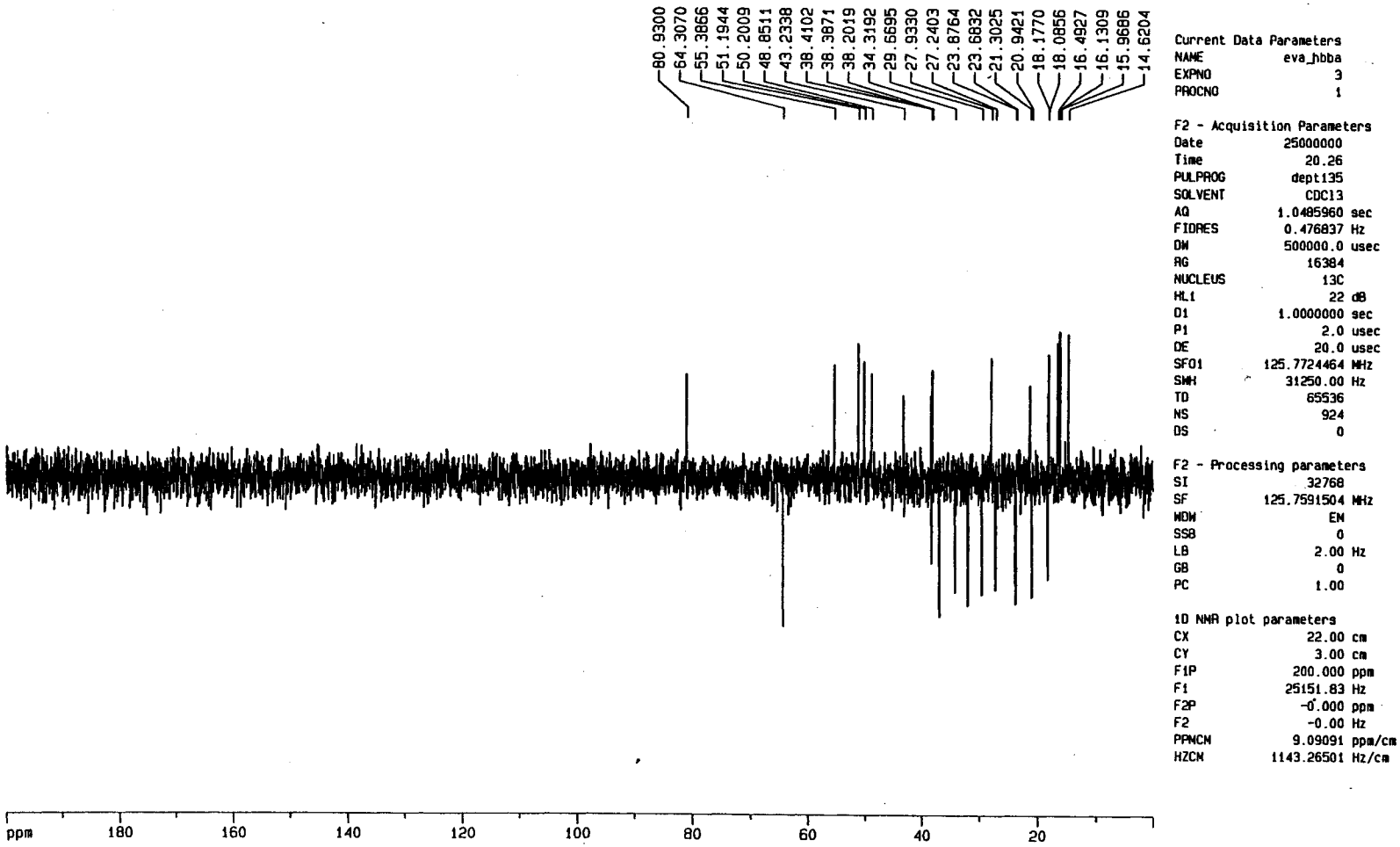


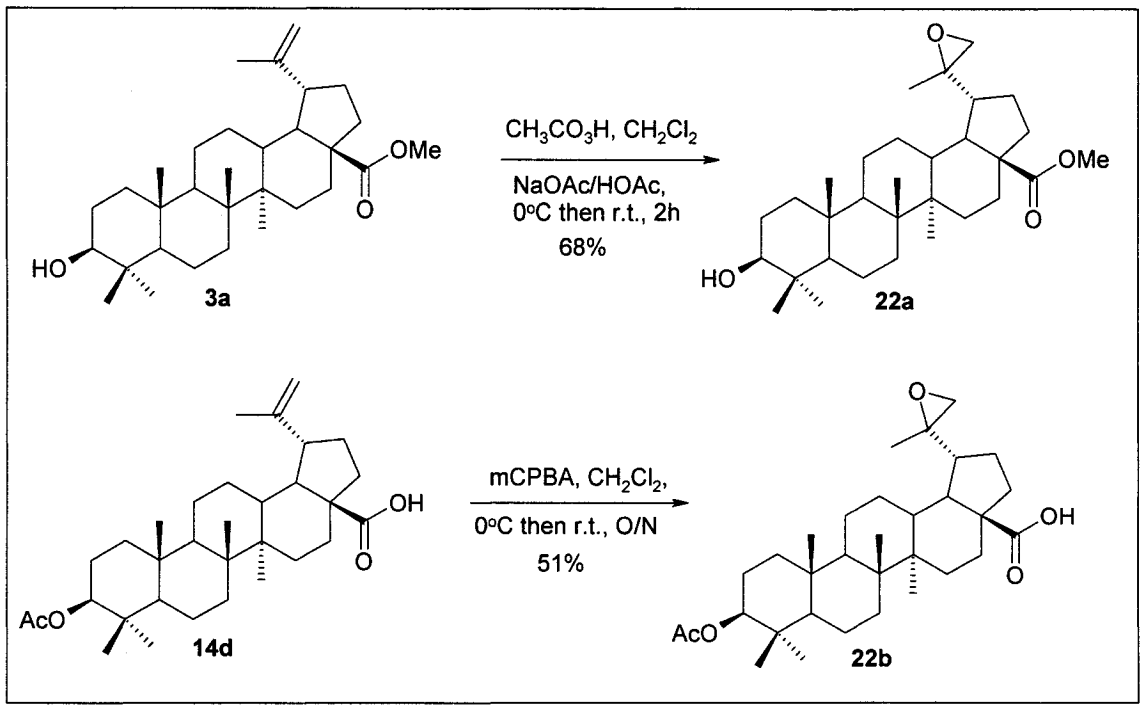
Figure 2.5.6.2d: DEPT 135 spectrum of Methyl 3β-acetyl, 29-hydroxy-lup-28-oate (**21b**).

2.5.7. Epoxidation of betulinic acid derivatives

It was anticipated that the action of meta-chloroperoxybenzoic acid (mCPBA) on the alkene functionality of betulinic acid would result in the formation of the corresponding epoxide, probably as a diastereomeric mixture. Indeed, whenmethyl betulinate (**3a**) and 3-acetyl betulinic acid (**14d**) were treated with mCPBA (CH_2Cl_2 at 0°C), (**22a**) and (**22b**) were formed.

However, the reaction of (**3a**) with mCPBA was not a very clean reaction and the yield of the epoxide was much lower compared to the reaction of (**17d**) with mCPBA. In each case, it appeared that the desired epoxide was quite unstable under the acidic reaction conditions.

Therefore, it was not unexpected that the epoxidation of (**3a**) to (**22a**) using peroxyacetic acid (CH_3CO_3H) in a buffered solution of NaOAc/HOAc in CH_2Cl_2 proved to be a superior route to (**22a**) giving (**22a**) in 68% isolated yield after rapid silica gel column chromatography (**Scheme 2.5.7a**).



Scheme 2.5.7a: Epoxidation of (**3a**) and (**14d**) to their respective epoxides (**22a**) and (**22b**).

Both epoxides (**22a**) and (**22b**) were unstable at room temperature and decomposed within several hours into about six spots on TLC. These transformation products were not isolated.

Interestingly, Patra and Chaudhur reported the same reaction of (**14d**) with mCPBA in 1988 which resulted in the formation of three main products, (**22c**), (**22d**) and (**22e**). The epoxide (**22b**) was not isolated (**Figure 2.5.7b**).[142]

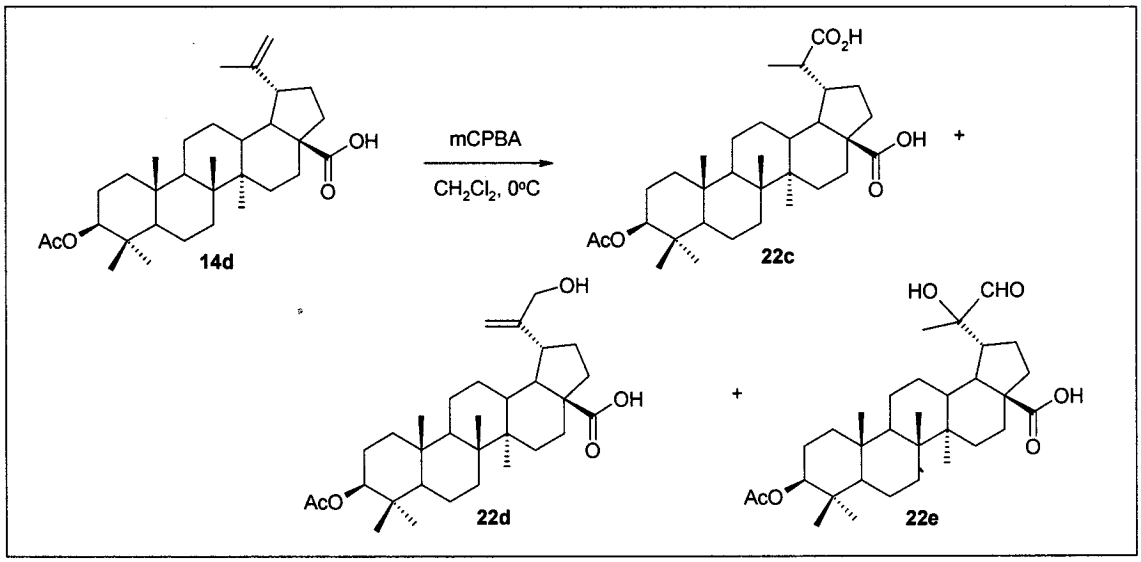


Figure 2.5.7b: Epoxidation products formed when 3β-acetyl betulinic acid (**14d**) was treated with mCPBA according to Patra and Chaudhur.[142]

On the other hand, when Dinda *et. al* subjected betulinic acid (**3**) to epoxidation conditions using mCPBA (CH_2Cl_2, r.t., 5h), two products were obtained, an aldehydic acid (**22f**) and a trihydroxy acid (**22g**), and no epoxide was found (**Figure 2.5.7c**).[141] It has been rationalized that both these products were formed through an epoxide intermediate, which on rearrangement gave (**22f**) and, on nucleophilic epoxide ring opening by water during work up of the reaction mixture produced (**22g**).

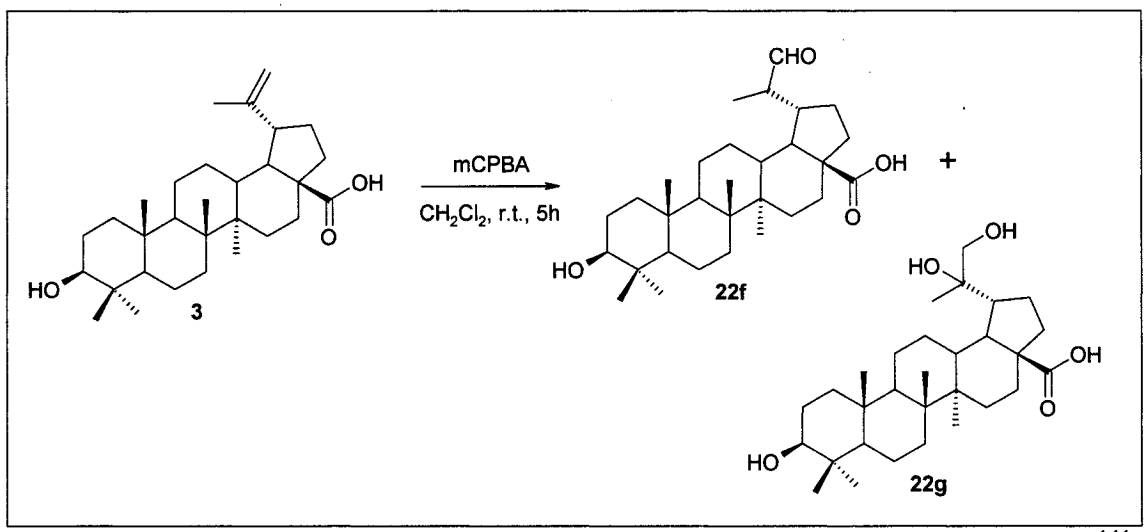


Figure 2.5.7c: Epoxidation products (**22f**) and (**22g**) obtained by Dinda *et. al.*[141] when betulinic acid (**3**) was treated with mCPBA.

2.5.7.1. Methyl 3β-hydroxy-lup-20(29)-epoxy-28-oate

Methyl 3β-hydroxy-lup-20(29)-epoxy-28-oate (**22a**) was isolated as a white solid, m.p.191-193°C, in 68% yield after silica gel column chromatography (**Scheme 2.5.7a**). An attempt to recrystallise (**22a**) from MeOH resulted in its complete loss through decomposition. The molecular formula $C_{31}H_{50}O_4$ was established by HRMS and by the $[M]^+$ of (**22a**) at m/z 468.

The ^1H NMR spectrum of (**22a**) showed a two proton singlet at δ 2.60 characteristic of the CH_2 part of the new epoxy ring. The signals of the olefinic hydrogens at C-29 were not observed. The C-30 methyl group, now attached to the epoxy ring appeared as a new methyl singlet at δ 1.20 (**Figure 2.5.7.1a**).

The key peaks in the ^{13}C NMR spectrum of (**22a**) were observed at δ 176.4 (CO_2Me), at δ 78.8 (C-3), and at δ 60.1 (C-29), (**Figure 2.5.7.1b**). The proton and carbon chemical shifts of (**22a**) have not been recorded in the literature.

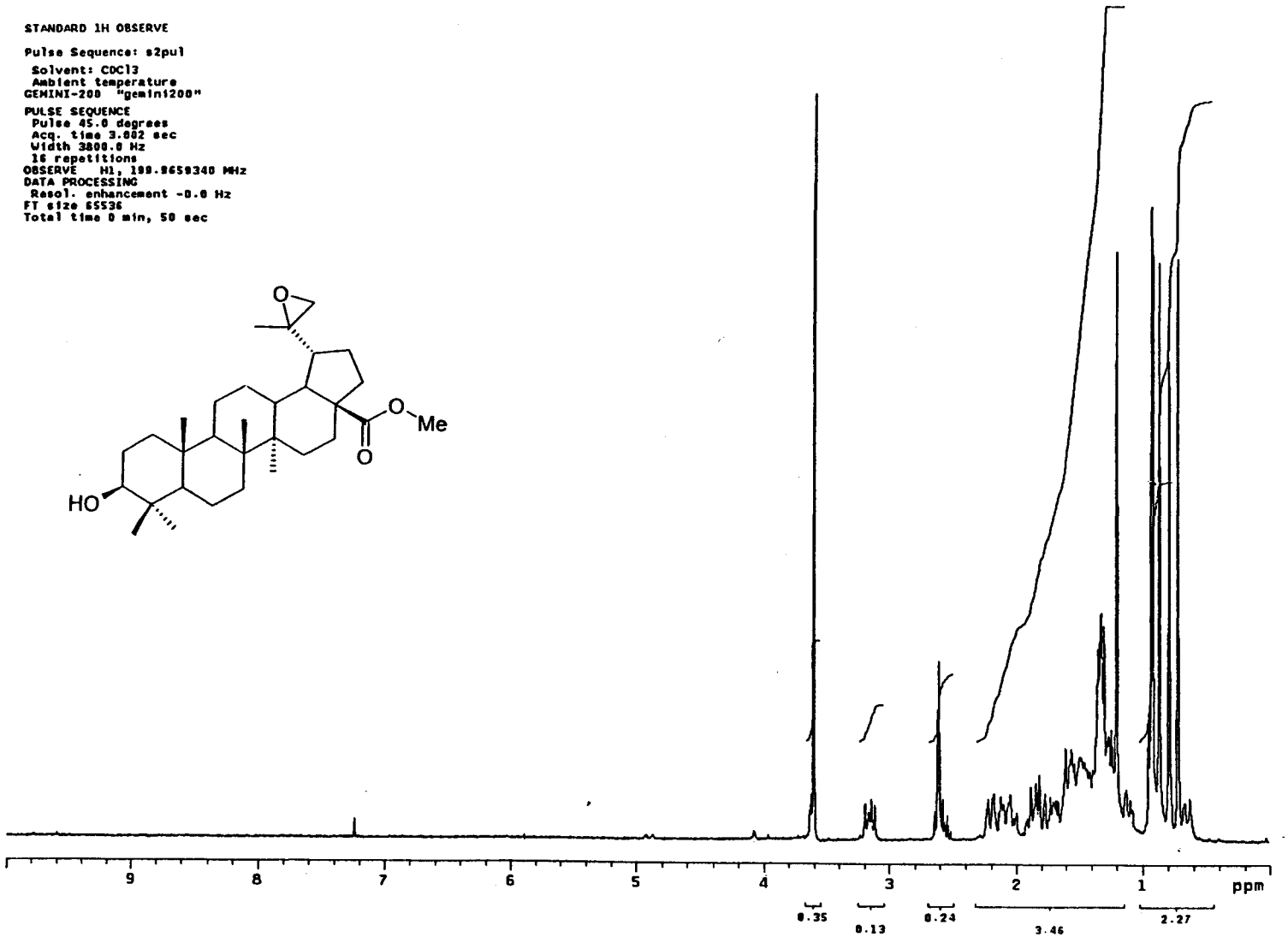


Figure 2.5.7.1a: ^{1}H NMR spectrum of Methyl 3β-hydroxy-lup-20(29)-epoxy-28-oate (**22a**).

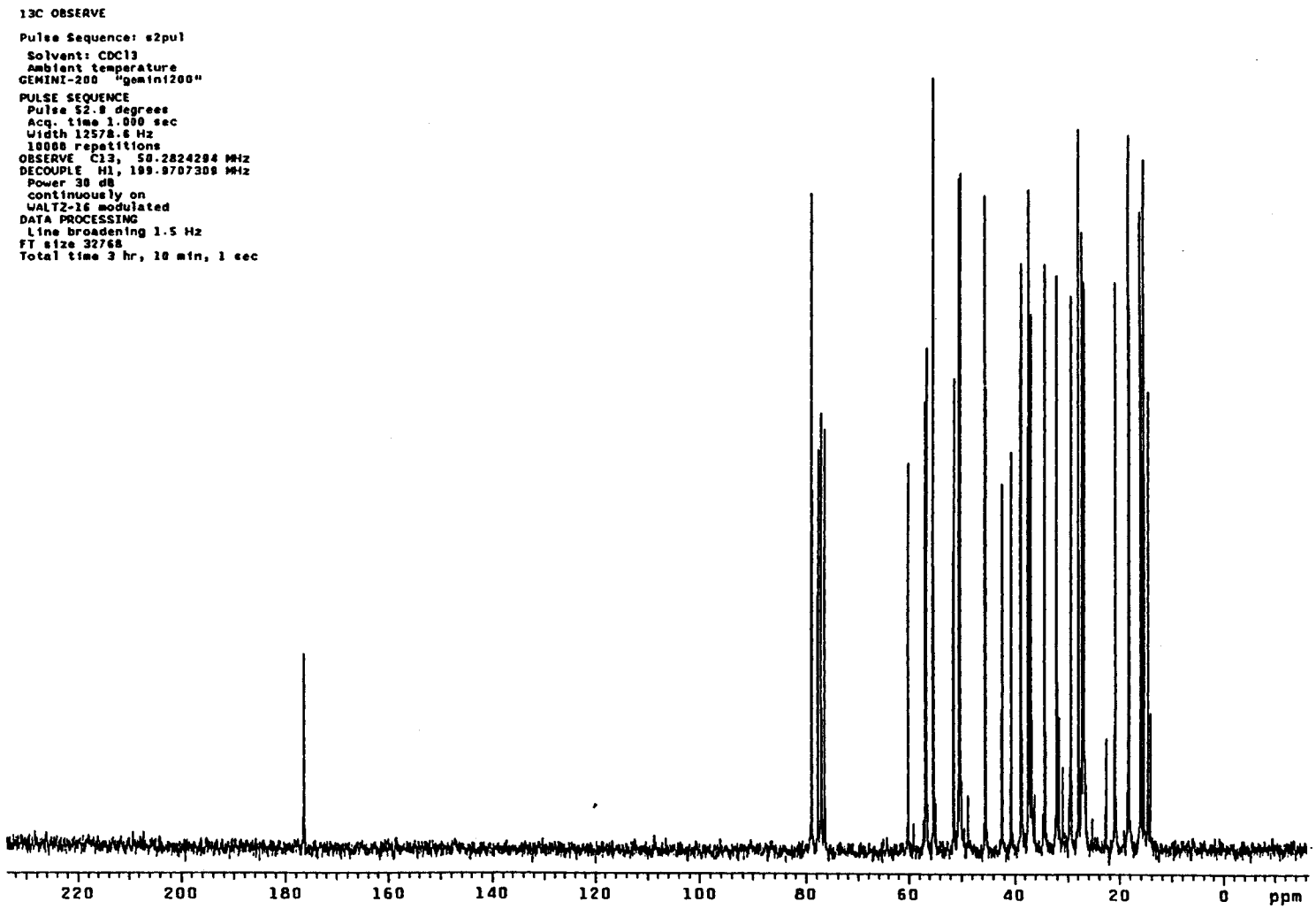


Figure 2.5.7.1b: ^{13}C NMR spectrum of Methyl 3β-hydroxy-lup-20(29)-epoxy-28-oate (**22a**).

2.5.7.2. 3β-Acetyl-lup- 20(29)-epoxy-28-oic acid

3β-Acetyl-lupan-(20)29-epoxy-28-oic acid (**22b**) was isolated as a white solid, m.p.290-293°C in 51% yield after silica gel column chromatography (**Scheme 2.5.7a**). Surprisingly, as for (**22a**), the epoxy ring protons in (**22b**) appeared as a two proton singlet at δ 2.64 and not the potential AB quartet (**Figure 2.5.7.2**). The physical and spectral data of (**22b**) listed in the experimental section have not been previously reported.

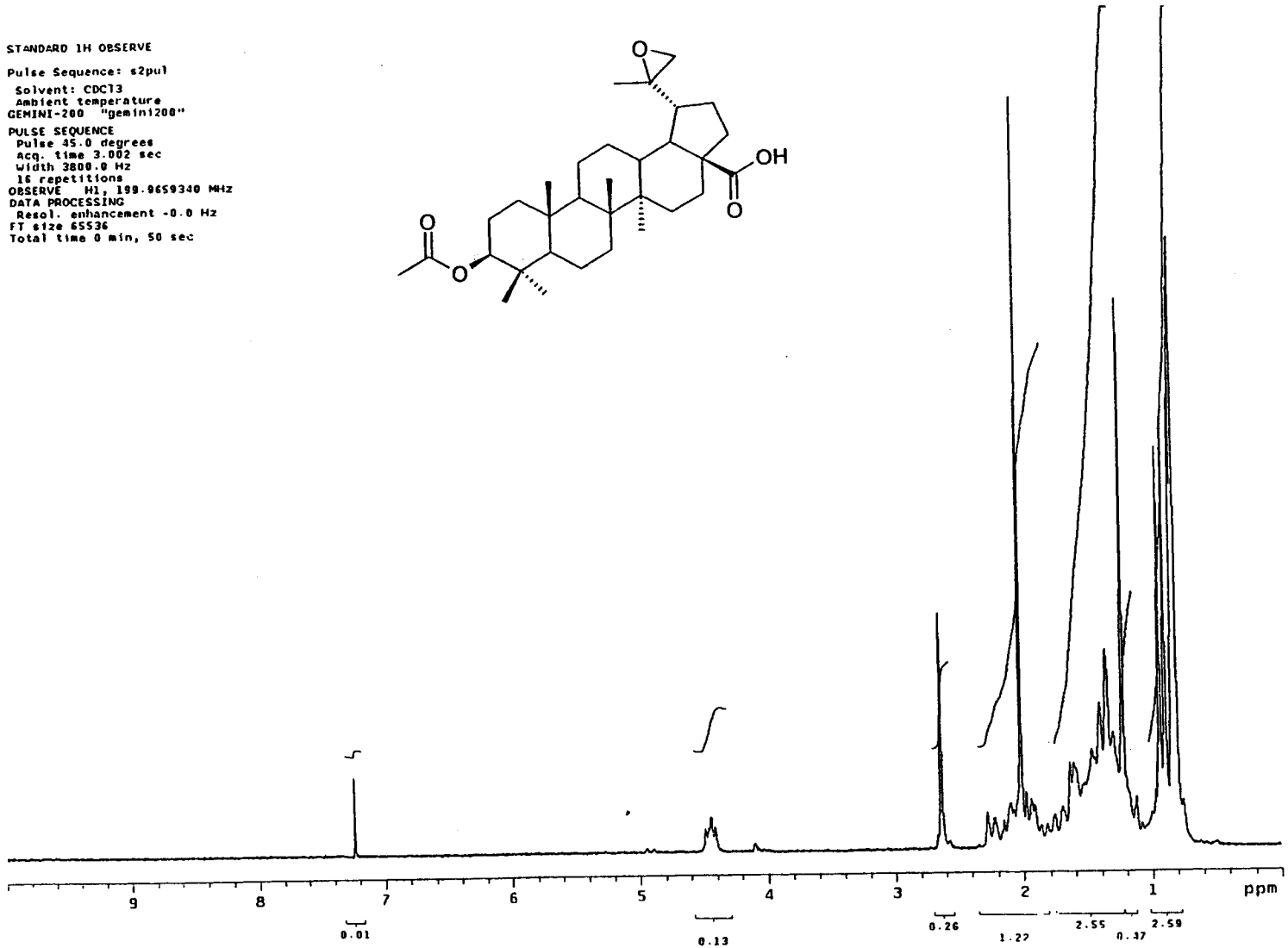


Figure 2.5.7.2: ^{1}H NMR spectrum of Methyl 3β-acetyl-lup-20(29)-epoxy-28-oate (**22b**).

2.5.8. Glycosylation of betulinic acid methyl ester

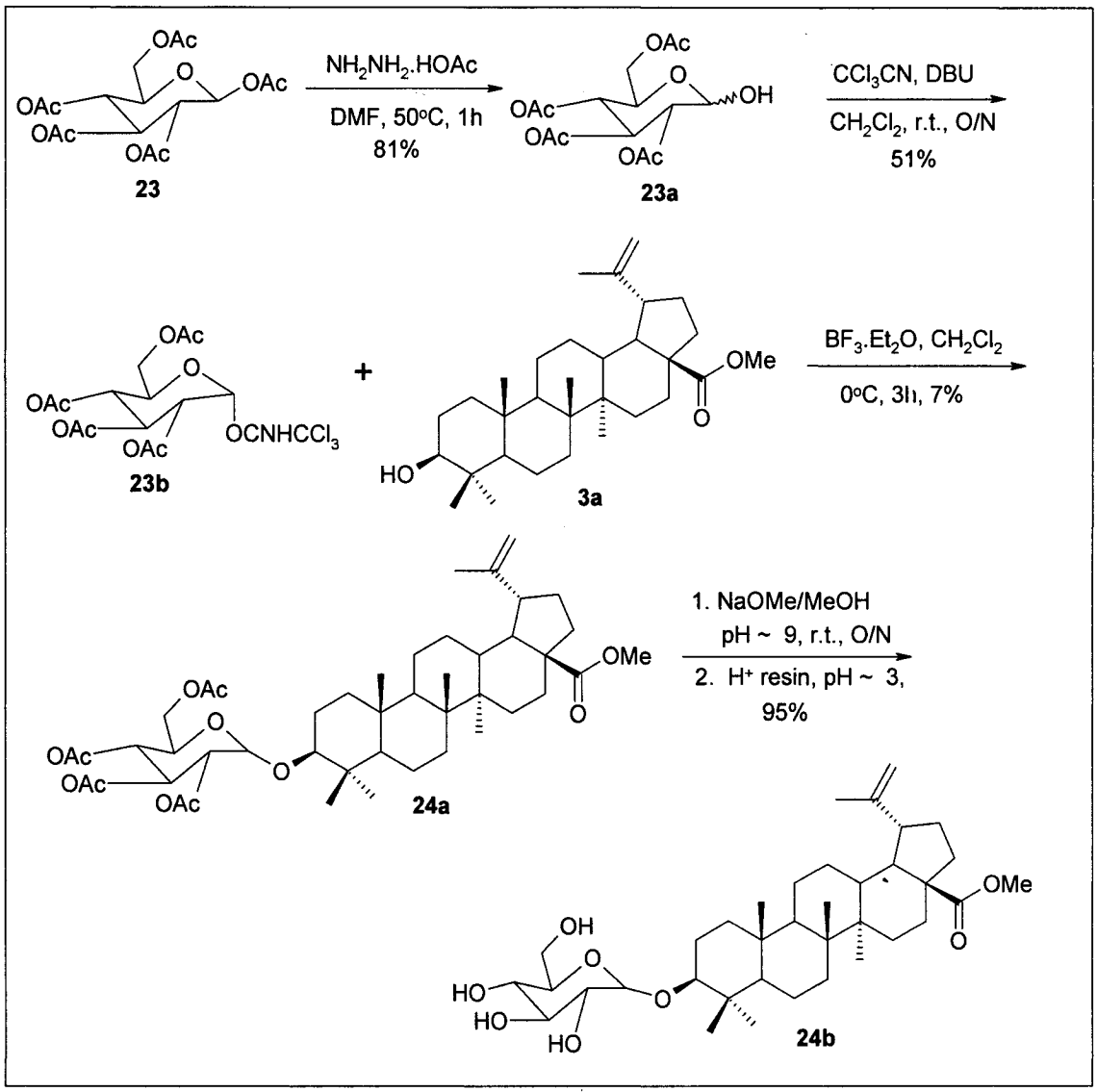
The low solubility of betulinic acid (**3**) in water and organic solvents meant inefficient biological efficacy. Therefore, it was anticipated that glycosylation of (3) will help improve aqueous solubility. Hence, glycosylation of (**3**) at the C-3 (OH) position employs acetyl protected-β-D-glucopyranose (**23**) as the starting material (**Scheme 2.5.8**).

Deprotection of (**23**) at the anomeric position in the presence of hydrazine acetate in DMF at 50°C afforded the C-1-unprotected glucospyranose (**23a**) as a mixture of anomers in 81% yield. The activation at the anomeric carbon in the form of trichloroacetimidate using trichloroacetonitrile and DBU in CH_2Cl_2 at room temperature led diastereospecifically to the α-imidate (**23b**) in 51% yield.[143]

The reaction of (**23b**) with methyl betulinate (**3a**) on catalysis with boron trifluoride etherate in CH_2Cl_2 at 0°C gave exclusively the β-glucoside (**24a**), unfortunately in low yield (7%). Zemplen de-O-acetylation of (**24a**) with NaOMe/MeOH/pH ~9 at room temperature furnished the glucoside derivative of betulinic acid (**24b**) in 95 % yield.

The glucosidation reaction of (**23b**) and (**3a**) was not a very clean reaction (showing about six spots on TLC) and, it did not appear to go to completion. A substantial amount of starting material (**3a**) was recovered (30%). Changing the number of equivalents of $BF_3.Et_2O$ from five to one did not to appear to improve the reaction but no further attempts was made at optimizing the reaction conditions.

In 1997, Klinotova *et. al* prepared glucoside (**24a**) in 53%, by reacting (**3a**) with acetobromoglucose in the presence of a soluble catalyst mercury (II) cyanide in acetonitrile and after refluxing for 34h.[144] Zemplen de-O-acetylation of (**24a**) furnished (**24b**) in 74% yield.



Scheme 2.5.8: Synthesis of the glycoside derivatives of betulinic acid (**24a**) and (**24b**)

The diagnostic signals observed in the ^{13}C NMR spectra of (**3a**), (**24a**) and (**24b**) are given **Table 2.5.8**. The published data for (**24a**) is also included for comparison ($\delta_{C'}$). The ^{13}C NMR data of (**24b**) has not been reported in the literature.

Table 2.5.8: Diagnostic ^{13}C NMR resonances for compounds (**3a**), (**24a**) and (**24b**). Comparison with published data ($\delta_{C'}$) for (**24a**).[144]

Carbon	**3a**	**24a**	$\delta_{C'}$	**24b**
C-3	79.0	90.7	90.7	90.1
C-17	56.5	56.5	56.6	56.5
C-20	150.6	150.6	150.6	150.5
C-28	176.7	176.6	176.7	176.6
C-29	109.6	109.5	109.6	109.6
OMe	51.3	51.3	51.2	51.3
Glucose				
C-1'		102.9	102.9	105.1
C-2'		71.4	71.5	73.8
C-3'		72.8	72.9	75.2
C-4'		68.7	68.8	69.6
C-5'		71.6	71.7	73.8
C-6'		62.2	62.2	61.8
Acetate				
C-2'		170.4	170.4	
C-3'		170.7	170.7	
C-4'		169.4	169.4	
C-6'		169.2	169.2	

2.5.8.1. Glucoside tetraacetate of betulinic acid methyl ester

The glucoside tetracetate of betulinic acid methyl ester (**24a**) was isolated as a white solid, m.p. 119-121°C (lit.[144] 120-123°C) in 7% yield after silica gel column chromatography. The electronspray ionization (ESI) of (**24a**) revealed a molecular ion at m/z 839 $[M+K]^+$ which was consistent with the molecular formula $C_{45}H_{68}O_{12}$ (calculated for $C_{45}H_{68}O_{12}$ + K). The IR spectrum of (**24a**) exhibited strong ester carbonyl absorptions at 1756 and 1734 cm^{-1}.

Both the ^1H and ^{13}C NMR data of (**24a**) were in excellent agreement with those reported by Klinotova *et. al.*[144] The glucose is attached to C-3 as proven by the significant downfield shift observed for C-3 (δ = 90.7) relative to the corresponding chemical shift (δ =79.0) in (**3a**).

The resonances at δ 102.9 (C-1'), 71.4 (C-2'), 72.8 (C-3'), 68.7 (C-4'), 71.6 (C-5') and δ 62.2 (C-6') were assigned to the glucose moiety whereas the four acetoxycarbons were assignable to the resonances at δ 170.7, 170.4, 169.4 and at δ 169.2. The resonances at δ 150.6 (C-20), 109.5 (C-29), 76.6 (C-28) and at δ 51.3 (OMe) proved the presence of the aglycone moiety.

2.5.8.2. Glucoside of betulinic acid methyl ester

The glucoside of betulinic acid methyl ester (**24b**) was isolated as a pale yellow solid, m.p. 196-198°C (lit.[144] 197-200 °C), after silica gel column chromatography in 95% yield.

The EIMS of (**24b**) displayed a molecular ion peak $[M]^+$ at 632 which was consistent with the molecular formula $C_{37}H_{60}O_8$. The clear loss of the glucose moiety was indicated by the prominent peaks at m/z 454 $[M\text{-}C_6H_{11}O_6]^+$ and m/z 453 $[M\text{-}C_6H_{112}O_6]^+$. The IR spectrum of (**24b**) revealed a broad hydroxyl band at 3398 cm^{-1} and a strong carbonyl absorption at 1721 cm^{-1}.

Both the ^1H and ^{13}C NMR of (**24b**) showed the disappearance of the four acetoxy groups. The anomeric CH part of the glucose moiety in (**24b**) was readily identified from the resonances at δ 105.1 (C-1) and at δ 4.30 (J=8.0 Hz, H-1'). The remaining protons within the glucose moiety were not very well resolved, but were assignable. Thus, resonances were observed at δ 3.77 (br s, 2H, H-6'), 3.53-3.46 (m, 3H, H-3', H-4', H-5') and δ 3.27 (br s, 1H, H-2'). Klinotova *et. al.,* reported the ^1H NMR data of (**24b**) in CD_3OD and not $CDCl_3$.[144]

The most significant resonance in the ^{13}C NMR spectrum of (**24b**) are listed in **Table 2.5.8**. The glucose moiety exhibited signals at δ 105.1 (C-1') for the anomeric carbon, at δ 75.2 (C-3'), 73.8 (C-5'/C-2'), 69.6 (C-4') and at 61.8 (C-6') for the carbons bearing hydroxyl groups. The aglycone moiety was indicated by one carboxyl carbon at δ 176.6, two olefinic carbons at δ 150.5 (C-20) and at δ 109.6 (C-29) and one carbon bearing the glucose moiety at δ 90.1 (C-3).

2.6. Evaluation of anti-anxiety activity: Models and data

The anti-anxiety activity of Margraviaceae leaf extracts, both the crude EtOH extract and the active EtOAc extract (f1), as well as the isolated bioactive constituent betulinic acid and some of its derivatives have been confirmed by in vivo bioassays.

As mentioned before (**Section 2.1.3**), tests were carried out on mice and rats using validated industry accepted tests namely, elevated plus- maze or EPM (rats and mice), fear potentiated startle (rats), punished drinking or Vogel test (rats), light-dark box (mice) and swim despair test (mouse). The results from these tests although carried out by students in Dr. Zul Merali's research group, are presented in this chapter in order to give an integrated picture of the importance of these compounds. The testing results also influenced the synthesis of analogs of betulinic acid. Due to cost, animal and time requirements, not all the derivatives synthesized have been evaluated.

2.6.1. Anti-anxiety activity of Margraviaceae leaf extracts

Investigation of the anti-anxiety activity of the crude EtOH extract (SS-CE) of Margraviaceae leaves revealed that it alleviates anxiety in the elevated plus maze (EPM) test (**Figure 2.6.1a**) and the fear potentiated startle paradigm (**Figure 2.6.1b**). SS-CE is code for the crude plant or crude EtOH extract [SS stands for "Sin-Susto" meaning no fear, and CE for the crude extract].

Figure 2.6.1a is a bar graph depicting the time (sec) spent in the open arms of the EPM by animals treated via gavage with SS-CE administered with condensed milk (control), EtOAc fraction (f1) and the aqueous fraction (f2) dissolved in condensed milk prior to test. The control group receive only milk.

It is clear from Figure **2.6.1a**, that rats treated with Margraviaceae leaf extracts, both SS-CE and the active ethyl acetate (EtOAc) extract or f1, spend significantly more time on the open arms of the maze as compared to the control,

indicating that the plant extracts (50 mg/rat) may impart potent anxiolytic effects. The aqueous fraction (f2) appears very much like the control and has no appreciable activity. The anxiolytic activity resides solely in the EtOAc fraction (f1).

In **Figure 2.6.1b,** the bar graph depicts the difference in startle response (paired with the fear cue) of animals that were administered the crude EtOH plant extract (SS-CE) and EtOAc fraction (f1) using peanut oil as the vehicle or control prior to test. The control shows the expected startle response (paired with the fear cue). This response is markedly attenuated in rats treated with the 95% EtOH crude Margraviaceae extract (SS-CE) or its bioactive fraction (f1).

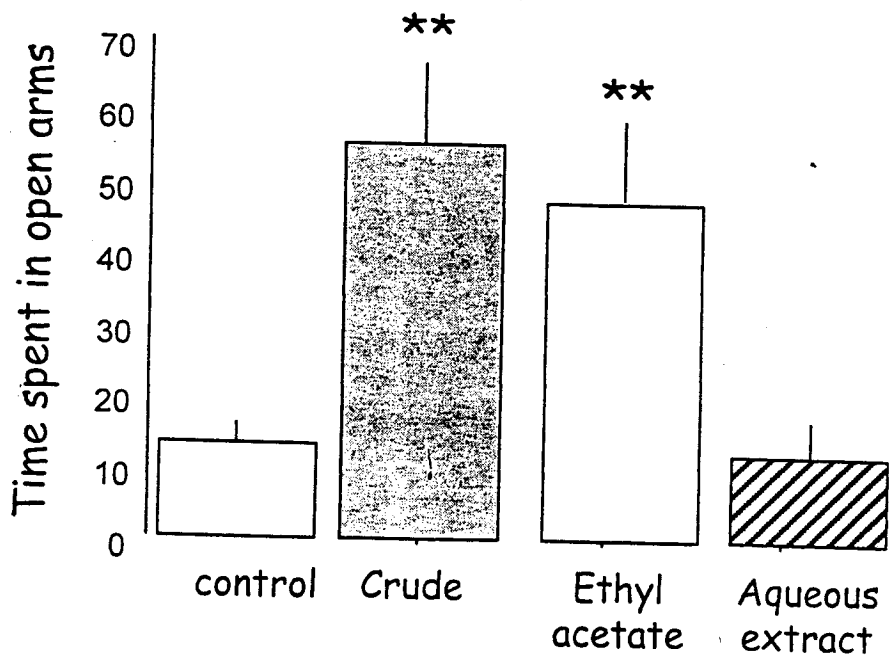


Figure 2.6.1a: Amount of time spent in the open arms of the EPM (sec) by animals administered condensed milk (control), crude plant extract (SS-CE), EtOAc extract (f1) and aqueous fraction (f2), all dissolved in condensed milk.
** Significantly different from control at $p<0.01$.

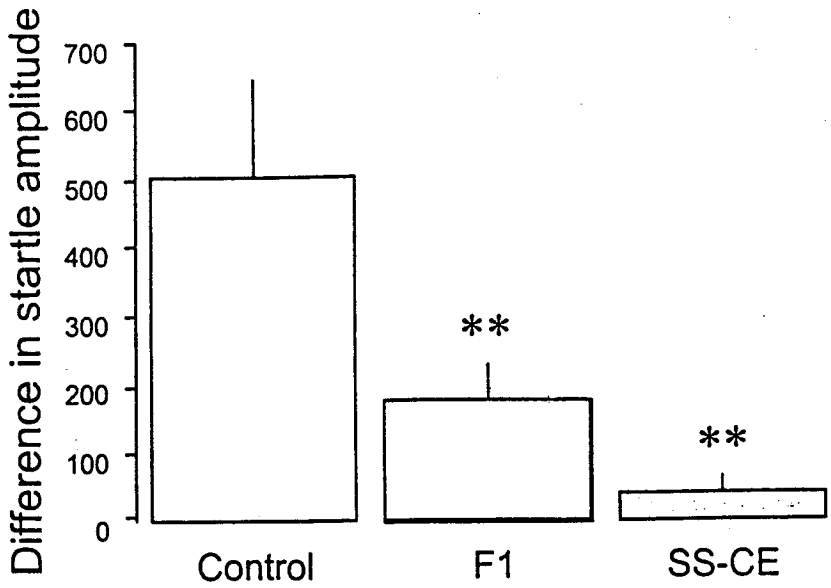


Figure 2.6.1b: Attenuation of fear-potentiated startle response. Rats were administered the crude plant extract (SS-CE), EtOAc fraction (f1) using peanut oil as vehicle (control), prior to test.
** Significantly different from control at $p<0.01$.

2.6.2. Anti-anxiety activity of betulinic acid

In the fear potentiated test of anxiety, anxiolytic drugs (benzodiazepines) reduce the startle amplitude when the sound (110 dB) is presented in the presence of a cue previously paired with footshock. Likewise, betulinic acid (1 mg/kg orally) attenuated the started response in the presence of the fear-cue.

Figure 2.6.2a is a bar graph depicting the difference in startle amplitude in the presence of a fear cue, which was markedly reduced in rats pretreated with betulinic acid (SS-01). SS-01 is the code for betulinic acid (**3**).

Figure 2.6.2b is a bar graph depiciting the effect of betulinic acid (SS-01) on the EPM test. The time spent on the open arms of the plus-maze by the rats pretreated with SS-01 is significantly increased, revealing that SS-01 is indeed

able to alleviate anxiety. These examples illustrate that betulinic acid (SS-01) is a major biologically active constituent of Margraviaceae.

When used at 0.5-1.0 mg/kg, the activity of pure betulinic acid is comparable to that of Valium. The anti-anxiety activity of betulinic acid is observed for at least 4h after oral administration. No other changes in behaviour were observed.

The effect of betulinic acid (SS-01) on CD-1 strain of mice was also tested. **Figure 2.6.2c** shows that mice treated with SS-01 (0.25 or 2.5 mg/kg) entered the open (anxiogenic) arms of the EPM more than the controls. The 0.25 mg/kg dose of SS-01 appears to be a maximally effective dose.

These data demonstrated that betulinic acid (SS-01) is effective as an anti-anxiety agent in both rats and mice.

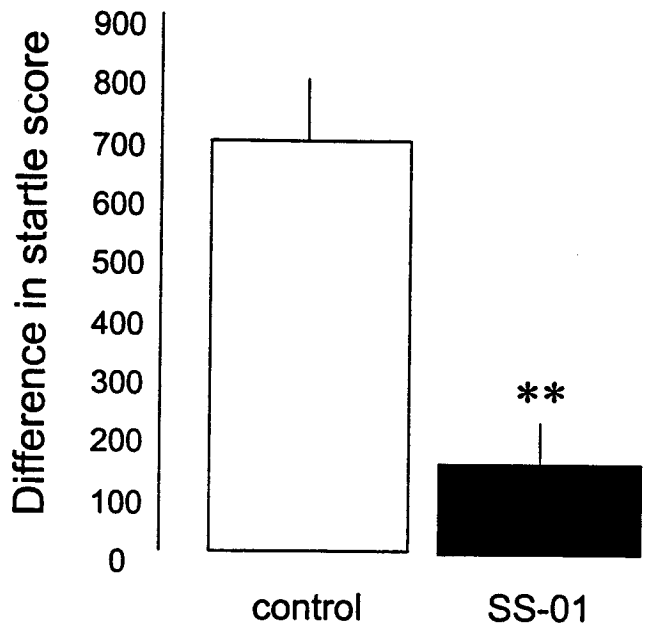


Figure 2.6.2a: Effect of betulinic acid (SS-01) on fear potentiated startle.
** Significantly different from control at $p<0.01$.

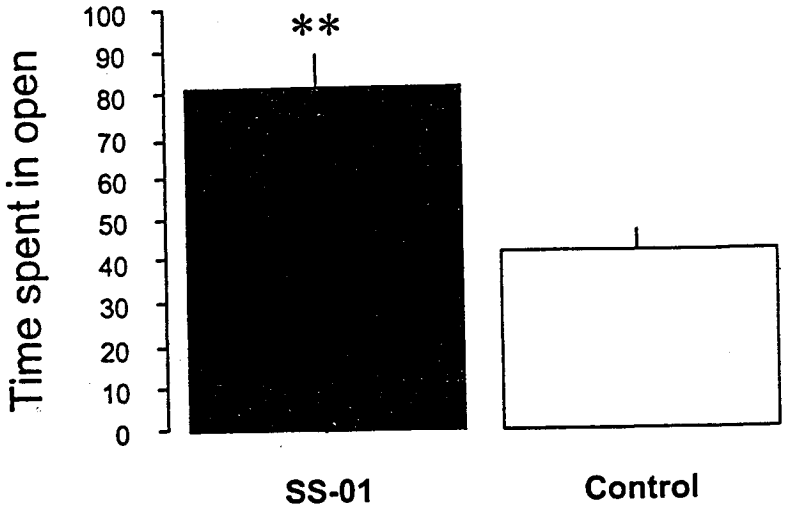


Figure 2.6.2b: Effect of betulinic acid (SS-01), (1 mg/kg by gavage) on the time spent on the open arms of the plus-maze.
** Significantly different from control at $p<0.01$.

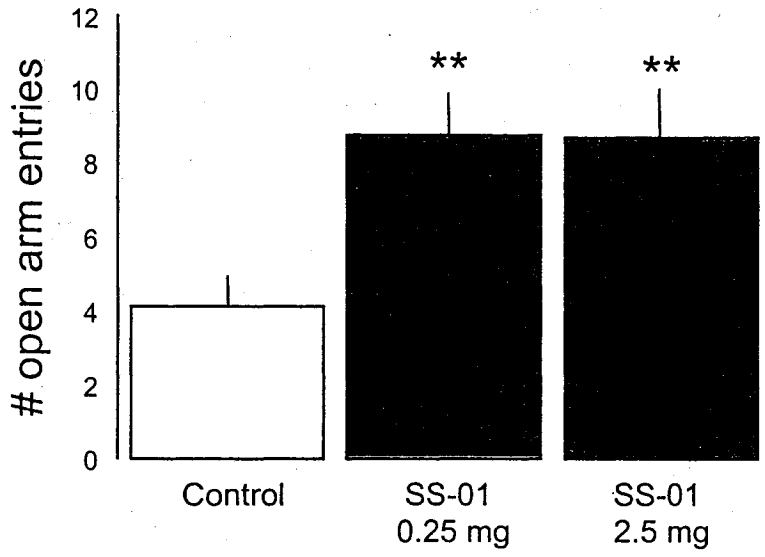


Figure 2.6.2c: Effect of betulinic acid (SS-01) on the time spent on the open arms of the plus maze by mice.
** Significantly different from control at $p<0.01$.

2.6.3. Anti-anxiety activity of betulinic acid derivatives

The anti-anxiety activity of some of the synthetic derivatives of betulinic acid was compared with betulinic acid itself and the well known standard anti-anxiety compound, Valium. Indeed, some of the derivatives of betulinic acid exhibited anti-anxiety effects that were equivalent to or even better than betulinic acid, particularly betulinic acid methyl ester (**3a**) or methyl betulinate (SS-05). Therefore, this compound was evaluated most intensely. SS-05 is the code for betulinic acid methyl ester.

In a Vogel test or punished drinking test for anxiety (**Figure 2.6.3**), thirsty rats were given access to water in an apparatus where every 5^{th} lick was punished with a mild shock. This suppressed the drinking behaviour. However, pretreatment with diazepam (2 mg/kg) alleviated this suppression. Likewise, betulinic acid (SS-01) also reversed punished drinking.

Moreover, many of the esters of betulinic acid showed an anxiolytic profile and so did the amides of betulinic acid. However, the amides of betulinic acid appeared to be less potent and/or efficacious than the parent compound and the esters. **Table 2.6.3** summarises the compounds which have been tested for anti-anxiety activity.

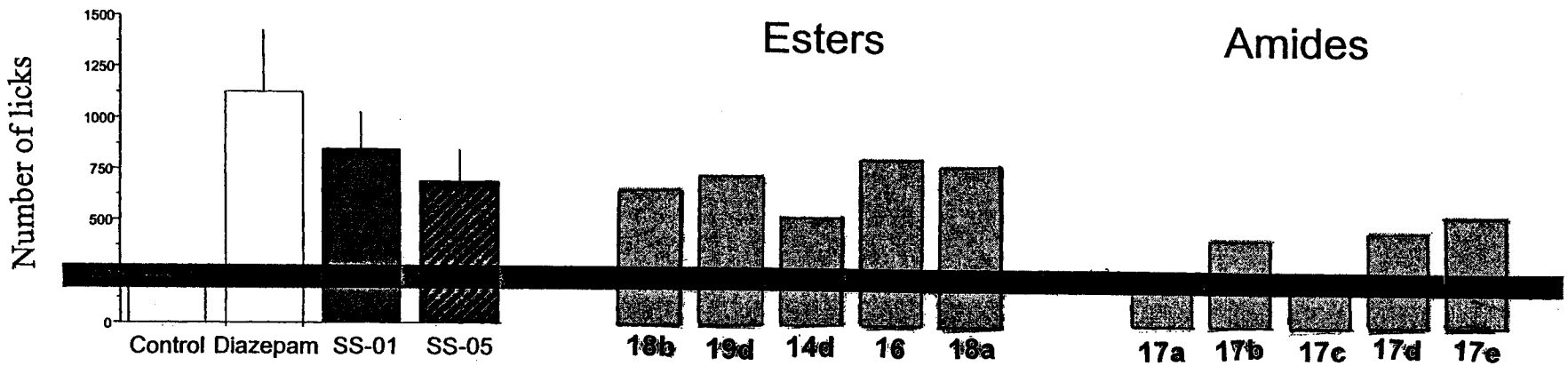


Figure 2.6.3: Effects of betulinic acid and derivatives on punished drinking (Vogel Test): Comparison to Diazepam.

Table 2.6.3: Summary of the synthetic derivatives of betulinic tested (T) or not tested (NT) for anti-anxiety activity.

Betulinic acid derivative	Anxiety activity
3a	T
3b	NT
3c	NT
3d	NT
3e	NT
3f	NT
5b*	T
14d	T
16	T
17a	T
17b	T
17c	T
17d**	T
17e***	T
18a	T
18b	T
19a	NT
19b	NT
19c	NT
19d	NT
19e	NT
20a	NT
20b	NT
21a	NT
21b	NT
22a	NT
22b	NT
23a	NT
23b	NT

*Oleanolic acid. **Betulinic acid glycine methyl ester amide. ***Betulinic acid glycine amide.

2.6.4. Anti-anxiety activity of betulinic acid methyl ester

Another test for anxiety in rodents is the amount of time they spend in social interaction which decreases when rats are anxious. On a test day, pairs of male rats were introduced into a familiar test arena (for 8 min.) 1h and 4h after drug administration (0.5 mg/kg; i.p).

It was found that both the rats treated with betulinic acid (SS-01) or betulinic acid methyl ester (SS-05) spent more time in social interaction at 1 or 4h after treatment (**Figure 2.6.4a**). Again, betulinic acid methyl ester (SS-05), exhibited anti-anxiety that was as effective as betulinic acid (SS-01), if not superior to betulinic acid. This is such that it could be considered as a viable drug candidate.

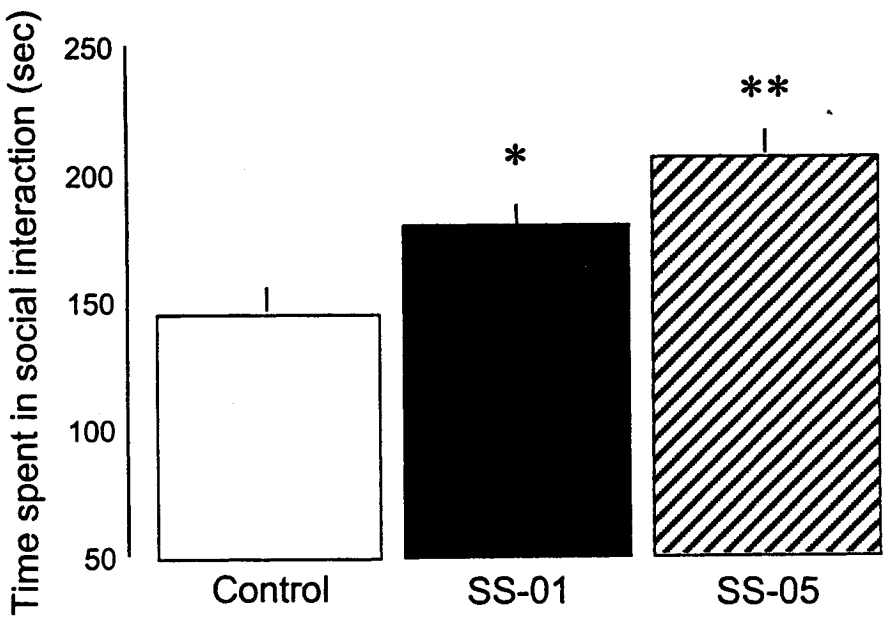


Figure 2.6.4: Effect of betulinic acid (SS-01) and betulinic acid methyl ester (SS 05) on social interaction. The score reflects the time in sec. the pairs of rats spent in social interaction.
*, ** Significantly different from control at $p<0.05$ and 0.01, respectively.

2.6.5. Assessment of chronic experiments

Betulinic acid (SS-01) and betulinic acid methyl ester (SS-ME, also SS01ME or SS-05) were administered chronically (0.5 mg/kg daily by oral gavage) for one month to a test group of rats (n=8). Four hours after the final dosage the rats were tested via the EPM test. **Figure 2.6.5a** illustrates that acute administration of the SS-01 and SS-ME to chronically treated rats maintained its anti-anxiety effects, as reflected by increased time spent on the open arms of the EPM test. The results were comparable, even slightly better than after a single dose. The animals did not develop tolerance or desensitization to the acute anti-anxiety effects of the drug. Hence, it did not require extra drug to achieve the same anti-anxiety effect.

The test animals were also monitored throughout the test for locomotor activity (**Figure 2.6.5b**) and weight gain (**Figure 2.6.5c**). It is apparent from **Figure 2.6.5b**, that chronic treatment with betulinic acid (SS-01) or betulinic acid methyl ester (SS-ME) did not alter 24 hour locomoter activity or patterns, suggesting no adverse locomoter effects and/or disturbances in sleeping cycle.

Figure 2.6.5c portrays no difference in body weight changes between the test and control groups during the one-month of chronic administration. The test and control groups were monitored after the end of one-month period to determine if the animals showed withdrawal symptoms. Cessation of chronic treatment failed to elicit overt withdrawal effect. Thus, no evidence of addiction was found.

Furthermore, it is known that a rise in the level of corticosterone (stress hormone) is observed for restraint-induced animals. However, acute and chronic treatment with betulinic acid methyl ester (SS-ME) resulted in a lower corticosterone level in restrain-induced animals (**Figure 2.6.5d**).

In general, the test animals and control groups behaved identically thoughout the test period. Both groups continued to preen themselves similarly indicating good health. The animals in the two groups could not be differentiated visually. This is in contrast to rats that have been administered Valium. Such

animals are lethargic due to the muscle relaxant property of the benzodiazepines. It is also known that the benzodiazepines become addictive and both humans and rats shows withdrawal symptoms such as agitation and restlessless.

The vital organs of the one-month administration test animals were harvested and are stored at -80°C for eventual examination of possible abnormalities by a pathologist. These examinations have not been done.

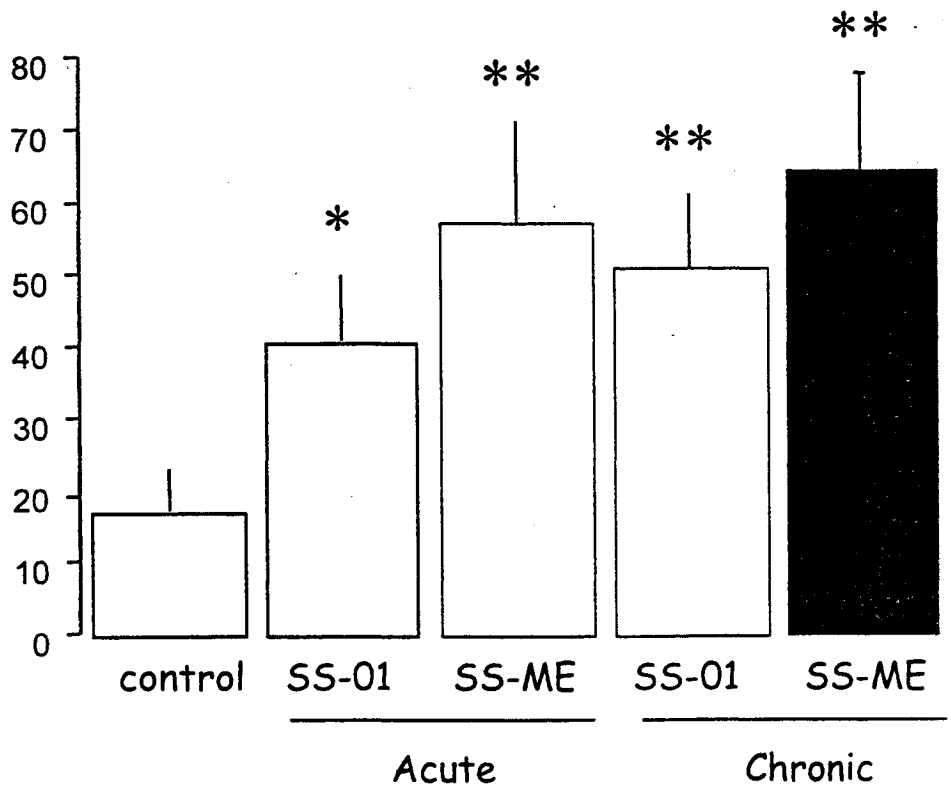


Figure 2.6.5a: Amount of time (sec) spend in the open arms of the EPM test by rats administered betulinc acid (SS-01) and methyl ester betulinic acid (SS-05). *, ** Significantly different from control at $p<0.05$ or $p< 0.01$, respectively.

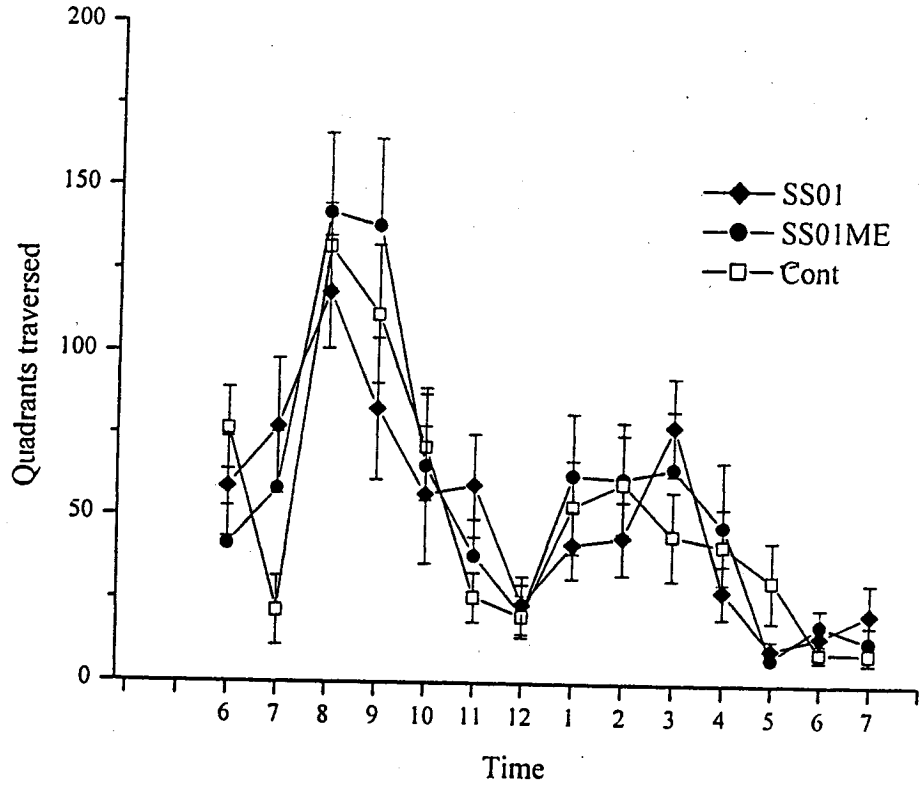


Figure 2.6.5b: Effects of chronic drug administration on locomotor activity.

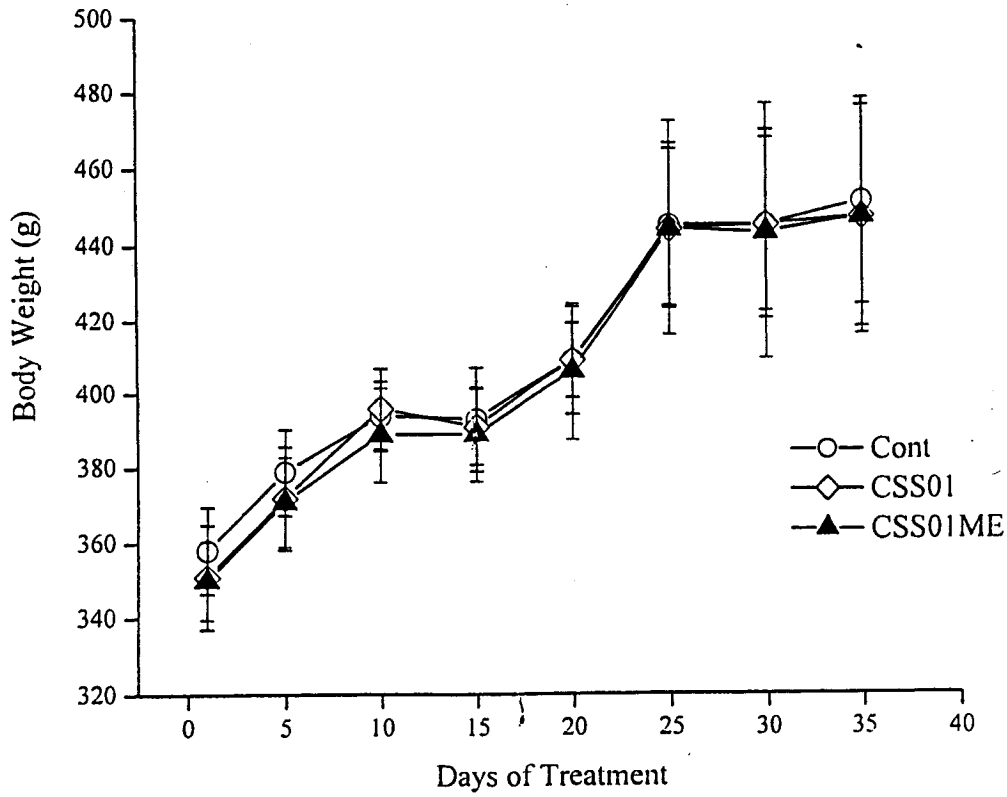


Figure 2.6.5c: Effects of chronic drug treatment on weight gain.

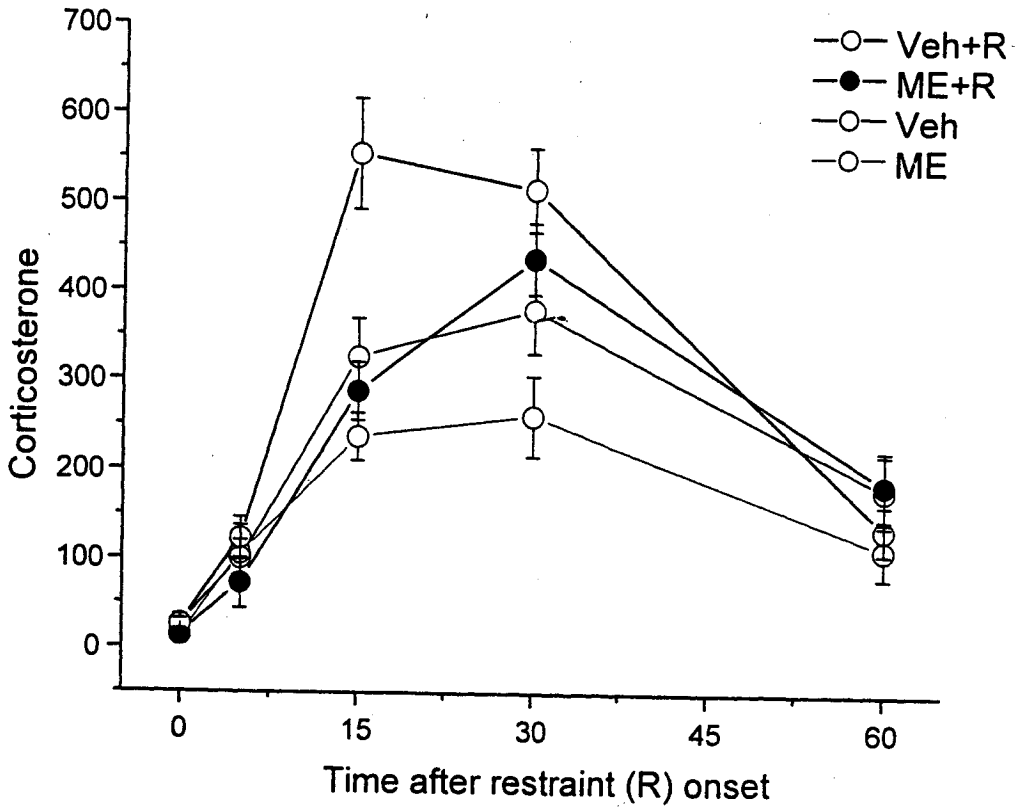


Figure 2.6.5d: Effects of chronic drug treatment on corticosterone level of restraint-induced animals.

2.6.6: Toxicity of Margraviaceae

There is no record of any toxic effects of the Magraviaceae plants in humans by the Kubuyuri, Karijona and Kekchi healers.[1] No overt behavioural effects (such as muscle flaccidity and motor effects seen in benzodiazepines) was observed in the rodents, other than anxiolytic effects. Gastrointestinal effects were not observed and the animals repeatedly consumed the extracts indicating no taste aversion. Animals appeared normal, continued to consume normal amounts of food and gain weight normally. Thus, no changes in food consumption was observed with short term use of the Magraviaceae plant extracts.

Betulinic acid showed no sign of acute or chronic activity, even at repeated doses up to 500 mg/kg (which is 1000 times higher than doses needed to reduce anxiety).

Betulinic acid has been shown to be non-toxic in a Hippocratic screen at doses of 200 and 400 mg/kg.[148] Others have reported other biological activities of betulinic acid such as anti-tumor activity at much higher doses (500 mg per kg body weight).[148] These investigations further supported the lack of toxicity of betulinic acid.

2.6.7. Developing a novel and effective anti-anxiety drug

Several of the simple betulinic esters appear to have ideal properties as new drug candidates since excellent bioassay results were obtained in all of the tests especialy betulinic acid methyl ester or methyl betulinate (**3a**). Thus, anti-anxiety activity is observed orally at 0.5 mg/kg (possibly lower), no adverse toxicity was observed even after long term administration (21-28 days), no signs of addiction, high therapeutic ratio, low cost compounds and facile one-step synthesis from betulinic acid.

Moreover, these simple esters are easily purified as white crystalline powder with high stability to heat and light. Assuming similar levels of activity in man compared to rats, at 0.5 mg/kg dosage for effective treatment, one would anticipate producing 25 and/or 50 mg tablets.

Based on current technology in our lab, 1 kg of ground birch bark yields about 60 g of betulinic acid (**3**). A 1 kg of drug would yield 20,000 (50 mg) and 40,000 (25 mg) tablets. The estimated cost of 1 kg of drug is $1000 and the chemical cost per 50 mg tablet is 5 cents and 2.5 cents per 25 mg tablet.

The fact that betulinic acid methyl ester (**3a**) is a known chemical entity makes its commercial development somewhat unlikely despite its excellent anti-anxiety profile. Pharmaceutical companies prefer to develop compounds that have both novel structures and good activity. In such cases, the intellectual

property position is stronger since both the chemical composition and the use can be claimed in a patent. In the case of methyl betulinate (**3a**), only the use can be claimed and companies are reluctant to spend up to $300 million required to get US FDA or Canadian Health Agency approval for a new drug.

Perhaps, one of the novel compounds described in **Section 2.5.1** has comparable or better activity than (**3a**). Such a compound might meet the patent requirements of the multinational drug companies. Unfortunately, the *in vivo* testing of these compounds is very time consuming and labour and animal intensive. Therefore, none of these tests have yet been carried out.

2.6.8. Mechanism of action

Betulinic acid (**3**) was sent for testing by MDS Inc. against a panel of forty receptors known to be involved in anxiety or potentially related (**Figure 2.6.8**). The tests revealed no significant binding of betulinic acid to any of these receptors currently implicated in anxiety.

If one accepts these results as correct [MDS is a highly regarded contract research organization used extensively by the pharmaceutical industry throughout the world] then it appears that betulinic acid and likely its derivatives reduce anxiety by a totally novel mechanism. This is plausible since betulinic acid is structurally distinct from currently marketed anti-anxiety drugs such as benzodiazepines, buspirones and SSRI inhibitors. All these compounds have a basic nitrogen atom(s) as a key structural feature (*see* **Figure 1.3.1**).

The discovery of a new mechanism of action for the treatment of anxiety has important scientific and commercial implications. It raises the possibility of a new screening target and the discovery of patentable new families of compounds for anti-anxiety therapy. That is, if the betulinic acid receptors can be identified (with the distinct possibility of discovering a new receptor), and if a high throughput screening method could be developed, then chemical libraries could be screened to uncover new leads and potentially novel anti-anxiety drugs.

Pharmacological preclinical assessment
MDS (Panlabs) Pharma Services

CATECHOLAMINES	$GABA_A$, agonist site	$5\text{-}HT_{1A}$
Adrenergic $\alpha 1$, $\alpha 2$ & β	$GABA_A$, BZP	$5\text{-}HT_2$
$MAO_{A\ \&\ B}$	GABA Cl^- channel	$5\text{-}HT_3$
COMT	$GABA_B$	Serotonin transporter
NE transporter	Glutamate non-sel	ACETYLCHOLINE
Dopamine D_1 & D_2	HISTAMINE	Muscarinic non-sel
DA transporter	Histamine H_1, H_2, H_3	Nicototinic
GABAERGIC	SEROTONIN (5-HT)	SIGMA
GABA transporter	$5\text{-}HT_1$	Sigma, non selective

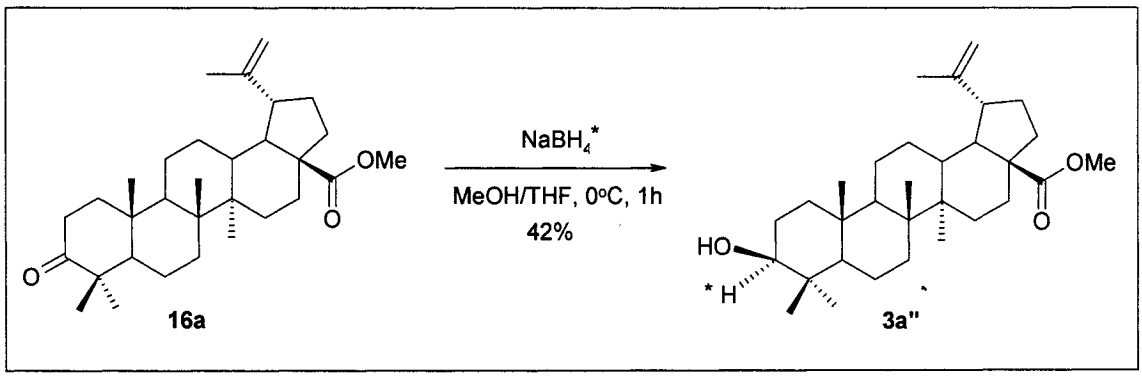
PEPTIDES	Neuropeptide Y_1 & Y_2	COX-1 & COX-2
Bombesin	Neurotensin	Interleukin IL-1α
Cannabinoid CB_1 & 2	Opiate non-selective	
Cholecystokinin(CCK)	Somatostatin	
CCK_A	Tachykinin $NK_{1,2\ \&3}$	
EGF	TNF non-selective	
Galanin $GalR_1$ & R2	VIP_1	
GLP-1	OTHER	
Melanocortin MC4	Cyclooxygenase	

Figure 2.6.8: List of receptors known to play a role in anxiety and fear.

2.6.8.1. Preparation of radioactive labelled betulinic acid methyl ester

In order to facilitate the search for the potential receptor, we have introduced tritium (^3H) into the 3α-position of the methyl ester of betulinic acid (**3a**), (**Scheme 2.6.8.1**).

The ^3H-labelling of betulinic acid methyl ester (**3a**) was effected by the reduction of methyl betulonate (**16a**) with ^3H-labelled sodium borohydride ($NaBH_4$*) in MeOH/THF at 0°C to afford the radioactive labelled betulinic acid methyl ester (**3a"**) in 42% yield (**Scheme 2.6.8.1**).



Scheme 2.6.8.1: Preparation of radioactive labelled betulinic acid methyl ester (**3a"**).

The radioative compound (**3a"**) was isolated as a white solid after silica gel column chromatography with EtOAc-hexane gradient elution. Approximately 300 mg of (**3a"**) with specific activity of 63.5 mCi/mmol has been prepared.

The starting material (**16a**) was prepared by methylation of betulonic acid (**16**) with diazomethane at 0°C in CH_2Cl_2 furnishing a white solid, m.p. 159-160°C (llit.[120] 146-148°C, lit.[117] 158-159°C, lit. [145]161-163°C, lit.[24] 165°C) in 80% yield after silica gel column chromatography. The HRMS of (**16a**) revealed a $[M]^+$ at m/z 468 which agreed with the desired molecular formula, $C_{31}H_{48}O_3$. The ^1H and ^{13}C NMR data of (**16a**) agreed with published data.[117]

2.6.9. Other biological activity of betulinic acid and its analogs

Other biological activities of betulinic acid and its analogs have been reported in the literature. This include anti-tumor activity against human melanoma and anti-AIDS or anti-HIV (human immunodeficiency virus) activity, anti-inflammatory activity, anti-malarial, cytotoxic activity, antimicrobial, inhibitor of amino-peptidase-N activity, mettaloproteinase inhibition, antiviral, antileishmanial, antineoplastic, spermicidal, anthelmintic and anti-angiogenic activities.[146] Antifeedant and allelopathic activity, has also been attributed to betulinic acid.[147]

2.6.9.1. Anti-cancer activity

In an effort to discover potential anticancer drugs from natural sources, betulinic acid isolated from the stem bark of *Ziziphus mauritiana* Lam. (Rhamnaceae), was shown to possess antitumor activity against human melanoma cells in both *in vitro* and *in vivo* models.[148,149] Betulinic acid exhibited selective cytotoxicity against cultured human melanoma (MEL-2) and animal models that function by induction of apoptosis (cell-suicide). Betulinic acid demonstrated highly effective tumor growth inhibitor in mice for each dose tested (50, 250 and 500 mg per kg body weight). Doses as low as 5 mg per kg body weight were employed and found effective.[148]

Betulinic acid is therefore, an attractive and promising new lead compound against human melanoma due to its high anti-tumor activity and favourable therapeutic index (TI) from the lack of toxicity towards normal cells. In fact, preclinical development is being conducted to explore the potential use of betulinic acid for the treatment or prevention of human melanoma.[150]

Chemical modifications of betulinic acid have been performed at the positions C-3 (OH), C-20 (C=C) and C-28 (COOH) for structure-activity relationship study (SARS).[126,151]

It was found that the C-20 position was sensitive to the size and electron density of the substituents in retaining the cytotoxicity of betulinic acid. So, C-20 was an undesirable position to derivatize.[126]

The free OH group at C-3 appeared to be important for biological activity in MEL-2 but a size limitation at this position was suggested. The hydrogen bonding capability and/or the acidity due to a free COOH at position C-28 appeared to be significant in the expression of biological effects in MEL-2. Hydrogenation of the methylene moiety in betulinic acid was unimportant for activity.[151] It seems that a combination of the size, nucleophilicity and strength of hydrogen bond formation is responsible for the biological effects of betulinic acid and its derivatives.

A number of amino acid conjugates of betulinic acid have demonstrated improved selective toxicity and water solubility which may be developed as antitumor agents. The free acid of alanine and glycine conjugates showed the best selective cytotoxicity and solubility profile (**Figure 2.6.9.1**).[152] However, results from a more extensive investigation using a greater number of derivatives are needed for SAR study and for the design and ultimate synthesis of a more effective betulinic acid-derived anti-tumor agent.

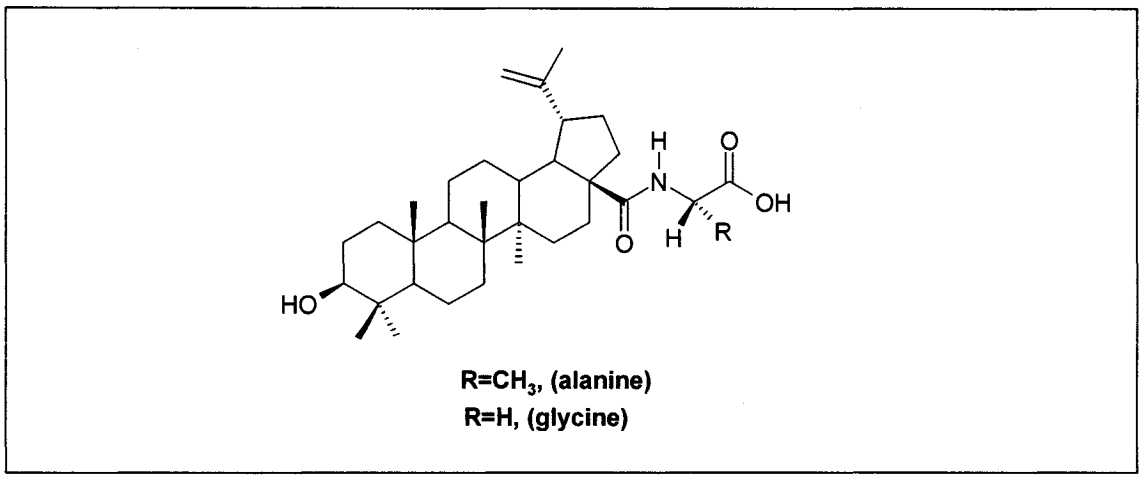


Figure 2.6.9.1: The alanine and glycine conjugate of betulinic acid as potential anti-tumor agents.

2.6.9.2. Anti-HIV activity

Betulinic acid (**3**) and its derivative platanic acid (**19a**), both isolated from the leaves of *Syzigium claviflorum* (Myrtaceae), are the first two identified triterpenes with a lupane skeleton to demonstrate anti-HIV activity.[19] Betulinic acid inhibited HIV replication in H-9 lymphocytes with EC_{50} value of 1.4μM and TI value of 9.3.

Subsequent derivatization of betulinic acid yielded 3-O-(3',3'-dimethylsuccinyl)-betulinic acid (**PA-457**) which was 4000-fold more active (EC_{50} value < 3.5×10^{-4} μM) and had a 2150-fold higher TI (TI > 20 000) than betulinic acid (**Figure 2.6.9.2**).[109] In fact, PA-457 is now in prelinical trial and could become the first example of a new class of AIDS drugs, maturation inhibitors.

Independently, Mayaux and Soler *et. al.*, reported RPR103611 as an anti-HIV agent that blocked HIV-1 entry into cells by inhibiting HIV-induced membrane fusion (**Figure 2.6.9.2**).[138]

A series of synthetic amides of betulinic acid have also been investigated as fusion inhibitors of HIV-1.[138] These betulinic acid derivatives represent the first low molecular weight compounds to be suggested as fusion inhibitors. Hence, they are new class of HIV-1 specific inhibitors with a new mode of action.

Last year, *Sun and Chen et. al.*, reported IC9564, a stereoisomer of RPR103611 to be equipotent and also a HIV-1 fusion inhibitor (**Figure 2.6.9.2**).[139] A series of synthetic IC9564 derivatives have also shown promising activity against HIV infection.

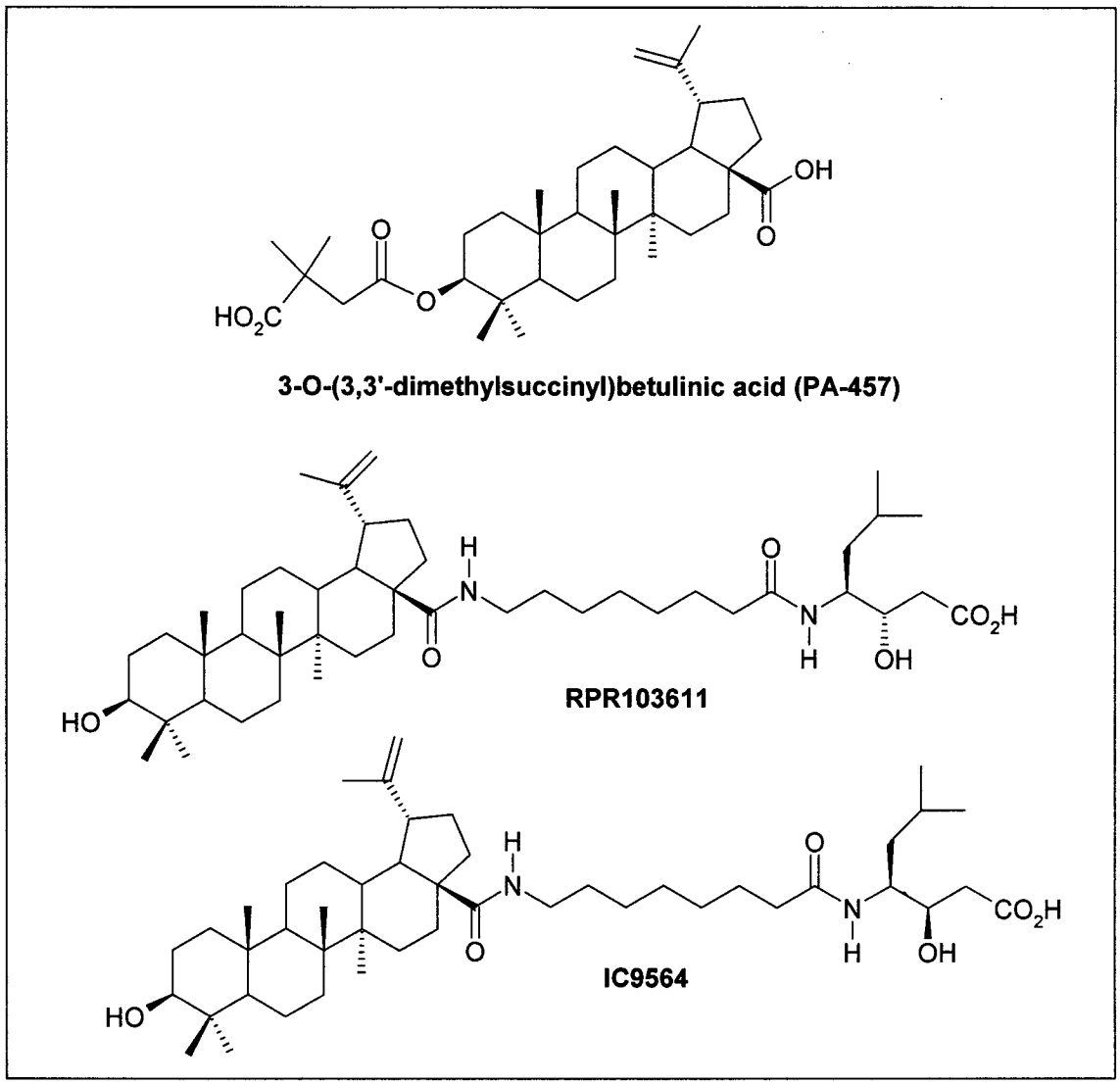


Figure 2.6.9.2: Betulinic acid derivatives with potent anti-HIV activity.

SAR studies have indicated that the free OH at C-3 and free COOH in the C-28 side chain were crucial for optimal anti-HIV potency.[132,133]

The presence of an amide function within the side chain and therefore, an aminoalkanoic chain with the appropriate chain length at C-28 was important for optimal activity.[138,139] When the ester group at C-3 in (**PA-457**) and its analogs were replaced with an amido group, it resulted in inactive or less potent compounds against HIV replication indicating that the acyl moiety at C-3 was essential for potent HIV activity.[153]

A lack of steric requirement was observed for groups introduced at C-30 but an acidic substituent such as 30-(carboxymethyl)thio was detrimental to potency.[132] The double bond at C-20 did not appear to play a key role in the HIV inhibitory activity since the saturated or dihydro analogue of IC9564 was equipotent with IC9564.[139] Moreover, almost all chemical modifications in ring A led to considerable loss of HIV inhibitory activity.[132]

2.6.9.3. Anti-malarial activity

Betulinic acid exhibited moderate-good *in vitro* antimalarial activity against asexual erythrocytic stages of the human malaria parasite *Plasmodium falciparum*.[154] The *in vitro* antiplasmodial IC_{50} value of betulinic acid against chloroquine resistant (K1) and sensitive (T9-96) *Plasmodium falciparum* were 19.6 ug/mL and 25.9 ug/mL respectively. When betulinic acid was tested for *in vivo* activity in a murine malaria model (*P. berghei*), the dosage emloyed of 250 mg/kg/day was ineffective at reducing parasitemia and exhibited some toxicity.[155]

2.6.9.4. Anti-feedant activity

Aryl derivatives of betulinic acid (**Figure 2.6.9.4**) have been reported to have antifeedant activities at low dosages of 25 ug/cm^2 against the agricultural pest tobacco caterpillar larvae *Spodoptera litura* F.[147] The EC_{50} values for both these compounds were 50 ug/cm^2.

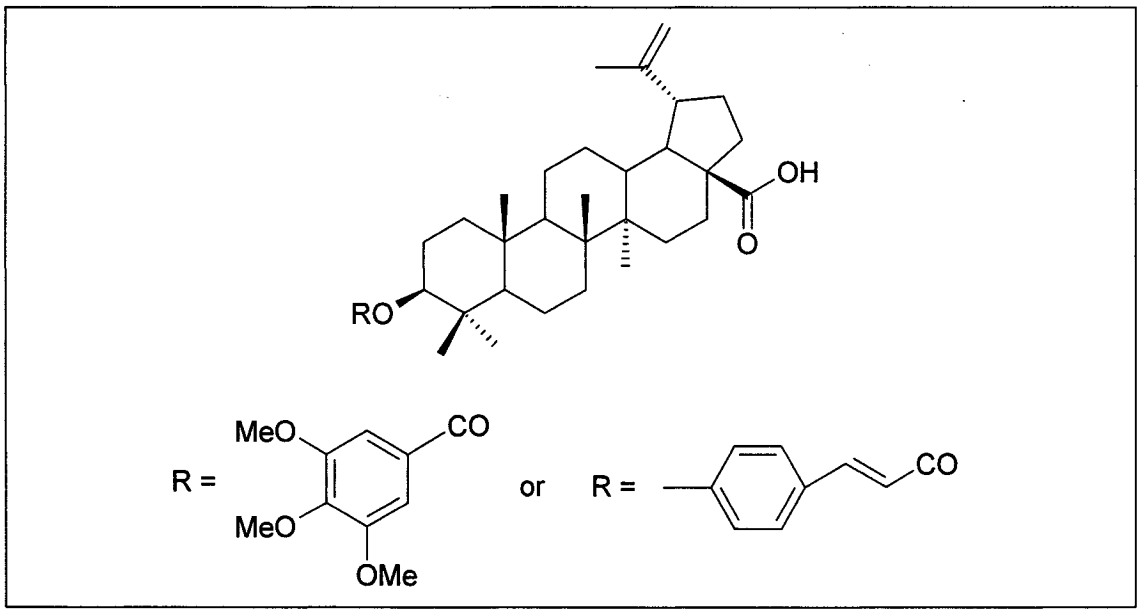


Figure 2.6.9.4: Aryl derivatives of betulinic acid with antifeedant activity against tobacco caterpillar.

2.6.9.5. Anti-inflammatory and anti-PLA_2 activity

The phospholipase A_2 (PLA_2) corresponds to a class of enzymes which catalyze the hydrolysis of membrane glycerophospholipids to release phospholipids and fatty acids. When the fatty acid is arachidonic acid, a complementary metabolism leads to pro-inflammatory mediators such as prostaglandins, leukotrienes, thromboxanes and platelet activating factors (PAF). Therefore, the modulation of pro-inflammatory lipid production by inhibiting PLA_2 activity remains a potential strategy for the development of new drugs for the treatment of inflammatory diseases.[156]

In an ethnobotanical study by Bernard *et.al.*, in South America, 56 plants were identified as anti-inflammatory remedies (often used to cure snake and dog bites or rheumatic pain) of which 7 were found to inhibit more than 50% of the PLA_2 activity, and betulinic acid was identified to be responsible for the activity.[156] In order to verify that betulinic acid was the candidate implicated in the PLA_2 inhibition, betulinic acid was docked into the PLA_2 crystal structure (**Figure 2.6.9.5**).[156]

The docking of betulinic acid presented in **Figure 2.6.9.5**, confirmed that betulinic acid can be inserted into the PLA_2 binding site with the correct energy values. The carboxylate group of betulinic acid was able to interact with the Ca^{2+} ion known to be present in the pharmacophore of PLA_2 structures.

Interestingly, the protein-based alignment of betulinic acid was compared to four other ligands and was found to fit well and occupied the same volume. Hence, betulinic acid represents a cylinder of about 13 A° long with a diameter of 5 A°, which occupies the place of the natural substrates, the phospholipids.[156]

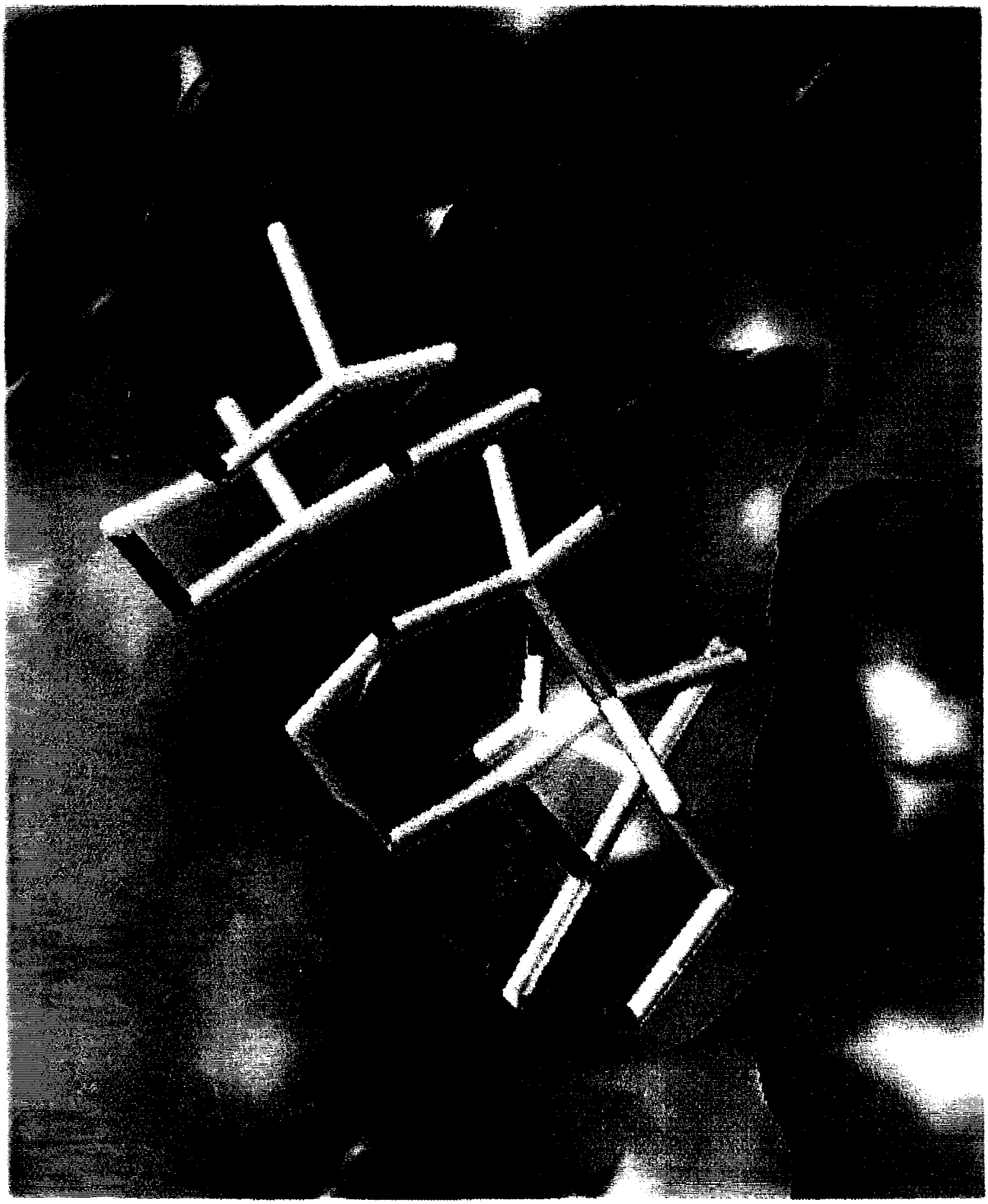


Figure 2.6.9.5: Docking of betulinic acid into the binding site of bovine PLA_2 structures. The carboxylate group (in red) interacts with Ca^{2+} in the bottom of the cavity (not visible in this view).[156]

2.6.10. Conclusions

The anti-anxiety activity of extracts from two species of Margraviaceae has been successfully determined and confirmed by standardized tests. The bioactive compound was identified to be betulinic acid (**3**). Its methyl ester derivative (**3a**) appeared to be more active.

Preliminary studies suggest that (**3**) is fairly safe and non-toxic. The lack of toxicity together with significant anti-anxiety activity leads to a favourable therapeutic index which is a definitive requirement for a new drug to be considered for deveopment.

Betulinic acid (**3**) has been synthesized from a local and abundant supply of natural product, betulin (**17**) in good yield. All the synthetic derivatives of (**3**) thus far are crystalline and indefinitely stable. These are additional aspects that are favourable in terms of drug development.

None of the classical neurotransmitter systems implicated in anxiety seemed to be affected by (**3**). Likewise, several of the peptidergic systems thought to play a role in fear and anxiety were unaffected. This suggests a novel mode of action possibly, via a yet to be identified receptor and a possibility for a novel receptor system to be patented. Radioactive labelled betulinic acid methyl ester (**3a"**) was synthesized in order to facilitate the search for potential anti-anxiety receptors.

Betulinic acid (**3**) is a business opportunity to develop new anti-anxiety remedies for pharmaceutical and potentially ethnobotanical markets.

Patent filing are in process covering the Magraviaceae extract and the derivatives of the active compound.

2.7. EXPERIMENTAL : Part II

GENERAL: As in Part I (**Section 2.3**).

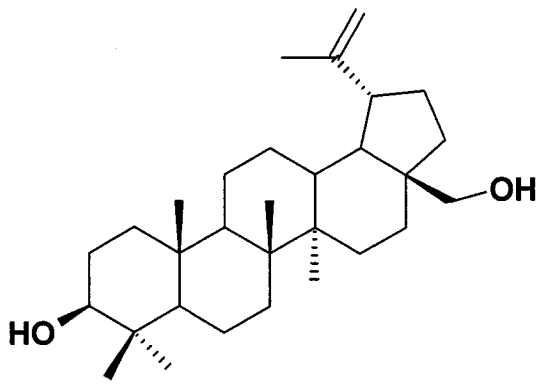
General procedures: Two general procedures were employed in the preparation of etheral diazomethane (CH_2N_2) solutions used in the methylation of betulinic acid its derivatives and the Magraviaceae extracts in **Section 2.3**.

Procedure 1: To a 20% solution of KOH (1.0 mL) in Et_2O (5.0 mL) at 0°C was added nitrosomethylurea ($CH_3N(NO)CONH_2$, 250 mg) furnishing a yellow etheral layer of CH_2N_2 on top of a clear aqueous layered solution. This was sufficient to methylate at least 300 mg of betulinic acid.

Procedure 2: To a solution of N-methyl-N-nitrosotoluene-*p*-sulfonamide ($CH_3N(NO)SO_2C_6H_4CH_3$, 3.5 g), in EtO_2 (40 mL) at 0°C was added 0.7M solution of KOH in EtOH (15 mL) until any precipitate that might be formed dissolved. After 5 minutes, the mixture was distilled at ~55-60°C (using a water bath) to furnish a yellow etheral solution of CH_2N_2 which was collected at 0°C.

2.7.1. Betulin

Other names: 3 Lup-20(29)-en-3β,28-diol, aH-Cyclopenta[a]chrysene, lup-20(30)-en-3β,28-diol, 3β,28-dihydroxylup-20(29)-ene, betuline, betulinol, betulol, trochol,



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The dried white birch bark was cut into small rectangular pieces, ground to powder (228.0 g), immersed in EtOAc (2 L) and left to stand overnight at room temperature. The mixture was filtered, rinsed with EtOAc (2 x 100 mL) and concentrated *in vacuo* to afford crude betulin (46.9 g, 21%).

White birch bark powder (138.0 g) was immersed in EtOAc (2 L) and left to stand overnight at room temperature. The mixture was filtered, rinsed with EtOAc (2 x 100 mL) and concentrated *in vacuo* to afford crude betulin (40.1 g). The bark residue was immersed again in EtOAc (800 mL) overnight at room temperature before it was heated to boiling, cooled, filtered and concentrated *in vacuo* to afford additional crude betulin (14.3 g) in 39% total crude yield. This extraction procedure was repeated on white birch bark powder (138.0 g) with CH_2Cl_2 (2 L) to afford crude betulin (49.0 g, 36%).

Using the Giner's procedure, the birch bark powder (138.0 g) was boiled in acetone (2 L) and filtered hot. The birch bark powder was re-extracted in hot acetone (1 L) and filtered hot again. The combined acetone extract was concentrated by boiling the solvent until about 700 mL was left then, water (500 mL) was added, cooled in ice (5h), filtered and dried to afford crude betulin (55.6 g, 40%), recrystallized from isopropyl alcohol as a pale yellow solid (16.3 g, 12%).

m.p. 248-249°C (lit.[60] 248-251°C, lit.[111] 254-256°C, lit.[112] 255-256°C, lit.[113] 257°C)

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm), 4.65 (d, J=2.0 Hz, H-29), 4.55 (d, J=1.8 Hz, H-29'), 3.77 (dd, J=10.9 Hz, J=1.4 Hz, H-19), 3.30 (d, J=10.8 Hz, H-28'), 3.16 (dd, J=10.9 Hz, J=5.1 Hz, H-19), 2.36 (ddd, J=10.6 Hz, J=10.6 Hz, J=5.8 Hz, H-19), 1.65 (s, 3H, H-30), 0.99 (s, 3H, H-27), 0.95 (s, 3H, H-26), 0.94 (s, 3H, H-23), 0.79 (s, 3H, H-25), 0.74 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 150.4 (C-20), 109.7 (C-29), 79.0 (C-3), 60.5, 55.2, 48.7, 42.7, 40.9, 38.8, 38.6, 37.2, 37.1, 34.2, 33.9, 29.7, 29.1, 27.9, 27.3, 27.0, 25.1, 20.8, 19.0, 18.3, 16.1, 15.9, 15.3, 14.7.

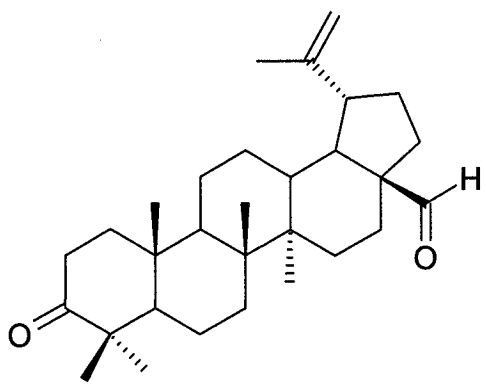
IR υ_{max} ($CHCl_3$, cm^{-1}): 3352, 3071, 2942, 2869, 1642, 1454, 1375, 1106, 1031, 984, 882, 757, 668.

MS (EI) *m/z* (rel. int.): 442 $[M]^+$ (19), 424 $[M-H_2O]^+$ (9), 411 $[M-CH_2OH]$ (50), 234 (18), 233 (17), 220 (19), 207 (73), 205 (13), 203 (63), 202 (13), 189 (100), 121 (48), 119 (38), 109 (42), 107 (47), 95 (71), 81 (58), 69 (46).

HRMS: *Calculated* for $C_{30}H_{50}O_2$, 442.38108 *found* 442.38230

2.7.2. Betulonic aldehyde

Other names: 3-Oxo-lup-20(29)-en-3β,28-al, 1H-Cyclopenta[a]chrysene, lup-20(30)-en-28-al, betulonaldehyde, liquidambronal.



15

Jones' reagent (35 mL) was added to a solution of betulin (20 g, 4.52 x 10^{-2} mol) in acetone (1 L) at 0°C until the solution remained persistently red. After 10 minutes the reaction was quenched with MeOH (300 mL) followed by water (200 mL) and the organic solvent was evaporated under reduce pressure. More water was added (200 mL) the residue was exctracted with EtOAc (5 x 200 mL). The combined EtOAc extract was washed successively with water (100 mL),

brine (200 mL) and water (200 mL). The organic layers were combined, dried $(MgSO)_4$ and concentrated *in vacuo* to afford 19.03 g of crude betulonic aldehyde (**15**) as a pale brown fluffy solid (92.5% crude yield). Purification of the crude amount of (**15**) (470 mg) via silica gel column chromatography using hexane-EtOAc gradient system as eluants furnished 240 mg of pure (**15**) as a white solid in 48% yield.

m.p. 163-165°C (lit.[28] 165-166°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 9.64 (d, J=1.7 Hz, H-28), 4.73 (dd, J=1.5 Hz, J=0.7 Hz, 1H, H-29), 4.60 (dd, J=1.4 Hz, J=0.7 Hz ,1H, H-29'), 2.85 (dt, J=11.6 Hz, J=5.9 Hz, 1H, H-19), 2.46 (m, H-2), 2.36 (m, H-2),1.67 (s, 3H, H-30), 1.04 (s, 3H, H-27), 0.99 (s, 3H, H-26), 0.96 (s, 3H, H-23), 0.90 (s, 3H, H-25), 0.89 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 217.9 (C-3), 206.4 (C-28), 149.6 (C-20), 110.1 (C-29), 59.2, 54.9, 49.8, 47.9, 47.4, 47.3, 42.6, 40.7, 39.6, 38.7, 36.8, 34.0, 33.6, 33.1, 29.8, 29.1, 28.7, 26.5, 25.5, 21.2, 21.0, 19.5, 18.9, 15.9, 15.7, 14.1.

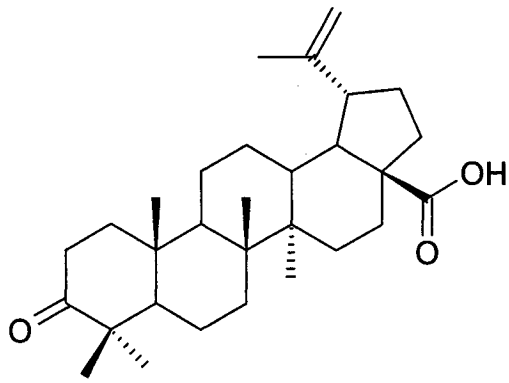
IR υ_{max} ($CHCl_3$, cm^{-1}): 3378, 3071, 2945, 2868, 2361, 2340, 2251, 1703 (C=O), 1642 (C=O), 1455, 1379, 1283, 1245, 1182, 1113, 989, 912, 843, 733, 647.

MS (EI) *m/z* (rel. int.): 438 $[M]^+$ (47), 420 $[M-H_2O]^+$ (11), 410 (52), 409 $[M-CHO]^+$ (64), 220 (21), 219 (45), 205 (92), 203 (55), 203 (39), 201 (53), 189 (81), 187 (52), (64), 147 (61), 135 (58), 133 (63), 121 (73), 119 (65), 109 (63), 107 (94), 105 (73), 95 (85), (83), 91 (67), 81 (100), 79 (79), 69 (77), 67 (78), 55 (90), 41 (64).

HRMS: *Calculated* for $C_{30}H_{46}O_2$, 438.34979, *found* 438.34964.

2.7.3. Betulonic acid

Other names: 3-Oxo-lup-20(29)-en-28-oic acid, lup-20(30)-en-28-oic acid, 3-oxobetulinic acid, liquidambaric acid, liquidambronic acid.



16

To a solution of the crude aldehyde (10 g, 2.28 x 10^{-2} mol) in tBuOH (160 mL) and H_2O (150 mL) was added 1-methyl-2-butene (30 mL) followed sequentially by $NaH_2PO_4.H_2O$ (31.48 g) and $NaClO_2$ (3.09 g). After 4h, the reaction mixture was extracted with ether (4 x 200 mL), dried ($MgSO_4$) and then concentrated *in vacuo*. The crude product was purified by silica gel column chromatography eluting with hexane-EtOAc gradient system to furnish betulonic acid (**16**) as a white solid (7.69 g, 74%).

m. p. 247-249 (lit.[120] 236-238°C, lit.[116] 247-249 °C, lit.[28] 253°C, lit.[111] 261-264°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 4.72 (s, 1H, H-29), 4.60 (s, 1H, H-29'), 2.99 (m, 1H, H-19), 2.46 (m, 2H, H-2), 1.67 (s, 3H, H-30), 1.05 (s, 3H, H-27), 0.99 (s, 3H, H-26), 0.97 (s, 3H, H-23), 0.95 (s, 3H, H-25), 0.90 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 218.4 (C-3), 182.5 (C-28), 150.3 (C-20), 109.8 (C-29), 56.4, 54.8, 49.8, 49.1, 47.3, 46.9, 42.4, 40.6, 39.5, 38.4, 37.0, 36.9, 34.1, 33.5, 32.0, 30.5, 29.6, 26.6, 25.4, 21.3, 21.0, 19.6, 19.3, 15.9, 15.8, 14.6.

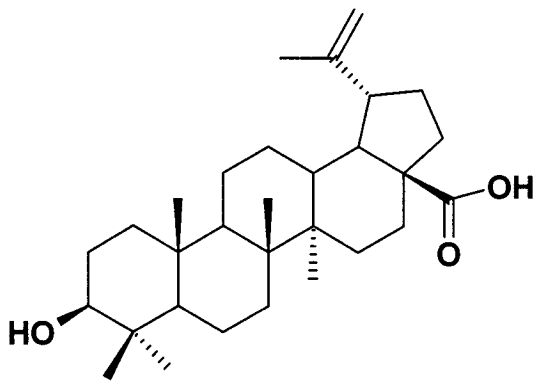
IR υ_{max} ($CHCl_3$, cm^{-1}): 3300 (OH), 3071, 2948, 2870, 2728, 2657, 1696 (C=O), 1642 (C=O), 1457, 1384, 1349, 1296, , 1237, 1215, 1193, 1114, 1061, 756.

MS (EI) *m/z* (rel. int.): 454 $[M]^+$ (58), 408 (34), 248 (57), 235 (42), 220 (14), 218 (25), 205 (84), 203 (38), 189 (99), 187 (43), 175 (44), 135 (58), 133 (44), 125 (44), 121 (55), 120 (29), 119 (57), 109 (45), 107 (61), 105 (59), 95 (65), 93 (61), 91 (59), 81 (87), 79 (56), 69 (64), 67 (70), 55 (100), 43 (51), 43 (47), 41 (91).

HRMS: *Calculated* for $C_{30}H_{46}O_3$, 454.34471, *found* 454.34580.

2.7.4. Betulinic Acid

Other names: 3β-Hydroxy-lup-20(29)-en-28-oic acid, betulic acid. 3aH-cyclopenta[a]chrysene.



3

To a stirred solution of betulonic acid (3g, 6.60 x 10^{-3} mol) in MeOH and THF (150 : 50 mL) at 0°C was added $NaBH_4$ (1.25 g). After 2h, the reaction mixture was quenched with NH_4Cl solution (100 mL) and the organic solvent was removed under reduced pressure. The residue was extracted with EtOAc (2 x 200 mL) and washed successively with H_2O (50 mL), brine (50 mL), H_2O (50 mL). The combined organic layers were dried ($MgSO_4$) and the solvent was evaporated *in vacuo* to furnish (**3**), (3.0 g, 99.7% crude yield). Recrystallisation of the crude betulinic acid from MeOH/$CHCl_3$ rendered pure betulinic acid as a white solid (2.2 g, 73.4%), (*see* **Section 2.3.5.6** for the physical and the spectroscopic data of betulinic acid).

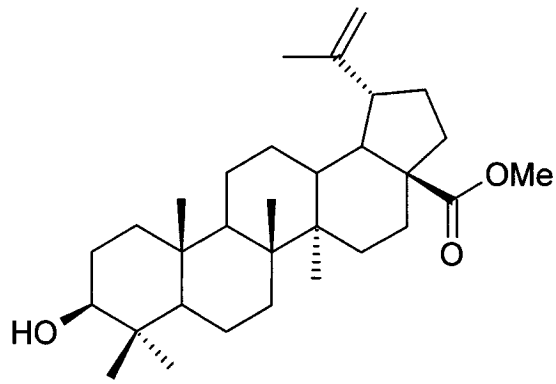
2.7.5. Esters of Betulinic Acid

General Procedure for the synthesis of esters (3b-3f): To a solution of betulinic acid (100 mg) in THF (5 mL) at room temperature (22°C) was added NaH (10 mol equivalent). The mixture was stirred for 30 mins before the appropriate alkyl halide (5 mol equivalent) was added, followed by 10 mol % of Bu_4NI. The solution was left to stir overnight (15h) before it was quenched with water (20 mL). THF was removed by rotary evaporation before it was extracted

with EtOAc (3 x 20 mL). The combined EtOAc extracts were washed with water (20 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The residue was purified by silica gel chromatography using a hexane-EtOAc gradient as eluant to furnish the desired ester.

2.7.5.1. Methyl betulinate

Other names: Betulinic acid methyl ester, methyl 3β-hydroxy-lup-20(29)-en-28-oate.



3a

The yellow etheral diazomethane solution [prepared by adding 250 mg of nitroso methyl urea into a solution of 20% KOH (1mL) in diethyl ether (5 mL) at 0°C] was added dropwise to a stirred solution of betulinic acid dissolved in CH_2Cl_2 (50 mL) and 5 drops of MeOH until the reaction mixture remained permanently yellow. The excess CH_2N_2 was left to evaporate in the fumehood overnight (15h) affording a clear solution. The solvent was removed *in vacuo* and the residue was re-dissolved in EtOAc (30 mL) and washed successively with H_2O (10 mL), brine (10 mL) and H_2O (10 mL). The organic layer was dried ($MgSO_4$) and the reaction mixture was concentrated *in vacuo* to afford (**3a**) as a white solid (100 mg, 97%) which was very clean by ^1H NMR. Further purification by recrystallisation from hexane/$CHCl_3$ yielded (**3a**), as white feathery solid (80 mg, 78%).

m.p. 220-221°C (lit.[111] 221-222°C, lit.[134] 222-224°C from MeOH and 224-225°C from benzene, lit.[23] 223-224°C, lit.[21,24,135] 224-225°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 4.70 (d, J=2.2 Hz, H-29), 4.56 (dd, J=2.2 Hz, J=1.4 Hz, H-29'), 3.63 (s, 3H, OMe), 3.15 (dd, J=10.3 Hz, J=5.5 Hz, H-3α), 2.97 (ddd, J=10.8 Hz, J=10.8 Hz, J=5.9 Hz, H-19), 1.65 (s, 3H, H-30), 0.93 (s, 6H, H-26/H-27), 0.88 (s, 3H, H-23), 0.79 (s, 3H, H-25), 0.72 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 176.7 (C-28), 150.6 (C-20), 109.6 (C-29), 79.0 (C-3), 56.5, 55.3, 51.3, 50.5, 49.4, 46.9, 42.4, 40.6, 38.8, 38.7, 38.2, 37.1, 37.0, 34.3, 32.1, 30.6, 29.7, 28.0, 27.4, 25.5, 20.9, 19.4, 18.3, 16.1, 15.9, 15.3, 14.7.

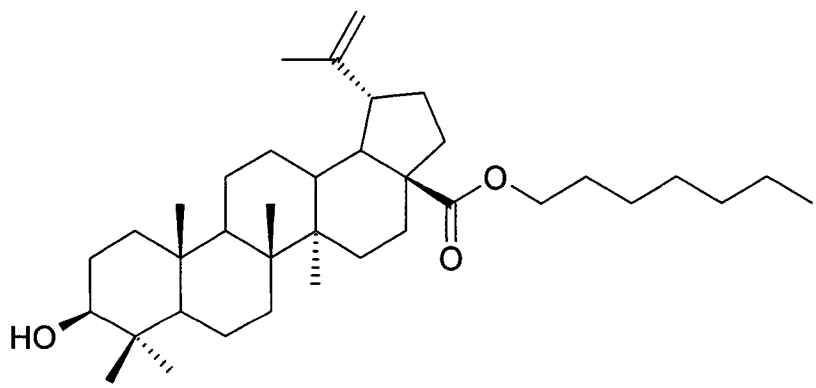
IR ($CHCl_3$, cm^{-1}): 3405 (OH), 2869, 2945, 1726 (C=O), 1462, 1453, 1377, 1320, 1189, 1136, 1011, 983, 911, 885, 734.

MS (EI) *m/z* (rel. int.): 470 $[M]^+$ (4), 452 $[M-H_2O]^+$ (7), 411 $[M-CO_2Me]^+$ (2), 410 $[M-CH_3CO_2H]^+$ (2), 262 (9), 233 (3), 220 (4), 207 (8), 203 $[262-CO_2Me]^+$ (11), 202 $[220-H_2O]^+$ (6), 189 (32), 86 (33), 69 (36), 57 (100), 56 (99), 43 (62), 41 (83), 29 (38).

HRMS: *Calculated* for $C_{31}H_{50}O_3$, 470.37590, *found* 470.37663

2.7.5.2. Heptyl betulinate

Other names: Betulinic acid heptyl ester, heptyl 3β-hydroxy-lup-20(29)-en-28-oate.



3b

Betulinic acid (100 mg, 2.19 x 10^{-4} mol), NaH (53 mg, 2.19 x 10^{-3} mol), 1-bromoheptane (196 mg, 1.10 x 10^{-3} mol) and 10 mol % of Bu_4NI (8.1 x 10^{-3} mol)

were reacted in THF (5 mL) at room temperature (22°C) for 15h according to the general procedure. The crude product, after usual workup, was purified by silica gel chromatography using a gradient of hexane-EtOAc as eluant to furnish (**3b**) as a white fluffy solid, (110 mg, 91%).

m.p. 54-56°C

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 4.70 (d, J=1.6 Hz, H-29), 4.57 (s, H-29'), 4.04 (m, 2H, CH_2-CO_2) 3.16 (dd, J=9.9 Hz, J=5.5 Hz, H-3α), 3.00 (ddd, J=10.8 Hz, J=4.8 Hz, H-19), 1.66 (s, 3H, H-30), 0.94 (s, 3H, H-27), 0.89 (s, 3H, H-26), 0.86 (s, 3H, H-23), 0.79 (s, 3H, H-25), 0.73 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 176.3 (C-28), 150.7 (C-20), 109.5 (C-29), 78 (C-3), 64.0, 56.5, 55.3, 50.5, 49.4, 47.0, 42.4, 40.7, 38.8, 38.7, 38.3, 37.1, 37.0, 34.3, 32.2, 31.8, 30.6, 29.6, 28.9, 28.7, 28.0, 27.4, 26.1, 25.5, 22.6, 20.9, 19.3, 18.3, 16.1, 16.0, 15.3, 14.7.

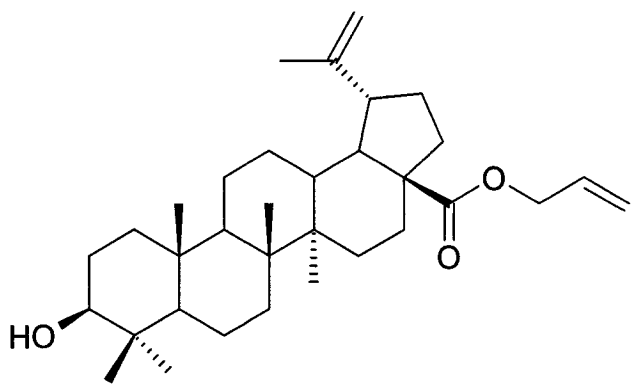
IR ($CHCl_3$, cm^{-1}): 3478 (OH), 3071, 2932, 2869, 2360, 2342, 1723 (C=O), 1642, 1454, 1318, 1294, 1270, 1216, 1135, 1107, 983, 945, 885, 758, 667.

MS (EI) *m/z* (rel. int.): 554 $[M]^+$ (21.2), 536 $[M\text{-}H_2O]^+$, 411 $[M\text{-}CH_3(CH_2)_5CH_2CO_2]^+$ (35), 410 $[M\text{-}CH_3(CH_2)_5CH_2CO_2H]^+$ (21), 346 (39), 220 (33), 207 (52), 203 (45), 202 (21), 189 (100), 95 (38), 81 (42), 69 (42), 57 (63), 55 (52), 43 (64), 41 (48).

HRMS: *Calculated* for $C_{37}H_{62}O_3$, 554.46990, *found* 554.47083

2.7.5.3. Allyl betulinate

Other names: Betulinic acid allyl ester, allyl 3β-hydroxy-lup-20(29)-en-28-oate.



3c

This ester was prepared in 89% as a white fluffy solid (90 mg), via the general procedure by reacting betulinic acid (100 mg, 2.19 x 10^{-4} mol), allyl bromide (132 mg, 2.19 x 10^{-3} mol), 60% NaH (88 mg, 2.19 x 10^{-3}) and 10 mol % of Bu_4NI (8 mg) in THF (5 mL) at 22°C for 15h. The crude product after usual workup was purified by silica gel chromatography eluting with a mixture of hexane-EtOAc of increasing polarity.

m. p. 65-68°C.

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 5.90 (m, CH_X=C), 5.31, (dd, J=17.2 Hz, J=1.4 Hz, CH_A=C), 5.20 (dd, J=10.3 Hz, J=1.4 Hz, CH_B=C), 4.70 (s, H-29), 4.54 (br s, 3H, H-29' and CH_2-CO_2), 3.15 (dd, J=10.2 Hz, J=5.5 Hz, H-3α), 3.00 (ddd, J=11.1 Hz, J=3.9 Hz, H-19), 1.65 (s, 3H, H-30), 0.93 (s, 6H, H-27 and H-26), 0.88 (s, 3H, H-23), 0.79 (s, 3H, H-25), 0.72 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 175.7 (C-28), 150.6 (C-20), 132.5 (C=C), 118.1 (C=C), 109.6 (C-29), 78.9 (C-3), 64.6, 56.5, 55.3, 50.5, 49.4, 46.9, 42.3, 40.7, 38.8, 38.7, 38.1, 37.1, 37.0, 34.3, 32.1, 30.5, 29.6, 27.9, 27.4, 25.5, 20.8, 19.3, 18.2, 16.1, 15.9, 15.3, 14.7.

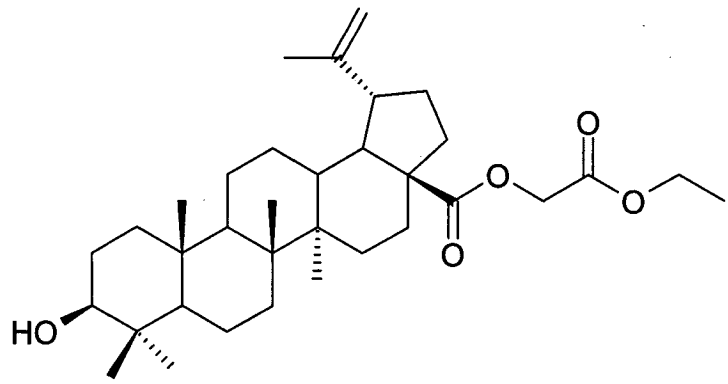
IR ($CHCl_3$, cm^{-1}): 34334 (OH), 3073, 2944, 2867, 2360, 2342, 1725 (C=O), 1643, 1453, 1389, 1361,1317, 1293, 1271, 1218, 1107, 1044, 982, 886, 757, 668.

MS (EI) *m/z* (rel. int.): 496 $[M]^+$ (36), 478 $[M\text{-}H_2O]^+$ (14), 411 $[M\text{-}CH_2CHCH_2CO_2]^+$ (37), 410 $[M\text{-}CH_2CHCH_2CO_2H]^+$ (27), 288 (52), 220 (30), 207 (54), 203 (42), 202 (15), 201 (25), 189 (100), 107 (35), 95 (42), 81 (50), 69 (42), 55 (46), 41 (90).

HRMS: *Calculated* for $C_{33}H_{52}O_3$, 496.39165, *found* 496.39220

2.7.5.4. Ethyl acetoxy betulinate

Other names: Betulinic acid ethyl acetoxy ester, ethyl acetoxy 3β-hydroxy-lup-20(29)-en-28-oate.



3d

The reaction of betulinic acid (100 mg, 2.19 x 10^{-4} mol) in THF (5 mL) at 22°C with 60% NaH (88 mg, 2.19 x 10^{-3} mol), ethyl bromoacetate (183 mg, 2.19 x 10^{-3} mol) and10 mol % of Bu_4NI (8.0 mg) for 15h according to the general procedure, furnished (**3d**) as a white fluffy solid (90 mg, 76%), after the same general work up and purification by silica gel column chromatography (hexane-EtOAc gradient).

m. p. 66-68°C

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 4.70 (d, J=2.0 Hz, H-29), 4.55 (s, 3H, H-29' and CO_2-CH_2-CO_2), 4.19 (q, J=7.2 Hz, 2H, CH_2-CO_2) 3.15 (dd, J=10.4 Hz, J=5.4 Hz, H-3α), 2.95 (ddd, J=10.8 Hz, J=4.5 Hz, H-19), 1.66 (s, 3H, H-30), 0.94 (s, 3H, H-27), 0.93 (s, 3H, H-26), 0.90 (s, 3H, H-23), 0.79 (s, 3H, H-25), 0.72 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 175.4 (C-28), 168.0 (C=O), 150.5 (C-20), 109.6 (C-29), 78.9 (C-3), 61.3, 60.2, 56.5, 55.3, 50.5, 49.3, 46.7, 42.4, 40.7, 38.8, 38.7, 38.0, 37.1, 36.9, 34.2, 31.9, 30.4, 29.5, 27.9, 27.3, 25.5, 20.8, 19.3, 18.2, 16.1, 15.9, 15.3, 14.6, 14.1.

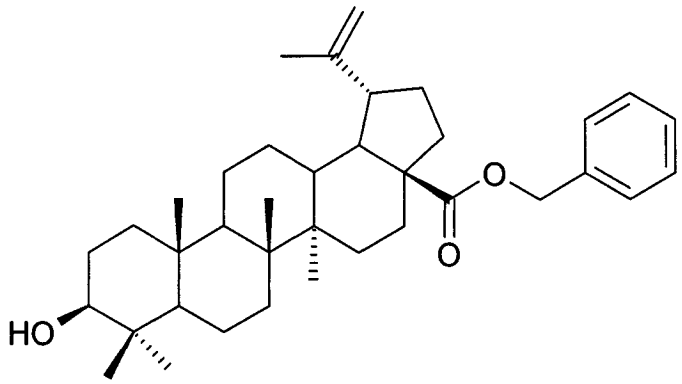
IR ($CHCl_3$, cm^{-1}): 3502 (OH), 3072, 2944, 28701, 2659, 1762 (C=O), 1738 (C=O) 1642, 1463, 1378, 1276, 1189, 983, 917, 863, 811, 757, 667.

MS (EI) *m/z* (rel. int.): 542 $[M]^+$ (29), 524 $[M\text{-}H_2O]^+$ (19), 411 $[M\text{-}CH_3CH_2OCOCH_2CO_2]^+$ (22), 410 $[M\text{-}CH_3CH_2OCOCH_2CO_2H]^+$ (31), 220 (14), 207 (40), 203 (33), 202 (23), 189 (100), 175 (47), 95 (44), 81 (49), 69 (43), 55 (47).

HRMS: *Calculated* for $C_{34}H_{54}O_5$, 542.39712, *found* 542.39682

2.7.5.5. Benzyl betulinate

Other names: Betulinic acid benzyl ester, benzyl 3β-hydroxy-lup-20(29)-en-28-oate.



3e

When betulinic acid (100 mg, 2.19 x 10^{-4} mol) in THF (5 mL) was reacted with 60% NaH (89 mg, 2.19 x 10^{-3} mol), benzyl bromide (187 mg, 2.19 x 10^{-3} mol) and10 mol % of Bu_4NI (8 mg) at 22°C for 15h (following the general procedure), ester (**3e**) was produced in 75% (90 mg) as a white solid after the usual work up and purification by silica gel column chromatography using eluting with hexane-EtOAc gradient.

m.p. 187-189°C (lit.[133] 184-185°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 7.32 (br s, 5H, Ph), 5.14 (d, J=12.3 Hz, CH-Ph), 5.06 (d, J=12.3 Hz, CH'-Ph), 4.70 (s, H-29), 4.57 (s, H-29'), 3.15 (dd, J=10.3 Hz, J=5.2 Hz, H-3α), 3.00 (ddd, J=11.0 Hz, J=4.9 Hz, H-19), 1.68 (s, 3H, H-30), 0.93 (s, 3H, H-27), 0.92 (s, 3H, H-26), 0.77 (s, 3H, H-23), 0.73 (s, 3H, H-25), 0.72 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 175.7 (C-28), 150.5 (C-20), 136.4, 128.4, 128.2, 128.0, 109.5 (C-29), 78.9 (C-3), 65.7, 56.5, 55.3, 50.5, 49.4, 46.9, 42.3, 40.6, 38.8, 38.6, 38.1, 37.1, 36.9, 34.2, 32.0, 30.5, 29.5, 27.9, 27.3, 25.5, 20.8, 19.3, 18.2, 16.1, 15.8, 15.3, 14.6.

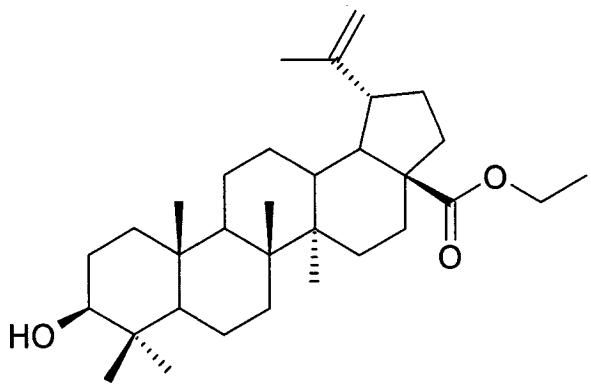
IR ($CHCl_3$, cm^{-1}): 3536 (OH), 3079, 2941, 2869, 2360, 2342.0, 1693 (C=O), 1645, 1586, 1482, 1387, 1320, 1282, 1190, 1107, 944, 915, 882, 757, 670, 602.

MS (EI) *m/z* (rel. int.): 546 $[M]^+$ (5), 528 $[M-H_2O]^+$ (4), 411 $[M-PhCH_2CO_2]^+$ (6), 410 $[M-PhCH_2CO_2H]^+$ (5), 220 (8), 207 (14), 203 (13), 189 (20) 91 (100).

HRMS: *Calculated* for $C_{37}H_{54}O_3$, 546.40720, *found* 546.40621.

2.7.5.6. Ethyl betulinate

Other names: Betulinic acid ethyl ester, ethyl 3β-hydroxy-lupan-20(29)-en-28-oate.



3f

Ester (**3f**) was obtained as a white solid (35 mg, 33%) after reacting betulinic acid (100 mg, 2.19×10^{-4} mol) with NaH (88 mg, 2.19×10^{-3} mol), ethyl iodide (171 mg, 1.10×10^{-3} mol) and 10 mol % of Bu_4NI (8.0 mg) in THF (5 mL) at 22°C for 15h using the general procedure and usual workup. The crude product was purified by silica gel column chromatography eluting with hexane-EtOAc gradient.

m.p. 197-198°C (lit.[23] 200-201°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 4.70 (s, H-29), 4.57 (s, H-29'), 4.11 (m, 2H, -CH_2-CO_2), 3.16 (dd, J=10.3 Hz, J=5.4 Hz, H-3α), 3.00 (ddd, J=10.7 Hz, J=4.6 Hz, H-19), 1.65 (s, 3H, H-30), 0.94 (s, 6H, H-27/H-26), 0.89 (s, 3H, H-23), 0.79 (s, 3H, H-25), 0.73 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 171.1 (C-28), 150.7 (C-20), 109.5 (C-29), 78.9 (C-3), 59.8, 56.4, 55.3, 50.5, 49.4, 47.0, 42.4, 40.7, 38.8, 38.7, 38.2, 37.1, 37.0, 34.3, 32.1, 30.6, 29.6, 28.0, 27.4, 25.5, 20.9, 19.4, 18.3, 16.1, 15.9, 15.3, 14.7, 14.3.

IR ($CHCl_3$, cm^{-1}): 3524 (OH), 3072, 2943, 2869, 2658, 2361, 1716 (C=O), 1642, 1558, 1463, 1296, 11351075, 983, 917, 886, 757, 667.

MS (EI) *m/z* (rel. int.): 484 $[M]^+$ (20), 466 $[M\text{-}H_2O]^+$ (19), 411 $[M\text{-}CH_3CH_2CO_2]^+$ (22), 410 $[M\text{-}CH_3CH_2CO_2H]^+$ (3), 276 (33), 247 (7), 220 (25), 207 (40), 203 (38), 202 (16), 201 (22), 189 (100), 175 (41), 81 (41), 69 (37), 55 (40), 41 (39).

HRMS: *Calculated* for $C_{32}H_{52}O_3$, 484.39165, *found* 484.38990.

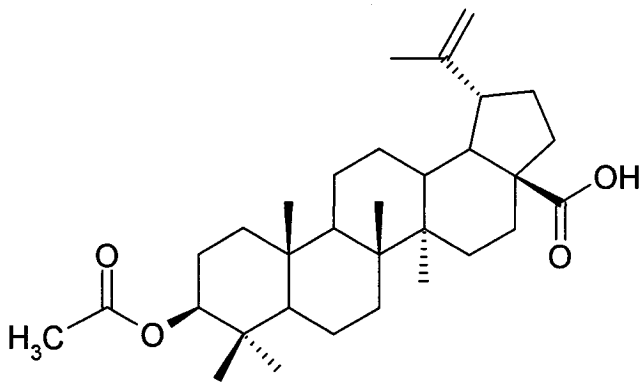
2.7.6. Amides of Betulinic Acid

General procedure for synthesizing the amides of betulinic acid: Oxalyl chloride (1.5 mol equivalent) and 1 drop of DMF was added dropwise to a solution of 3β-acetyl betulinic acid (**14d**, 100 mg) in CH_2Cl_2 (10 mL) and stirred for 6h at room temperature (22°C). The solvent and the DMF were removed *in vacuo*, the residue was re-dissolved in CH_2Cl_2 (2 mL) and added dropwise to another solution containing the appropriate amine (1.1 mol equivalent) and triethyl amine (Et_3N), (1.1 mol equivalent) in CH_2Cl_2 (8 mL) at 0°C. Stirring was continued for 1h . The reaction mixture was washed successively with water (5.0 mL), 1% HCl (5 mL), water (5 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The 3-hydroxyl group was deprotected by stirring the residue with excess K_2CO_3 in MeOH (20 mL) at 30-35°C overnight (15h). The reaction mixture was then filtered, concentrated *in vacuo* and chromatographed over silica gel using hexane-ethyl acetate gradient as eluant to afford the desired amides in good yields (64-97%).

Preparation of 3β-acetyl betulinic acid (14d): A solution of betulinic acid (1.12 g, 2.45 x 10^{-3} mol), Et_3N (500 mg, 0.7 mL) and DMAP (10 mg) in CH_2Cl_2 (50 mL) at 22°C was stirred for 10 mins before acetic anhydride (500 mg, 0.5 mL) was added and stirring was continued overnight (15h). The reaction mixture was washed successively with water (20 mL), 5% HCl (20 mL), water (20 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The crude product was purified by silica gel chromatography eluting with hexane-EtOAc gradient to furnish (**14d**) as a white solid, (810 mg, 66%).

3β-Acetyl betulinic acid (14d).

Other names: 3β-Acetoxy-lup-20(29)-en-28-oic acid.



14d

m.p. 275-277°C (lit.[137] 258°C, lit.[16] 269-271°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 4.71 (s, H-29), 4.59 (s, H-29'), 4.45 (dd, J=9.4 Hz, J=6.4 Hz, H-3α), 2.98 (m, H-19), 2.02 (s, 3H, CH_3CO_2-), 1.67 (s, 3H, H-30), 0.95 (s, 3H, H-27), 0.90 (s, 3H, H-25), 0.82 (s, 6H, H-26/23), 0.80 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 181.3 (CO_2H), 171.1 (OAc), 150.4 (C-20), 109.8 (C-29), 80.9 (C-3), 56.3, 55.4, 50.3, 49.2, 46.9, 42.4, 40.6, 38.3, 37.1, 37.8, 34.2, 32.1, 30.5, 29.7, 27.9, 25.4, 23.7, 21.3, 20.8, 19.3, 18.1, 16.4, 16.2, 16.0, 14.6.

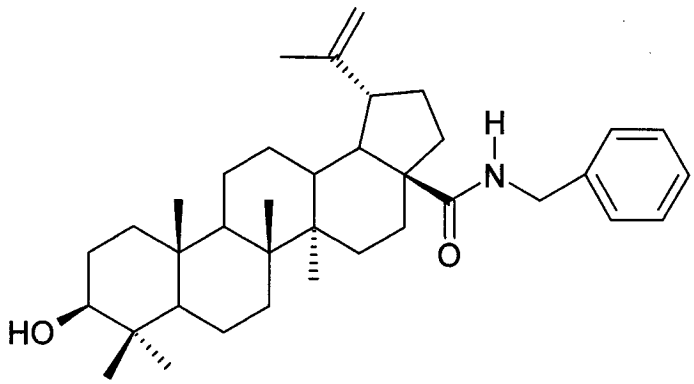
IR ($CHCl_3$, cm^{-1}): 3609 (OH), 2946, 2872, 2361, 2340, 1735 (C=O), 1696 (C=O), 1643, 1454, 1245, 1193, 1028, 979, 905, 881, 734.

MS (EI) *m/z* (rel. int.): 498 $[M]^+$ (5), 439.4 (18), 438 $[M-CH_3CO_2H]^+$ (56), 423 (23), 395 (30), 262.2 (4), 248 (19), 220 (5), 219 (9), 205 (16), 203 (24), 202 (15), 191 (23), 189 (100), 187 (41).

HRMS: *Calculated* for $C_{32}H_{50}O_4$, 498.37091, *found* 498.37201

2.7.6.1. Betulinic acid benzyl amide

Other names : N-[3β-hydroxylup-20(29)-en-28-oyl]-benzylamine .



17a

The reaction of (**14d**) (100 mg, 2.01 x 10^{-4} mol) in CH_2Cl_2 (10 mL) with oxalyl chloride (38.2 mg, 3.01 x 10^{-4} mol, 0.03 mL), 1 drop of DMF, and benzylamine (23.7 mg, 2.21 x 10^{-4} mol, 0.02 mL) in the presence of Et_3N (22.4 mg, 2.21 x 10^{-4} mol, 0.03 mL) in CH_2Cl_2 (8 mL) according to the general procedure and workup outlined above afforded (**17a**) as a white solid (70 mg, 62%) after the crude product was subjected to silica gel column chromatography (EtOAc-hexane gradient) followed by recrystallisation from $MeOH/CH_2Cl_2$.

m.p. 239-242°C,

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 7.29 (m, 5H, Ph), 5.85 (t, J=5.7 Hz, CONH), 4.72 (d, J=2.0 Hz, H-29), 4.58 (d, J=2.1 Hz, H-29'), 4.47, (dd, J=14.7 Hz, J=5.8 Hz, CONHCH-Ph), 4.35 (dd, J=14.0 Hz, J=5.6 Hz, CONHCH'-Ph), 3.16 (m, 2H, H-3α/H-19) 2.47 (dt, J=12.2 Hz, J=3.2 Hz, H-13), 1.67 (s, 3H, H-30), 0.94 (s, 6H, H-27/H-26), 0.89 (s, 3H, H-23), 0.80 (s, 3H, H-25), 0.74 (s, 3H, H-24).

^{13}C NMR (500 MHz, $CDCl_3$): δ (ppm) 175.9 (C=O), 151.0 (C-20), 139.2, 128.7, 127.8, 127.3, 109.3 (C-29), 79.0 (C-3), 55.7, 55.4, 50.7, 50.2, 46.7 (C-19), 43.3 ($NHCH_2$), 42.5, 40.8, 38.9, 38.8, 38.4, 37.8 (C-13), 37.2, 34.5, 33.8, 30.9, 29.9, 29.5, 27.9, 27.4, 25.7, 20.9, 19.5, 18.3, 16.2, 15.3, 14.7.

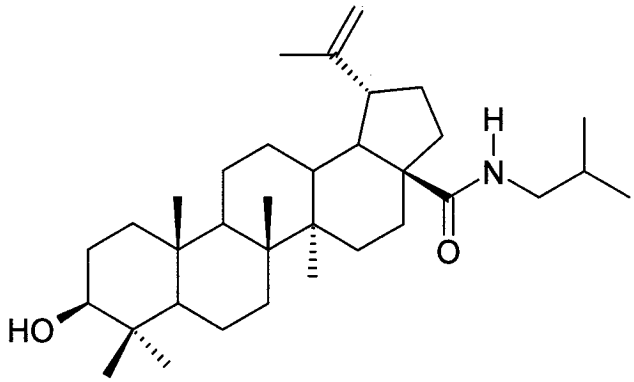
IR υ_{max} ($CHCl_3$, cm^{-1}): 3448 (OH), 2942, 2868, 2360, 2342, 1638 (C=O), 1522, 1455, 1376, 1189, 982, 884, 757, 699, 668.

MS (EI) *m/z* (rel. int.): 545 $[M]^+$ (58), 530 (17), 528 (26), 527 $[M-H_2O]^+$ (49), 518 (17), 517 (40), 512 (22), 502 (17), 499 (21), 484 (21), 411.4 $[M-CONHCH_2Ph]^+$ (11), 410 $[M-HCONHCH_2Ph]^+$ (4), 337 (4), 324 (35), 227 (54), 220 (2), 207 (6), 203 (38), 202 (7), 189 (74), 91 (100).

HRMS: *Calculated* for $C_{37}H_{55}NO_2$, 545.42329, *found* 545.42218.

2.7.6.2. Betulinic acid isopropyl amide

Other names : N-[3β-hydroxylup-20(29)-en-28-oyl]-isobutylamine.



17b

The amide (**17b**) was isolated as a white solid (100 mg, 97%), after reacting **14d** (100 mg, 2.01 x 10^{-4} mol) in CH_2Cl_2 (10 mL) with oxalyl chloride (38.2 mg, 3.01 x 10^{-4} mol, 0.03 mL), 1 drop of DMF, isobutylamine (16.2 mg, 2.21 x 10^{-4} mol, 0.02 mL) in the presence of Et_3N (22.4 mg, 2.21 x 10^{-4} mol, 0.03 mL) in CH_2Cl_2 (8 mL) according to the general procedure and work up. Purification of the crude product was achieved by silica gel column chromatography (hexane-EtOAc solvent gradient) followed by recrystallisation from MeOH. .

m. p. 216-217°C

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 5.61 (t, J=5.8 Hz, CONH), 4.71 (d, J=2.2 Hz, H-29), 4.56 (d, J=2.3 Hz, H-29'), 3.12 (m, 3H, H-3α, H-19, CONHC-H), 2.98 (m, 1H, CONHC-H'), 2.46 (dt, J=12.3 Hz, J=3.2 Hz, H-13), 1.65 (s, 3H, H-30), 0.91 (s, 3H, H-27), 0.89 (s, 1H, H-26), 0.88 (s, 3H, H-23), 0.79 (s, 3H, H-25), 0.73 (s, 3H, H-24).

^{13}C NMR (500 MHz, $CDCl_3$): δ (ppm) 175.9 (C=O), 150.9 (C-20), 109.2 (C-29), 78.9 (C-3), 55.6, 55.4, 50.8, 50.2, 46.7 (C-19), 46.6 ($NHCH_2$), 42.5, 40.7, 38.8, 38.7, 38.5, 37.7 (C-13), 37.2, 34.4, 33.9, 30.9, 29.4, 28.7, 27.9, 27.4, 25.6, 20.9, 20.2, 20.1, 19.4, 18.3, 16.1, 16.0, 15.3, 14.6.

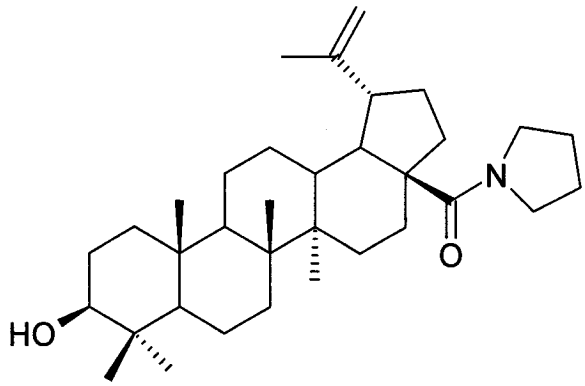
IR υ_{max} ($CHCl_3$, cm^{-1}): 3449 (OH), 2949, 2869, 2361, 2343, 1638 (C=O), 1509, 1388, 1195, 1044, 983, 909, 882, 733.

MS (EI) *m/z* (rel. int.): 511 $[M]^+$ (100), 509 (15), 496 (34), 493 $[M\text{-}H_2O]^+$ (34), 484 (34), 483 (92), 468 (33), 411 $[M\text{-}CONHCH_2CH(CH_3)_2]^+$ (25), 410 $[M\text{-}HCONHCH_2CH(CH_3)_2]^+$ (13), 290 (37), 203 (25), 193 (39), 189 (35), 57 (36).

HRMS: *Calculated* for $C_{34}H_{57}NO_2$, 511.43918, *found* 511.44049.

2.7.6.3. Betulinic acid pyrrolidenyl amide

Other names : N-[3β-hydroxylup-20(29)-en-28-oyl]-pyrrolidine.



17c

The reaction of compound **14d** (100 mg, 2.01 x 10^{-4} mol) in CH_2Cl_2 (10 mL) with oxalyl chloride (38.2 mg, 3.01 x 10^{-4} mol, 0.03 mL),1 drop of DMF and pyrrolidine (15.7 mg, 2.21 x 10^{-4} mol, 0.02 mL) in the presence of Et_3N (22.4 mg, 2.21 x 10^{-4} mol, 0.03 mL) in CH_2Cl_2 (8 mL) according to the general procedure and usual workup furnished (**17c**) as a white solid (80 mg, 78%). The crude product was purified by silica gel column chromatography using hexane-ethyl acetate gradient as eluant. Further purification was carried out by recrystallisation from MeOH.

m. p. 223-226°C

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 4.69 (d, J=2.2 Hz, H-29), 4.54 (s, H-29'), 3.40 (m, 4H, CONH-$(CH_2)_2$-), 3.09 (m, 2H, H-3α/ H-19), 2.81 (dt, J=12.1 Hz, J=3.4 Hz, H-13), 1.65 (s, 3H, H-30), 0.93 (s, 6H, H-27/H-26), 0.91 (s, 3H, H-23), 0.79 (s, 3H, H-25), 0.72 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 173.6 (C=O), 151.6 (C-20), 108.9 (C-29), 78.9 (C-3), 55.4, 52.4, 50.8, 46.3, 42.0, 40.6, 38.8, 38.7, 37.2, 37.1, 35.2, 34.4, 31.2, 30.8, 29.7, 27.9, 27.4, 25.6, 21.1, 19.6, 18.3, 16.2, 15.3, 14.7.

IR υ_{max} ($CHCl_3$, cm^{-1}): 3424 (OH), 3070, 2942, 2868, 2362, 1637 (C=O), 1451, 1406, 1390, 1246, 1168, 1137, 1108, 982, 917, 881, 754, 665.

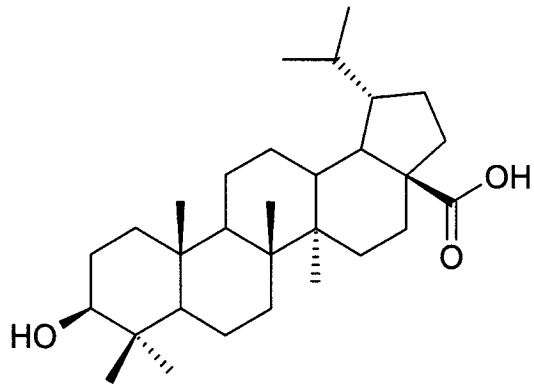
MS (EI) *m/z* (rel. int.): 509 $[M]^+$ (61), 495 (28), 494 (80), 491 $[M-H_2O]^+$ (8), 482 (28), 481 (79), 412 (17), 411 $[M-CON(CH_2)_8]^+$ (56), 410 $[M-HCON(CH_2)_8]^+$ (14), 207 (8), 204 (20), 191 (100), 189 (31).

HRMS: *Calculated* for $C_{34}H_{55}NO_2$, 509.42329, *found* 509.42312

2.7.7. Hydrogenated betulinic acids

2.7.7.1. Dihydrobetulinic acid.

Other names: 3β-Hydroxy-lup-28-oic acid, 3aH-cyclopenta[a]chrysene lupan-28-oic acid.



18a

30% Pd on carbon (20 mg) was added to a solution of **14d** (100 mg, 2.01 x 10^{-4}) in MeOH (25 mL) and was degassed before it was stirred under hydrogen gas overnight (15h) at room temperature (22°C). The reaction mixture was filtered, the filtered cake rinsed with MeOH (2 x 10 mL) and the solvent was removed *in vacuo*. The crude product was was stirred with excess K_2CO_3 in MeOH at room temperature overnight at 25-30°C. The MeOH was evaporated under reduced pressure and the residue was re-dissolved in EtOAc (25 mL), washed with water (2 x 10 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude product was recrystallised from MeOH affording (**18a**) as a white solid, (79 mg, 86%).

m.p. 297-299°C (lit.[19] >300°C, lit.[21] 311-315°C) .

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 3.13 (m, H-3α), 0.95 (s, 3H, H-27), 0.93 (s, 3H, H-26), 0.91 (s, 3H, H-23), 0.81 (s, 3H, H-25), 0.74 (s, 3H, H-24).

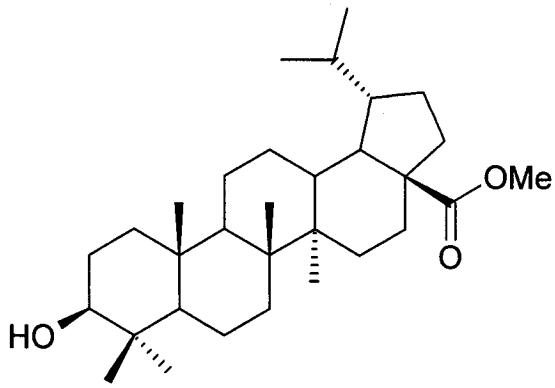
IR υ_{max} ($CHCl_3$, cm^{-1}): 3415 (OH), 2951, 2867, 1694 (C=O), 1655, 1452, 1385, 1365, 1313, 1299, 1272, 1241, 1207, 1096, 1044, 1025, 984.

MS (EI) *m/z* (rel. int.): 458 $[M]^+$ (14), 440 $[M-H_2O]^+$ (38), 425 $[M-CO_2H]^+$, 220 (3), 207 (100), 203 (21), 191 (47), 190 (43), 189 (91), 177 (57), 136 (47), 135 (56), 121 (48), 107 (40), (44), 93 (41), 81 (45), 69 (51), 55 (41), 43 (39).

HRMS: *Calculated* for $C_{30}H_{50}O_3$, 458.37599, *found* 458.37656

2.7.7.2. Methyl dihydrobetulinate.

Other names: Methyl 3β-hydroxy-lupane-28-oate, lupan-28-oic acid, 3β-hydroxy methyl ester.



18b

The solution of methyl betulinate **3a** (100 mg, 2.13 x 10^{-4} mol) in MeOH (25 mL) and 30% Pd on carbon (20 mg) was degassed before it was stirred under hydrogen gas overnight (15h) at room temperature (22°C). The reaction mixture was filtered, the filtered cake rinsed with MeOH (2 x 10 mL) and the solvent was removed *in vacuo*. The crude product was recrystallised from MeOH to afford (**18b**) as a white solid, (80 mg, 80%).

m.p. 236-238°C (lit.[23] 238-240°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 3.63 (s, 3H, OMe), 3.16 (dd, J=10.3 Hz, J=5.5 Hz, H-3α), 0.95 (s, 3H, H-27), 0.93 (s, 3H, H-26), 0.89 (s, 3H, H-23), 0.81 (s, 3H, H-25), 0.74 (s, 3H, H-24).

IR υ_{max} ($CHCl_3$, cm^{-1}): 3370 (OH), 2951, 2869, 2361, 2340, 1762 (C=O), 1559, 1456, 1387, 1318, 1221, 1189, 1040, 983, 910, 734.

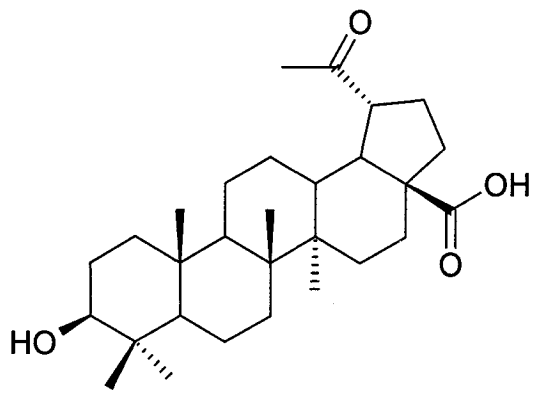
MS (EI) *m/z* (rel. int.): 472 $[M]^+$ (22), 454 $[M-H_2O]^+$ (35), 413 $[M-CH_3CO_2]^+$ (86), 412 $[M-CH_3CO_2H]^+$ (21), 250 (20), 207 (67), 205 (21), 203 (33), 191 (84), 190 (47), 189 (100), 177 (68), 175 (44), 107 (40), 69 (44).

HRMS: *Calculated* for $C_{31}H_{52}O_3$, 472.39165, *found* 472.39230

2.7.8. Ozonized betulinic acids

2.7.8.1. Platanic acid

Other names: 3β-Hydroxy-20-oxo-norlupan-28-oic acid.



19a

Ozone was passed through a stirred solution of betulinic acid (200 mg) in 5% MeOH/CH_2Cl_2 (100 mL) at –78°C until the characteristic blue color of ozone appeared after 30 mins. Dimethyl sufide (2 mL) was added and the resulting solution was allowed to warm to room temperature (22°C). After stirring for 10 min. at room temperature (22°C), the solvent was removed *in vacuo*. The residue was then dissolved in EtOAc (100 mL), washed with water (2 x 10 mL), dried ($MgSO_4$), filtered and then concentrated *in vacuo*. The crude product was purified by silica gel chromatography eluting with hexane-EtOAc gradient to afford (**19a**) as a white solid (120 mg, 60 %).

m.p. 279-282°C (lit.[19] 279-282°C).

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 3.20 (m, 2H, H-3α, H-19), 2.15 (s, 3H, CH_3CO), 0.98 (s, 3H, H-27), 0.94 (s, 3H, H-26), 0.89 (s, 3H, H-23), 0.80 (s, 3H, H-25), 0.73 (s, 3H, H-24).

^{13}C NMR (500 MHz, $CDCl_3$): δ (ppm) 212.2 (CH_3CO), 181.3 (C-28), 78.9 (C-3), 56.2, 55.3, 51.2, 50.3, 49.2, 42.2, 40.6, 38.8, 38.6, 37.5, 37.2, 36.7, 34.2, 31.4, 30.1, 29.7, 28.3, 28.0, 27.3, 27.2, 20.8, 18.2, 16.1, 15.9, 15.3, 14.7.

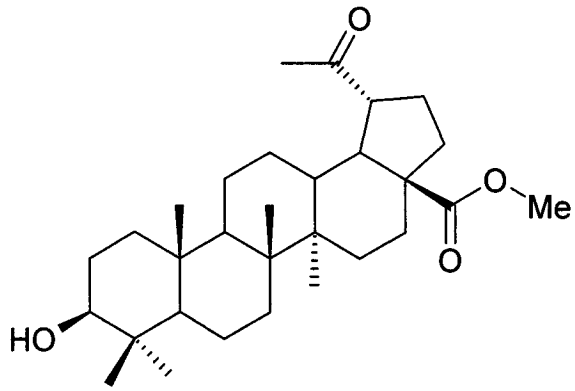
IR υ_{max} ($CHCl_3$, cm^{-1}): 3467 (OH), 2944, 2871, 2252, 1702 (C=O), 1560, 1451, 1388, 1357, 1278, 1137, 1108, 1074, 734, 647.

MS (EI) *m/z* (rel. int.): 458 $[M]^+$ (9), 440 $[M-H_2O]^+$ (37), 413 $[M-CO_2H]^+$ (2), 369, (54), 207 (76), 203 (15), 190 (38), 190 (38), 189 (100), 147 (56), 136 (44), 135 (70), 121 (52), 107 (40), 95 (48), 43 (49).

HRMS: *Calculated* for $C_{29}H_{46}O_4$, 458.33963, *found* 458.33860.

2.7.8.2. Methyl platanate

Other names: Platanic acid methyl ester, 3β-Hydroxy-20-oxo-30-norlupan-28-oic acid methyl ester.



19c

A solution of ethereal CH_2N_2 was added to a solution of platanic acid (80 mg, 1.75 x 10^{-4} mol) already dissolved in CH_2Cl_2 and 5 drops of MeOH (50 mL) at room temperature (22°C) until the reaction mixture remained permanently yellow. Excess CH_2N_2 was evaporated in the fumehood overnight before the solvent was removed *in vacuo*. The residue was re-dissolved in EtOAc (30 mL), then washed successively with 10 mL of water, brine, water, dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude product was chromatographed over silica gel eluting with hexane-EtOAc gradient to afford (**19c**) as a white solid (50 mg, 60%).

m.p. 250-251°C (lit.[134] 250-251°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 3.65 (s, 3H, O-CH_3), 3.20 (m, 2H, H-3α, H-19), 2.15 (s, 3H, CH_3CO), 0.98 (s, 3H, H-27), 0.94 (s, 3H, H-26), 0.89 (s, 3H, H-23), 0.80 (s, 3H, H-25), 0.73 (s, 3H, H-24).

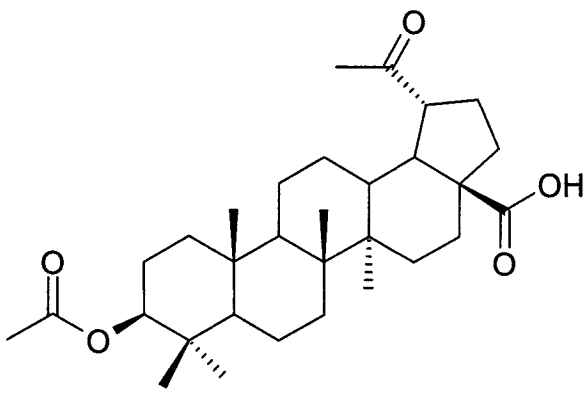
^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 212.4 (CH_3CO), 176.5 (C-28), 78.8 (C-3), 56.4, 55.2, 51.4, 51.1, 50.3, 49.4, 42.1, 40.5, 38.8, 38.6, 37.3, 37.1, 36.6, 34.1, 31.4, 30.1, 29.7, 28.2, 27.9, 27.3, 27.2, 20.8, 18.2, 16.0, 15.8, 15.3, 14.7.

MS (EI) *m/z* (rel. int.): 472 $[M]^+$ (13), 454 $[M-H_2O]^+$ (45), 412 $[M-CH_3CO_2H]^+$ (9), 411 (39), 207 (55), 203 (14), 190 (36), 189 (100), 161 (40), 147 (69), 136 (43), 135 (69), 133 (41), 121 (57), 107 (44), 95 (47), 81 (45), 43 (66).

HRMS: *Calculated* for $C_{30}H_{48}O_4$, 472.35528, *found* 472.35078.

2.7.8.3. 3β-Acetyl platanic acid

Other names: 3β-Acetyl-20-oxo-30-norlupan-28-oic acid, 30-norlupan-28-oic acid, 3β-hydroxy-20-oxo-acetate.



19b

Ozone was passed through a stirred solution of 3β-acetyl betulinic acid, **14d** (1.45 g) in CH_2Cl_2 (150 mL) at -78°C until the characteristic blue color of ozone appeared after 3h. Dimethyl sufide (2 mL) was added and the resulting solution was allowed to warm to room temperature (22°C). After stirring for 10 mins. at room temperature, the solvent was removed *in vacuo*. The residue was dissolved in EtOAc (100 mL), washed with water (2 x 30 mL), dried ($MgSO_4$), filtered and concentrated under diminished pressure. The crude product was purified by silica gel column chromatography (hexane-EtOAc gradient) to give (**19b**) as a white solid (900 mg, 62 %).

m.p. 252-255°C (lit.[140] 252-255°C, lit.[132] >250°C).

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 4.44 (m, H-3α), 3.23 (m, H-19), 2.16 (s, 3H, CH_3CO), 2.02 (s, 3H, CH_3CO_2), 0.98 (s, 3H, H-27), 0.89 (s, 3H, H-26), 0.82 (s, 6H, H-25/ H-23), 0.81 (s, 3H, H-24).

^{13}C NMR (500 MHz, $CDCl_3$): δ (ppm) 212.2 (CH_3CO), 182.3 (C-28), 171.1 (OAc), 80.8 (C-3), 56.2, 56.1, 55.3, 51.2, 50.2, 49.7, 49.1, 48.5, 47.6, 42.2, 40.5, 38.3, 37.7, 37.5, 37.0, 36.7, 34.0, 31.4, 30.1, 29.6, 28.2, 27.9, 27.1, 23.6, 21.3, 20.8, 18.1, 16.4, 16.1, 15.9, 14.6.

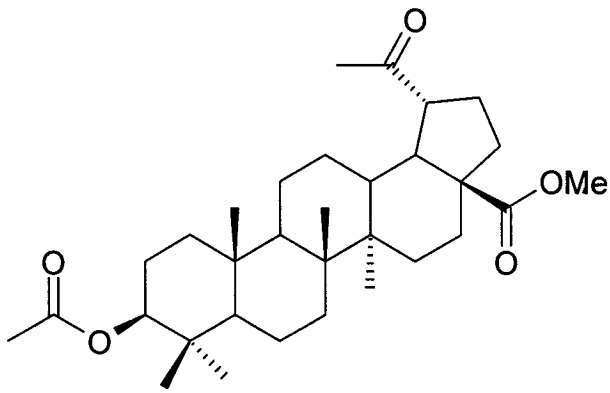
IR υ_{max} ($CHCl_3$, cm^{-1}): 3200 (OH), 2946, 2872, 2359, 1728 (C=O), 1694 (C=O), 1464, 1453, 1391, 1318, 1248, 1108, 1028, 979, 734, 668, 648.

MS (EI) *m/z* (rel. int.): 500 $[M]^+$ (1), 441 $[M\text{-}CO_2CH_3]^+$ (20), 440 $[M\text{-}CH_3CO_2H]^+$ (61), 425 (25), 397 (31), 205 (15), 203 (13), 190 (45), 189 (100), 147 (38), 136 (56), 135 (54), 121 (43), 95 (37), 93 (27), 81 (37), 43 (69).

HRMS: *Calculated* for $C_{31}H_{48}O_5$, 500.35018, *found* 500.34948.

2.7.8.4. 3β-Acetyl methyl platanate

Other names: Methyl 3β-acetyl-20-oxo-30-norlupan-28-oate, 30-norlupan-28-oic acid, 3β-(acetyloxy)-20-oxo-methyl ester, 30-norbetulinic acid, 3β-hydroxy-20-oxo-methyl ester acetate.



19d

Ethereal CH_2N_2 solution was added to a stirred solution of **19b** (100 mg) in CH_2Cl_2 with 5 drops of MeOH (50 mL) at room temperature (22°C) until the reaction mixture remained permanently yellow. Excess CH_2N_2 was evaporated in the fumehood overnight before the solvent was removed *in vacuo.* The residue was re-dissolved in EtOAc (30 mL), then washed successively with 10 mL of water, brine, water, dried ($MgSO_4$), filtered and concentrated under

reduced pressure. The crude product subjected to silica gel column chromatography (hexane-EtOAc gradient) affording (**19d**) as a white solid (80 mg, 78 %).

m.p. 204-206°C (lit.[134] 204-206°C, lit.[140] 205-207°C, lit.[134] 206-207°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 4.44 (dd, J=9.8 Hz, J=6.2 Hz, H-3α), 3.64 (s, 3H, OMe), 3.21 (m, H-19), 2.15 (s, 3H, CH_3CO), 2.01 (s, 3H, OAc), 0.96 (s, 3H, H-27), 0.86 (s, 3H, H-26), 0.80 (s, 9H, H-23/H-25/H-24).

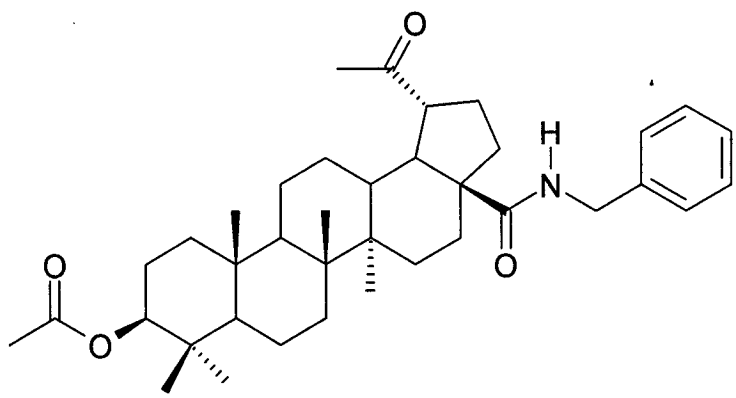
^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 212.3 (CH_3CO), 176.5 (C-28), 170.9 (OAc), 80.8 (C-3), 56.3, 55.3, 51.4, 51.1, 50.2, 49.3, 42.1, 40.5, 38.2, 37.7, 37.2, 37.0, 36.5, 34.0, 31.4, 30.1, 29.6, 28.2, 27.8, 27.1, 25.6, 21.3, 20.8, 18.1, 16.4, 16.1, 15.8, 14.6.

IR υ_{max} ($CHCl_3$, cm^{-1}): 2947, 2871, 2360, 2342, 1728 (C=O), 1453, 1432, 1391, 1368, 1329, 1318, 1222,1108, 1027, 980, 900, 757, 668.

MS (EI) *m/z* (rel. int.): 514 $[M]^+$ (1), 455 $[M\text{-}CH_3CO_2]^+$ (20), 454 $[M\text{-}\ CH_3CO_2H]^+$ (61), 439 (28), 411 (39), 204 (18), 203 (14), 189 (100), 147 (57), 136 (44), 135 (50), 121 (43), 107 (37), 43 (62).

HRMS: *Calculated* for $C_{32}H_{50}O_5$, 514.36586, *found* 514.36725

2.7.8.5. N-[3β-Acetoxy-nor-20-oxolupan-28-oyl]-benzylamine.



19e

Compound (**19e**) was prepared by reacting **19b** (100 mg, 2.0×10^{-4} mol) in CH_2Cl_2 (5 mL) with oxalyl chloride (38.0 mg, 3.0×10^{-4} mol, 0.03 mL,), five drops of DMF and benzylamine (24.0 mg, 2.2×10^{-4} mol, 0.02 mL) in the presence of Et_3N (22.0 mg, 2.2×10^{-4} mol, 0.03 mL) in CH_2Cl_2 (5 mL) according to the general

procedure used for amide formation previously described. The usual work up followed by silica gel column chromatography eluting with hexane-EtOAc gradient system furnished (**19e**) as a white solid (60 mg, 51%), m.p. 280-283°C.

m.p. 280-283°C.

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 7.32 (m. 5H, Ph), 5.91 (t, J=5.8 Hz, CONH), 4.58 (dd, J=14.8 Hz, J=5.9 Hz, CONHCH-Ph), 4.44 (dd, J=11.5 Hz, J=4.8 Hz, H-3α), 4.32 (dd, J=14.7 Hz, J=5.6 Hz, CONHCH'-Ph), 3.46 (dt, J=11.3, J=4.3 Hz, H-19), 2.24 (dt, J=12.0 Hz, J=4.2 Hz, H-13), 2.15 (s, CH_3CO), 2.01 (s, OAc), 0.96 (s, 3H, H-27), 0.86 (s, 3H, H-26), 0.82 (s, 3H, H-23), 0.81 (s, 3H, H-25), 0.80 (s, 3H, H-24).

^{13}C NMR (500 MHz, $CDCl_3$): δ (ppm) 212.9 (CH_3CO), 175.7 (C-28), 170.9 (OAc), 139.0, 128.7, 127.8, 127.4, 80.9, 55.5, 55.4, 51.0, 50.4, 50.0, 43.3, 42.3, 40.7, 38.4, 38.0, 37.8, 37.1, 36.8, 34.3, 33.0, 29.5, 28.6, 27.9, 27.2, 23.7, 21.3, 20.9, 18.2, 16.5, 16.2, 16.1, 14.7.

IR υ_{max} ($CHCl_3$, cm^{-1}): 3376 (CONH), 2946, 2869, 2360, 1733 (C=O), 1713 (C=O), 1647 (C=O), 1522, 1467, 1422, 1248, 1195, 1108, 917, 732, 699, 647.

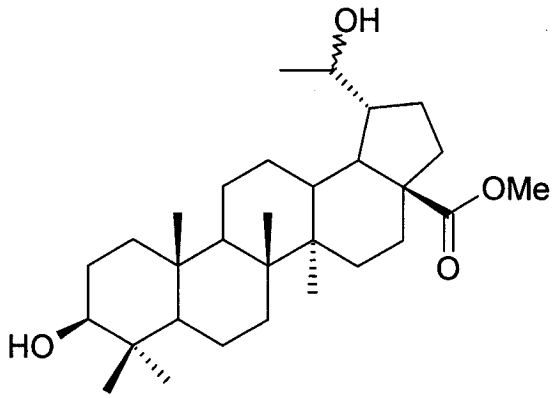
MS (EI) *m/z* (rel. int.): 589 $[M]^+$ (22), 546 $[M\text{-}CH_3CO]^+$ (6), 530 $[M\text{-}CH_3CO_2]^+$ (54), 529 $[M\text{-}CH_3CO_2H]^+$ (100), 415 (43), 486 (62), 460 (35), 205 (7), 203 (9), 189 (30), 91 (85), 43 (37).

HRMS: *Calculated* for $C_{38}H_{55}NO_4$, 589.41332, *found* 589.41409.

2.7.9. Derivatives of platanic acid

2.7.9.1. Methyl 3β, 20- dihydroxy-lup-28-oate

Other names: 3β, 20-dihydroxy-30-norlupan-28-oic acid methyl ester.



20a

To a solution of **19c** (50 mg, 1.06 x 10^{-4} mole) in MeOH/THF (6 : 2 mL) at 0°C was added $NaBH_4$ (40 mg, 1.1 x 10^{-3} mol) and the reaction mixture was stirred at room temperature overnight (15h) before it was quenched with NH_4Cl solution (5 mL), and the organic solvent removed *in vacuo*. The resulting solution was extracted with EtOAc (2 x 10 mL) and the combined EtOAc extracts were washed successively with 5 mL of water, brine, water, dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using hexane-EtOAc gradient as eluant to furnish (**20a**) as a white solid (30 mg, 60%).

m.p. 194-196°C.

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 3.84 (q, J=6.3 Hz, H-20), 3.63 (s, 3H, OMe), 3.17 (dd, J=10.4 Hz, J=5.6 Hz, H-3α), 0.94 (s, 6H, H-27/ H-26), 0.88 (s, 3H, H-23), 0.80 (s, 3H, H-25), 0.73 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 176.9 (C-28), 78.9 (C-3), 68.9 (C-20), 56.9, 55.2, 51.2, 50.2, 47.9, 45.6, 42.4, 40.6, 38.8, 38.6, 37.9, 37.1, 37.0, 34.3, 31.7, 29.6, 27.9, 27.3, 27.0, 23.3, 22.2, 20.8, 18.3, 16.1, 15.9, 15.4, 14.7.

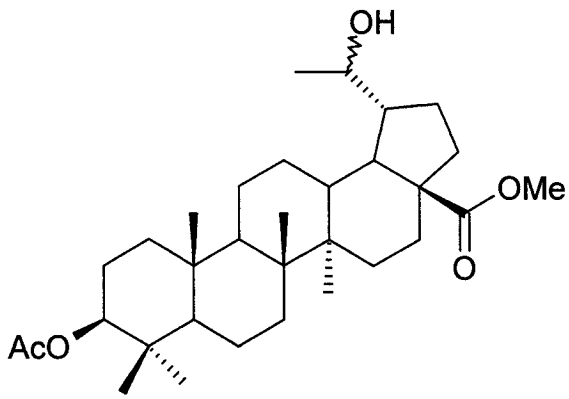
IR υ_{max} ($CHCl_3$, cm^{-1}): 3407 (OH), 2945, 2869, 1714 (C=O), 1655, 1561, 1454, 1390, 1320, 1276, 1217, 1034, 1002, 983, 945, 817, 757.

MS (EI) *m/z* (rel. int.): 474 $[M]^+$ (0.6), 456 $[M-H_2O]^+$ (21), 414 $[M-CH_3CO_2H]^+$, (0.6), 234 (34), 220 (10), 207 (55), 203 (13), 189 (85), 175 (100), 164 (70), 138 (58), 134 (43), 121 (44), 118 (48), 107 (46), 95 (55), 93 (38), 81 (54), 69 (44).

HRMS: *Calculated* for $C_{30}H_{50}O_4$, 474.37090, *found* 474.37130

2.7.9.2. Methyl 3β-acetyl-20-hydroxy-lup-28-oate.

Other names: 3β-(acetyloxy)-20-hydroxy-30-norlupan-28-oic acid methyl ester.



20b

The reduction of compound **19d** (110 mg, 2.14 x 10^{-4} mole) with $NaBH_4$ (81 mg, 2.14 x 10^{-3} mol in MeOH/THF (9 : 3 mL) at 0°C mol) following the same procedure and work-up described for compound **20a** above, resulted in the formation of (**20b**) as a white solid (60 mg, 54%), after purification by silica gel column chromatography (hexane-EtOAc gradient).

m.p. 256-259°C.

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 4.45 (dd, J=10.8, J=5.5 Hz, H-3α), 3.88 (q, J=6.4 Hz, H-20), 3.63 (s, 3H, O-CH_3), 2.02 (s, OAc), 0.94 (s, 3H, H-27), 0.88 (s, 3H, H-26), 0.83 (s, 3H, H-23), 0.82 (s, 3H, H-25), 0.81 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 176.9 (C-28), 171.1 (OAc), 81.0 (C-3), 68.9 (C-20), 56.9, 55.3, 51.2, 50.1, 47.9, 45.6, 42.4, 40.6, 38.3, 38.0, 37.7, 37.0, 34.3, 31.7, 29.6, 27.9, 27.0, 23.6, 23.3, 22.2, 21.3, 20.8, 18.3, 16.4, 16.1, 15.9,14.6.

IR υ_{max} ($CHCl_3$, cm^{-1}): 3538 (OH), 2948, 2871, 2453, 2281, 1720 (C=O), 1655, 1561, 1458, 1392, 1370, 1030, 980, 945, 902, 857, 757, 666.

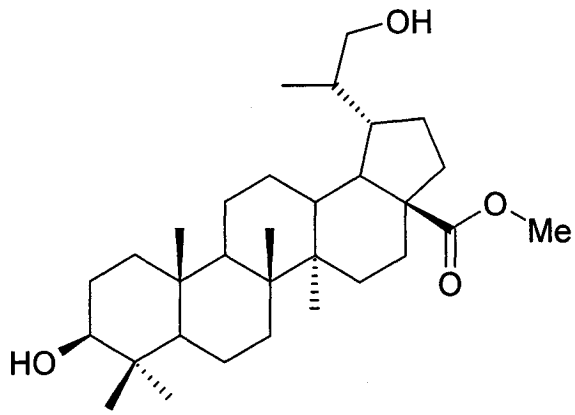
MS (EI) *m/z* (rel. int.): 516 $[M]^+$ (2), 498 $[M-H_2O]^+$ (7), 457 $[M-CH_3CO_2]^+$ (11), 456 $[M-CH_3CO_2H]^+$ (25), 203 (14), 202 (10), 189 (100), 175 (81), 161 (60), 136 (50), 135 (44), 121 (45), 107 (38), 95 (41).

HRMS: *Calculated* for $C_{32}H_{52}O_5$, 516.38150, *found* 516.38129.

2.7.10. Hydrated betulinic acid derivatives

2.7.10.1. Methyl 3β, 29-dihydroxy-lup-28-oate.

Other names: 3β, 29-dihydroxy-lupan-28-oic acid methyl ester.



21a

To a solution of **3a** (200 mg, 4.25 x 10^{-4} mol) in dry THF (15 mL) at room temperature was added $(CH_3)_2S.BH_3$ (64.6 mg, 8.50 x 10^{-4} mol) under nitrogen. After 7h, 3N NaOH (0.43 mL) was added dropwise, followed by 30% w/w H_2O_2 while keeping the temperature between 30-35°C and then slowly warmed to 50°C. The reaction mixture was then stirred overnight (15h) at 50-60°C before it was diluted with diethyl ether (20 mL), washed with brine (10 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude product was purified by silica gel chromatography using hexane-EtOAc gradient system as eluant to afford (**21a**) as a white solid (140 mg, 67%).

m.p. 218-220°C (lit.[141] 212-214°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 3.76 (dd, J=10.4 Hz, J=4.6 Hz, H-29), 3.62 (s, 3H, O-CH_3), 3.39 (dd, J=10.5 Hz, J=8.1 Hz, H-29'), 3.17 (dd, J=10.1 Hz, J=5.6 Hz, H-3α), 0.94 (s, 3H, H-27), 0.92 (s, 3H, H-26), 0.88 (s, 3H, H-23), 0.80 (s, 3H, H-25), 0.73 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 176.6 (C-28), 78.9 (C-3), 64.2 (C-29), 56.8, 55.2, 51.2, 50.2, 48.7, 43.1, 42.5, 40.6, 38.8, 38.6, 38.3, 38.1, 37.1, 37.0, 34.3, 32.0, 29.6, 27.9, 27.3, 27.2, 23.8, 20.9, 18.2, 18.1, 16.0, 15.9, 15.4, 14.6.

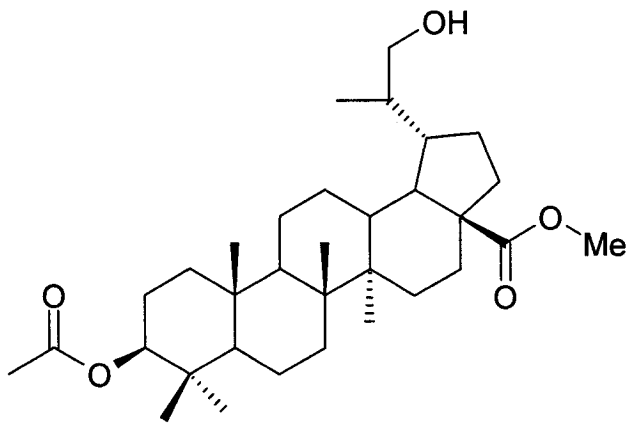
IR υ_{max} ($CHCl_3$, cm^{-1}): 3386 (OH), 2948, 2870, 2361, 1716 (C=O), 1455, 1390, 1377, 1290, 1106, 1041, 983, 946, 757.

MS (EI) *m/z* (rel. int.): 488 $[M]^+$ (6), 470 $[M-H_2O]^+$ (18), 429 $[M-CH_3CO_2]^+$ or $[M-CH_3CHCH_2O]^+$ (31), 428 $[M-CH_3CO_2H]^+$ or $[M-CH_3CHCH_2OH]^+$ (14), 220 (9), 207 (87), 205 (20), 203 (22), 202 (7), 190 (36), 189 (100), 175 (47), 161 (43), 147 (52), 135 (63), 133 (43), 121 (53), 119 (53), 109 (53), 107 (55), 105 (50), 95 (58), 93 (57), 91 (44), 81 (63), 79 (44), 69 (46), 67 (46), 55 (56), 40 (51).

HRMS: *Calculated* for $C_{31}H_{52}O_4$, 488.38658, *found* 488.38549.

2.7.10.2. Methyl 3β-acetyl, 29-hydroxy-lup-28-oate.

Other names: 3β-acetoxy- 29-hydroxy-lupan-28-oic acid methyl ester.



21b

3β-Acetoxy methyl betulinate (**3h**, 50 mg, 9.75 x 10^{-5} mol) was reacted with $(CH_3)_2S.BH_3$ (8.9 mg, 1.17 x 10^{-4} mol, 0.05 mL) in dry THF (5 mL) at room temperature (under nitrogen). After 5.5h, $(CH_3)_2S.BH_3$ (8.9 mg, 1.17 x 10^{-4} mol, 0.05 mL) was added again and the solution was stirred overnight before a solution of 3N NaOH (0.10 mL) was added dropwise, followed by 30% w/w H_2O_2 (0.10 mL) and keeping the temperature between 30-35°C. The solution was warmed to 50°C and stirred for 5h before it was worked up following the same procedure used for (**21a**) and purified by silica gel chromatography (hexane-EtOAc gradient) to give (**21b**) as a white solid (25 mg, 48%).

m.p. 235-237°C.

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 4.45 (dd, J=10.8 Hz, J=5.6 Hz, H-3α), 3.76 (dd, J=10.4 Hz, J=4.6 Hz, H-29), 3.62 (s, 3H, O-CH_3), 3.40 (dd, J=10.4 Hz, J=8.0 Hz, 1H, H-29'), 2.02 (s, 3H, OAc), 0.94 (d, J=6.9 Hz, 3 H, H-30), 0.92 (s, 3H, H-27), 0.88 (s, 3H, H-26), 0.83 (s, 3H, H-23), 0.82 (s, 3H, H-25), 0.81 (s, 3H, H-24).

^{13}C NMR (500 MHz, $CDCl_3$): δ (ppm) 176.7 (C-28), 171.0 (OAc), 80.9 (C-3), 64.3 (C-29), 56.9, 55.4, 51.2, 50.2, 48.9, 43.2, 42.5, 40.7, 38.4, 38.2, 38.0, 37.1, 37.0, 34.3, 32.0, 29.7, 27.9, 27.2, 23.9, 23.7, 21.3, 20.9, 18.2, 18.1, 16.5, 16.1, 15.9, 14.6.

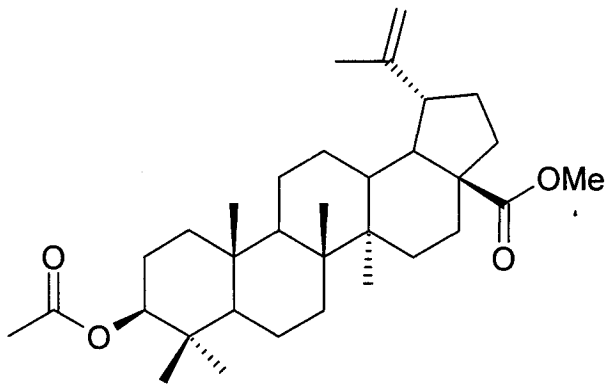
IR υ_{max} ($CHCl_3$, cm^{-1}): 3438 (OH), 2872, 2359, 2989, 1717 (C=O), 1642 (C=O), 1558, 1455, 1368, 1249, 1159, 1135, 1040, 1025, 979, 913., 736.

MS (EI) *m/z* (rel. int.): 530 $[M]^+$ (6), 512 $[M\text{-}H_2O]^+$ (0.4), 471 $[M\text{-}CH_3CO_2]^+$ or $[M\text{-}CH_3CHCH_2OH]^+$ (24), 470 $[M\text{-}CH_3CO_2H]^+$ (42) 411 (27), 203 (17), 189 (100), 136 (45), 135 (51).

HRMS: *Calculated* for $C_{33}H_{54}O_5$, 530.39715, *found* 530.39731.

3β-Acetyl methyl betulinate (3h)

Other names: 3-O-acetyl betulinic acid, 3β-acetoxy methyl betulinate



3h

Et_3N (4.30 x 10^{-2} g, 4.25 x 10^{-4} mol, 59.2 uL) and a catalytic amount of DMAP (1 x 10^{-2} g) was added to a solution of methyl betulinate **3a** (1 x 10^{-1} g, 2.12 x 10^{-4} mol) in CH_2Cl_2 (5 mL) and stirred for 15 mins before acetic anhydride (4.34 x 10^{-2} g, 4.25 x 10^{-4} mol, 40.1 uL) was added. Stirring continued overnight at room temperature. The reaction mixture was washed succesively with 5.0 mL of water 5 % HCl, water before it was dried ($MgSO_4$), filtered and concentrated *in*

vacuo to afford 105 mg (96%) of a white solid which was clean by NMR and TLC and was not purified further.

m.p. 200-201°C (lit.[111] 201-202°C, lit.[21] 203°C, lit.[135] 210-212°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 4.72 (d, J=1.5 Hz, H-29), 4.59 (d, J=1.5 Hz, H=29'), 4.20 (dd, J=10.0 Hz, J=4.7 Hz, H-3α), 2.99 (ddd, J=11.0 Hz, J=5.5 Hz, H-19), 3.73 (s, OMe), 2.03 (s, OAc), 1.67 (s, 3H, H-30), 0.96 (s, 3H, H-27), 0.94 (s, 3H, H-23), 0.93 (s, 3H, H-26), 0.83 (s, 3H, H-25), 0.82 (s, 3H, H-24).

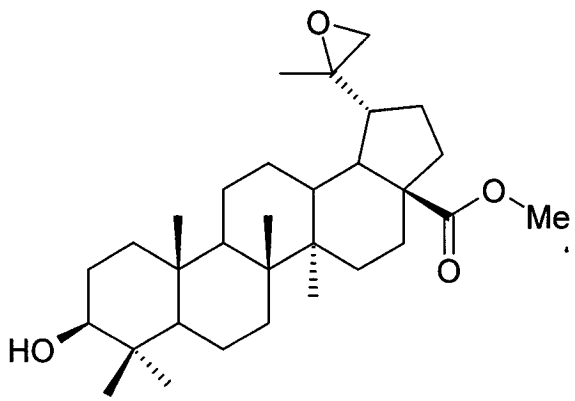
MS (EI) *m/z* (rel.int.): 512 $[M]^+$ (1), 453 $[M\text{-}CH_3CO_2]^+$, 452 $[M\text{-}CH_3CO_2H]^+$, 409 (6), 249 (5), 248 (4), 233 (4), 203 (11), 202 (6), 190 (10), 189 (34), 69 (100).

HRMS: *Calculated* for $C_{33}H_{52}O_4$, 512.38656, *found* 512.38960.

2.7.11. Epoxides of betulinic acid derivatives

2.7.11.1. Methyl 3β-hydroxy-lup-20(29)-epoxy-28-oate.

Other names: 3β-hydroxy-20,29-epoxy-lupan-28-oic acid methyl ester.



22a

To a stirred solution of **3a** (100 mg, 2.13 x 10^{-3} mol) in CH_2Cl_2 (10 mL) was added NaOAc (100 mg, 1.22 x 10^{-3} mol), and the solution was cooled to 0°C before the peracetic acid (0.2 mL, 2.97 mmol) was added. The ice bath was removed and the reaction mixture was warmed to room temperature. Stirring continued for 3h before the reaction was quenched with 15% solution of Na_2SO_3 (20 mL). The solvent was removed *in vacuo* and the residue was extracted with ethyl acetate (3 x 20 mL). The combined organic extract was washed with

saturated solution of K_2CO_3 (2 x 20 mL), brine (20 mL) then dried ($MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica gel chromatography eluting with hexane-EtOAc gradient to furnish (**22a**) as a white solid (70 mg, 68%).

m.p. 191-193°C.

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 3.60 (s, 3H, OMe), 3.15 (dd, J=10.4 Hz, J=5.5 Hz, H-3α), 2.60 (d, J=2.1 Hz, 2H, H-29), 1.20 (s, 3H, H-30), 0.92 (s, 3H, H-27), 0.91 (s, 3H, H-26), 0.86 (s, 3H, H-23), 0.79 (s, 3H, H-25), 0.72 (s, 3H, H-24).

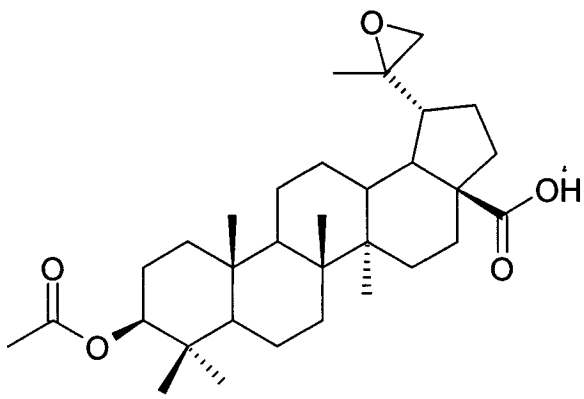
^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 176.4 (C-28), 78.8 (C-3), 60.1 (C-29), 56.8, 56.5, 55.2, 51.3, 50.3, 50.1, 45.4, 42.3, 40.6, 38.8, 38.7, 37.4, 37.1, 36.8, 34.2, 32.0, 29.3, 27.9, 27.3, 27.0, 26.8, 20.9, 18.3, 18.2, 16.0, 15.8, 15.3, 14.5.

MS (EI) *m/z* (rel. int.): 486 $[M]^+$ (1), 468 $[M-H_2O]^+$ (1), 429 $[M-CH_3OCH_2]^+$ (2), 427 $[M-CH_3CO_2]^+$ (3), 426 $[M-CH_3CO_2H]^+$ (5), 220 (20), 207 (75), 203 (16),190 (39), 189 (100), 175 (41), 161 (39), 147 (51), 135 (55), 119 (40), 107 (42), 95 (49).

HRMS: *Calculated* for $C_{31}H_{50}O_4$, 486.37093, *found* 486.36242.

2.7.11.2. 3β-Acetyl-lup-20(29)-epoxy-28-oic-acid.

Other names: 3β-Acetoxy-20,29-epoxy-lupan-28-oic acid.



22b

To a solution of **14d** (530 mg, 1.06 x 10^{-3} mol) in CH_2Cl_2 (20 mL) at 0°C was added mCPBA (280 mg, 1.60 x 10^{-3} mol). The ice-bath was removed and the solution was stirred at room temperature overnight. The reaction mixture was washed successively with 10% solution of $NaHSO_3$ (2 x 10 mL), saturated solution of Na_2CO_3 (2 x 10 mL), brine (2 x 10 mL), dried ($MgSO_4$), filtered and

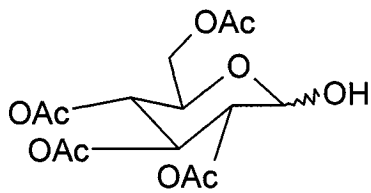
concentrated *in vacuo*. The crude product was purified by silica gel chromatography using hexane-EtOAc solvent gradient as eluant yielding (**22b**) as a white solid (280 mg, 51%), m.p. 290-293°C.

m.p. 290-293°C.

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 4.44 (dd, 1H, J=9.3 Hz, J=6.4 Hz, H-3α), 2.64 (s, 2H, H-29), 2.02 (s, 3H, CH_3-CO_2), 1.22 (s, 3H, H-30), 0.93 (s, 3H, H-27), 0.89 (s, 3H, H-26), 0.83 (s, 3H, H-23), 0.82 (s, 3H, H-25), 0.81 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 182.2 (C-28), 171.1 (OAc), 80.9 (C-3), 60.2 (C-29), 56.6, 55.3, 50.2, 49.9, 45.4, 42.3, 40.7, 40.6, 38.3, 37.7, 37.5, 37.0, 36.8, 34.1, 31.9, 29.3, 27.9, 26.9, 26.7, 23.6, 21.3, 20.8, 18.1, 18.0, 16.4, 16.1, 16.0, 14.5.

2.7.12. Glucosides of betulinic acid methyl ester

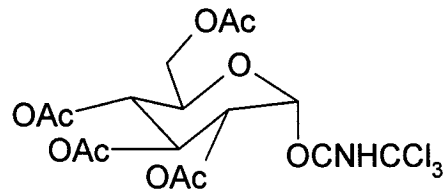


23a

To a solution of β-D-glucose pentaacetate **23** (6.0 g, 1.54 x 10^{-2} mol) in DMF (20 mL) was added hydrazine acetate (1.8 g, 1.99 x 10^{-2} mol) and the reaction mixture was stirred at 50°C for 30 mins before it was poured into ice-NaCl solution (100 mL). The resulting solution was extracted with CH_2Cl_2 (3 x 50 mL) and the combined organic extract was washed successively with 50 mL of 5% HCl, sat. $NaHCO_3$, water, dried ($MgSO_4$) and concentrated *in vacuo*. The crude product was subjected to silica gel column chromatography eluting with EtOAc-hexane mixtures of increasing polarity to afford (**23a**) as a white sticky solid (4.34 g, 81%).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm): 5.54 (d, J=10.0 Hz, H-1), 5.45 (d, J=9.6 Hz, H-2), 5.06 (dd, J=9.9 Hz, J=9.5 Hz, H-3), 4.88 (dd, J=10.1 Hz, J=3.7 Hz, H-6), 4.17 (m, 3H, H-3, H-4, H-5, H-6'), 2.07 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.99 (s, 3H, OAc).

2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl trichloroacetimidate



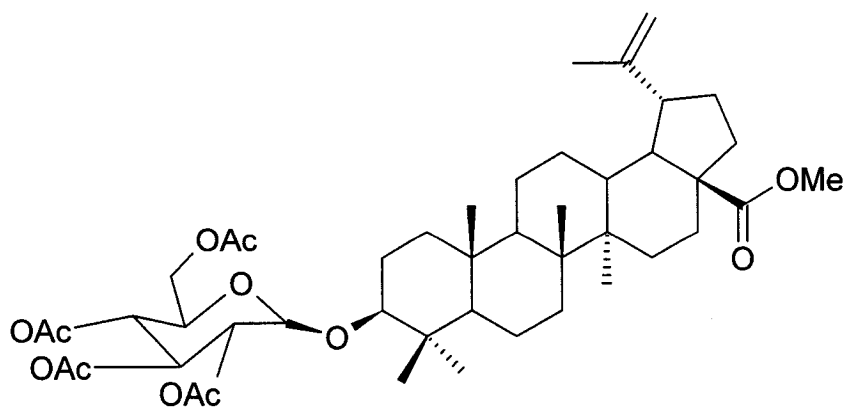
23b

To a solution of the acetyl-protected glucopyranose **23a** (500 mg, 1.44 x 10^{-3} mol) in dry CH_2Cl_2 (10 mL) was added trichloroacetonitrile and DBU (22 mg, 1.44 x 10^{-4} mol, 0.02 mL) and was stirred at room temperature overnight before it was concentrated *in vacuo*. The residue was purified by silica gel chromatography using hexane-EtOAc gradient as eluant to afford (**23b**) as a pale brown gum (360 mg, 51%).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 8.66 (s, NH), 6.52 (d, J=3.7 Hz, H-1), 5.53 (d, J=10.1 Hz, H-2), 5.12 (m, 2H, H-3/H-4), 4.16 (m, 3H, H-5/H-6), 2.04 (s, 3H, OAc), 2.01 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.98 (s, 3H, OAc),.

MS (EI) *m/z* (rel. int.): 491 $[M]^+$ (0.1), 434 (23), 432 $[M\text{-}OAc]^+$ (23), 333 (37), 332 $[M\text{-}CONHCCl_3]^+$ (100), 271 (44), 229 (37), 222 (60), 211 (43), 70 (39), 169 (49), 109 (42).

2.7.12.1. Methyl 3-O-β-D-2',3',4',6'-tetraacetoxy-glucopyranosyl-lup-20(29)-en-28-oate.



24a

To a solution of betulinic acid methyl ester (500 mg, 1.06×10^{-3} mol) and the acetyl-protected imidate **23b** (783 mg, 1.59×10^{-3} mol) in dry CH_2Cl_2 (30 mL) under nitrogen was added powdered molecular sieves 4 Å (3.0 g) and stirred for 3h at room temperature then cooled to 0°C before $BF_3.Et_2O$ (754 mg, 5.31×10^{-3} mol, 0.67 mL) was added dropwise. After 3h, the reaction mixture was neutralized with saturated $NaHCO_3$ solution (50 mL) and diluted with CH_2Cl_2 (30 mL). Theaqueous layer was extracted with CH_2Cl_2 (3 x 20 mL) and the combined organic layer was dried ($MgSO_4$), filtered through celite and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography eluting with hexane-EtOAc gradient system to afford 7% (60 mg) of glycoside (**24a**) as a white solid.

m.p. 119-121°C (lit.[144] 120-123°C).

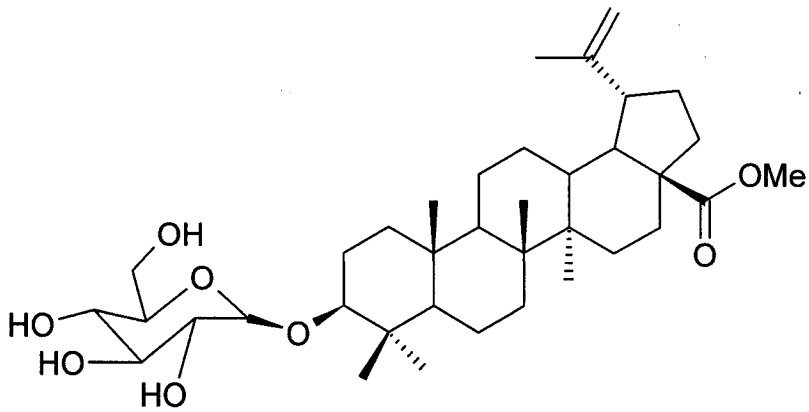
^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 5.15. (t, J=9.4 Hz, H-3'), 5.00 (dd, J=9.5 Hz, J=8.8 Hz, 2H, H-2'/H-4'), 4.70 (br s, H-29), 4.56 (br s, H-29'), 4.50 (d, J=8.0 Hz, H-1'), 4.22 (dd, J=12.2 Hz, J=5.5 Hz, H-6'), 4.06 (dd, J=12.0 Hz, J=2.6 Hz, H-6"), 3.67 (m, H-5'), 3.63 (s, 3H, OMe), 3.01 (m, 2H, H-3α/H-19), 2.04 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.97 (s, 3H, OAc), 1.65 (s, 3H, H-30), 0.91 (s, 3H, H-27), 0.86 (s, 3H, H-26), 0.85 (s, 3H, H-23), 0.77 (s, 3H, H-25), 0.67 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 176.6 (C-28), 170.7 (OAc), 170.4 (OAc), 169.4 (OAc), 169.2 (OAc), 150.6 (C-20), 109.5 (C-29), 102.9 (C-1'), 90.7 (C-3), 72.8 (C-3'), 71.6 (C-5'), 71.4 (C-2'), 68.7 (C-4'), 62.2 (C-6'), 56.5, 55.6, 51.3, 50.5, 49.4, 46.7, 42.3, 40.6, 38.9, 38.6, 38.2, 36.9, 36.8, 34.3, 32.1, 30.6, 29.6, 27.6, 25.9, 25.5, 20.8, 20.7, 20.6, 20.5, 19.4, 18.1, 16.1, 16.0, 15.9,14.6.

IR υ_{max} ($CHCl_3$, cm^{-1}): 3436, 3354, 3301, 2947, 2870, 2871, 1756 (C=O), 1734 (C=O), 1641 (C=O), 1601, 1451, 1434, 1376, , 1168, 909, 886, 826, 757, 668.

ESI *m/z* (rel. int.): *Calculated* for $C_{45}H_{68}O_{12}$, 800.47114, *found* 839.3 $[M+K]^+$ (24).

2.7.12.2. Methyl 3-O-β-D-glucopyranosyl-lup-20(29)-en-28-oate.



24b

To a solution of **24a** (40 mg, 5.0 x 10^{-5} mol) in MeOH (1 mL) and CH_2Cl_2 (0.2 mL) was added a 0.5M solution of NaOMe/MeOH (2 mL) until the pH ~ 9 and stirred at room temperature overnight. The solution was then neutralized with acidic resin Amberlite until the pH was ~ 3 before it was filtered and concentrated *in vacuo*. The crude material was subjected to silica gel column chromatography eluting with hexane-EtOAc gradient system to furnish glycoside (**24b**) as a pale yellow solid (30 mg, 95%).

m.p. 196-198°C (lit.[144] 197-200 °C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 4.72 (s, H-29), 4.59 (s, H-29'), 4.30 (d, J=6.0 Hz, H-1'), 3.77 (br s, 2H, H-6'/H-6"), 3.64 (s, 3H, OMe), 3.53-3.46 (m, 3H, H-3', H-4', H-5'), 3.27 (br s, 1H, H-2'), 3.01 (m, 2H, H-3α/H-19), 1.67 (s, 3H, H-30), 0.94 (s, 3H, H-27), 0.93 (s, 3H, H-26), 0.88 (s, 3H, H-23), 0.78 (s, 3H, H-25), 0.76 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 176.6 (C-28), 150.5 (C-20), 109.6 (C-29), 105.1 (C-1'), 90.1 (C-3), 75.2 (C-3'), 73.8 (C-5'/C2'), 69.6 (C-4'), 61.8 (C-6'), 56.5, 55.6, 51.3, 50.5, 49.4, 46.7, 42.3, 40.6, 39.1, 38.8, 38.2, 36.9, 34.3, 32.2, 30.6, 29.7, 27.9, 26.3, 25.5, 20.9, 19.4, 18.1, 16.5, 16.1, 15.9,14.7.

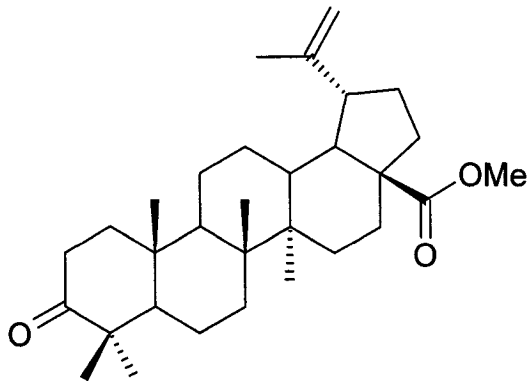
IR υ_{max} ($CHCl_3$, cm^{-1}): 3398 (OH), 3069, 2943, 2870, 2361, 2342, 1721 (C=O), 1638, 1602, 1451, 1388, 1363, 1165, 1135, 1077, 1020, 980, 880, 757.

MS (EI) *m/z* (rel. int.): 632 $[M]^+$ (9), 573 $[M\text{-}CH_3CO_2]^+$ (0.3), 453 $[M\text{-}C_6H_{11}O_6]^+$ (42), 454 $[M\text{-}C_6H_{12}O_6]^+$ (24), 262 (3), 233 (3), 220 (2), 207 (6), 205 (28), 203 (13), 202 (4), 189 (24), 187 (100), 145 (56), 133 (50), 131 (44), 95 (44).

2.7.13. Radioactive labelled betulinic acid methyl ester

Methyl betulonate (16a).

Other names: Methyl 3-oxo-lup-20(29)-en-28-oate, 3-Oxo-lup-20(29)-en-28-oic acid methyl ester.



16a

The yellow ethereal solution of CH_2N_2 was added to the solution of betulonic acid, **16** (340 mg, 7.45 x 10^{-4} mol) in CH_2Cl_2 (50 mL) at 0°C was until the colour remained permanently yellow and the excess CH_2N_2 was allowed to evaporate in the fumehood overnight before the solvent was removed *in vacuo*. The residue was redissolved in EtOAc (70 mL) and washed successively with 30 mL of water, brine, water, dried ($MgSO_4$) and concentrated *in vacuo*. The crude product was subjected to silica gel column chromatograpy eluting with EtOAc-hexane gradient as to afford (**16a**) as a white feathery solid (280 mg, 80%).

m.p. 159-160°C (lit.[120] 146-148°C, lit.[117] 158-159°C, lit. [145]161-163°C, lit.[24] 165°C).

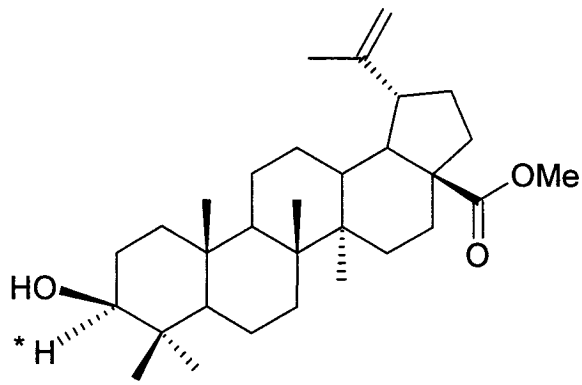
^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 4.71 (d, J=1.7 Hz, H-29), 4.58 (d, J=1.3 Hz, H-29'), 3.65 (s, 3H, OMe), 2.97 (ddd, J=10.8 Hz, J=4.4 Hz, H-19), 2.47 (m, H-2), 2.37 (m, H-2'), 1.66 (s, 3H, H-30), 1.04 (s, 3H, H-27), 1.00 (s, 3H, H-26), 0.95 (s, 3H, H-23), 0.93 (s, 3H, H-25), 0.90 (s, 3H, H-24).

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 176.6 (C-28), 150.4 (C-20), 109.6 (C-29), 56.5, 55.0, 51.2, 49.9, 49.4, 47.3, 46.9, 42.4, 40.6, 39.6, 38.3, 36.9, 34.1, 33.6, 32.1, 30.5, 29.6, 26.6, 25.5, 21.4, 21.0, 19.6, 19.3, 15.9, 15.7, 14.6.

IR ($CHCl_3$, cm^{-1}): 3437, 2947 2869, 2360, 2342, 1727 (C=O), 1707 (C=O) 1642, 1377, 1319, 1187, 1156, 1140, 985, 884, 756, 669.

MS (EI) *m/z* (rel. int.): 468 $[M]^+$ (13), 409 $[M\text{-}CH_3CO_2]^+$ (22), 408 $[M\text{-}CH_3CO_2H]^+$, (16), 262 (18), 249 (19), 205 (25), 203, (31), 202 (13), 201 (24), 189 (100), 175 (41).

HRMS: *Calculated* for $C_{31}H_{48}O_3$, 468.36036, *found* 468.35649.



3a"

To a solution of methyl betulonate, **16a** (707.19 mg, 1.51 x 10^{-3} mol) in MeOH (60 mL) and THF (20 mL) at 0°C, was added tritiated $NaBH_4$* (14.28 mg, 3.7748 x 10^{-4} mol) and the reaction followed by TLC. After 1h, the reaction was quenched with saturated NH_4Cl solution (60 mL). The organic solvent was removed *in vacuo* and the residue was re-extracted with EtOAc (3 x 80 mL). The combined EtOAc extracts were washed with water (60 mL), dried ($MgSO_4$), filtered andconcentrated *in vacuo*. The crude product was subjected to Si gel column chromatography using hexane-EtOAc gradient as eluant to afford 42% of the radioactive labelled (^3H)-betulinic acid methyl ester (**3a"**) as a white solid (300 mg).

2.8. References for Chapter 2

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Chapter 3

NATURAL INSECTICIDE FROM A NEOTROPICAL PIPER SPECIES

3.1. Introduction

In a recent study by Professor Arnason's group here at the University of Ottawa, it was demonstrated that extracts from the leaves and fruits of *Piper tuberculatum* (*P.tuberculatum*) were the most active among the 12 neotropical *Piper* species tested for insecticidal activity against their model insect, the European corn borer ECB), *Ostrinia nubilalis.*[1] This study also established that the tropical American *Piper* species have a chemical and insecticidal profile comparable to the well-studied and well-documented Asian (*P. nigrum*) and African (*P. guineense)* species.

The bioassay guided isolation and purification of the leaf extracts of *P.tuberculatum* by silica gel column chromatography (hexane-EtOAc solvent gradient) followed by recrystallization provided 5,6-dihydropiperlonguminine (**25**), a known piperamide, as the major chemical constituent (**Figure 3.2a**).

Since the insecticides from *Piper* species are generally regarded as safe and suitable for registration, these findings, together with our interest in biologically active natural products prompted us to identify the chemical components in the active fruit extracts of *P. tuberculatum.*

This chapter details the isolation and characterization of the piperamides found in the active extract from the fruits of *P. tuberculatum*, as well as their syntheses. A radioactive labelled piperamide was also synthesized for toxicokinetic studies. The results of the bioassays and synergistic studies carried out on the synthetic piperamides are also presented in this chapter as well as some of the results from toxicokinetic studies.[2-4]

3.2. Isolation and identification of piperamides from *Piper tuberculatum*

The fresh fruits of *P. tuberculatum* collected in Costa Rica were immediately immersed in 95% EtOH in 1L Nalgene bottles where they were stored until they were processed.

In our laboratory, the fruit and the EtOH were poured into a blender. Additional 95% EtOH was added to facilitate the blending process. The macerate was then filtered in a Buchner funnel and the filtrate was evaporated under diminished pressure until mostly water was left. More water was added to it before it was extracted successively with hexane (twice) and EtOAc (thrice) in a separating funnel. Each fraction was concentrated *in vacuo* to afford a hexane and a EtOAc-soluble extract.

Part of the EtOAc extract was subjected to silica gel column chromatography (hexane-EtOAc gradient) furnishing two major fractions. Fraction 1 (light green solid) furnished 5,6-dihydropiperlonguminine (**25**) in 0.2% of dry weight, as the major component, after recrystallisation from hexane/acetone. This is the same piperamide previously isolated from the leaf extracts of *P. tuberculatum* by Bernard *et. al.*[1] Fraction 2 (yellow material) was further purified using a recycling preparative HPLC to afford three known piperamides, 5,6-dihydropiperine (**26**), piperine (**27**) and piperlonguminine (**28**), each in 0.08% of dry weight (**Figure 3.2a**).

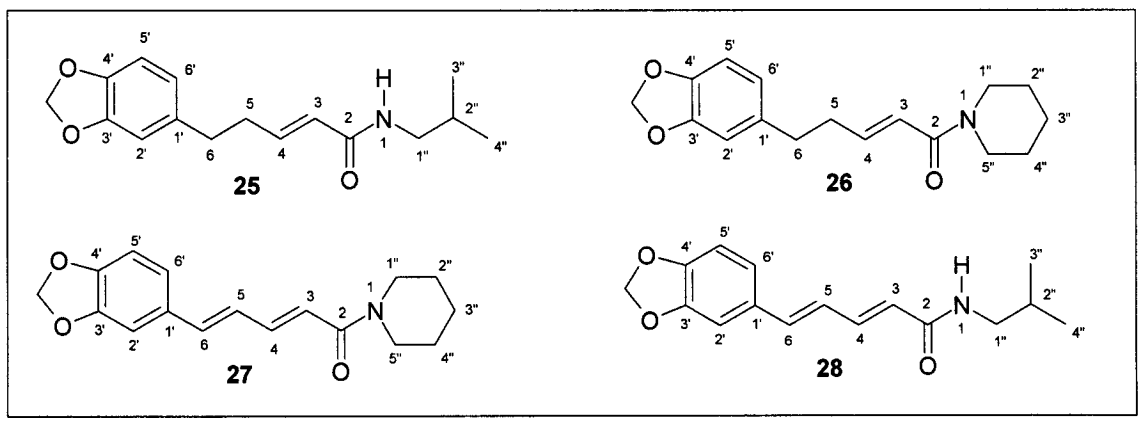


Figure 3.2a: Piperamides isolated from the fruits of *P.tuberculatum.*

The identification of these piperamides was accomplished by comparing their physical properties and spectroscopic data with literature data.[5-9] The synthetic piperamides (**25-28**) were also identical (TLC, IR, ^{1}H and ^{13}C NMR) to the natural piperamides isolated from *P. tuberculatum*.

Inspection of the ^{1}H NMR spectra of piperamides (**25-28**) revealed resonances attributable to the methylenedioxyphenyl or MDP group at δ ~5.90 (s, OCH_2O) and three aromatic protons (H-2', H-5', H-6'). In the case of piperamides (**25**) and (**26**) two olefinic protons (H-3, H-4) were observed compared to four olefinic protons in (**27**) and (**28**) (H-3, H-4, H-5, H-6), indicative of an α, β, γ, δ unsaturated system. (**Table 3.2a**). The values of the coupling constants (J>12 Hz) confirmed that both the double bonds possess the E due to the N-H of the isobutyl moiety in (**25**) and (**28**), respectively. This was absent in (**26**) and (**27**) which contained the corresponding piperidine unit.

The ^{13}C NMR spectra of all four piperamides (**25-28**) exhibited characteristic signals for the CONH group at δ ~165-166, MDP group at δ ~101, and six aromatic carbons (C-1 'to C-6'). Piperamides (**25**) and (**26**) exhibited two olefinic carbons (C-3, C-4) in their ^{13}CNMR spectra compared to four olefinic carbons (C-3, C-4, C-5, C-6) in (**27**) and (**28**). Moreover, (**25**) and (**28**) revealed three saturated carbons (two equivalent carbons) due to the isopropyl moiety (C-1", C-2", C-3"/C-4") whereas (**26**) and (**27**) displayed five saturated carbons ascribed to the piperidinyl moiety (C-1", C-2", C-3", C-4", C-5"), (**Table 3.2b**).

The IR spectra of piperamides (**25-28**) revealed strong absorption bands at ~1634-1667 cm^{-1} due to the conjugated carbonyl group. The IR bands at ~3420 cm^{-1} were attributed to the N-H stretch in (**25**) and (**28**).

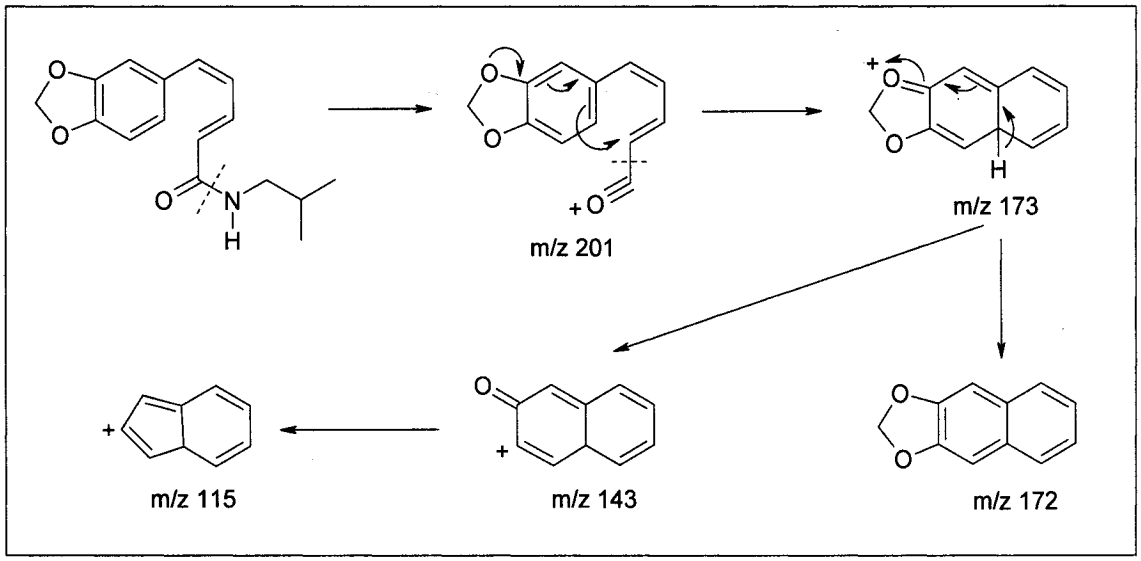
Table 3.2a: ^{1}H NMR data of piperamides **25-28** (δ, ppm, $CDCl_3$).

Proton	**25**	**26**	**27**	**28**
CONH	5.51, br s	-	-	5.59, br s
H-3	5.75, dt (J=15.3,1.5 Hz)	6.17, dt (J=15.0, 1.5 Hz)	6.39, d (J=14.7 Hz)	5.90, d (J=14.8 Hz)
H-4	6.82, dt (J=15.2, 6.7 Hz)	6.76, dt (J=15.1, 7.0 Hz)	7.35, ddd (J=14.7, 8.5, 1.5 Hz)	7.33, dd (J=14.9, 10.8 Hz)
H-5	2.44, dq, (J=6.4, 1.6 Hz)	2.43, dq (J=7.3, 1.3 Hz)	6.73, dd J=14.6, 8.6 Hz)	6.64, dd (J=15.4, 10.8 Hz)
H-6	2.65, dd (J=8.5, 6.3 Hz)	2.66, t (J=7.6 Hz)	6.72, d (J=14.6)	6.74, d (J=13.7 Hz)
H-2'	6.66, d, (J=1.5 Hz)	6.64, d (J=1.7 Hz)	6.94, d (J=1.4 Hz)	6.94, d (J=1.5 Hz)
H-5'	6.70, d (J=8.2 Hz)	6.69, d (J=7.8 Hz)	6.74, d (J=8.0 Hz)	6.75, d (J=8.1 Hz)
H-6'	6.58, dd (J=7.8, 1.7 Hz)	6.59, dd (J=7.8, 1.7 Hz)	6.84, dd (J=8.0, 1.5 Hz)	6.85, dd (J=8.0, 1.5 Hz)
H-1"	3.11, t (J=6.7 Hz)	3.38, br s	3.49, br s	3.16, t (J=6.5 Hz)
H-2"	1.77, septet (J=6.7 Hz)	1.56, m	1.55, q (J=5.5 Hz)	1.79, septet (J=6.7 Hz)
H-3"	0.89, d (J=6.6 Hz)	1.56, m	1.62, t (J=5.4 Hz)	0.92, d (J=6.7 Hz)
H-4"	0.89, d (J=6.6 Hz)	1.56, m	1.55, q (J=5.5 Hz)	0.92, d (J=6.7 Hz)
H-5"	-	3.55, br s	3.60, br s	-
OCH_2O	5.90, s	5.88, s	5.93, s	5.94, s

Table 3.2b: ^{13}C NMR spectral data of amides **25-28** (δ, ppm, $CDCl_3$).

Carbon	**25**	**26**	**27**	**28**
2	166.5	165.4	165.4	166.1
3	121.7	121.2	121.4	123.2
4	143.8	144.0	142.4	141.0
5	34.7	34.4	125.3	124.7
6	35.0	34.5	138.1	138.8
1'	135.5	131.2	131.0	130.9
2'	108.9	108.1	105.6	105.7
3'	146.8	147.5	148.1	148.2
4'	148.2	145.7	148.1	148.2
5'	108.8	108.8	108.4	108.5
6'	124.9	121.4	122.4	122.6
1"	47.5	43.0	43.2	47.0
2"	29.2	26.6	26.7	28.6
3"	20.7	24.6	24.6	20.1
4"	20.7	25.5	46.8	20.1
5 "		46.8	43.2	
OCH_2O	101.8	101.1	101.2	101.3

The EIMS of piperamides (**25-28**) showed fragment ions due to the cleavage of the N-CO bond.[10,11] In the fully conjugated amides (**27**) and (**28**), this resulted in the formation of the acyl ion at m/z 201 as the base peak. The base peak at m/z 135 for (**25**) and (**26**) is due to the methylenedioxytropylium ion.[10-13] The genesis of the fragment ions observed at m/z 173, 172, 143 and 115 in the mass spectrum of (**27**) and (**28**) have been rationalized as follows, (**Scheme 3.2**).[10,12]



Scheme 3.2: Some characteristic fragment ions observed for piperamides (**27**) and (**28**).

3.2.1. 5,6-Dihydropiperlonguminine

5,6-Dihydropiperlonguminine (**25**) was isolated as a white solid, m.p. 117-118°C (lit.[5] 90-94°C. lit.[10] 110°C, lit.[1] 116-118°C) with a molecular formula of $C_{16}H_{21}NO_3$ based on HRMS data and $[M]^+$ at m/z 275. The fragmentation pattern agreed with reported values.[5,10] The ^1H NMR and ^{13}C NMR data (**Tables 3.2a** and **3.2b**) were also in accordance with those published in the literature.[5,6]

Piperamide (**25**) was first isolated from the fruit of *P.guineense* in 1976[6] and has been found in 5 other different *Piper species*.[14] Compound (**25**) has recently been reported to be present in stems and seeds of *P.tuberculatum* and to be active against the fungus *Cladosporium sphaerospermun*.[6]

3.2.2. 5,6-Dihydropiperine

5,6-Dihydropiperine (**26**) was isolated as a pale yellow gum 0.08 % dry weight. It was subsequently synthesized as a pale yellow solid, m.p. 78-79°C (lit.[15] 74°C, lit.[7,10] 78°C, lit.[16] 79-80°C).

The EIMS displayed the $[M]^+$ at m/z 287 which agreed with the anticipated molecular formula $C_{17}H_{21}NO_3$, was confirmed by HRMS. The observed mass spectral ions were consistent with previously reported MS data for (**26**).[7,10] The ^1H NMR and ^{13}C NMR data agreed with those published by Schobert *et. al.*, and Navickiene *et. al.*, (**Tables 3.2a** and **3.2b**).[6,7]

Compound (**26**) was first isolated as a gum from the wood of *P.novae-hollandiae* in 1969[10] and has been found two other *Piper species*.[17] Antifungal and activity has been reported for (**26**).[6]

3.2.3. Piperine

Piperine (**27**) was obtained as a yellow solid 0.08% yield (dry weight), m.p. 129-130°C (lit.[7] 126-127°C, lit.[12] 128°C, lit.[10,18] 129°C, lit.[9] 128-129°C, lit.[19] 128-130°C, lit.[20,21] 129-130°C, lit.[22] 130-131°C) after silica gel column chromatography followed by preparative recycling HPLC.

The molecular formula $C_{17}H_{19}NO_3$ agreed with the observed $[M]^+$ 285. This was verified by HRMS. The mass spectral data was almost identical with literature values.[7,10] The ^1H NMR and ^{13}C NMR data (**Tables 3.2a** and **3.2b**) were also consistent with published data for piperine.[7,8]

Piperine (**27**), the pungent principle in pepper (*P. nigrum*) was the first amide to be isolated from *Piper* species and whose structure was established by classical chemical methods in the era before chromatography and spectroscopy.[14]

Compound (**27**) acts as CNS depressant (antagonism of electroshock induced seizures and muscle relaxation in mice), antipyretic (typhoid vaccinated

rabbits), analgesic (taildip presence and writhing in mice) and to display anti-inflammatory activity (carrageenan-induced edema in rats).[17]

Compound (**27**) was the most potent antifeedant amongst the isolated amides in tests on fifth instar larvae of chilopartellus. This study concluded that the presence of a MDP and an alicyclic amide group may be crucial for high antifeedant activity.[14]

Su and Hovarth who investigated the insecticidal activity of amides from *P. nigrum* observed that (**27**) is a synergist for pyrethrins, but contrary to an earlier report, it is not responsible for contact toxicity in insects.[14] Moreover, (**27**) has been shown to affect mammalian polysubstrate monooxygenase (PSMO) that detoxify insecticides. Specifically, (**27**) inhibited the activities of arylhydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin deethylase (7ECDE) activities in rat lung microsomes.[4]

Piperine (**27**) has also been recently patented as a bioavailability enhancer[23] and a non-nicotine smoking cessation aid.[24]

3.2.4. Piperlonguminine

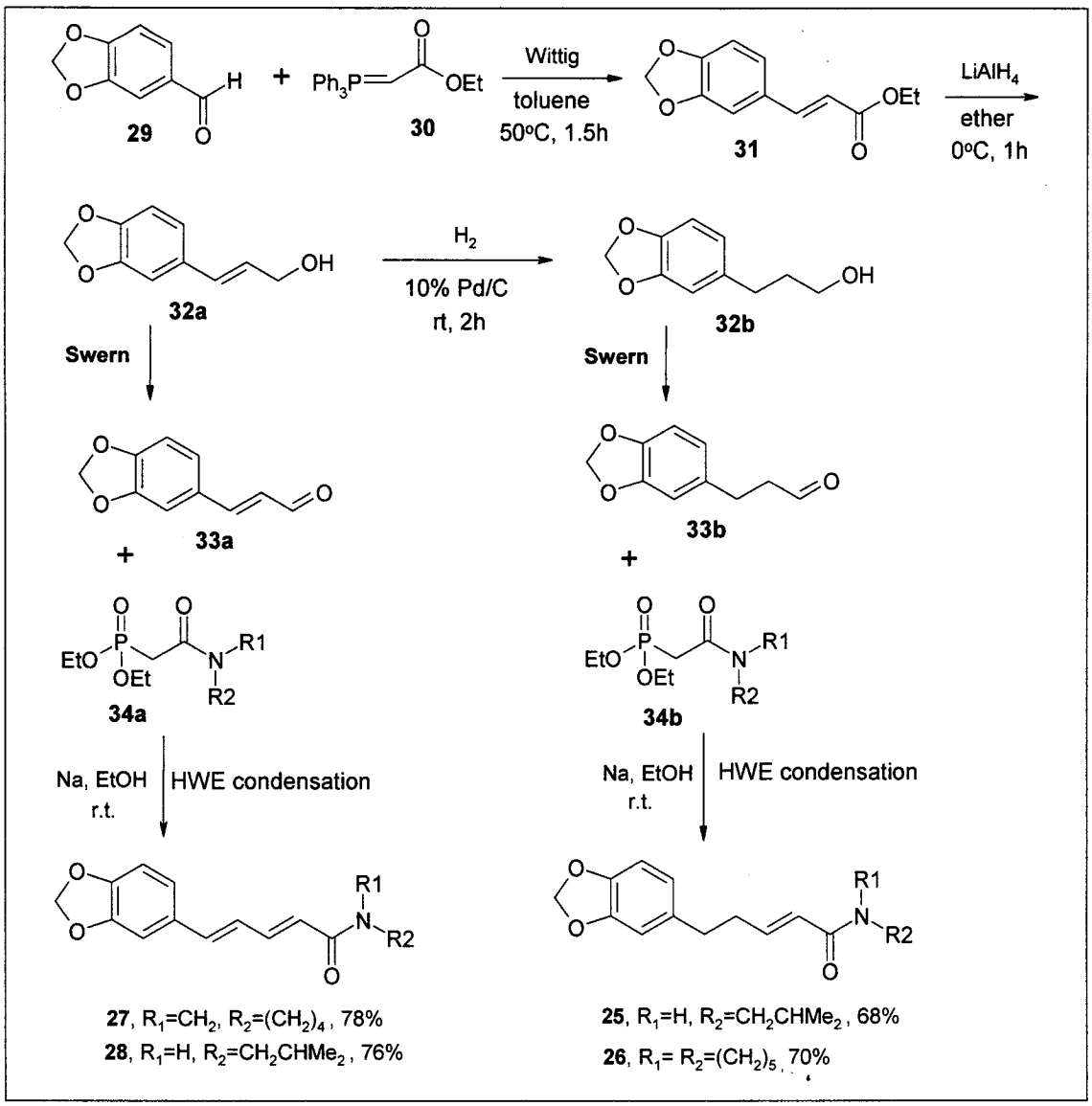
Piperlonguminine (**28**) the major component in *P.tuberculatum* fruit and leaf and extracts was isolated as a white feathery solid, m.p.165-166°C (lit.[10] 161-162°C, lit.[19] 162-164°C, lit.[21] 165-167°C, lit.[11,12,20] 166-168°C, lit.[10] 167°C, lit.[18] 167-168°C) in 0.2% yield from the dried plant material, after silica gel column chromatography followed by recrystallisation from hexane/acetone.

The EIMS of (**28**) displayed a $[M]^+$ at m/z 273 which coincides with the its molecular formula $C_{16}H_{15}NO_3$. This was further established by HRMS. The fragment losses agreed with previously published MS data for piperlonguminine.[10,11] The ^1H NMR data of (**28**) (**Tables 3.2a**) were consistent with literature values.[9] The carbon chemical shifts of (**28**) has not been previously reported.

Piperlonguminine (**28**) was first isolated from the roots of *P.longum* Linn in 1966 by Chatterjee and Dutta[11] and has been found in 16 different other *Piper* species.[17] A recent report revelaed (**28**) to be highly active against one particular species of bacteria, *Bacillus subtilis*.[25]

3.3. Synthesis of Piperamides

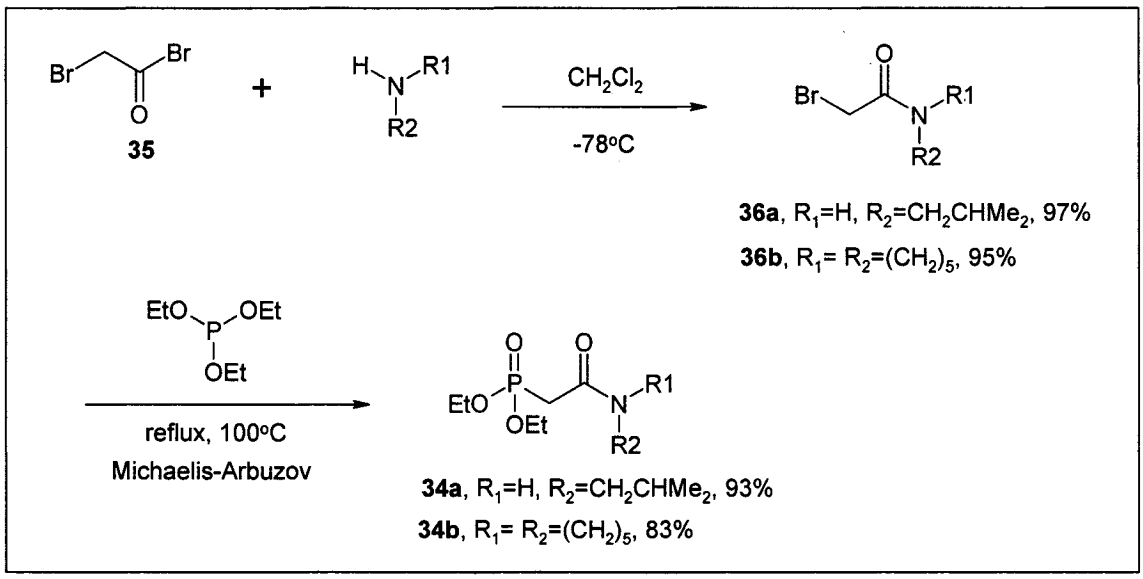
The synthetic pathway to piperamides (**25**)-(**28**) is outlined below (**Scheme 3.3a**). The Wittig olefination[26,27] of piperonal (**29**) with ethoxycarbonylmethylenetriphenyl phosphorane (**30**) in toluene at 110°C afforded ethyl (E)-3,4-(methylenedioxy)cinnamoate (**31**) in 50% isolated yield. Subsequent $LiAlH_4$ reduction gave the corresponding alcohol (**32a**) in 98% crude yield which was clean enough for the next reaction.[28] Hydrogenation of this *trans* allylic alcohol furnished the expected alcohol (**32b**) in 81% crude yield. Both these alcohols were then subjected to Swern oxidation[29] to afford their respective aldehyes (**33a**) and (**33b**) in good yields. The Horner-Wadworth-Emmons (HWE)[30,31] condensation of (E)-3,4-(methylenedioxy)cinnamaldehyde (**33a**) with the anion of either (isobutyl carbamoyl-methyl)-phosphonic acid diethyl ester (**34a**) or oxo-2-piperidin-1-yl-ethyl phosphonic acid diethyl ester (**34b**) methylpropyl)amino]-2-oxoethyl]diester yielded piperamides (**28**) and (**27**) respectively. Analogous treatment of (E)-3,4-(methylenedioxy)hydrocinnamaldehyde (**33b**) gave piperamides (**25**) and (**26**) respectively in good isolated yields.



Scheme 3.3a: Synthesis of piperamides (**25**)-(**28**).

The β-amide phosphonated (**34a**) and (**34b**) were prepared in two steps from bromoacetyl bromide (**35**) as shown in **Scheme 3.3b**.

Our synthesis compares favourably both with respect to length, yield and accessibility of starting components to most of the previous syntheses described below (*see* **Section 3.4**).

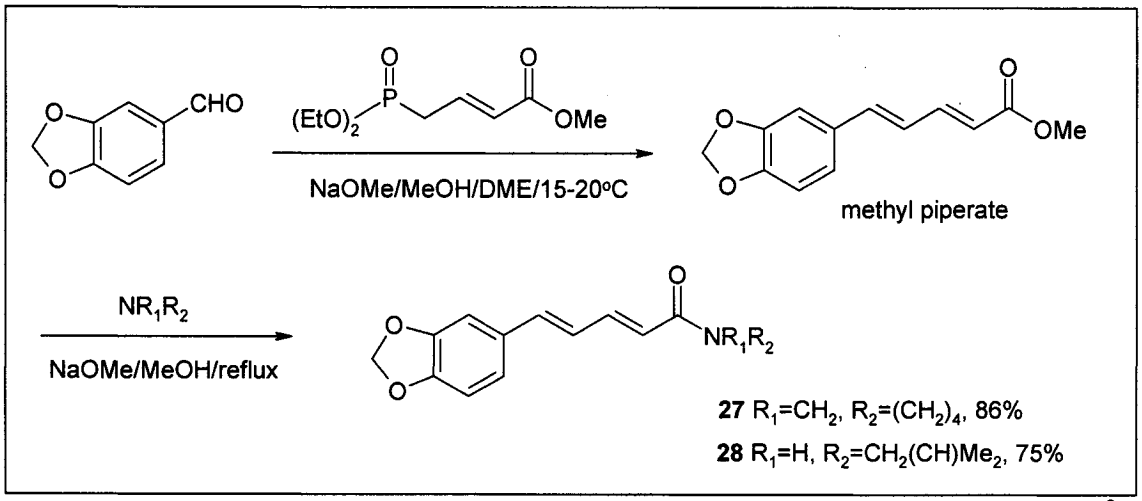


Scheme 3.3b: Preparation of phosponates (**34a**) and (**34b**) for the HWE condensation reactions.

3.4. Previous syntheses of piperamides

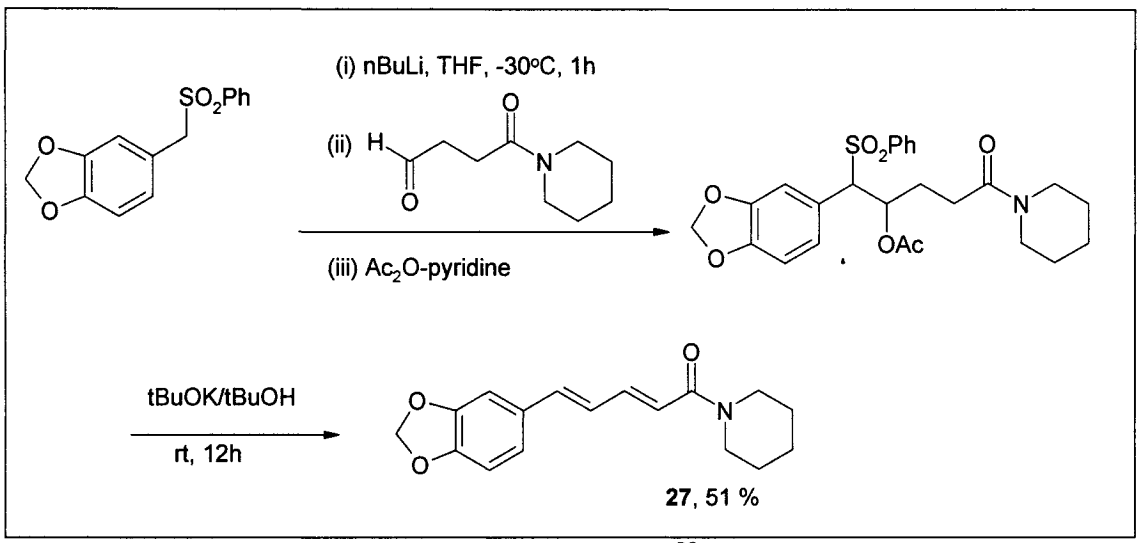
Chatterjee and Dutta first reported the synthesis of these types of piperamides in 1966, where (**28**) was obtained by reacting the acid chloride of piperic acid (prepared by treating piperic acid with PCl_3) with isobutylamine in the presence of pyridine in dry benzene.[11] In 1969, Loder *et. al.*, synthesized (**25**) and (**26**) using the same procedure but with acid chloride of dihydropiperic acid and the appropriate amine.[10]

In 1981, Olsen and Spessard reported a two step stereoselective approach to (**27**) and (**28**) involving the Wadsworth-Horner modified Wittig condensation of piperonal with the anion derived from methyl (E)-4-diethylphosphono-2-butenoate to afford methyl piperate (**Scheme 3.4a**).[9] The methoxide-catalyzed aminolysis of methyl piperate with isobutylamine produced (**28**) in 74% crude yield. Analogous treatment of methyl piperate with piperidine gave (**27**) in 86% crude yield.



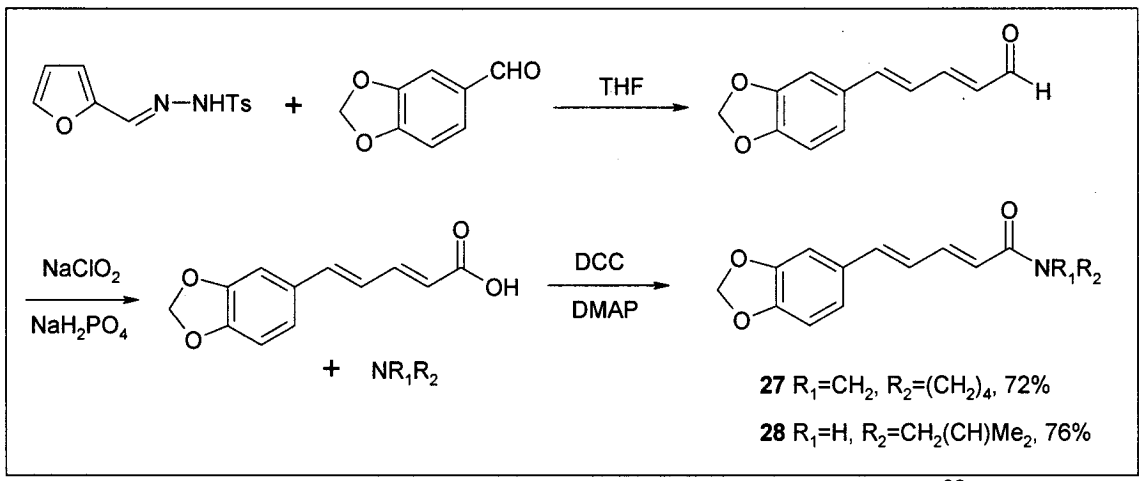
Scheme 3.4a: Synthesis of piperamides (**27**) and (**28**) by Olsen and Spessard.[9]

In 1986, Madai *et. al.*, reported the synthesis of (**27**) through double elimination reaction of β-acetoxy sulfones (**Scheme 3.4b**).[22]



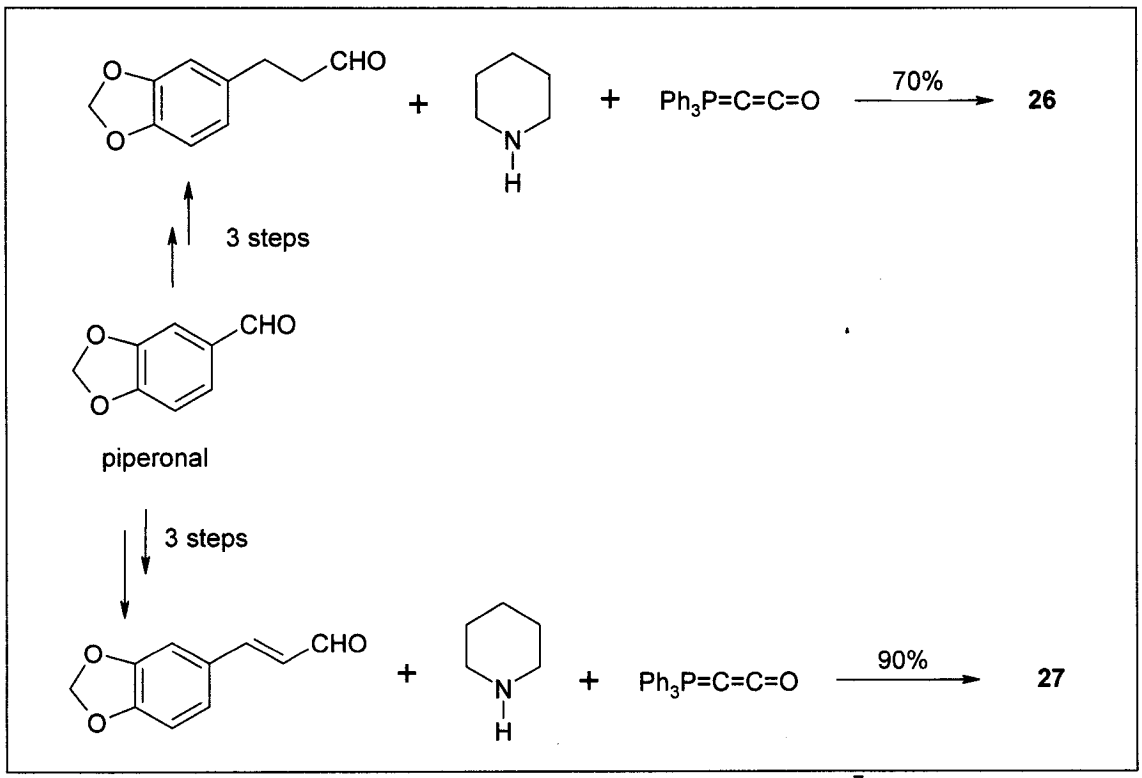
Scheme 3.4b: Synthesis of (**27**) by Madai *et. al.*[22]

In the year 2000, a three step synthesis of (**27**) and (**28**) from tosylhydrazones was reported by Chandrasekhar *et. al.* (**Scheme 3.4c**).[32]



Scheme 3.4c: Synthesis of (**27**) and (**28**) by Chandrasekhar *et. al.*[32]

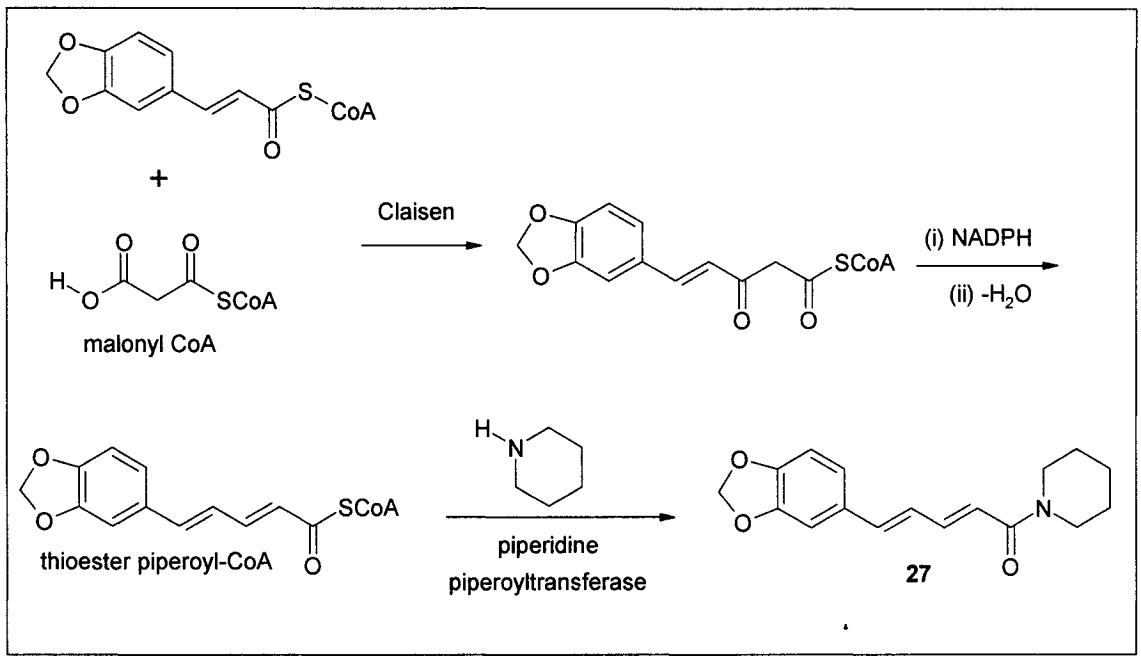
Finally in the year 2001, Schobert *et. al.*, reported a three-component synthesis of (**26**) and (**27**) from piperonal, (**Scheme 3.4d**).[7]



Scheme 3.4d: Synthesis of (**26**) and (**27**) by Schobert *et. al.*[7]

3.5. Biosynthesis of piperine

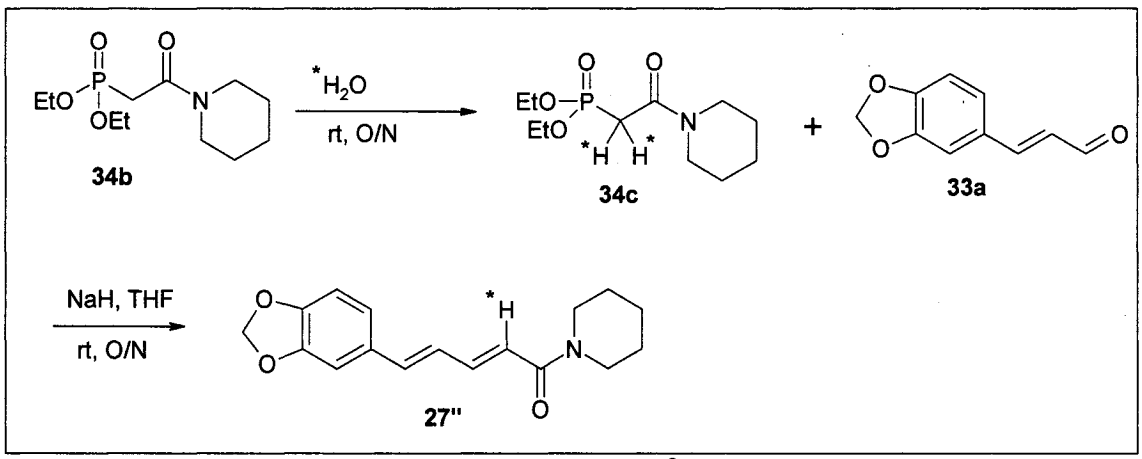
Piperine (**27**) originates from the condensation of piperidine with thioester piperoyl CoA catalyzed by piperidine piperoyl transferase (**Scheme 3.5**).[33] The piperic acid portion of piperine is derived from a cinnamoyl-CoA precursor with chain extension using malonyl-CoA in a Claisen reaction followed by reduction/dehydration reaction leading to the thioester piperoyl CoA.[34]



Scheme 3.5: Biosynthesis of piperine (**27**).

3.6. Synthesis of radioactive labelled ^{3}H-piperine

Tritiated labelled ^{3}H-piperine for toxicokinetic studies was synthesized in a two steps process (**Scheme 3.6**). Firstly, (**34b**) was reacted with tritiated water ($^{3}H_2O$) in the presence of Et_3N at room temperature gave tritiated diethyl phosphonate (**34c**). Secondly, the HWE condensation of (**34c**) with aldehyde (**33a**) in the presence of NaH/THF at room temperature produced the desired radioactive labelled ^{3}H-piperine (**27"**) as a pale yellow solid in 62% yield.



Scheme 3.6: Synthesis of radioactive labelled (^{3}H)-piperine (**27"**).

3.7. Bioassays and synergistic trials of piperamides

Bioassays with mosquito Aedes astropalpus L. assessed the relative toxicity of extracts of *P. tuberculatum* and the four piperamides (**25**)-(**28**).[2] The effective concentration for 50% mortality (EC_{50}) determined with early fourth-instar larvae for the extracts of *P.tuberculatum*, *P.nigrum* and *P.guineense* did not differ significantly (**Table 3.7a**). Since the three sources of *P.nigrum* were also not significantly different, the results further indicate that the source of the seed material did not affect the insecticidal activity This demonstrated that *P.tuberculatum* extracts are comparable in activity to the Asian (*P. nigrum*) and African (*P.guineense*) pepper species.

The EC_{50} values for mosquitoes with each of the four synthetic piperamides (**25**)-(**28**) are also shown in **Table 3.7a**. These results show that they are insecticidal principles. Compound (**25**) had a lower EC_{50} than the other three compounds, hence the most toxic, but overlapping confidence limits indicate that there is no statistical difference with either (**25**) or (**27**). The EC_{50} for (**28**) could not be calculated because the mortality of A.astropalpus larvae did not exceed 50% at the highest concentration tested, 45 ug/mL.

Table 3.7a: EC_{50} values for Piper extracts and piperamides (**25-28**).

Extract	EC_{50} (ug/mL)
P. tuberculatum	1.5
P. guineense	0.8
P. nigrum	
From Vietnam	2.4
From Sarawak	1.8
From Lombok	1.6
Piperamide	
25	6
26	13.5
27	11.1
28	>45

Binary mixtures containing equal weights of the various piperamides either produced less than the expected or approximately the 50% of the expected mortality (**Table 3.7b**). Combinations of either amides (**27**), (**26**) and (**28**) or (**27**) and (**28**) were equal in toxicity to single piperamides alone.

All equimolar tertiary mixtures and the quarternary mixture caused mortality greater than the 50% mortality produced by single piperamide treatment (**Table 3.7b**). This suggests that potentiation is occurring when three or more piperamides are combined. Piperlonguminine (**26**) by definition, acts to synergise the toxicity of the other piperamides since it increases the toxicity of the mixture as a dose that is itself not toxic.

Table 3.7b: Mean % mortality of *A.atropalpus* L. +/- SE after 24h exposure to binary, tertiary and quaternary mixtures with combinations of four piperamides

Binary mixture (1:1)	n	Percent mortality (+/- SE)	Joint activity
25+26	5	6 (2.9)	-
25+27	5	9 (1.9)	-
25+28	4	17 (4.3)	-
26+27	5	42 (14.7)	ND
26+28	4	43 (8.8)	ND
27+28	4	49 (14.5)	ND
Tertiary or Quaternary mixture (1:1:1 or 1:1:1:1)			
25+26+27	5	63 (7.7)	+
26+27+28	5	73 (6.2)	+
25+27+28	5	74 (2.9)	+
25+26+28	5	61 (4.3)	+
25+26+27+28	5	84 (4.8)	+

n = number of trials completed to determine average percent mortality for each combination of piperamides.
- = less than 50% mortality
+ = greater than 50% mortality
ND = no different from EC_{50}.

3.8. Toxicokinetic of radioactive labelled ^{3}H-piperine

An investigation of the time course of tritiated piperine (**27a**) distribution into the exoskeleton, hemolymph and the soft tissures of adult female American cockroaches, *Periplaneta americana* was carried out after topical application to the sternites of the abdomen.[3] The objective was to measure the toxicokinetic parameters of piperine and the identify the piperine metabolites depurated by *P. americanas*.

It was found that (**27a**) rapidly penetrated the cockroach exoskeleton and underlying epidermal layers ($k=-0.05\ h^{-1}$) over a 24-hour period. The flow of (**27a**) through the hemolymph was triphasic, T_{max} was measured 2 hours after

dosing, and AUC_{0-96} was 21.4h x ug/insect. The value of k and $t_{1/2}$ during the terminal elimination phase was –0.01 hr^{-1} and 49.5h, respectively.

Radioactive piperine (**27a**) was slowly accumulated in the soft tissues (T_{max}= 48h) and AUC_{0-96} for (**27a**) in soft tissues was greater than in the hemolymph. Compound (**27a**) was continuously eliminated from the cockroach body and minimally transformed by the insect over a 24h period. No metabolites were identified although water and EtOAc soluble fractions of collected depuration samples indicated that both polar and non-polar types of metabolites were present.

This study concluded that piperine (**27**) rapidly penetrated into the cockroach body, was minimally transformed and slowly eliminated over time periods ranging from 0-96h.

These experiments, their description and the conclusions represent the works of Scott[2,4] and Leduc.[3]

3.9. Experimental

GENERAL: As in Section 2.3.

3.9.1. Plant materials

Fruits of *P. tuberculatum* (Piperaceae) collected in Costa Rica were immersed in EtOH in 1L Nalgene bottles before they were transferred to Ottawa.

3.9.2. Extraction and isolation of piperamides

The mixture of EtOH and the fruits of *P. tuberculatum* was blended together in a blender before it was filtered and the filter cake washed with additional EtOH. The filtrate was evaporated *in vacuo* until mostly water was left, and the mixture was further diluted with water (100 mL) before it was extracted twice with hexane (2 x 50 mL) and thrice with EtOAc (3 x 50 mL). The combined

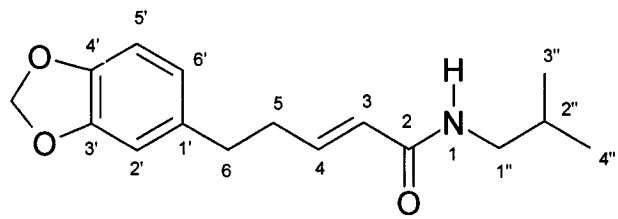
extracts for each extraction were concentrated under diminished pressure, placed in a pump to dry overnight to give a hexane extract (1.7 g) and a EtOAc extract (3.6 g). The weight of the dry plant material was 91.7 g. 1.0 g of the EtOAc extract (3.6 g) was subjected to silica gel column chromatography using EtOAc-hexane gradient as eluants to afford two major fractions.

Fraction 1 (90 mg) was a greenish solid which was recrystallised from hexane/CH_2Cl_2 to afford 50 mg of pure white feathery solid of 3,4-dihydropiperlonguminine (**25**) in 0.2% dry weight). Fraction 2 was a yellow gum (290 mg) which was purified further by using a JAIGEL recycling HPLC equipped with a reverse phase column, eluted with a solvent system of acetonitrile/water (60 : 40) at a flow rate of 2.5 mL/min and monitored by a UV detector set at 128 nm, furnishing 20 mg each of 3,4-dihydropiperine (**26**), piperine (**27**) as a yellow solid, 3,4-dihydropiperine also a yellow solid, and piperlonguminine (**28**) as a pale yellow solid.

3.9.3. Preparation of piperamides

3.9.3.1. 5,6-Dihydropiperlonguminine

Other names: (E)-5-(1,3-Benzodioxol-5-yl)-N-(2-methylene propyl)-2-pentenamide, N-Isobutyl-5-[3,4-(methylenedioxy)phenyl-2-pentenamide.



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(Isobutyl carbamoyl-methyl)-phosphonic acid diethyl ester, **34a** (5.64 x 10^{-1} g, 2.25 x 10^{-3} mol) dissolved in 99% EtOH (1.0 mL) was added to a stirred solution prepared by dissolving sodium metal (5.17 x10^{-2} g, 2.25 x 10^{-3} mol) in 99% EtOH mL) at room temperature (22°C). The solution was stirred for 30 mins before 3-benzo[1,3]dioxol-5-yl-propionaldehyde, **33b** (4.0 x 10^{-1} g, 2.24 x 10^{-3}

mol) dissolved in 99% EtOH (1.0 mL) was added. Stirring continued overnight under a nitrogen atmosphere before EtOH was removed *in vacuo*. The resulting mixture was dissolved in CH_2Cl_2 (10 mL), washed with water (3 x 5 mL), dried ($MgSO_4$), filtered and concentrated under diminished pressure.

The crude product was purified by silica gel column chromatography eluting successively with mixtures of hexane-EtOAc of increasing polarity to afford (**25**) as a pale yellow solid, m.p. 117-118°C (412 mg, 68%). Further purification was achieved by recrystallisation from hexane/acetone.

m.p. 117-118°C (lit.[5] 90-94°C. lit.[10] 110°C, lit.[1] 116-118°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 6.82 (dt, J=15.2, 6.7 Hz, H-4), 6.70 (d, J=8.2 Hz, H-5'), 6.66 (d, J=1.5 Hz, H-2'), 6.58 (dd, J=7.8, 1.7 Hz, H-6'), 5.90 (s, 2H, OCH_2O), 5.75 (dt, J=15.3,1.5 Hz, H-3), 5.51 (br s, CONH), 3.11 (t, J=6.7 Hz, 2H, H-1"), 2.65 (dd, J=8.5, 6.3 Hz, H-6), 2.44 (dq, J=6.4, 1.6 Hz, H-5), 1.77 (septet, J=6.7 Hz, 1H, H-2") 0.89 (d, J=6.6 Hz, 6H, H-3"/H-4").

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm): 166.5 (CONH), 148.2, 146.8, 143.8, 135.5, 124.9, 121.7, 108.9, 108.8, 101.8 (OCH_2O), 47.5, 35.0, 34.7, 29.2, 20.7, 20.7.

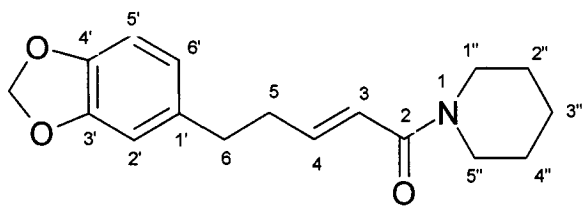
IR υ_{max} ($CHCl_3$, cm^{-1}): 3450, 3302, 2957, 2927, 2870, 1667 (CONH), 1626, 1550, 1503, 1489, 1466, 1443, 1388, 1368, 1334, 1248, 1188, 1098, 1039, 978, 858.

MS (EI) *m/z* (rel. int.): 275 $[M]^+$ (49), 203 $[M\text{-}NHCH_2CH(CH_3)_2]^+$ (4), 175 $[M\text{-}CONHCH_2CH(CH_3)_2]$ (4), 143 (6), 135 (100), 77 (5), 29 (7), 28 (18).

HRMS: *Calculated* for $C_{16}H_{21}NO_3$, 275.15219, *found* 275.15192

3.9.3.2. 5,6-Dihydropiperidine

Other names: 1-[(2E)-5-(1,3-benzodioxol-5-yl)-1-oxo-2-pentenyl]-iperidine, 1-[5-[3,4-(Methylenedioxyphenyl]-2-pentenoyl]-piperidine.



26

Phosphonic acid-(2-Oxo-2-piperidin-1-yl-ethyl) diethyl ester, **34b** (2.35 x 10^{-1} g, 1.01 x 10^{-3} mol) dissolved in 99% EtOH (1.0 mL) was added to a stirred solution prepared by dissolving sodium metal (2.32 x 10^{-2} g, 1.01 x 10^{-3} mol) in 99% EtOH 1.0. mL) at room temperature (20°C). The solution was stirred for 30 mins before 3-benzo[1,3]dioxol-5-yl-propionaldehyde, **33b** (1.80 x 10^{-1} g, 1.01 x 10^{-3} mol) dissolved in 99% EtOH (1.0 mL) was added. Stirring continued overnight under a nitrogen atmosphere before EtOH was removed *in vacuo*.

The resulting mixture was worked up and purified as for compound (**25**) to furnish (**26**) as a pale yellow solid, m.p. 78-79°C (200 mg, 70%). Further purification was achieved by recrystallisation from hexane/EtOAc.

m.p. 78-79°C (lit.[15] 74°C, lit.[7,10] 78°C, lit.[16] 79-80°C),

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 6.76 (dt, J=15.1, 7.0 Hz, H-4), 6.69 (d, J=7.8 Hz, H-5'), 6.64 (d, J=1.7 Hz, H-2'), 6.59 (dd, J=7.8, 1.7 Hz, H-6'), 6.17 (dt, J=15.0, J=1.5 Hz, H-3), 5.88 (s, 2H, OCH_2O), 3.55 (br s, 2H, H-5"), 3.38 (br s, 2H, H-1"), 2.66 (t, J=7.6 Hz, H-6), 2.43 (dq, J=7.3, 1.3 Hz, H-5), 1.56 (m, 6H, H-2"/H-3"/H 4").

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 165.4 (CONH), 147.5, 145.7, 144.0, 135.0, 125.3, 121.4, 108.8, 108.1, 101.1 (OCH_2O), 46.8, 43.0, 34.5, 34.4, 26.6, 25.5, 24.6.

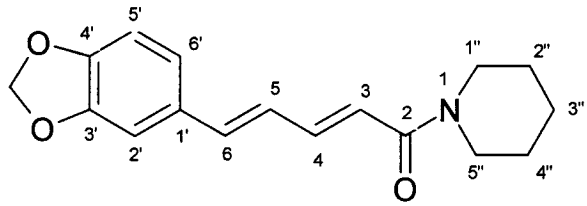
IR υ_{max} ($CHCl_3$, cm^{-1}): 3000, 2934, 2855, 2780, 1638 (CONH), 1611, 1503, 1489, 1443, 1354, 1249, 1190, 1136, 1122, 1098, 1038, 975, 929, 853, 810.

MS (EI) *m/z* (rel. int.): 287 $[M]^+$ (26), 202 (5), 203 $[M\text{-}N(CH_2)_5]^+$ (1), 175 $[M\text{-}CON(CH_2)_5]$ (4), 174 (7), 136 (13), 135 (100), 77 (9), 69 (7), 41 (4).

HRMS: *Calculated* for $C_{17}H_{21}NO_3$, 287.15219, *found* 287.14956.

3.9.3.3. Piperine

Other names: 1-[(2E, 4E)-5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]-piperidine, (E, E)-1-[5-(1,3-Benzodioxol-5-yl)-2,4-pentadienoylpiperidide.



27

Using the same reaction procedure, (2-oxo-2-piperidin-1-yl-ethyl)-phosphonic acid diethyl ester **34b** (3.96 x 10^{-1} g, 1.70 x 10^{-3} mol) dissolved in 99% EtOH (1.0 mL) was added to a stirred solution prepared by dissolving sodium metal (3.93 x 10^{-2} g, 1.71 x 10^{-3} mol) in 99% EtOH (1.0 mL) at room temperature (20°C). The solution was stirred for 30 mins before 3-benzo[1,3]dioxol-5-yl-propionaldehyde, **33a** (3.0 x 10^{-1} g, 1.7 x 10^{-3} mol) dissolved in 99% EtOH (1.0 mL) was added. Stirring continued overnight under a nitrogen atmosphere before EtOH was removed *in vacuo*.

The resulting mixture was subjected to the usual work up and purification procedures to give (**27**) as a pale yellow solid (380 mg, 78%). Further purification was achieved by recrystallisation from hexane/acetone.

m.p. 129-130°C m.p. 129-130°C (lit.[7] 126-127°C, lit.[12] 128°C, lit.[10,18] 129°C, lit.[9] 128-129°C, lit.[19] 128-130°C, lit.[20,21] 129-130°C, lit.[22] 130-131°C),

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 7.35 (ddd, J=14.7, 8.5, 1.5 Hz, H-4), 6.94 (d, J=1.4 Hz, H-2'), 6.84, (dd, J=8.0, 1.5 Hz, H-6'), 6.74 (d, J=8.0 Hz, H-5'), 6.73 (dd, J=14.6, 8.6 Hz, H-5), 6.72 (d, J=14.6 Hz, H-6), 6.39 (d, J=14.7 Hz, H-3), 5.93 (s, 2H, OCH_2O), 3.60 (br s, 2H, H-5"), 3.49 (br s, 2H, H-1"), 1.62 (t, J=5.4 Hz, 2H, H-3"), 1.55 (q, J=5.5 Hz, 4H, H-2"//H 4").

^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 165.4 (CONH), 148.1, 145.7, 142.4, 138.1, 131.0, 125.3, 122.4, 121.1, 108.4, 105.6, 101.2 (OCH_2O), 46.8, 43.2, 26.7, 25.6, 24.6.

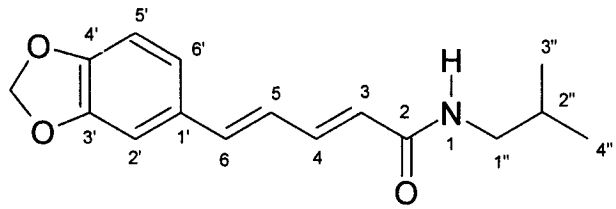
IR υ_{max} ($CHCl_3$, cm^{-1}): 3010, 2939, 2863, 1634 (CONH), 1586, 1493, 1366, 1349, 1254, 1229, 1202, 1151, 1135, 1106, 1067, 894.

MS (EI) *m/z* (rel. int.): 285 $[M]^+$ (73), 202 (27), 201 $[M\text{-}N(CH_2)_5]^+$ (100), 200 (13), 174 (17), 174 (18), 173 $[M\text{-}CON(CH_2)_5]$ (24), 143 (13), 135 (36), 115 (41).

HRMS: *Calculated* for $C_{17}H_{19}NO_3$, 285.13654, *found* 285.13649.

3.9.3.4. Piperlonguminine

Other names: (2E, 4E)-(1,3-Benzodioxol-5-yl)-N-(2-methylpropyl)-2,4-pentadienamide.



28

(Isobutyl carbamoyl-methyl)-phosphonic acid diethyl ester, **34a** (2.57 x 10^{-1} g, 1.02 x 10^{-3} mol) dissolved in 99% EtOH (1.0 mL) was added to a stirred solution prepared by dissolving sodium metal (2.35 x 10^{-2} g, 1.02 x 10^{-3} mol) in 99% EtOH (1.0 mL) at room temperature. The solution was stirred for 30 mins before 3-benzo[1,3]dioxol-5-yl-propionaldehyde, **33a** (1.80 x 10^{-1} g, 1.03 x 10^{-3} mol) dissolved in 99% EtOH (1.0 mL) was added. Stirring continued overnight under a nitrogen atmosphere before EtOH was removed *in vacuo*.

The usual work up and purification provided (**28**) as a pale yellow solid, (208 mg, 76%). Further purification was achieved by recrystallisation from hexane/acetone.

m.p.165-166°C (lit.[10] 161-162°C, lit.[19] 162-164°C, lit.[21] 165-167°C, lit.[11,12,20] 166-168°C, lit.[10] 167°C, lit.[18] 167-168°C),

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 7.33 (dd, J=14.9, 10.8 Hz, H-4), 6.94 (d, J=1.5 Hz, H-2'), 6.85, (dd, J=8.0, 1.5 Hz, H-6'), 6.75 (d, J=8.1 Hz, H-5'), 6.74 (d, J=13.7 Hz, H-6), 6.64 (dd J=15.4, 10.8 Hz, H-5), 5.94 (s, 2H, OCH_2O), 5.90 (c, J=14.8 Hz, H-3), 5.59 (br s, CONH), 3.16 (t, J=6.5 Hz, 2H, H-1"), 1.79 (septet, J=6.7 Hz,H-2"), 0.92 (d, J=6.7 Hz, 6H, H-3"/H-4").

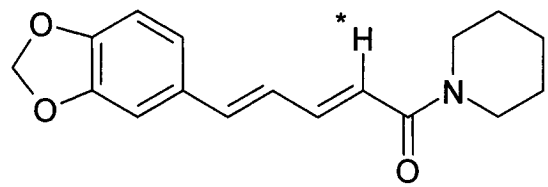
^{13}C NMR (200 MHz, $CDCl_3$): δ (ppm) 166.1 (CONH), 148.2, 145.7, 141.0, 138.8, 130.9, 124.7, 123.2, 122.6, 108.5, 105.7, 101.3 (OCH_2O), 47.0, 28.6, 20.1.

IR υ_{max} ($CHCl_3$, cm^{-1}): 3440, 3300, 2957, 1644, 1606, 1556, 1503, 1488, 1445, 1372, 1254, 1192, 1153, 1037, 988, 930, 853.

MS (EI) *m/z* (rel. int.): 273 $[M]^+$ (56), 216 (13), 202 (17), 201 $[M\text{-}NHCH_2CH(CH_3)_2]^+$ (100), 174 (17), 173 $[M\text{-}CONHCH_2CH(CH_3)_2]$ (50), 172 (20), 171 (12), 162 (14), 143 (17), 135 (14), 115 (50), 96 (14), 29 (13), 28 (31).

HRMS: *Calculated* for $C_{16}H_{19}NO_3$, 273.13654, *found* 273.13497

3.9.3.5. Radioactive labelled piperine



27"

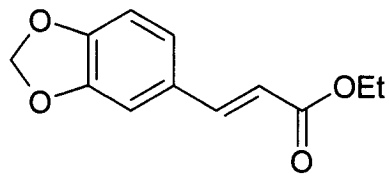
A solution of the phosphonic acid -(2-oxo-piperidin-1-yl-ethyl)- diethyl ester, **34b** (1.29 g, 4.91 x 10^{-3} mol) in triethylamine (3.4 mL) was stirred at room temperature for 1h before the tritiated water, $^3H_20$ (0.5 mL) was added and stirring continued overnight. EtOAc (30 mL) was added to the reaction mixture before it was washed successively with 10 mL water, 10 % HCl (3 x), 5 % Na_2CO_3, water, dried ($MgSO_4$) and concentrated *in vacuo* to afford radioactive labelled ^{3}H-phosphonic acid -(2-oxo-piperidin-1-yl-ethyl)- diethyl ester (**34c**), (*see* **Scheme 3.6**)

To a solution of (**34c**) (6.20 x 10^{-1} g, 2.31 x 10^{-3} mol) in THF (20 mL) was added excess NaH and stirred for 15 mins at room temperature before the 3-benzo[1,3] dioxol-5-yl-propenal, **33a** (5.40 x 10^{-1} g, 3.07 x 10^{-3} mol) previously dissolved in THF (10 mL) was added and stirring was continued overnight. The reaction mixture was quenched with water (30 mL). The THF was removed *in vacuo* and the reaction mixture was re-dissolved in CH_2Cl_2 (50 mL). The aqueous layer was extracted with CH_2Cl_2 (3 x 50 mL) and the combined organic

layers were dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford the crude piperine (680 mg). After silica gel column chromatography, pure radioactive ^3H - piperine (**27"**) was obtained as a pale yellow solid (411 mg, 62%).

3.9.4. Ethyl (E)-3,4-(methylenedioxy)cinnamoate

Other names: (2E)-3-(1,3-Benzodioxol-5-yl)-2-propenoic acid ethyl ester, Cinnamic acid 3,4-(methylenedioxy)-ethyl ester



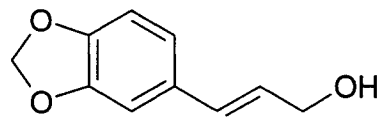
31

Ethoxy carbonylmethylenetriphenyl phosphorane, $Ph_3P{=}CHCO_2Et$ (4.64 g, 1.33×10^{-2} mol) was added to a solution of piperonal (2 g, 1.33×10^{-2} mol) in toluene (150 ml) and stirred to reflux at 110°C for 1.5 h. The solution was left to stir overnight at room temperature before toluene was removed *in vacuo*. The mixture was re-dissolved in ether (150 mL), washed with water (3 x 50 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude product was Kelgulrohr distilled to afford (**31**) as a light yellow solid (1.45 g, 50%).

^1H NMR (200 MHz, $CDCl_3$): δ (ppm) 7.56 (d, J=15.9 Hz, CH=C), 7.00 (d, J=0.9 Hz, 1H, Ar), 6.98 (d, J=8.0, Ar), 6.78 (dd, J=7.7 Hz, J=0.7 Hz, 1H, Ar), 6.23 (dd, J=15.9 Hz, J=1.2 Hz, CH=C), 5.97 (s, 2H, OCH_2O), 4.22 (q, J=7.2 Hz, 2H, CO_2CH_2), 1.30 (t, J=7.1 Hz, 3H, CH_3).

3.9.5. (E)-(1,3-Benzodioxol-5-yl)-2-propen-1-ol

Other names: (2E)-3-(1,3-Benzodioxol-5-yl)-2-propen-1-ol, (E)-3,4-Methylenedioxycinnamyl alcohol.



32a

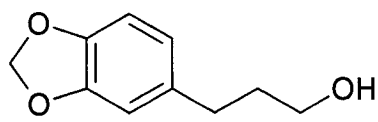
A solution of ester **31** (1.43 g, 6.5 x 10^{-3} mol) in dry ether (20 mL) was added dropwise to a stirred solution of $LiAlH_4$ (4.20 x 10^{-1} g, 1.10 x 10^{-2} mol) in ether (20 mL) under nitrogen at 0°C. The reaction mixture was stirred at room temperature for 1h. The reaction was quenched with saturated solution of NH_4Cl (2.8 mL), dried ($MgSO_4$), filtered, rinsed well with ether and concentrated under diminished pressure to give (**32a**) as a white solid (1.13 g, 98%),

m.p. 71-73°C (lit.[7] 71°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 6.94 (s, 1H, Ar), 6.78 (m, 2H, Ar), 6.52 (d, J=15.8 Hz, CH=C), 6.18 (dt, J=15.8 Hz, J=5.9 Hz, CH=C), 5.95 (s, 2H, OCH_2O), 4.22 (d, J=5.5 Hz, CH_2OH), 2.52 (br s, OH).

3.9.6. 1,3-Benzodioxole-5-propanol

Other names: 1,3-Benzodioxole-5-yl)propanol, 3-(3,4-Methylenedioxyphenyl) propanol, 3,4-(Methylenedioxy)hydrocinnamyl alcohol.



32b

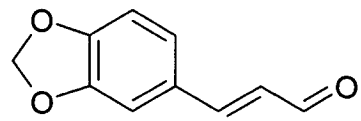
10% Pd on carbon (30 mg) was added to a solution of **32a** (1.50 x 10^{-1} g, 8.42 x 10^{-4} mol) in MeOH (5.0 mL). The solution was degassed before it was stirred under hydrogen gas at room temperature for 2h. The reaction mixture

was filtered, rinsed well with MeOH and concentrated *in vacuo* to afford **32b** as a colorless oil (150 mg, 99%).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 6.67 (m, 3H, Ar), 5.90 (s, 2H, OCH_2O), 3.65 (t, J=6.4 Hz, 2H, CH_2-Ar), 2.62 (t, J=6.8 Hz, 2H, CH_2OH), 1.82 (m, 2H, CH_2).

3.9.7. 3-Benzo[1,3]dioxol-5-yl-propenaldehyde

Other names: (2E)-3-Benzo[1,3]dioxol-5-yl-2-propenal, (E)-3,4-(Methylenedioxy)-cinnamaldehyde.



33a

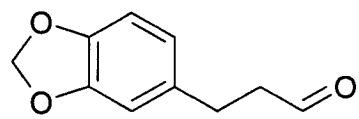
A solution of DMSO (8.77×10^{-1} g, 1.12×10^{-2} mol, 0.8 mL) in CH_2Cl_2 (5.0 mL) was added dropwise to a stirred solution of oxalyl chloride, CO_2Cl_2 (7.12×10^{-1} g, 5.61×10^{-3} mol, 0.49 mL) in CH_2Cl_2 (15 mL) at -78°C. Alcohol, **32a** (8.0×10^{-1} g, 4.49×10^{-3} mol) was dissolved in CH_2Cl_2 (10 mL) and then added slowly dropwise to the reaction mixture. After 30 mins, triethylamine, Et_3N (2.27 g, 2.24×10^{-2} mol, 3.1 mL) was added and the reaction mixture was warmed to room temperature (3h). Water (30 mL) was added and the aqueous layer was re-extracted with CH_2Cl_2 (15 mL) and the combined organic layers were washed successively with 10 mL solutions of saturated NaCl, 1% HCl, water, 5 % Na_2CO_3 and water, dried ($MgSO_4$), filtered and concentrated under reduced pressure furnishing (**33a**) as a white solid (750 mg, 95 %).

m.p. 77-79°C (lit.[7] 77°C).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 9.62 (d, J=7.8 Hz, CHO), 7.36 (d, J=15.8 Hz, CH=C), 7.05 (m, 2H, Ar), 6.84 (d, J=8.0 Hz, 1H, Ar), 6.54 (dd, J=15.8 Hz, J=7.7 Hz, CH=C), 6.02 (s, 2H, OCH_2O).

3.9.8. 3-Benzo[1,3]dioxol-5-yl-propionaldehyde

Other names: 1,3-Benzodioxole-5-propanal, 3,4-(Methylenedioxy)-hydrocinnamaldehyde, 3(3,4-Methylenedioxyphenyl)-1-propanal, 3(3,4-Methylenedioxyphenyl)-propionaldehyde.



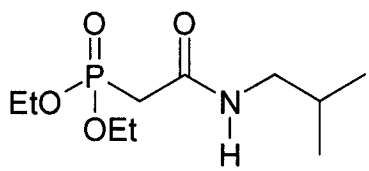
33b

Alcohol **32b** (1.50×10^{-1} g, 8.32×10^{-4} mol) was subjected to the same Swern oxidation procedure described for (**33a**) with CO_2Cl_2 (1.32×10^{-1} g, 1.04×10^{-3} mol, 0.09 mL) in CH_2Cl_2 (1.0 mL), DMSO (1.63×10^{-1} g, 2.08×10^{-3} mol, 0.15 mL) in CH_2Cl_2 (1.0 mL), and Et_3N (4.21×10^{-1} g, 4.16×10^{-3} mol, 0.58 mL). Work up with water (5.0 mL), and 1.0 mL solutions of saturated solution of NaCl, 1% HCl, water, 5 % Na_2CO_3 and water. Compound (**33b**) was obtained as a colorless oil (140 mg, 95 %).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 9.78 (d, J=4.8 Hz, CHO), 6.68 (m 3H, Ar), 5.92 (s, 2H, OCH_2O), 2.83 (t, J=7.2 Hz, 2H, CH_2-Ar), 2.72 (m 2H, CH_2CHO).

3.9.9. (Isobutyl carbamoyl-methyl)-phosphonic acid diethyl ester

Other names: Phosphonic acid, 2-[(2-methyl)amino]-2-oxoethyl]-diethyl ester.



34a

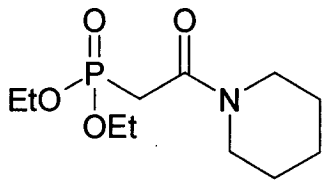
Triethyl phosphite, $(C_2H_5O)_3$ P (8.56×10^{-1} g, 5.15×10^{-3} mol, 0.86 mL) was added to compound **36a** (1.0 g, 5.15×10^{-3} mol) and the mixture was

refluxed at 155-157°C for 1h. The crude product was Kelgulrohr distilled to afford **34a** as a pale yellow liquid (1.05 g, 93%).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 6.81 (br s, CONH), 4.11 (q, J=7.0 Hz, 4H, OCH_2 x 2), 3.08 (t, J=6.4 Hz, 2H, NCH_2), 2.87 (s, 1H, CH-P), 2.76 (s, 1H, CH-P), 1.77 (septet, J=6.7 Hz, CH), 1.31 (t, J=6.9 Hz, 6H, CH_3 x 2), 0.90 (J=6.7 Hz, 6H, CH_3 x 2).

3.9.10. Phosphonic acid-(2-oxo-2-piperidin-1-yl-ethyl) diethyl ester

Other names: Phosphonic acid, [2-oxo-2(1-piperidinyl)ethyl]-diethyl ester, Phosphonic acid, [(piperidinocarbonyl)methyl]-diethyl ester.



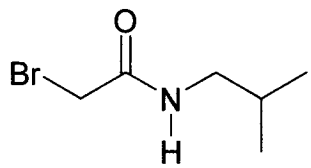
34b

The reaction of triethyl phosphite (3.90 g, 2.35 x 10^{-2} mol, 4.0 mL) with **36b** (4.84 g, 2.35 x 10^{-2} mol) according to the procedure described for **34a** yielded **34b** as a pale yellow liquid (4.55 g, 83%) after Kelgulroh distillation.

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 4.18 (m 2H, OCH_2 x 2), 3.51 (m, 4H, CH_2-N-CH_2), 3.01 (s, 1H, CH-P), 2.98 (s, 1H, CH-P), 1.58 (m, 6H, $(CH_2)_3$), 1.30 (t, J=7.1 Hz, CH_3 x 2).

3.9.11. 2-Bromo-N-(2-methylpropyl) acetamide

Other names: N-Isobutyl bromoacetamide



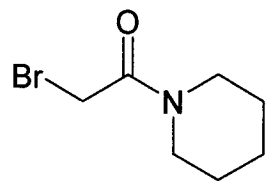
36a

Bromoacetyl bromide (5 g, 2.48 x 10^{-2} mol, 2.32 mL) was added slowly dropwise to a solution of isobutylamine (3.62 g, 4.95 x 10^{-2} mol, 4.92 mL) in CH_2Cl_2 (20 mL) at -78°C under nitrogen over a period of 10 minutes. The reaction mixture was warmed to room temperature (3h) before it was washed with water (3 x 5.0 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford **36a** as a colorless oil in 97% yield (4.70 g).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 6.55 (br s, CONH), 3.90 (s, 2H, CH_2Br), 3.11 (t, J=6.5 Hz, 2H, CH_2N), 1.80 (septet, J=6.4 Hz, CH), 0.92 (d, J=6.5 Hz, 6H, 2 x CH_3).

3.9.12. 1-(Bromoacetyl)-piperidine

Other names: 2-Bromo-1-piperidinoethanone, Bromoacetopiperidide, N-(bromoacetyl)-piperidine



36b

The reaction of bromoacetyl bromide (5 g, 2.32 x 10^{-2} mol, 2.16 mL) with piperidine (4.22 g, 4.96 x 10^{-2} mol, 4.90 mL) in CH_2Cl_2 (20 mL) at -78°C following the procedure described for (**36a**) gave (**36b**) as a cream colored liquid (4.84g, 95%).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 3.85 (s, 2H, CH_2Br), 3.45 (m 4H, CH_2-N-CH_2), 1.59 (m, 6H, $(CH_3)_2$).

3.10. References for chapter 3

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CLAIMS TO ORIGINAL RESEARCH

1. The evaluation of the anti-anxiety activity of EtOH extracts of two species of the Margraviaceae family (*Souroubea gilgi* and *Souroubea sympetala*) collected in Costa Rica, and the subsequent identification of an EtOAc active fraction (f1).

2. The isolation and identification of betulinic acid (**3**) as the bioactive anti-anxiety agent from the active fraction (f1), and the development of (**3**) as an anti-anxiety therapy.

3. The isolation and identification of the chemical components in the leaves and fruits of the Margraviaceae family, which was previously unknown.

4. An efficient and reliable relay synthesis of betulinic acid (**3**) from betulin (**14**) that is comparable to literature methods was realized.

5. The synthesis of a number of new betulinic acid analogs and the evaluation of their anti-anxiety activity.

6. Synthesis of radioactive labelled ^{3}H-betulinic acid methyl ester (**3a"**) for future receptor studies.

7. The isolation and identification of four piperamides [(**25**)-(**28**)] from the fruit extracts of *P. tuberculatum*, and the evaluation of their insecticidal activity and synergistic effects.

8. Synthesis of radioactive labelled ^{3}H-piperine (**27"**) for utilization in toxicokinetic studies.

PUBLICATIONS

1. Scott, I. M., Puniani, E., Durst, T., Phelps, D., Merali, S., Assabgui, R. A., Sanchez-Vindas, P., Poveda, L., Philogene, B. J. R., and Arnason, J. T., *Agricultural and Forest Entomology*, **2002**, 4, 1. "*Insecticidal Activity of Piper tuberculatum Jacq. Extracts: Synergistic Interaction of Piperamides*".

2. Scott, I. M., Leduc, R., Puniani, E., Durst, T., Budzinski, J., Blais, J., Philogene, B. J. R., Arnason, J. T., "*Toxicokinetics of piperamides from Piper spp. (Piperaceae) in the cockroach Periplaneta americana L*", **2003**, In preparation; To be submitted to the American Chemical Society Botanicals Symposium Prodeedings.

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Patent

Patent filing of the anti-anxiety activity of Magraviaceae plant extracts, the bioactive constituent betulinic acid (**3**), and its derivatives are in process.