PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES OF SPECIES OF AFRICAN *COMBRETACEAE*

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By

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to these three,
deceased:

Peter Sharples d. 8/1998
sponsor and mentor

Sekuru Samson Mhlanga d. 8/1999
grandfather and friend

Bernard Munyaradzi Katerere d. 7/2000
brother and friend

i am the poorer from your loss
yet,
richer still for having known you.

also to Monica Bess and Simon Chitseya
who sired me, and sire me, still.
(makandibereka, munondibereka)

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ABSTRACT

The isolation of pharmacologically active stilbenoids like combretastatin and its analogues from the African Combretaceae in previous studies prompted the work described in the present study. It was postulated that secondary metabolites with chemotherapeutic potential first isolated in *Combretum caffrum* would also occur in related species from Southern Africa. Starting from this premise, a general search for these and other compounds was mounted on six of twelve plants gathered from Zimbabwe in May 1999. These were *Combretum albopunctatum* (a mixture of fruit and leaves was investigated), *C. apiculatum* (leaves), *C. collinum* (leaves), *C. hereroense* (fruit) and *C. imberbe* (leaves), as well as *Terminalia stulhmanii* (stem bark).

Phytochemical fractionation was carried out on ground plant material in a search for both polar and non-polar compounds. Once the powdered plant material was defatted with *n*-hexane, the non-polar compounds were extracted by macerating with dichloromethane (DCM). These were subsequently fractionated by Sephadex LH-20 and preparative HPLC using acetonitrile-water as eluents in a gradient system. Thirty-two compounds were isolated by this method and are reported here. Of these compounds, the vast majority are known but have been isolated from Combretaceae for the first time in this study. Seven compounds were previously unknown, among them a bibenzyl from *C. apiculatum*, two cyclobutane chalcone dimers from *C. apiculatum* and *C. albopunctatum*, and three acetylated rhamnosides from *C. imberbe*.

There was an attempt to isolate polar constituents by routine HPLC techniques as well as a general search for evidence to suggest the presence of polyhydroxylated alkaloids. The former yielded only one pure compound, quercetin galactoside and it was concluded that the methods used are not suited for this type of work and the development of better methods is required. GC-MS and High Voltage Electrophoresis (HVE) gave preliminary data suggesting that polyhydroxylated alkaloids do occur in the Combretaceae. No further work was done to isolate pure compounds employing these methods due to limited time.

The biological work performed was to evaluate the antimicrobial potency of all the natural products isolated and characterized in the first phase of the project. Two microbiological assays were used - the well-diffusion assay (WDA) and the microdilution assay (MDA). The former showed that the bibenzylic compound B14-7, isolated here for the first time from *C. apiculatum* was active against two yeasts NCYC 2251 and NCYC 327, and two novel bidesmosides from *C. imberbe* and *T. stulhmanii* were both active against *Listeria monocytogenes*.The most active compounds in the MDA were those aglycone and monodesmosides from *C. imberbe* which showed very low minimum inhibitory concentrations (MICs) against *Mycobacterium fortuitum* and *Staphylococcus aureus*. All the other compounds only mustered moderate activity against *Candida albicans* and *S. aureus* and were inactive against *Proteus vulgaris* and *E. coli*.

Further work will involve evaluation of these compounds for anti-tumour activity. They will be submitted for the screening programme of National Cancer Institute (NCI) Natural Products Branch (NPB).

This is the single largest phytochemical and pharmacological survey of species from both *Combretum* and *Terminalia* of the African Combretaceae growing in southern Africa to date. Compounds similar to the combretastatins were isolated only in three of the six plant species investigated, and two species gave only terpenoid constituents. There is need for a broader evaluation of the biological activity of the compounds herein isolated with a view to tapping into the potential of the Combretaceae to yield compounds which may be of pharmaceutical and agrochemical interest

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GLOSSARY OF ABBREVIATIONS

Acetone-d_6	Deuterated acetone
APT	Attached proton test
C_5D_5N	Deuterated pyridine
$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
COSY	^{1}H-^{1}H COrrelation SpectroscopY
DBE	Double Bond Equivalence
DCM	Dichloromethane
DMSO	Dimethyl sulphoxide
EtOAc	Ethyl acetate
EtOH	Ethanol
FABMS	Fast Atom Bombardment Mass Spectrometry
FTIR	Fourier Transforming Infra-red spectroscopy
GOESY	^{1}H-^{1}H Gradient nuclear Overhauser Effect SpectroscopY
HMBC	^{1}H-^{13}C Heteronuclear Multiple Bond Connectivity
HMQC	^{1}H-^{13}C Heteronuclear Multiple Quantum Correlation
HPLC	High Performance Liquid Chromatography
HREIMS	High Resolution Electron Impact Mass Spectrometry
HRESIMS	High Resolution Electron Spray Ionization Mass Spectrometry
IR	Infra Red spectrum
MDA	Microdilution Assay
Mp	Melting point
NMR	Nuclear Magnetic Resonance
NOESY	^{1}H-^{1}H Nuclear Overhauser Effect SpectroscopY
PENDANT	Polarisation Enhancement Nurtured During Attached Nuclei Testing
PPO	Polyphenoloxidase
TFA	Triflouroacetic acid
TLC	Thin Layer Chromatography
WDA	Well Diffusion Assay

NOMENCLATURE

The naming of some of the compounds is based on the system used in most of the reference literature and not strictly according to IUPAC system. In every case the IUPAC name has also been included in the summary.

CHAPTER ONE

1.1. GENERAL INTRODUCTION

The war against ill-health in the 21st century will have to be fought simultaneously on two fronts – infectious diseases and chronic non-communicable diseases (WHO, World Health Report, 1998).

In the past, non-communicable diseases such as heart disease, cancer and diabetes have been confined to the industrialized world, but it is predicted that these will shift to affect developing countries as well, adding to the burden of infectious diseases typical of poorer economies such as malaria and tuberculosis

As of 1998, cardiovascular and cerebrovascular disease accounted for at least 15 million deaths world-wide, while cancer, a collection of about 100 diseases, of which 4 anatomical sites account for most of the mortality and morbidity, was the second most important non-communicable disease. It is also estimated that in 1996 more than 10 million people world-wide developed cancer and at least 6 million died from it. (WHO Biennial Report, 1997).

Previously associated with affluent western societies (Pratt and Ruddon, 1979), cancer is a growing threat in the developing world. The incidence of cancer is increasing because of increased life expectancy, general reduction in mortality, and

industrialisation and attendant environmental pollution. It was estimated by Parkin *et al* (1988) that the world total of new cancer cases stood at 6.35 million in 1980 and 7.6 million in 1985 all almost evenly distributed between developed and developing countries. More recent estimates show that of the more than 10 million cases newly diagnosed annually, up to 57% are in the Third World (WHO Director General's Annual Report, 1996).

The diseases traditionally associated with poor tropical countries, malaria and tuberculosis (TB) are making a world-wide comeback (WHO, 1997). At least 3 million people a year die from these diseases which are also spreading into new geographical areas. It is known that 1.8 billion people are infected with the TB bacillus world-wide and the burden will grow with the expanding global population and co-infection with HIV/AIDS. HIV/AIDS is only one of at least 30 new and highly infectious diseases to emerge in the last 20 years. Some of these other poorly understood diseases include Legionnaire's disease, a potentially fatal pneumonia, Ebola haemorrhagic fever, Lyme's disease, Dengue, *E. coli* 0157:H7 and new variant CJD (*nv*CJD).

Over one billion people enter the 21st century without hope – their lives remain short and scarred by disease and their death, premature (WHO, 1999). There is a need for research and development to find new cures for both age-old and emerging diseases. This study will focus on the preliminary phytochemical isolation and pharmacological testing of plant metabolites as chemotherapeutic agents.

Chemotherapy, in its broadest sense, can be defined as the use of chemical compounds to eliminate cells that are perceived as being foreign to a physiological system i.e. oncological and infective disease (Bowman *et al*, 1986). Thus chemical entities with anti-microbial, anti-viral and anti-tumour activity can be described as chemotherapeutic.

1.1.1. ANTI-CANCER AGENTS

The indispensable need for new and more effective anti-cancer agents cannot be over-emphasized. At present, anti-tumour drugs in clinical usage, though the mainstay of treatment in metastatic cancer, have numerous drawbacks (Armand, 1997). Serious adverse effects such as leucopenia, neutropenia and subsequent immunosuppression and vulnerability to infection, neurotoxicity, cardiotoxicity, alopecia, and serious allergic manifestations are common (Rang, Dale and Ritter, 1995).

The efficacy of these agents is low and most patients have a low probability of cure (Armand, 1997). These drugs are most beneficial when used as adjuvants to radical local therapy (Pinido and Giacione, 1997) in combined modality therapy which also involves surgery and radiotherapy.

1.1.2 ANTIMICROBIAL AGENTS

The so-called "Golden Era" of antibiotic discovery and development in the 1940s and 1950s (Chicarelli-Robinson *et al,* 1997) marked a sustained assault on microbiological pathogens. However, the gains made for human health during that period have been partly lost primarily due to the emergence of multiply antibiotic-

resistant organisms (Torres-Viera *et al,* 2000) and increased incidence of apparently new pathogenic organisms of fungal and viral nature (Hongsa and Bobek, 2000). Medical advances have led to increased numbers of immuno-compromised patients living longer which has, in turn, led to an increase in the incidence of fungal infections which, in general, have not always been amenable to treatment (Torres-Vierra *et al,* 2000).

In recent years, the emergence of retroviruses as a significant cause of morbidity and mortality has added to the complexity of the picture and thrown down one more gauntlet that research into chemotherapy has to deal with.

In order to circumvent some, or all, of these limitations much work to synthetically modify currently available drugs to improve their therapeutic profile, has achieved little (Dewick 1996). There arises the need for new prototypes; novel anti-tumour and anti-infective compounds with greater selectivity and less general toxicity. Herein lies the challenge to phytochemists who have the natural materia medica as their armamentarium.

1.2. NATURAL PRODUCTS IN DRUG DISCOVERY

The potential of higher plants as the source not only of useful chemical compounds but also as unique templates of synthetic analogues (Farnsworth, 1984) remains largely unexplored (Hostettmann *et al,* 1996). Of the 500,000 different species of plants estimated to exist, only a small percentage has been investigated

phytochemically and an even smaller proportion of this has been screened for pharmacological efficacy (Mahidol *et al*, 1997).

Crude natural products have a long history of usage in the management and treatment of cancer in traditional medical practice (Cassady and Douros, 1980) and thus present a rational source of potential cytotoxic compounds with the added advantage of the possibility of discovering new modes of action and novel structures which may then be synthesized or semi-synthesized (Saunders and Carter, 1978).

Over the past 40 years, at least 300,000 chemical compounds of natural origin have been submitted to, and screened for anti-tumour activity by, the National Cancer Institute of the United States of America (Cragg *et al,* 1996). Between 1960 and 1982, at least 180,000 microbial fermentation products, 114,000 plant-derived and 16,000 marine organism-derived extracts were tested for *in vivo* anti-tumour activity using L1210 and P388 mouse leukemia models. Since 1985, the NCI has turned its attention to the search for natural products with anti-HIV activity (Cragg *et al*, 1997). Many successes have resulted from these extremely taxing and time-consuming screens which have seen the interdisciplinary collaboration of phytochemists, medicinal chemists, pharmacologists and ethnobotanists as well as traditional healers and/or herbalists.

Despite the expense involved in the development of a drug today, at least US$230 million and a timespan between 10 - 20 years (Cragg *et al,* 1996), nature remains the most reliable and most important source of novel drug molecules (Choudhary and

Rahman, 1997). Nature provides 80% of all pharmacological and therapeutic lead compounds (Potier *et al,* 1996) and the NCI estimates that over 60% of the compounds currently in pre-clinical and clinical development in its laboratories are of natural origin (Cragg *et al*, 1996). Thus higher plants remain as an important and reliable source of potentially useful chemical compounds not only for direct use as drugs, but also as unique prototypes for synthetic analogues and as tools that can be used for a better understanding of biological processes (Farnsworth, 1984).

CHAPTER TWO

2.1 HYPOTHESIS

Species of the African Combretaceae are used in traditional medicine for the management of disease and are likely to possess secondary metabolites which demonstrate potent *in vitro* biological and pharmacological activity.

From the foregoing, the isolation and biological testing of a number of plants of *Combretum* and *Terminalia* species has met with considerable success. This suggests that analogous evaluation of related species may prove to be quite productive. This, in short, is the brief of this project as expounded on in the following section. The section examines the motivation, and argument, for the phytochemical and pharmacological interest in the African Combretaceae.

2.2. AIMS AND OBJECTIVES

Traditional medicine has a long history of existence spanning several millenia and it was the cornerstone of ancient civilisations. An estimated 90% of the world's population relies almost exclusively on herbal and traditional medicine because of the non-availability of allopathic medicine (Ayensu, 1985). This has probably not changed tremendously since the macro-economic state of the world has not shifted over the last two decades.

The World Health Organisation (WHO, 1976) defines traditional medicine as, "the sum total of all knowledge and practices whether explicable or not, used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observations handed down from generation to generation verbally or in writing".

The treatment is concentrated on the primary cause, often believed to be supernatural, but it may be directed at the symptoms as well (Okpako, 1991). The advantage of non-allopathic medicine is that "it treats the patient, and not the disease" (Chavunduka, 1978) i.e. it can be described as being "holistic" and "spiritual" while modern medicine is "materialistic". While there appears to be no scientific basis for diagnosis, the use of herbal medicine appears to be quite rational.

In their book "*The Traditional Medical Practitioner in Zimbabwe*", Gelfand *et al* (1985) advocate the investigation of the chemical and pharmacological properties of African plants in order to determine their medicinal value and clinical efficacy as well as the potential dangers posed to the general public by their indiscriminate use.

The Organisation of African Unity has advocated the "scientific exploration of African fauna and flora for the benefit of the peoples of Africa" and the "diversification of the sources of herbal medicaments in order to reduce dependence on importation of drugs" (OAU, 1975).

This study aims to carry out a critical evaluation of species of the African Combretaceae, which is ecologically ubiquitous in sub-Saharan Africa and widely

used in traditional African medical practice (Gelfand, Mavi *et al*, 1985; Banskota, Tezuka *et al*, 2000).

Despite the widespread use of the species of *Combretum* and *Terminalia* in herbal medicine, these are also known to be toxic to humans and animals (Rogers, 1996). The actual cause of toxicity has yet to be unambiguously established while the case for a thorough phytomedical investigation remains.

Thus the aim of this study is two–pronged:

- to provide information on the phytochemical constituents of the chosen species,and
- to evaluate their pharmacological efficacy and toxicological potential.

The main thrust is to isolate phytochemical compounds which possess selective activity against a battery of cancer cell lines and microbial organisms. In general, there is an ethnopharmacological rationale for screening the plants this way: decoctions of these plants are used in traditional medical practices to manage symptoms which are of infective aetiology (Martini and Eloff, 1998) and some species of this family have been used as traditional cancer treatments.

Previous studies on *Combretum caffrum* (Pettit *et al,* 1982, 1987; Rodgers, 1996) have been extremely successful in finding novel anti-tumor compounds and it is plausible that further work on more species of African Combretaceae will yield secondary metabolites of pharmacological interest and medicinal importance.

This study is important in helping to build a pharmacological and phytochemical profile of indigenous resources which is of immediate scientific interest, and possible clinical potential. The chemical and pharmacological profiling of medicinal plants is an important first step to their scientific validation and conventional usage. It presents an opportunity to discover new and useful drugs (Kyerematen and Ogunlata, 1985) and is vital for the safe and rational use of such medicines in herbal, botanical or pharmaceutical form (Hylands, 1985).

The motivation for carrying out this work, while being intimately personal, also arises from the need to contribute to the current body of knowledge on the biology and phytochemistry of African plants in general, and Southern Africa's biodiversity in particular. And perhaps to make a small but marked contribution to the health care of the people in this region.

CHAPTER THREE

3.1. INTRODUCTION TO THE PRESENT STUDY

This study undertakes to investigate the phytochemistry and biological activity of plants of the African Combretaceae for which the botanical features, folklore usage and previous work are discussed below.

3.1.1. TAXONOMY

The family Combretaceae belongs to the order Myrtales and consists of 600 species of trees and shrubs in 20 genera which include *Anogeisssus, Bucida, Combretum, Quisqualis, Terminalia* and *Thiloa,* found throughout the tropics and sub-tropics (Watson and Dallwitz, 1992). The genus *Combretum* Loefl contains 250 species found in all tropical regions except Australia, while another 250 species make up the genus *Terminalia.* These two are the largest genera of the African Combretaceae and consist of climbers, shrubs , shrublets and trees , rarely subherbaceous (Exell, 1970). They occur in most parts of Africa and are often the dominant vegetation (Rogers and Verotta, 1996).

In general, the genus of *Combretum* has 4-5-winged, -ridged, -angled, sessile or stipitate fruit while *Terminalia* has 2-winged fruit. Hydridization is a common occurrence in both genera because of the considerable resemblance among some of the species. This results in the formation of numerous subspecies which look, on visual inspection, quite different. Thus *C. albopunctatum* and *C. apiculatum* are

similar in many respects, and so are *C. psisiodes* and *C. molle*, and *T. mollis* and *T. stenostachya* (Exell 1970).

The botanical data below are extracted from the review of Combretaceae in *Kirkii,* Journal of the Government Herbarium (Southern Rhodesia) by Exell, 1970. It is limited to the species of interest in this study. Where possible the local (Shona) language name is give as well as the common English name. The plant material was collected at the end of the wet season in May 1999.

3.1.1.1. *Combretum albopunctatum* Suesseng

C. albopunctatum occurs as a thicket-forming shrub or small tree up to 3m high with a tendency to climb. It is found in hotter drier areas on grits and sandstones or in alluvial or riverine thickets. It was collected on the Zambezi Escarpment, a hot, sandy and dry low-lying area in Northern Zimbabwe.

The leaves are opposite or subopposite and the flowers are yellow and glutinous when young. The fruit is reddish-brown and usually satiny.

3.1.1.2. *Combretum apiculatum* Engl. & Diels *(mugodo)* (Red bushwillow)

This is a small tree, 4 – 10 m. high with greyish-black bark and leaves opposite. The flowers are yellow and the leaf–buds black or dark-brown. See Fig. **3.02**. This tree is very common at low altitudes in dry savanna and savanna woodland particularly in rocky places, but may also grow in medium altitudes. It was collected in a peasant field at Mahuwe, Zambezi Valley, Northern Zimbabwe.

3.1.1.3. *Combretum collinum* Engl. & Diels

This is a small tree growing up to 12 m. high or may grow as a shrub. It has a reddish-brown to pale yellow bark and leaves which are opposite, alternate or verticillate and variable in shape. See Fig. **3.01**. The flowers are white or yellow-green. It is widespread in tropical Southern Africa in low to medium altitudes and occasionally found in copper bearing soils. It was collected from Impinge Mine near Guruve in Northern Zimbabwe.

3.1.1.4. *Combretum hereroense* Engl. & Diels *(mutechani)*

This is a small tree growing up to 9 m. high or more, rarely a shrub, with arching stems, frequently flowering before the leaves with grey–black bark. See Fig. **3.03**. The leaves are opposite and with yellow flowers, there is great variability in leaf-shape and size of fruit. It is found in thickets on termite mounds, dry deciduous shrub savanna, edges of seasonal swamps, in copper bearing soils and widespread in low to medium altitudes. It was collected at Mbuya Mhlanga's plot, Nyanga in the Eastern Highlands, Zimbabwe.

3.1.1.5. *Combretum imberbe* Engl. & Diels *(mutsviri)* (Leadwood)

The tree grows up to 30 metres tall (usually 6- 10m) or shrub with spiny/spiky branchlets and silvery leaves, opposite or subopposite. The flowers are yellow in colour. See Fig. **3.02**. This tree is found in sandy karoo soils of the savanna. This was collected from a peasant plot at Mahuwe, Zimbabwe.

3.1.1.6. *Terminalia stuhlmannii* Engl. & Diels

This a small tree or shrub up to 12 m. high with brownish–grey or whitish–grey bark and leaves clustered at the ends of spur shoots. The flowers are whitish and the fruit purple–pink or brown. It is found in *Acacia* savanna in hotter drier areas. It was collected outside Mahuwe, Zambezi Valley, Zimbabwe.

3.1.2. ETHNOPHARMACOLOGY

Species of the Combretaceae are widely used in traditional medicine but have largely remained unexplored as a source of new bioactive substances (Pettit *et al*, 1987; Rogers, 1996). In East and Southern Africa, the species most used are those of the genera *Combretum* and *Terminalia*, and these are used for a variety of complaints ranging from treating infertility and sexual impotence, management of stomach disorders including diarrhoea and helminthiasis, to haematuria (Gelfand, Mavi *et al,* 1985). It appears that the more widespread the plant species is located, the more commonly it is used: see Table **3.01a** and **b**.

At least seven species of this genus have been used in traditional treatments of cancer (Hartwell, 1982). Claims of treating oncological disease in traditional medicine are usually fatuous because of diagnostic limitations (Marston and Hostettman, 1987).



Fig. 3.01. *Combretum collinum* in fruit. The photograph was taken at the end of the rainy season in Zimbabwe, May 1999.

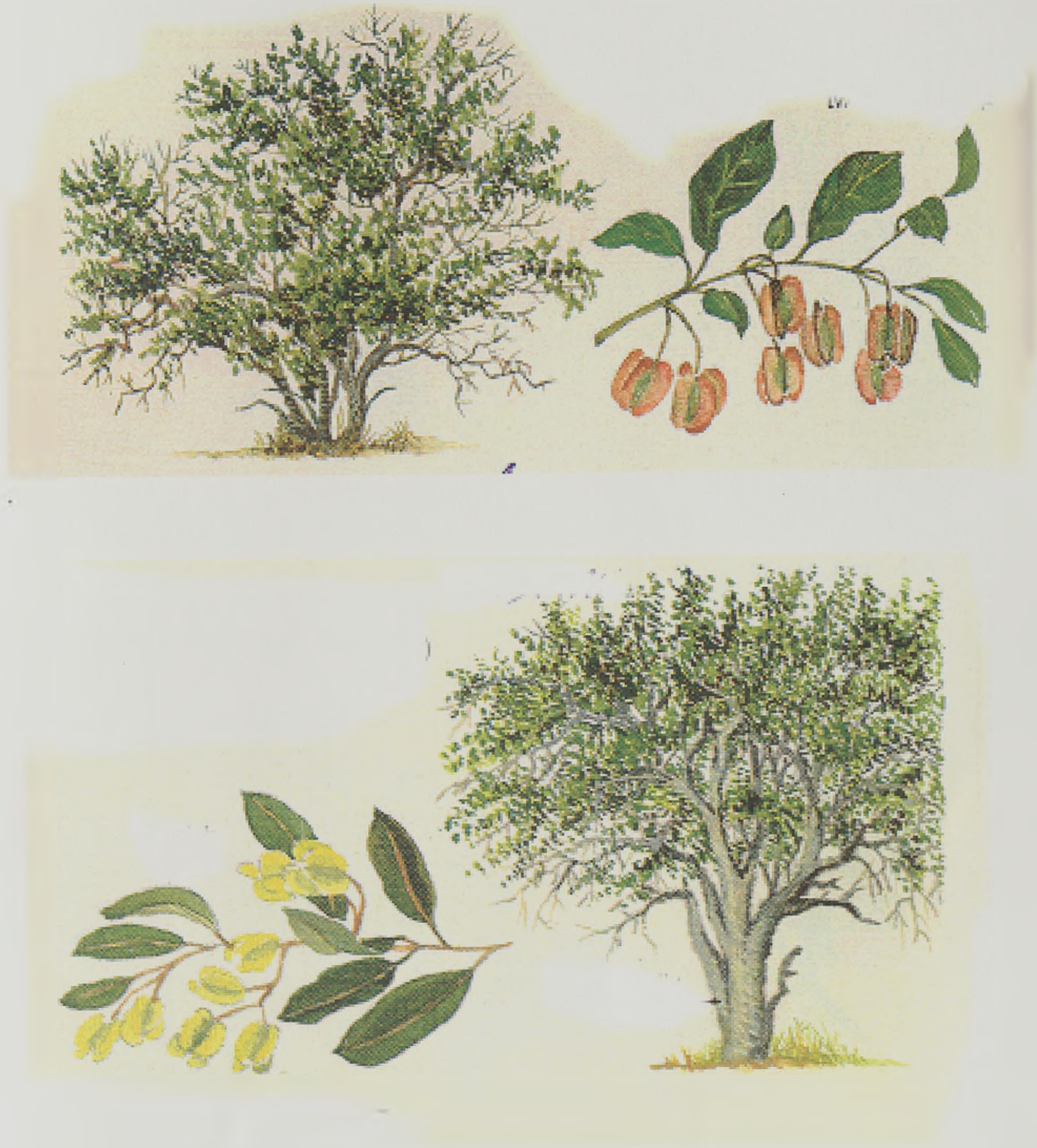


Fig. 3.02. Sketches of *C. apiculatum* (top) and *C. imberbe* (above) (Moll 1994)



Fig. 3.03. Photograph of *Combretum hereroense* Schint. in fruit, Zimbabwe, May 1999 (top), and below is a sketch of the plant adapted from Moll 1994.

Table 3.01a. Some Uses of Combretaceae in Traditional Medicine in Southern Africa (adapted from Gelfand *et al*, 1985; Hutchings *et al*, 1996)

Plant species	Medicinal uses
Combretum apiculatum	Abdominal disorders Conjuctivitis General malaise
C. erythrophleum (has been recorded as poison)	Abdominal disorders To reduce the size of the vaginal orifice Venereal disease
C. fragans	Aphrodisiac
C. hereroense	Abdominal disorders Headache Infertility Venereal disease
C. imberbe	Bilharziasis Diarrhoea Drive away bad spirits
C. molle	Abdominal pain Aphrodisiac Diarrhoea (with or without blood) Depressed fontanelle in infants Convulsions "Fatten babies" (presumably anthelminthiasis) General malaise Headache Infertility Post-partum bleeding Wounds
C. *paniculatum* subsp. *microphyllum*	Lucky charm

Table 3.01b. Some Uses of Combretaceae in Traditional Medicine in Southern Africa (adapted from Gelfand *et al*, 1985; Hutchings *et al*, 1996) (continued)

Plant species	Medicinal uses
C. platypetalum subsp. *oatessii* (has been recorded as poison)	Burns Diarrhoea Dilation of birth canal Dysmenorrhoea Earache Epistaxis Haemoptysis Infertility Pneumonia
C. zeyheri	Diarrhoea (with blood)
T. brachystemma	Bile emesis Constipation Haematuria
T. sericea	Abscesses and wounds Abdominal pain Anti-emetic Anthelmintic Bilharziasis Diarrhoea Dilatation of birth canal Depressed fontanelle in infants Epistaxis General malaise Gonorrhoea Infertility Illness from sex with woman who has recently aborted Prevention of abortion Sore throat Tonic
T. mollis	Bile emesis

While traditional healers have no problems diagnosing external and gross anatomical complaints, the diagnosis of cancers is vague and not reliable unless there is an obvious physical aberration.

The use of these plants in treating wounds, haematuria or diarrhoea is probably scientifically rational. It may be attributed to their anti-microbial action (including anti-vibrio and anti-amoebic activity) which has since been established in various studies (Martini and Eloff, 1998; Sato *et al*, 1997; Fabry *et al*, 1996; Silva *et al*, 1996).

However, this work has been carried out mainly on *Terminalia* from West Africa, the Indian sub-continent or Far East Asia. Little or no studies are reported on the Southern African varieties.

The work emanating from Southern Africa has been confined to general pharmacological screening concentrating mainly on the widely distributed *Combretum* species in the Republic of South Africa. This study will concentrate on species growing in the northern subcontinent in the Republic of Zimbabwe and will involve the development of phytochemical screening methods, isolation and pharmacological testing. It will initially look at plants that have been previously studied and then progress to plants in both genera which have had little or no attention before.

An interesting observation that has been made is that the fruits of *Terminalia* or *Combretum* species are rarely used in traditional medical practice, or ingested for food by humans or animals. In fact, they have all been found to be highly toxic (Panzini *et al*, 1993) and ingestion leads to violent and prolonged hiccoughing (hence the name "hiccup nut" given to many of the species). The actual toxic constituents remain unidentified. The general screening to be undertaken in this study should help to close this gap.

3.1.3. PHARMACOLOGY

There appears to be an increasing interest in the Combretaceae, particularly of Southern African and South Asian origin. This recent interest has arisen due to the isolation of the phytochemical constituents from the family which have shown good biological activity and the potential for clinical usage.

3.1.3.1. *Combretum* Loefl species

Many constituents, mainly stilbenoids and terpenoids, have been isolated and the chemical structures elucidated. The most interesting of these are the stilbenoids isolated from *C. caffrum* (also known as *C. salicifolium*) which is used in Zulu traditional healing. These compounds, which have been called combretastatins, are now undergoing pre-clinical development as anti-neoplastic agents.

The combretastatins have demonstrated an array of potent actions with therapeutic potential including the following (Pettit *et al*, 1982; Pettit *et al,* 1995):

- Activity against murine P388 leukemia *in vitro*
- Activity against 9ASK system
- Significant rise in the mitotic index of L1210 (i.e. effective mitotic agent)
- Inhibition of tubulin polymerization
- Stimulation of tubulin-dependent GPT hydrolysis
- Competitive inhibition of the binding of colchicine to tubulin

Analogues of the most active of the combretastatins have been synthesized to improve aqueous solubility and found to show potent anti-tubulin activity and cytotoxicity against murine colon 26 adenocarcinoma (Ohsumi, Nakagawa *et al*, 1998). It has been found that the analogues lead to a high tumour cell kill by causing a shutdown of existing tumour blood vessels depriving the tumours of oxygen and nutrients (Siemann *et al*, 1998).

Combretastatin A-4, in particular, appears to potentiate the effects of radiotherapy as well as those of anti-cancer drugs (Li *et al*, 1998). It is one of the first compounds to be classified as an angiogenesis inhibitor due to its effect on blood supply to the tumour, and hence becomes one of the new generation of anti-neoplastic agents.

Angiogenesis, the proliferation of blood vessels, is a basic physiological function frequently occurring in tumour growth and dissemination (Armand, 1997). The mode of

action of anti-angiogenesis agents may involve inhibition of production and/or binding of angiogenic factors e.g. growth factors or the inhibition of nuclear enzymes.

Combretum quadrangulare was shown to possess cycloartane-type triterpenes which are active against murine colon 26-l5 carcinoma cells (Banskota, Tezuka *et al*, 1998). The flavonoids, kumatakenin and isokaempferide were also isolated from this plant species (Ganzera *et al*, 1998).

Seven novel triterpene acids with a 9-β, 19-cyclopropyl-1-en-3-one skeleton have been isolated from *C. erythrophyllum* (Rogers, 1998). A crude extract of the leaves showed antibacterial activity (Martini and Eloff, 1998) while the methanolic extract of *C. micranthum* showed *in vitro* anti-viral activity against Herpes Simplex Virus Type 1 and Type 2 (Ferrea, Canessa *et al*, 1993). Activity appeared to be due to catechinic tannins which underwent rearrangement in alkaline conditions. The aqueous fraction of the leaf of C. *glutinosum,* traditionally used for the treatment of hepatic disease, was found to be able to inhibit hepatitis B virus antigen (HbsAg) as well as the Angiotensin Converting Enzyme (ACE) (Pousset, Rey *et al,* 1993). A hydrolyzable tannin, combreglutinin as well as punicalin, punicalagin and 2,3-(S)-hexahydroxydiphenyl-D-glucose were isolated from this plant (Jossang *et al,* 1994) and it may be possible that they would elicit such bioactivity.

While the *Combretum* species have received a fair amount of attention with success resulting in the isolation of the combretastatins a lot of the work has centred on *C. caffrum*. The full potential of this genus, which contains up to 250 different species, remains unexplored (Nandy, Banerjee *et al*, 1991).

3.1.3.2. *Terminalia* Species

The genus *Terminalia* contains about 250 species of which only a handful have been investigated phytochemically and/or for pharmacological efficacy. Of these, most work has been done on *Terminalia arjuna.* This plant is widely used in India by Ayurvedic doctors for managing cardiovascular disorders. The tannins isolated from the plant were found to exert a cholinergic hypotensive effect in rats (Takahashi, Tanaka *et al,* 1997). 3-β-Hydroxyolean-12-ene and related triterpenoids were isolated from the leaves (Chauhan *et al,* 1997). The bark powder has apparent hypolipidaemic effects (Khanna *et al*, 1996), and in another study by Pettit and colleagues (1988), the bark constituents were found to be gallic acid, ethyl gallate and luteolin which were found to be inhibitors of cancer cell growth. Gallic acid as well as chebulagic acid and chebulinic acid isolated from *T. chebula* were found to have moderate activity against cultured tumour cell lines including A-549, SK-OV-3, SK-MEL-2, XF-498 and HCT-15 *in vitro* (Lee, Ryu *et al*, 1995).

Gallic acid and its ethyl derivative are also responsible for the potent anti-microbial activity which this plant exhibits even against Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Sato, Oketami *et al,* 1997). *T. chebula* was also found to inhibit HIV-1

protease enzyme (Xu, Wan *et al*, 1996). This may have been due to the chelating effects of the tannins.

Plants of this genus are used in African traditional medicine for a variety of conditions which may be of an infective nature e.g. bilious vomiting, diarrhoea, bilharziasis, sore throat, gonorrhoea, hepatitis and malaria (See section **3.1.2**). The putative anti-microbial actions of the species in this class seem to be validated by data from various studies. Young branches of *T. spinosa* have been found to possess anti-fungal activity (Fabry *et al*, 1996) as well as activity against *Helicobacter pylori* and *Candida* species. *T. macroptera* root extracts showed some activity against *Candida albicans* and enteropathogen bacteria. The best results were obtained against clinical strains of *Shigella dysenteriae* and *Vibrio cholerae* (Silva, Duarte *et al*, 1996). Ellagitannins are the main constituents in the active fractions. O-Glycosyl flavonoids have been isolated from the flowers of the plant (Nongonierma *et al*, 1990). Tannins isolated from the methanol extract of *T. citrina* have been tested for anti-microbial activity (Burapadaja and Bunchoo, 1995); *T. avicennoides* shows activity against *Vibrio cholerae* (Akinside and Olukoya, 1995).

In general, biological activity arising from condensed tannins is not reproducible *in vivo* but some ellagitannins can enter the blood stream and have shown biological activity which may have potential for use in chemotherapy (Nash, personal communication, 2000).

The dearth of detailed information on this genus compared to *Combretum* species makes work into this area important and challenging.

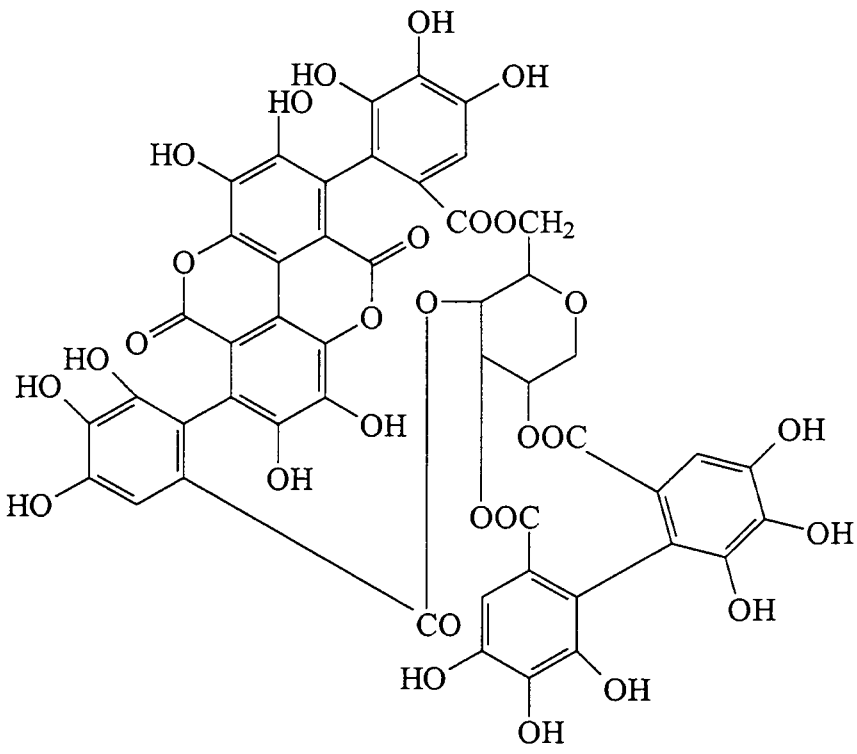
3.1.5. PHYTOCHEMISTRY

Phytochemical work on *Combretum* and *Terminalia* dates back to the early 1970s and stretches well into present publications. This section outlines the phytochemical constituents that have been isolated in detail and considers the sources.

3.1.5.1. TANNINS

Tannins are polyphenolic compounds of sufficiently high molecular weight which have the ability to precipitate proteins (Horvath, 1981) and may also complex with starch, cellulose and minerals. They are synthesized via the shikimate pathway, which is the same pathway which results in the formation of isoflavones, coumarins, stilbenoids and other phenolic metabolites.

Gallic acid and its derivatives are commonly occuring constituents of the Combretaceae. A number of elaborate tannins have been isolated mainly from *Terminalia* e.g the diphenoyl-gallagylglucose (**1**) isolated from *T. oblongata* and an ellagitannin from the leaf of *T. calamansanai* as well as derivatives from *T. catappa* and *T. chebula* (Tanaka *et al*, 1986 and 1991; Lui *et al*, 1990).



(1)

Fig.3.04. 2,3- (S)-Hexahydroxydiphenoyl-4,6-(S,S)-gallagylglucose (punicalagin) from *Terminalia oblongata*

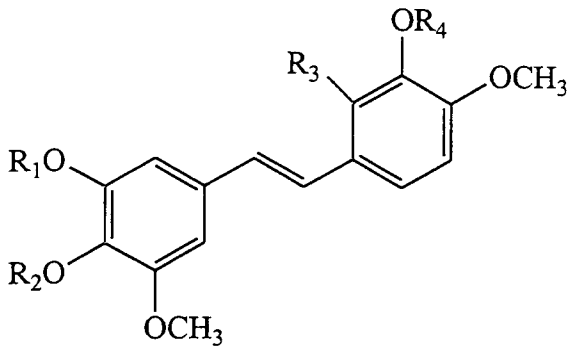
These compounds have been shown to have anti-tumour activity in numerous studies (Pettit *et al,* 1996). It is worth repeating here that pharmacological activity induced by tannins (with the recent exception of ellagitannins) is, in general, non-specific and thus of little clinical potential.

3.1.5.2. STILBENOIDS

These compounds which are biogenetically related have generated immense interest because of their biological potency and structural simplicity. See section **3.1.3.1.** They were first isolated from *Combretum* species by Letcher and Nhamo in 1971. However,

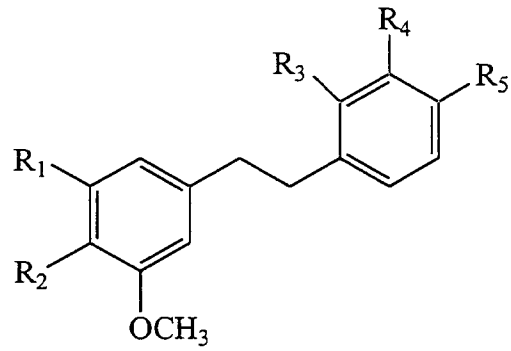
their biological activity was only discovered by Pettit and colleagues in the early 1980s who isolated stilbenes from a methylene chloride–methanol extract of the leaves, fruit and stemwood of *C. caffrum* (Cape bushwillow tree) (Pettit *et al,* 1982). They called these compounds combretastatins (**2**)-(**25**) and they have since been isolated from *C. kraussi, C. molle, C. psidiodes* and *C. apiculatum* (Malan and Swinny, 1993; Schwikkard *et al*, 2000). See Table **3.02a** and **3.02b**. They have been designated as A, B, C and D according to their chemical structures. Combretastatins A and B are almost identical but differ in that the former has an ethylene bridge joining the two benzyl groups and is chemically identified as a stilbene. The latter, meanwhile, has an ethane-type bond and is based on the dihydrostilbenes (also called bibenzyls).

The pioneering work by Letcher and Nhamo, carried out at the University of Rhodesia (now Zimbabwe) was mainly concerned with the chemical analysis (including organic synthesis) of constituents of *C. apiculatum, C. psidiodes, C. molle* (Letcher and Nhamo, 1971; Letcher *et al*, 1972; Letcher and Nhamo, 1972). In addition to isolating a few stilbenes, their work was successful in yielding several hitherto unknown substituted phenanthrenes and 9,10-dihydrophenanthrenes (**26**)-(**44**). See Table **3.03**. Some of these compounds have since been also isolated from related species (Malan and Swinny, 1993) and from the leaves of *Cannabis sativa* (Crombie and Crombie, 1975).



(2)-(6)

Primary structure of Combretastatins A



(7)-(25)

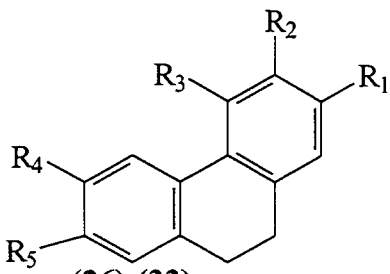
Primary structure of Combretastatins B

	R_1	R_2	R_3	R_4		PLANT SOURCE
2	CH_3	CH_3	OH	H	C-A1	*C. caffrum* *C. kraussi*
3	$-CH_2-$	$-CH_2-$	OH	H	C-A2	*C. caffrum*
4	H	CH_3	H	OH	C-A3	*C. caffrum*
5	H	CH_3	H	CH_3	C-A6	*C. caffrum*
6	CH_3	CH_3	OH	OGlc		*C. kraussi*

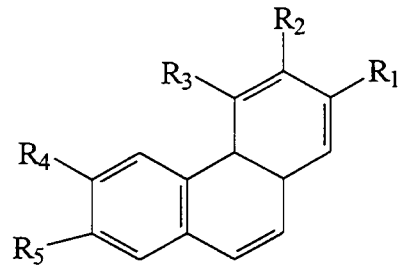
Table 3.02a. Derivatives of stilbenes (combretastatins) isolated from Combretaceae.

	R_1	R_2	R_3	R_4	R_5		PLANT
7	OCH_3	OCH_3	OH	OH	CH_3	C-B1	*C. caffrum* *C. kraussi*
8	O-CH_2-O	O-CH_2-O	OH	OH	CH_3	C-B2	*C. caffrum*
9	OCH_3	OCH_3	H	OH	H	C-B3	*C. caffrum*
10	OCH_3	H	H	OH	H	C-B4	*C. caffrum*
11	OCH_3	H	H	OH	CH_3		*C. caffrum*
12	OCH_3	H	H	H	H		*C. caffrum*
13	OCH_3	OCH_3	H	H	H		*C. psidiodes* *C. caffrum*
14	OH	H	-	H	H		*C. apiculatum*
15	OH	H	H	OH	CH_3		*C. apiculatum*
16	OH	OCH_3	H	OCH_3	H		*C .apiculatum*
17	OH	OH	H	OCH_3	CH_3		*C. apiculatum*
18	OH	OCH_3	O-Glc	OH	H		*C. apiculatum*
19	OH	OH	OH	H	CH_3		*C. apiculatum*
20	OH	OCH_3	O-Glc	H	H		*C. apiculatum* *C. molle*
21	OCH_3	OH	H	H	H		*C. apiculatum* *C. psidiodes*
22	OCH_3	OCH_3	CH_3	OH	O-Glc		*C. kraussi*
23	OCH_3	OH	CH_3	OH	OH	C-B5	*C. kraussi*
24	OCH_3	OCH_3	CH_3	OH	O-Glc		*C. kraussi*
25	OCH_3	OH	CH_3	OH	O-Glc		*C. kraussi*

Table 3.02b. Derivatives of dihydrostilbenes (combretastatins) isolated from Combretaceae.



(26)-(33)



(34)-(44)

	R_1	R_2	R_3	R_4	R_5		PLANT
26	OCH_3	OCH_3	OH	OH	OH		*C. apiculatum*
27	OH	OCH_3	OCH_3	OH	OCH_3		*C. apiculatum*
28	OCH_3	OCH_3	OH	OCH_3	OH		*C. apiculatum, C. molle*
29	OCH_3	OCH_3	OCH_3	OCH_3	OH		*C. caffrum*
30	OH	OH	OCH_3	OCH_3	OH		*C. apiculatum*
31	OCH_3	OH	OCH_3	OH	OH		*C. apiculatum*
32	OCH_3	OH	OCH_3	OCH_3	OH		*C. apiculatum*
33	OCH_3	OH	OCH_3	OCH_3	OCH_3		*C. apiculatum*
34	OCH_3	H	OH	OCH_3	OH		*C. apiculatum*
35	OCH_3	OCH_3	OH	OCH_3	OH		*C. apiculatum, C. molle*
36	OH	OCH_3	OCH_3	OH	OCH_3		*C. apiculatum C. molle*
37	OCH_3	OCH_3	OH	OH	OH		*C. apiculatum, C. molle*
38	OH	OCH_3	OCH_3	OH	OCH_3		*C. apiculatum, C. molle*
39	OCH_3	OCH_3	OH	OH	OH		*C. apiculatum, C. molle*
40	OCH_3	OCH_3	OCH_3	OH	OH		*C. apiculatum, C. caffrum, C. hereroense*

41	OH	OCH_3	OCH_3	OCH_3	OCH_3		*C. caffrum*
42	OH	OCH_3	OCH_3	OCH_3	OH		*C. caffrum*, *C. apiculatum*, *C. psidiodes*
43	OCH_3	OCH_3	OCH_3	OCH_3	OH		*C. apiculatum*, *C. psidiodes*, *C. caffrum*
44	OCH_3	OH	OCH_3	OCH_3	OCH_3		*C. apiculatum*

Table 3.03. Derivatives of phenanthrenes isolated from Combretaceae.

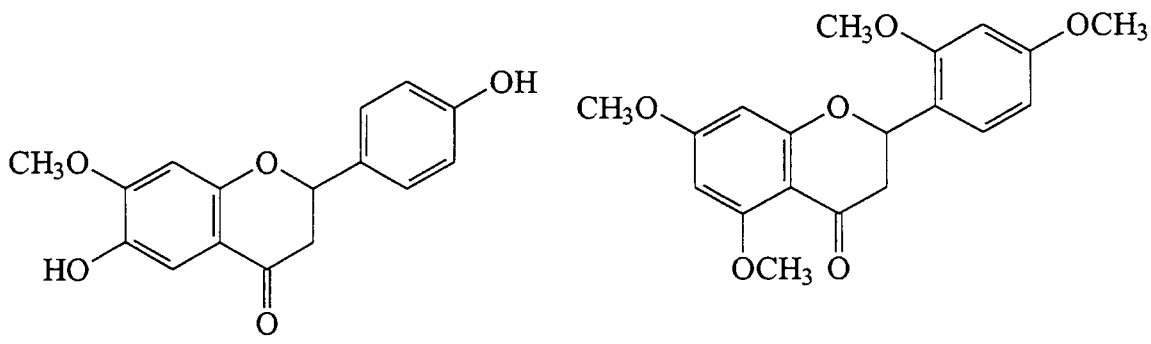
Stilbenoids have also been isolated in members of Orchidaceae - *Pleine bulbocodioides* (Li Bai *et al*, 1996), *Bulbophyllum vaginatum* ((Leong *et al*, 1997), *Cymbidium aloifolium* (Juneja, 1987) and *Dendrobium amoenum* (Majumder *et al*, 1999). The taxonomic significance of this has not been established but it could be surmised that there may be a close evolutionary relationship with Combretaceae.

3.1.5.3. FLAVONOIDS

Flavonoids are conjugated aromatic phenols which may occur in glycosidic combinations or as aglycones (Harborne, 1973). At least nine classes of flavonoids are recognised, all of which are biogenetically related and originate from the amino acid, phenylalanine. They are one of the most diverse and widespread groups of natural constituents, are of great interest in phytochemistry (Agrawal, 1989), and may represent an interesting new therapeutic approach with low toxicity and a wide variety of chemical structures (Anton *et al,* 1987). Flavonoids are also widely used taxonomic markers.

The flavanoid, luteolin, has been isolated from *Terminalia arjuna* and shown to be active against a murine P388 lymphocytic leukemia and human cancer cell line as well as inhibiting the growth of *Neisseria gonorrhoeae* (Pettit *et al,* 1996). Luteolin has been shown to be both an anti-tumour promotor and mutagen.

Arjunolone (**45**) and arjunone (**46**), two flavanones, have also been isolated from *T. arjuna* which is widely used in India and surrounding countries for many medical conditions arising from cardiovascular insufficiency (Kumar and Prabhakar, 1987)



(45) Arjunolone

(46) Arjunone

Fig. 3.05. Some dihydroflavones isolated from *Terminalia arjuna.*

A study of the leaves of the South American plant, *Combretum leprosum* showed two flavonoids, 3-O-methylquercertin and 3-O-α-L-rhamnopyranosylquercetrin (quercetrin) (Facundo *et al*, 1993). This, along with the isolation of cycloartenoid and dammarane terpenoids from South American species establishes an interesting cross-continental chemotaxonomic relationship between African and Latin American species of Combretaceae (Rogers and Verotta, 1996). See section **3.1.5.4.** below.

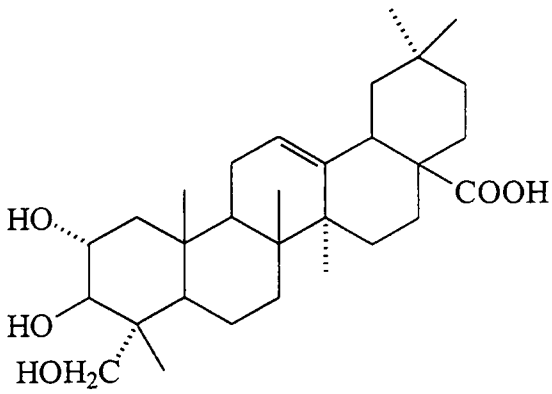
3.1.5.4. TERPENOIDS

Terpenoids are lipid-soluble secondary metabolites which are biosynthetically derived from isoprenoid units. There are at least five classes which are of importance in the growth, metabolism and ecology of plants (Harborne, 1973). Triterpenoids are the most ubiquitous non-steroidal secondary metabolites in terrestrial and marine flora and fauna (Mahato *et al,* 1992). Their medicinal use is rather limited but there remains some potential for application.

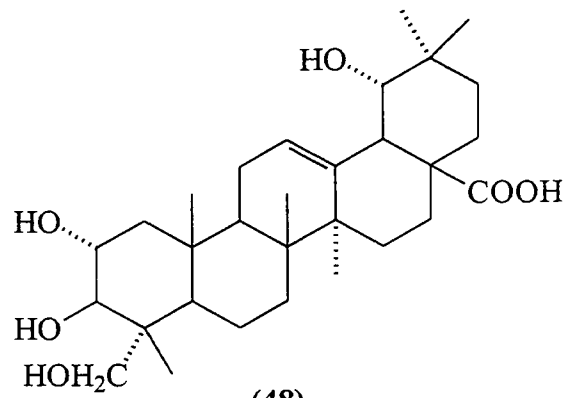
The Combretaceae has yielded a number of different pentacyclic triterpenoid structures including oleanoic and ursanoic acids, friedelins, cycloartanes and dammaranes.

The fruit of *Combretum molle*, when extracted with acetone, yielded arjunolic acid (**47**) arjungenin (**48**) and arjunglucoside (**49**), pentacylic triterpenoids previously found in *Terminalia arjuna* (Panzini *et al*, 1993; Kumar and Prabhakar, 1987). See Fig. **3.06**.

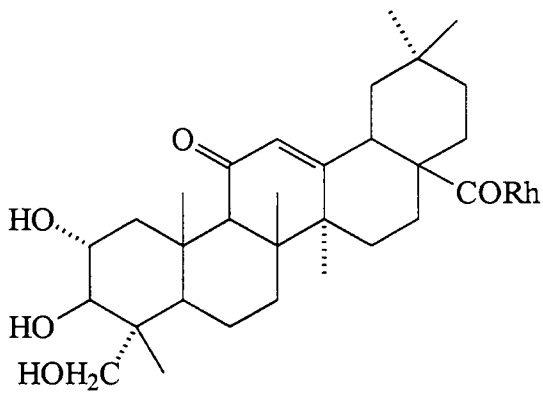
The leaf ether extract of *C. imberbe,* commonly called the leadwood tree, has yielded a complex mix of triterpenoids and their glycosides (Rogers, 1988, 1989). Rogers and Subramony (1988) isolated friedelin, epifriedelin and betulinic acid from the bark of *C. imberbe* and an oleanene-based pentacyclic triterpene with its glycosides from the leaves (**50**)-(**51**), Fig. **3.07**.



(47)



(48)

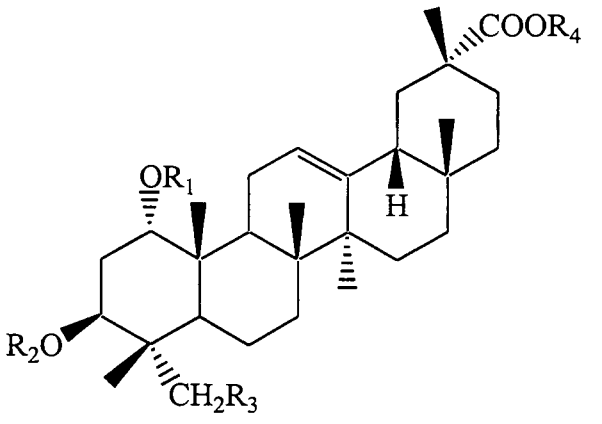


(49)

Fig 3.06. Some pentacyclic triterpenes from *C. molle* and *T. arjuna*

The latter, characterized as 1α, 3β-dihydroxyolean-12-en-29-oic acid, and named imberbic acid, is of chemotaxonomic significance because of the occurrence of the 1α-hydroxy substitution pattern.

The ether extract of *C. edwardsii* leaves yielded the xyloside and arabinoside of mollic acid, previously isolated from *C. molle* (Rogers, 1988) and also reported to be present in a South American species *C. leprosum* (Facundo *et al*, 1993). See Fig. **3.08.**



Compound	R_1	R_2	R_3	R_4
50	H	H	O-Rh	H
51	Ac	H	O-Rh	H

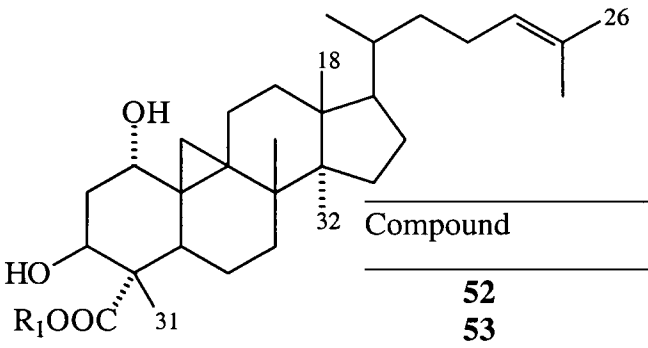
23-hydroxyimberbic acid 23-O-α-L-rhamnopyranoside (**50**)

23-hydroxyimberbic acid 23-O-α-L-rhamnopyranoside-1-acetate (**51**)

Fig. 3.07. Triterpenoids from *C. imberbe*

The isolation of oleanene- type pentacyclic triterpenoids containing 29–carboxy-1α-hydroxy groups from *C. molle*, *C. edwardsii, C. eleagnoides, C. imberbe*, *C. apiculatum, C. kraussi* and *C. padoides* confirms chemotaxonomically significant bifurcation in triterpenoid synthesis in the *Combretum* species (Rogers and Verotta, 1996). Both imberbic acid and mollic acid have anti-inflammatory and molluscicidal activity (Panzini *et al*, 1993).

Jessic acid (**54**)-(**55**) was previously isolated by Osborne and Pegel (1984) from *C. eleagnoides*. It was later found in the acetone fraction of *C. molle* fruit as the 3-O-β-D-xylopyranoside. See Fig. **3.09**.



Compound	R_1
52	H
53	Me

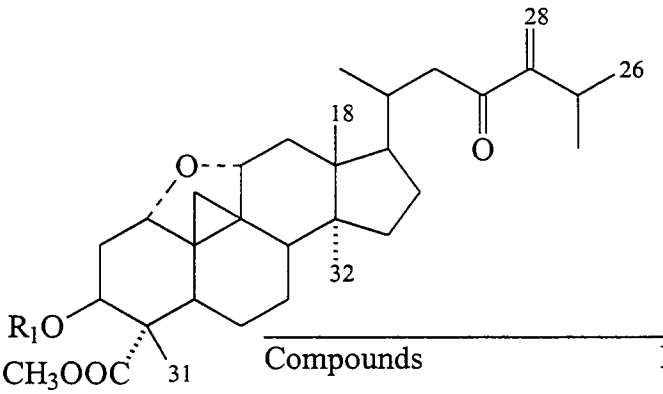
Fig.3.08. Mollic acid and derivatives from *C. molle*

Several other derivatives of jessic acid, a cycloartane triterpenoid, have been found; the most chemically interesting being methyl jessate 1α, 11α-oxide (**54**) isolated from the hexane fraction of dry leaves and which shows a boat-like oxide bridge.

C. nigricans, a small tree from the savanna of Central Africa was investigated by Jossang and others (1995). A methanol extract of the stem bark yielded four pentacyclic triterpenic acids and their glucosides. Two of these, arjungenin and arjunglucoside have been previously found in *T. bellerica, C. molle* and *T. arjuna* as well as in some species of Rosaceae and Apocynaceae.

Combretum erythrophyllum has yielded some unusual cycloartane dienone lactones (**56**) and (**57**) from the leaves (Rogers, 1996). See Fig. **3.10.** The cycloartane-type

triterpenoids are widely found in the Combretaceae. Several novel cycloartane-type triterpenoids, of which many were previously unreported, have recently been isolated from *C. quadrangulare* (Banskota *et al*, 2000). See Fig. **3.11**.



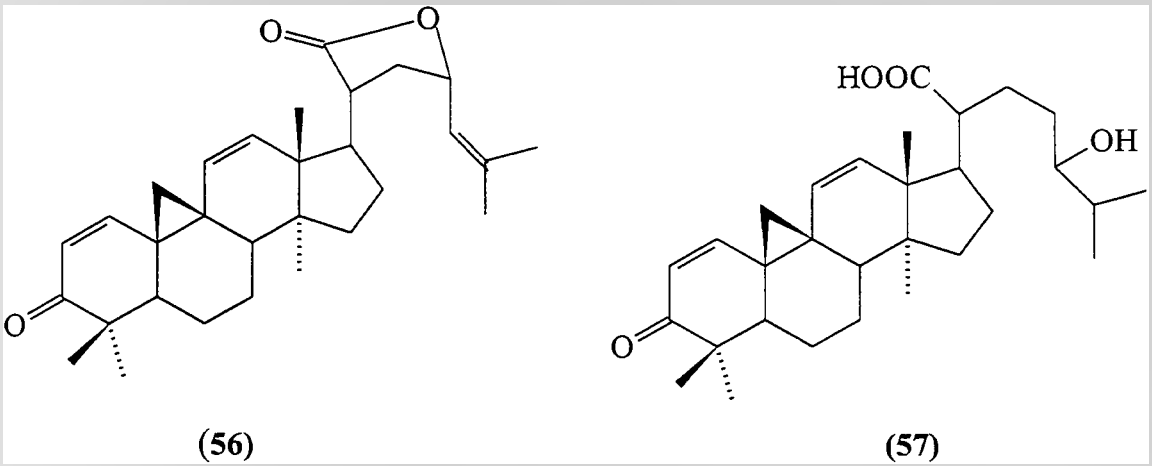
Compounds	R
54	**H**
55	**Ac**

Fig.3.09. Methyl jessate 1α, 11α-oxide (i.e. 1α, 11α-epoxy-3β-hydroxy-23-oxo-24-methylenecycloartan-30-oate) from *C. elaeagnoides.*

A study of South American species of Combretaceae has yielded many similar pentacyclic terpenoids (as well as flavonoids) to those found in the African Combretaceae. Derivatives of 3β,6β,16β-trihydroxylup-20(29)-ene (**65**)-(**71**), hitherto unknown, and dammarane arabinofuranosides (**72**)-(**73**) were isolated from *C. leprosum* and *C. rotundifolium* respectively (Rogers, 1995; Facundo *et al*, 1993). See Fig. **3.12**. and **3.13**.

The phytochemical similarities between African and Latin American Combretaceae establish an intriguing phytochemical and phytogeographical link in the ancestry of

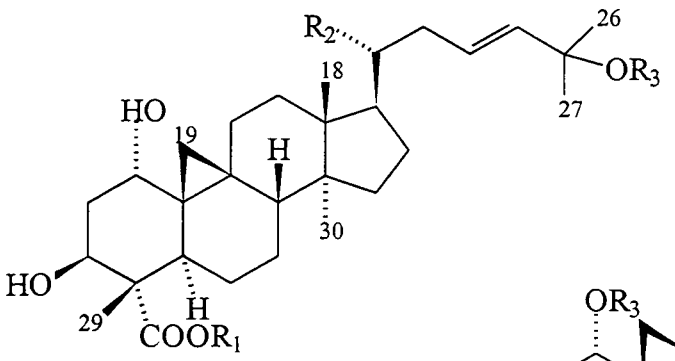
this family (Rogers, 1996). From this survey of phytochemical constituents it may be concluded that terpenoids not only appear to be taxonomic markers to establish biogenetic relationships, but are the most commonly occurring compounds in the Combretaceae.



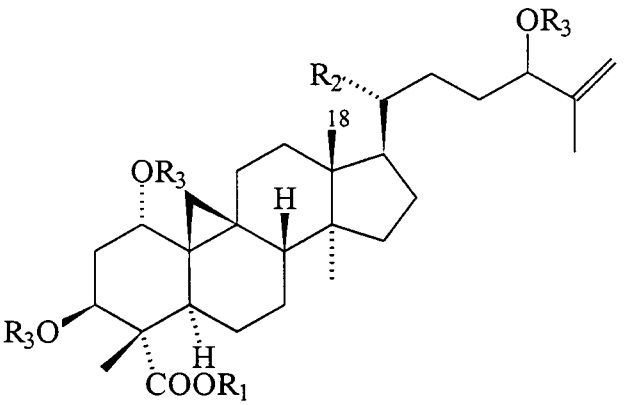
(56) **(57)**

Fig.3.10. Cycloartane dienone lactones from *C. erythrophyllum*

However, their apparent ubiquitous occurrence in the Combretaceae may be a result of the ease with which terpenoids can be isolated because of their moderate polarity. In general, it is true to say that phytochemical literature is surfeit with non-polar compounds because of this.



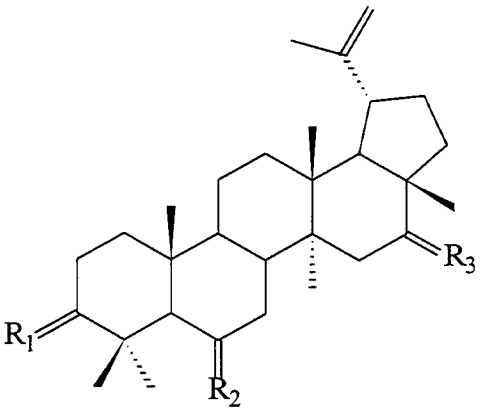
(58)-(62)



(63)-(64)

Compound	R_1	R_2	R_3
58	CH_3	COOH	H
59	CH_3	$COOCH_3$	H
60	H	CH_3	CH_3
61	CH_3	CH_3	CH_3
62	CH_3	CHO	H
63	CH_3	COOH	H
64	CH_3	$COOCH_3$	H

Fig.3.11. Novel cycloartane-type triterpenes from *C. quadrangulare*



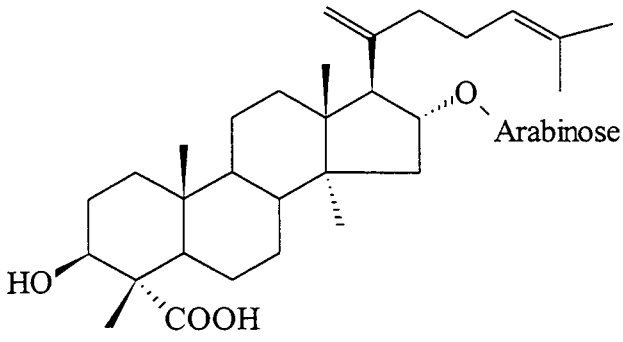
(65)-(71)

Compound	R_1	R_2	R_3
65	β - OH, H	β - OH, H	β - OH, H
66	β - OAc, H	β - OH, H	β - OAc, H
67	O	O	O
68	β - OAc, H	O	α - OAc, H
69	β - OH, H	O	α - OH, H
70	β - OH, H	H, H	H, H
71	O	β - OH, H	H, H

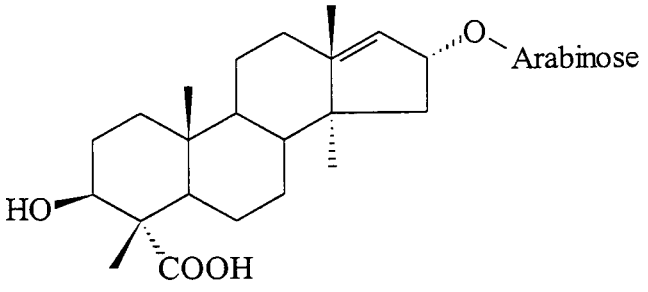
Fig.3.12. Trihydroxyl pentacyclic lup-20 (29)-ene derivatives from *C. leprosum*

It can be seen from the foregoing that the Combretaceae possess a diverse class of triterpenoids. In general, those that have been isolated have been either tetracyclic e.g. the dammaranes, or pentacyclic e.g. oleanolate-based, in nature and also acidic.

Panzini *et al* (1993) did report, however, the co-occurrence of the two types together in *C. molle*, which is unusual.



(72)



(73)

Fig. 3.13. Acidic dammarane arabinofuranosides from *C. rotundifolium*.

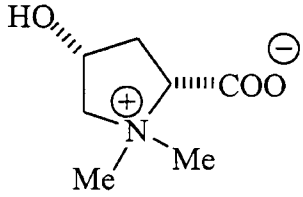
It should also be noted that the glycosides of all these terpenoids are generally isolated with their aglycones. Doubts have been expressed in general concerning the isolation of aglycones as they may in fact be artefacts of the separation process arising from cleavage of the native glycosides.

3.1.5.5. ALKALOIDS

Alkaloids have been defined as cyclic nitrogen-containing compounds of limited occurrence produced by living organisms (Pelletier, 1982). They are the most structurally diverse class of secondary metabolites with over 5000 known compounds

(Mann, 1994) and they are variously used as poisons, euphoriants, psychedelics, stimulants and medicines.

Apart from the simple betaine (**74**) isolated from *C. micrathum* (Ogan, 1973), and simple indole alkaloids from the species of a related genus, *Guiera senegalensis,* no other alkaloids are known to have been isolated from Combretaceae. This may be attributed to the inability of the separation methods to detect polar constituents. This study will attempt to search for highly polar compounds (including complex alkaloids) in this family.

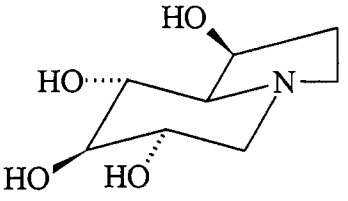


(74)

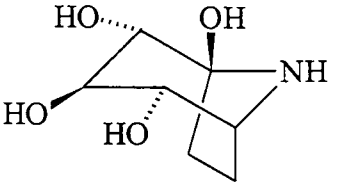
Fig. 3.14. 4-Hydroxyproline betaine, an alkaloid from the leaves of *C. micranthum*

Alkaloids may possess a large range of heterocyclic ring systems - pyrrolidine, piperidine, pyrrolizidine (two fused pyrrolidines), octahydroindolizine or indolizidine (fused piperidine and pyrrolidine) and *nor*tropane. The polyhydroxylated alkaloids (**75**) and (**76**) are of immediate interest in this study because of their potential as chemotherapeutic agents (Nash *et al,* 1997). In most cases these go undetected because they do not react with commonly used spray reagents such as Dragendorff's and iodoplatinate, they are not readily soluble in solvents such as chloroform (Watson, 1999) and they are not easily detectable by UV/visible detectors in standard HPLC.

Because of improving techniques in the isolation of polyhydroxylated alkaloids, they have been recently shown to occur in some species of Moraceae, Myrtaceae, Solanaceae, Araceae and Leguminosae. The increase in new reports of this sort suggests that there is a lot of potential for similar discoveries of novel alkaloids if the search is widened to other species.



(75)



(76)

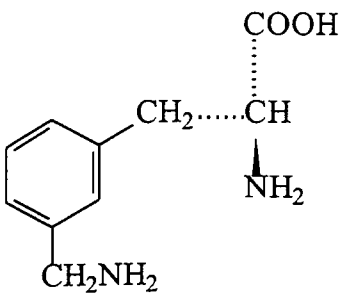
Fig.3.15. Some *nor*tropane polyhydroxy alkaloids, castanospermine (**75**) from *Castanospermum* and *Alexia* sp and calystegine B2 (**76**) isolated from *Calystegia sepium* and *Ipomoea* sp.

To a large extent, the search for alkaloids in this study is a stab in the dark – success depends on chance and serendipity. However, it will be extremely useful even if the results are negative because this will establish the case for future work.

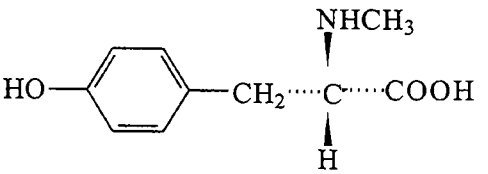
3.1.5.6. OTHER CONSTITUENTS

The fruit of *C. zeyheri* has shown the presence of the amino acids, L-3-(3'–hydroxymethylphenyl) alanine (**77**), its glucoside and L-N-methyltyrosine β-D-glucopyranoside (**78**) (Panzini *et al,* 1993).

The hexane extract of *C. apiculatum* fruit produced a compound identified as the anti-oxidant, 2,6-di-*ter*-butyl-p-cresol (BHT), also isolated from *C. zeyheri* leaves (Panzini, 1993).



(77)



(78)

Fig.3.16. (Aminomethyl) phenylalanine and N-methyltyrosine isolated from the seeds of *C. zeyheri.*

This compound is toxic to brine shrimp but there are doubts about its authenticity as a plant metabolite, though it is reported to have been isolated from the family Annonaceae (Yang *et al*, 1992). It is likely to an accidental contaminant which has been passed as a natural product (Waigh, personal communication, 2001).

This study is not bioassay-guided, so any compounds isolated will be of use in the pharmacological work involved and also will be an addition to the phytochemical survey of the Combretaceae, this being one of the objectives as discussed previously in Chapter Two.

CHAPTER FOUR

4.0. MATERIALS AND METHODS

4.1.1. GENERAL

The plant material for this study was collected in Zimbabwe during the month of May 1999, which is after the harvest and before the cooler, dry season. Twelve species were collected in this sub-tropical region of Southern Africa. Geographically, Zimbabwe ranges from high altitude, clay loamy soils and high rainfall to drier, hotter, sandy, low-lying climes and has intensive agricultural and mining activity traversing the country from south-west to the north and east towards Zambia and Mozambique. This region is ecologically classified as sub-tropical savanna (Moll and Moll, 1994).

The plants were collected in the Eastern Highlands, in Beatrice and Chivhu to the south-east Harare which are areas of intensive crop farming, and in the drier, sandy and hotter locations in the Northern Lowveld, Zambezi Valley, bordering Zambia around Guruve and Mahuwe, areas adjacent to the mining belt.

The plant material, once collected, was sun-dried *in loco*, with further oven-drying where appropriate after shipment to the Phytochemistry Research Laboratory at the University of Strathclyde, Glasgow. Where possible, all the major anatomical parts of the plant were collected as it is known that different plant parts may have different constituents. There was no particular reference to ethnomedical information concerning the plant parts used

by herbalists because a generalised phytochemical and biological screening was envisaged. Voucher specimens were deposited with the National Herbarium at Harare, Zimbabwe as well as in the plant store, Phytochemistry Research Laboratories at the University of Strathclyde.

4.1.2. PROCESSING OF NON-POLAR CONSTITUENTS

The phytochemical work was carried out by routine separation and chromatographic techniques. In all cases the material (leaves, fruit or stem bark) was ground separately (unless otherwise stated). It was then defatted by *n*-hexane for 1-2 hours with agitation. Thereafter the material was macerated in dichloromethane (DCM) and subsequently ethanol (50%) in water (E/W) over several days. The mixture of solvent and extract was decanted off, and maceration repeated with fresh solvent in order to increase the extracted yield. After filtering the material was re-extracted to increase the extracted yield. In general, approximately 3 litres of solvent was used to extract 2 kilograms of crude material. The fractions were dried down using a rotary evaporator and weighed in order to ascertain the yield which was found to be generally between 10-30% of the crude material in DCM fractions and approximately 25-40% in the E/W fractions.

Various experimental methods were used to purify the constituents. The main purpose of the phytochemical work was to isolate pure compounds, but an underlying aim was to try to find different methods of doing this and settling for the most efficient and effective of them. In the non-polar isolation work, various methods were tried out at different times.

Some of them were discarded as soon as problems appeared due to lack of time. Two of the more successful methods are described below:

1. DCM extracts were loaded on to Sephadex LH-20 and slowly eluted initially with trichloromethane (TCM), then increasing amounts of methanol. TLC plates were used to analyse the components of the fractions. Further separation was attempted by running smaller columns or by crystallization.

2. An initial clean up of the DCM extract was carried out by drying on pretreated Dialon HP-20 resin. This is a styrene-divinylbenzene which binds to organic compounds of small molecular weight e.g. chlorophylls and fatty acids.

The sample material was dried onto loose HP20 resin and dried on a rotary evaporator until free-flowing. This was then packed into a Buchner funnel and eluted with 10% acetone in methanol under vacuum. The eluent was dried using a rotary evaporator and then fractionated by gel filtration on Sephadex LH-20. In this case the fractionation was rather fast, done over a few hours resulting in crude 'cuts' which were analysed by TLC. Waters Preparative (Prep) 2000 HPLC (on NovaPak C18 reversed-phase preparative column) coupled with a UV-Vis detector was used for isolation of compounds after a suitable method was developed on an analytical column. LC-MS-ESI was used as a quality monitoring process and the chromatograms were obtained using a Waters Integrity system.

4.1.3. PROCESSING OF POLAR CONSTITUENTS

The polar extracts were more demanding in their isolation. The 50% ethanol/water (E/W) fractions were passed through a Dowex-50 H^+ packed column. An ion exchange material is defined as an insoluble matrix containing labile ions able to exchange with ions in the surrounding medium without any major physical change taking place in its structure (BDH Laboratory Manual, 1981). An advantage of the technique is that it allows the isolation of non-solvent extractable natural products, long neglected because of the difficulties associated with their isolation (Dufresne, 1998).

Ion exchange resins fall into two categories – cation and anion exchangers. These may further be divided into strongly acidic and weakly acidic cation exchangers and strongly basic and weakly basic anion exchangers. The terms "strong" and "weak" are used to refer to the extent of the variation of ionisation with pH (Watson, 1999). Strong exchangers are completely ionised over a wide range of pH, whereas the degree of dissociation of weak ion exchangers varies markedly with pH and this can affect their ability to take up exchangeable counter-ions. The extent of ion exchanging is affected by the type and accessibility of the functional groups in the exchanger, the nature of counter-ions and the selectivity towards them of the charged functional groups and the concentration and ionic strength of the eluent.

The separation of similar compounds in ion exchange chromatography is obtained by reversible binding. Samples are applied to the ion exchanger typically packed in a glass

column where adsorption occurs and the unbound substances are washed out from the exchanger bed before sequentially eluting the bound substances using a suitable eluent. Compounds can be separated since they display different affinities for the ion exchanger due to differences in their charge. The affinities can be controlled by varying conditions e.g. ionic strength by using a different type of resin or by varying the pH of the eluent. The matrix used in this study for the initial bulk extraction (Dowex-50W-X8) is a cross-linked polystyrene with sulphonic ($-SO_3H$) functional groups. It is a strongly acidic cation exchanger which can be used in either the free acid or hydrogen (H^+) form or in the salt form e.g. sodium (Na^+) or ammonium (NH_4^+) salts. Both forms of the resin adsorb cations from solution and release an equivalent number of counter-ions back into solution (H^+, Na^+, or NH_4^+ ions, depending on the form of the resin used). The NH_4^+ resin adsorbs basic compounds and thus when it is used in the form of Dowex 50W-X8 can be used to separate polyhydroxylated alkaloids and other basic compounds from acidic and neutral compounds.

The Dowex-50 column was prepared thus: the volume of the column was determined by the quantity of material to be loaded, typically 10ml per 10g of plant material. Once packed, an excess of 2M HCl was used to wash the Dowex 50W-X8 resin (50 – 100 mesh size) to ensure complete conversion to the H^+ form. The excess acid was removed by extensive rinsing with distilled water. The 10ml column of resin was washed with 75ml of 50% ethanol solution. The filtered extract was run down the column slowly, the pH being constantly checked to ensure that it remained acidic.

The unbound fraction was collected and processed as reported in Section **4.1.3.2.**

The bound fraction was processed as described below.

4.1.3.1 Bound Fraction

The column was washed with 150ml of 50% ethanol solution followed by 150ml of distilled water. It was then eluted with 100ml of 2M ammonium hydroxide (NH_4OH) to retrieve the bound fraction (^{P}X -1). This was collected and quickly reduced to dryness by using a rotary evaporator and freeze-drying. A small amount was transferred to vials for analysis by GC-MS. Further separation of these fractions was done on CG50 resin.

CG50 is a weak cation exchanger which was prepared by soaking the resin in 2M HCl and then rinsing with water in a beaker overnight. It is then converted to the NH_4^+ form by adding 2M NH_4OH. It was then packed into an appropriate size column and allowed to settle. The finer particles were removed from the top after the sedimentation to avoid blockage. Distilled water was then run down until the pH was between 9 and 10.
The sample was dissolved in a minimum of water and filtered, then packed into the column and eluted with 9 x 50ml aliquots of distilled water, and 250ml of 2M NH_4OH in that order. The fractions were dried on a rotary evaporater at less than 45°C and then finally by freeze drying. Their purity was checked by GC-MS and High Voltage Paper Electrophoresis (HVE). The resins chosen for further purification depended on the electrophoretic properties of the compounds selected for further work (Nash, personal communication, 2000).

4.1.3.1.1. High Voltage Paper Electrophoresis (HVE)

High Voltage Paper Electrophoresis (HVE) was used in the study initially for obtaining a profile to determine the presence of polyhydroxy alkaloids and other amines, and then subsequently to monitor the progress of the isolation process.

HVE uses an electric field to separate a mixture of ionic compounds on paper moistened with buffer. Each ion will migrate towards an attracting pole according to its charge and size with cationic and anionic compounds migrating towards opposite electrodes at varying speeds. The larger the hydrated ion the slower its electrophoretic mobility.

In practice, a vesssel is set up with two buffer compartments which each contain an electrode and a filter paper moistened with a buffer solution is positioned to complete the circuit by making contact with the solution in each of the compartments. When an electric field is applied to the system, a current flows across the paper with the buffer salts acting as conductors. The pH of the buffer can greatly influence the electrophoretic mobility of the substances to be separated, especially when amphoterics (e.g. amino acids) are involved. Therefore, the buffer pH can be chosen to favour separation of specifically targetted ions, and the current is applied for controlled periods to enable optimum separation to discrete spots to occur. The paper is then rapidly dried and sprayed with suitable reagents to locate the separated compounds in much the same way as analytical TLC plates are treated.

The test substances were spotted onto Whatman chromatography paper, grade 1 (23 x 57cm), which had been previously wetted in a buffer at pH 1.9 of a mix of ferric chloride/acetic acid. A standard solution of 1mg/ml aqueous arginine and aspartic acid was also spotted as a control.

The prepared paper was then placed on the flat plate of an high voltage electrophoresis apparatus (Shandon Southern, Runcorn, UK) and connected to electrode compartments containing the same buffer using wicks made from Whatman 3MM chromatography paper. The paper was subjected to electrophoresis at 3-5kV for 30- 40 minutes and then oven-dried at 105°C for 10 minutes.

The papers were stained by dipping in a solution of ninhydrin in acetone (0.2%w/v) and heating at 105°C for 2-3 minutes (Smith, 1969). Compounds containing a primary or secondary amino group attached to an aliphatic carbon tend to react strongly with ninhydrin. A purple colour is generally given by primary amines but secondary amines produce yellow or green colours. Tertiary amines sometimes give weak gray or brown colours (Nash, personal communication, 2000).

4.1.3.1.2. Gas Chromatography - Mass Spectrometry (GC-MS)

Specific identification of molecules is more certain with the use of gas chromatography coupled to a mass spectrometer (GC-MS). The information on MS is adapted from Williams and Fleming, 1995.

In electron impact mass spectrometry (EIMS) the effluent from the gas chromatograph (GC), which contains the separated and vaporised compounds, passes into the ion chamber of the mass spectrometer which is under a high vacuum. The molecules are bombarded by a beam of electrons accelerated from a filament which ionises and fragments them. Initially, one electron is removed from each molecule to form a positively charged molecular ion (M^+). Breakage of bonds relative to bond strength occurs rapidly in the molecular ion to generate fragment ions. The various ions are accelerated into the analyser portion of the mass spectrometer where they are sorted according to their mass to charge ratios (*m/z* values) which are equivalent to the molecular weights of the fragments. The ion signal is amplified by an electron multiplier and the mass spectrum is plotted from low to high mass. The *m/z* values are plotted against relative abundance of the ions. The most abundant ion (base peak) in the spectrum is assigned as 100%.

The conditions for the GC separation of polyhydroxy alkaloids were fine-tuned to improve resolution. A capillary column was used instead of a packed column because of their superior resolving power for complex mixtures. In capillary columns the stationary phase is coated on the inner wall of the tube. The optimum sample volume required for a capillary column is less than that required for analyses using packed columns (less than 0.1μl). Since it is not possible to accurately measure such small volumes manually, split valve injectors are generally used with capillary columns in order to reduce the proportion of the sample injected which actually enters the column in a controlled

manner. A temperature programme was also used as this allowed the separation of compounds of a very wide boiling range more rapidly than could be achieved by isothermal operation of the column.

Experimentally, the samples were prepared thus: after freeze-drying the samples in the vials they were sealed to prevent water entering and treated with a mixture of the silylating agent, Sigma-Sil A™ (trimethylchlorosilane-hexamethyldisilazane-pyridine, 1:3:9) (Sigma, UK) which was added to the sample (bound fraction) in sufficient quantity to make up a final concentration of 1μg/100μl. The sample was shaken well and then heated at 60°C for 30 minutes. It was then centrifuged at 2000rpm at 15°C for 25 minutes. The supernatant was transferred to a clean GC vial and centrifuged again at the same settings. It was then put into the GC-MS autosampler to run.

GC analyses were performed using a Perkin-Elmer Model 8500 Gas Chromatograph fitted with a BPX-5 capillary column (12m x 0.22mm i.d. x 0.25μm polydimethylsiloxane stationary phase, Phase separations, UK.). The pressure of the helium carrier gas was 10psi. The method used was modified from that of Nash *et al* (1986). The temperature programme for the column consisted of an initial isothermal period (at 180°C for 2min), followed by a linear temperature rise to 300°C at 20°C/minute, which was then held up to 26 minutes when cooling to 100°C occurred for the next injection. The total length of the programme was 30 minutes. Electron impact mass spectrometry (EIMS) of the column effluent was carried out using a QMASS 910

quadrupole MS (Perkin-Elmer) set at a constant temperature of 290°C. The mass spectrometer was programmed to incorporate a filament delay of 3.33 minutes in order to prolong the life of the filament and increase sensitivity. The samples were introduced onto the column of the GC using a split valve injector set at 100% (sample size 1.0μl) and a temperature of 260°C. Data were collected up to 20 minutes and re-injection occurred after 30 minutes. After the filament delay of 3 minutes, mass spectral data were acquired for 17 minutes.

4.1.3.2. Unbound Fraction

The unbound fraction from Dowex 50 (H^+) was collected and cleaned up by the HP-20 'scavenging' method. This involves passing the unbound solution through a Buchner funnel packed with Dialon HP-20. The unretained fraction (^{P}X-2) was further fractionated by liquid – liquid partition resulting in ethyl acetate (^{P}X –2–E), *n*-butanol (^{P}X–2–B) and water (^{P}X–2–W) fractions. This was analysed by HPLC using a Waters Analytical 2000 HPLC and considered for preparative HPLC. The HP-20 – bound extract was eluted with 10% acetone in methanol and then dried by rotary evaporater (^{P}X -3) at 20°C.

^{P}X -3 was further fractionated by flash chromatography using the Flash 40 (Biotage ™) 12mm x 15.0cm system using polyethylene cartridges pre-packed with KP-Sil ™ 32 - 63μm, 60Λ silica. In brief, the Biotage ™ method involves the following protocol:

The sample (^{P}X -3) was packed into the sample injection module (SIM) as a slurry mix with silica gel (60H) for column chromatography (Merck, UK). Approximately 1:3 sample-silica ratio. The system (set up as shown in Figure **4.01**) operates under pressure of nitrogen 5–10psi which pushes the solvent through the system.

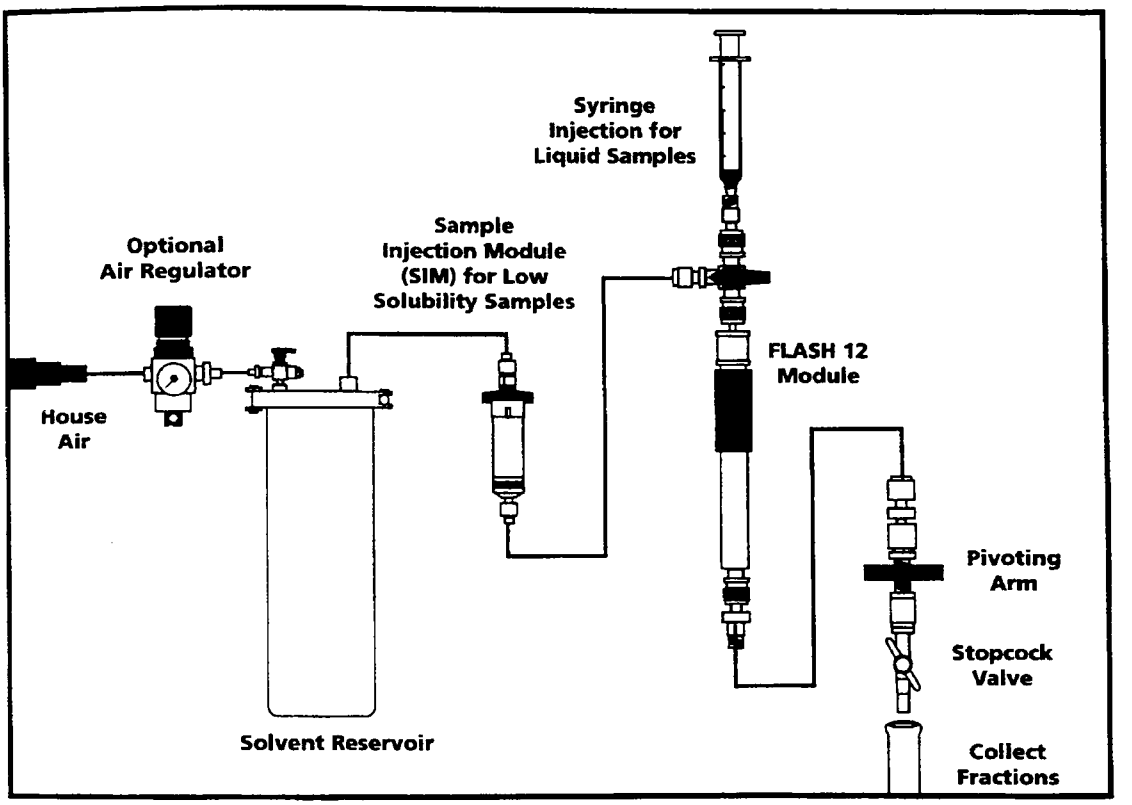


Fig. 4.01. The Flash 40 Biotage ™ System

The sample was dissolved in a little solvent and then silica added. The mix was dried on a rotary evaporator and the slurry tightly packed into the SIM and held in place by a frit.

The SIM was then inserted into the hold and hooked up to the solvent lines. A gradation of solvents of increasing polarity was used starting with 400ml of 75% hexane in ethyl acetate, then 200ml of increasingly polar hexane/ethyl acetate and ethyl acetate/methanol mix. All in all 11 fractions were collected, dried using the rotary evaporater and analysed by HPLC for possible preparative chromatography work.

4.2. SPECTROSCOPIC METHODS

4.2.1 GENERAL

NMR spectra were recorded with either a Bruker DRX500 NMR spectrometer equipped with an auto-tune probe and using the automation mode aided by the Bruker programme, Icon-NMR, or Bruker AMX 400MHz NMR instruments.

Ultra-violet (UV) spectra (where necessary) were obtained along with the mass spectra from a methanol solution of the sample by Waters Integrity™ LC-MS System with ThermaBeam Mass Detector and 996 Photodiode Array Detector

.

Infra-red spectra were recorded as KBr discs or drying trichloromethane dissolved sample on NaCl windows by a Matson Genesis series FT-IR spectrophotometer.

Melting point (Mp) measurements were done using the Reichert Apparatus (Austria). A small sample is mounted on a heating element with a thermometer under a microscope.

The sample is observed until it melts and the temperature reading at this point is recorded as the Mp. This method is rather subjective because it depends on the observer deciding almost arbitrarily the point at which melting first occurs and different samples appear to melt differently under the microscope. It was used because of the severe limitation of the quantity of sample available.

HREIMS were run on a JEOL JMS-AX505HA double focusing instrument at 70eV. FABMS were also run on this instrument using glycerol as the matrix.

4.2.2. NMR SPECTROSCOPY

The phenomenon of nuclear magnetic resonance (NMR), first observed by Purcell *et al* in 1946 (Ganssen, 1989), has become a most versatile and powerful technique for application in organic chemistry (Williams and Fleming, 1995). The details given below are adapted in the main from Williams and Fleming (1995), Friebolin (1990), Kemp (1986), Homans (1989) and Agrawal (1989).

4.2.2.1 ONE-DIMENSIONAL SPECTROSCOPY

Proton magnetic resonance (PMR / ^{1}H NMR) is widely employed. The spectrum appears predominantly in the range 0–10ppm downfield from the reference signal of trimethylsilane. The integral of the signal is proportional to the number of protons it represents, and the nature of the hydrogen is established by the chemical shift.

Carbon magnetic spectroscopy (CMR/ ^{13}C NMR) has developed into the most valuable tool available for structure elucidation of organic molecules. Due to the low abundance of the C-13 isotope, sensitivity in acquisition is always a problem. The pendant and J-modulated techniques were utilised in this study. These have the advantages of being able to distinguish and segregate the evenly protonated carbon (i.e. quaternary carbon and methylene signals), which are normally displayed pointing upwards, from the oddly protonated carbon (i.e. methines and methyls) which will point downwards.

4.2.2.2 TWO-DIMENSIONAL SPECTROSCOPY

Two-dimensional (2-D) spectroscopy is a more recent innovation. The spectrum contains signals dispersed according to two characteristic frequencies rather than one so that the number of distinct signals which can be resolved are more than in a normal 1D spectrum.

In 'resolved' 2-D experiments, chemical shifts and hetero-or homonuclear spin couplings are separated into two dimensions. The 'correlation' experiments meanwhile differ from resolved experiments in that they contain a mixing period during which coherence is transferred or evolves in the spin system. The result is a 2-D spectrum exhibiting connectivities, i.e. cross peaks between signals from coupled spins. The most common method of presenting this data is by contour plot as it is able to cope with crowded spectra and also allows easier determination of the frequency co-ordinates of peaks.

4.2.2.2.1. HOMONUCLEAR CORRELATIONS

a) ^{1}H- ^{1}H Correlation Spectroscopy (COSY)

COrrelated SpectrocopY (or COSY) is a 2-D experiment which shows all the spin-spin coupled protons. It consists of two non-selective radiofrequency (rf) pulses between which there is a variable delay, t_1. Data are acquired after the second pulse, during a further period, t_2. Two essentially identical chemical shift axes are plotted orthogonally. The diagonal peaks and cross peaks form the corners of a square and the cross peaks always occur when two nuclei interact with each other through a scalar coupling.

4.2.2.2.2. HETERONUCLEAR CORRELATIONS

Heteronuclear correlation spectroscopy is analogous to COSY. However, a different experimental regime is required since two types of nuclei with different Larmor frequencies are involved. It differs from COSY only in that the second pulse is applied simultaneously to both nuclei. It can be used to work out one-bond correlations or longer distance correlations and thus aids in building up a picture.

a) Heteronuclear Multiple Quantum Coherence (HMQC) (^{1}H-^{13}C COSY)

This ^{1}H-^{13}C COSY spectrum allows the use of the carbon spectrum to unambiguously assign often severely overlapping proton signals. It has the advantage that even in complicated molecules there is little overlapping of the correlation peaks which combine the large chemical shifts of ^{13}C NMR and those of ^{1}H NMR spectroscopy.

b) Heteronuclear Multiple Bond Connectivity (HMBC) (Long range ^{13}C-^{1}H COSY)

In this sequence, a time delay of about 50ms in the pulse sequence is used to determine two and three bond coupling. It is crucial in constructing extended pieces of the molecular skeleton which can be joined together to complete structure elucidation.

4.2.2.2. NOESY SPECTROSCOPY

The nuclear Overhauser effect (n.O.e) results from through-space interactions of magnetic nuclei. It is defined as a change in the integrated NMR absorption intensity of a nuclear spin when the NMR absorption of another spin is saturated. It allows moieties that are spatially proximal (but may be too far to couple through bonds) to be located and it makes a major contribution to the determination of stereochemistry.

4.3. BIOLOGICAL ASSAYS

For the investigation of plants to establish their biological activity, a suitable and robust test procedure must be used (Hostettmann *et al*, 1996). The test should ideally be simple, rapid, reproducible, reasonably priced and sensitive while at the same time it limits the false positives to a minimum. Of special relevance in phytochemical work is the solubility of the sample as this determines the outcome.

4.3.1. ASSAYING FOR ANTI-MICROBIAL ACTIVITY

4.3.1.1 Well Diffusion Assay (WDA)

Seeded strains were prepared either as a cell suspension from a fresh slope in quarter-strength Ringers solution (OXOID BR52) or from a fresh stationary culture, in both cases diluted to approximately 1.0 OD_{600nm} (approx. 1.5ml–8.5ml ringers).

The molten agar was inoculated to give a final inoculum of 1ml/50ml. The agar was poured into square plates and allowed to settle into a completely flat surface and solidify for 15-20 minutes. Nine wells were cut using a sterile 9mm borer and the plugs removed using sterile cocktail sticks. The test samples were made up by solubilizing 1mg of compound in no more than 0.5ml of DMSO and making up to 6.5ml with distilled water to give a 150µg/ml solution. 100µl of this solution was then pipetted into the wells. The plates were duplicated to give a repeatable result and a mixture of 0.5ml DMSO in 6.0ml of water was used as a control. The plates were incubated at the required temperature for 24- 48 hours. The clearing zone (zone of inhibition) was measured using a Biorad GS-710 scanning densitometer on transmission mode.

For the yeast assays, Yeast Methylene Blue agar was used, for Salmonella, Listeria and *E. coli,* iso-sensitest agar (OXOID CM 471) was used while MRS agar (OXOID CM 361) was used for lactic acid bacteria. The positive controls were pimaricin 0.25% for yeasts, and chloramphenicol 0.025% for *E.coli*, Salmonella, Listeria and lactic acid bacteria. Listed below are the test micro-organisms.

Yeasts

NCYC 1006 – *Saccharomyces cerevisiae*
NCYC 388 – *Candida glabrata*
NCYC 575 – *Kluyveromyces lactis*
NCYC 1466 – *Candida albicans*
NCYC 1400 – *Zygosaccharomyces bailii*
NCYC 327 – *Candida valida*
NCYC 492 – *Torulaspora delbruecki*
NCYC 137 – *Candida holmii*
NCYC 389 – *Candida famata*

Bacteria

3704 – *Escherichia coli* 0157 (non-toxigenic)
NCIMB 13034 – *Salmonella typhimurium*
Listeria monocytogenes (IGER culture)
Powerstart (GENUS silage inoculant – lactic acid bacteria)

Yeasts isolated from spoiled silage

KL 36 – *Pichia anomala*
KL 128, KL 133, KL 102 - *Unidentified*
KL 6 –*Saccharomyces servazzii (*NCYC 2775*)*

4.3.2.2. Microtitre Dilution Assay (MDA)

The Well Diffusion Assay has various limitations in phytochemical work. These include the possible non-diffusion of the test samples into the agar, its laborious and time consuming nature and the relative cost of material. Drummond and Waigh (unpublished 1999) have adapted the Microtitre Dilution Assay for use with phytochemical compounds. It is partly based on methods used previously by Fabry *et al* (1998), Silva *et al* (1997) and Martini and Eloff (1998) and adapted from the susceptibility tests recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (1990).

This method uses 96-well microtitre plates and a dye which changes colour depending on the inhibition of growth. It is an economical low-technology approach and has the other advantages of being easy to set up, rapid in the production of results and uses small

amounts of sample. It is, therefore ideal for high through-put screening of plant constituents. It was used in this study to complement the work done with the Well Diffusion Assay (WDA).

In brief, the indicator solution (resazurin) was prepared by adding one tablet to 40ml of sterile water. The test organism was prepared by diluting overnight broth culture with sterile diluent (0.1% Peptone water). The turbidity was adjusted to match 0.5 McFarland standard. The broth culture was then diluted hundred-fold to give a concentration 10^8 CFU/ml. The test sample was made up as a stock solution of 200µg/ml solubilized with less than 20µl of DMSO in sterile water.

A mixture of indicator and broth medium was prepared by adding 1ml of indicator solution to 7.5ml of broth medium. 100µl of this mixture was pipetted into sterility control wells in the 96-well plate. 5ml of the broth culture was then added to the mixture of indicator and broth medium made earlier and 100µl of this pipetted into test and growth control wells in plate. The test solution (100µl) was pipetted into well one of the plate, mixed well and 100µl was transferred into well two. This mixing and transferring was repeated until well ten and then the pipette tip was discarded with its contents.

The plates were incubated at 31°C for at least five hours and taken out at the time the dye changed colour for the first time to pink. The test wells were read high to low concentration and compared with the sterility and growth controls. The concentration in

the last well showing no colour change (blue) was taken as the minimum inhibitory concentration (MIC).

The organisms used in this study and their incubation times are indicated below:

Candida albicans	8-24hrs
Esherichia coli	8hrs
Mycobacterium fortuitum	24-48hrs
Proteus vulgaris	8hrs
Staphylococcus aureus	8hrs

CHAPTER FIVE

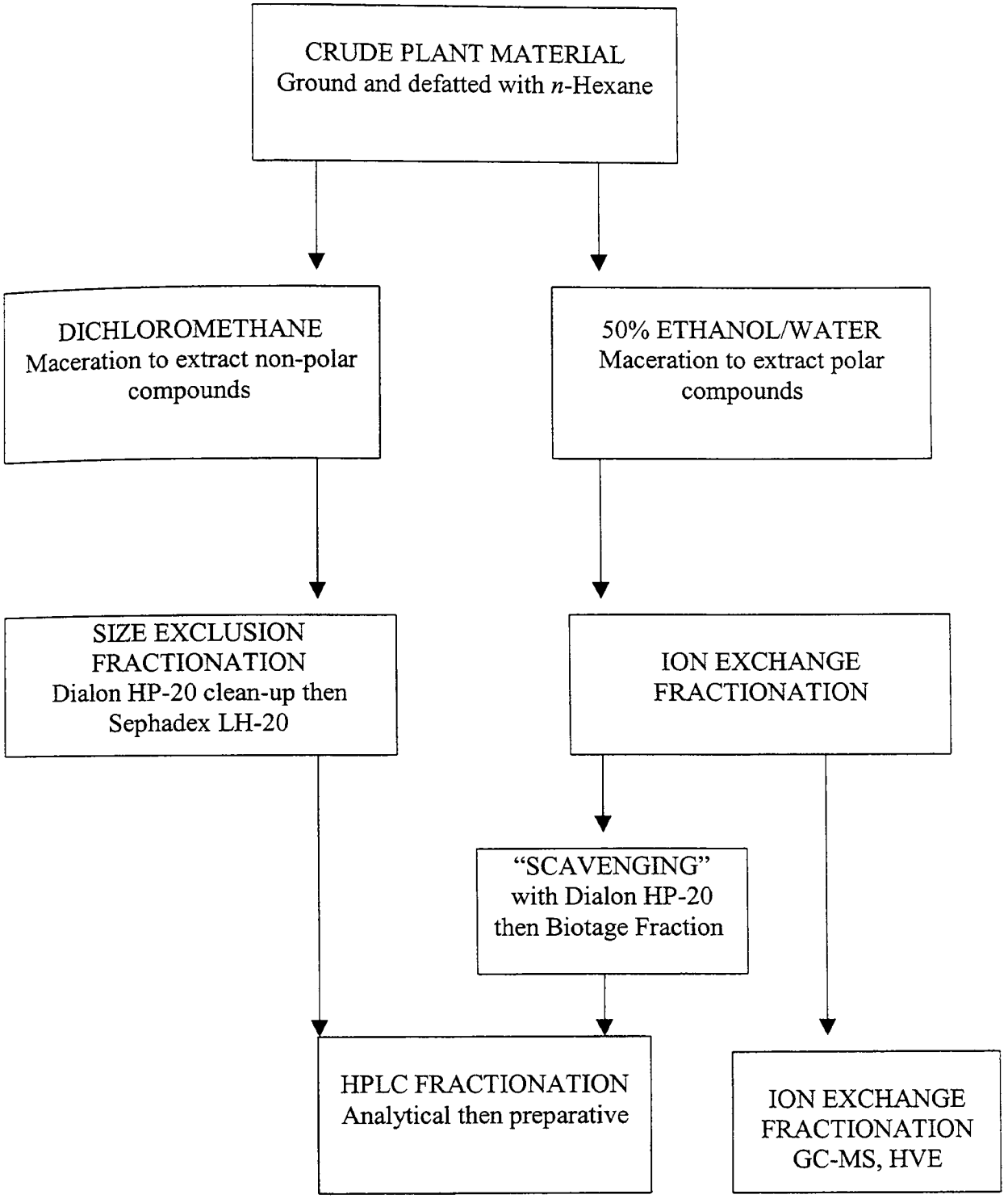
5.0. RESULTS AND DISCUSSION

The results of the phytochemical work are given, interpreted and discussed in this chapter. Scheme **5.01** illustrates the methods used in fractionating the crude plant extract as outlined in Chapter Four.

More compounds were isolated from the non-polar work than from the polar work because the methods used are routine, rapid and more conventional. The attempt to isolate polar constituents was less successful in terms of through-put, but the few compounds isolated are reported in Combretaceae for the first time here.

5.1 NON-POLAR FRACTIONATION

The compounds reported here were isolated from the DCM fraction using the methods outlined in Section **4.1.2**. Method 1 described earlier was used only in the work on *Combretum albopunctatum* and discontinued because it was found to be too slow. Method 2, which was faster and more efficient was then used for all subsequent work. In all cases the preparative HPLC method used was a gradient of water / acetonitrile with 0.1% Triflouroacetic acid (TFA) (90%/10%) to 0%/100% for 20-30 minutes



Scheme 5.01. The processing of plant material in this study.

Several compounds were isolated and characterized to be either phenolics or isoprenoids by these methods. The detailed information of the isolation work is described below for each one of the plants involved in this study

5.1.1. REPORT OF EXPERIMENTAL WORK

Combretum albopunctatum (AOD)

Leaves and fruit were collected weighing a combined total of 419.3g after drying. Of this, I57.0g was defatted and extracted, yielding 4.2g of DCM fraction, designated AOD. It was loaded onto a Sephadex LH-20 column and fractionated resulting in flavonoids AO-15, AO-14 and AO-18 on elution with 100% TCM. 10% MeOH/TCM gave AO-66 by recrystallization. When this fraction was put through preparative HPLC it yielded AO-68. These compounds are both chalcone dimers.

C. apiculatum (BID)

A dry weight of 1.134kg of leaves was collected and ground. Of this, 213.6g was defatted and extracted yielding 9.4g of DCM extract coded BID. This was put through the Dialon HP-20 clean up process resulting in 5.2g which was then loaded onto a Sephadex LH-20 column. HPLC separation of the fractions from Sephadex LH-20 yielded seven flavonoids and four stilbenoids.

C. collinum (DID)

Of 1.822kg of dry leaf mass, 270.5g was defatted and extracted resulting in 6.5g of DCM extract. This was cleaned up by Dialon HP-20 to give 2.85g which was then loaded on a Sephadex LH-20 column. Three compounds are reported here from the HPLC fractionation.

C. hereroense (CHE)

This is the only plant of which work was done on the fruit rather than the leaves. 234.1g yielded 17.1g of DCM fraction. Of this 4.2g was loaded on Sephadex LH-20. HPLC yielded four phenanthrenes.

C. imberbe (CIM)

Dry leaf material (762.0g) was collected and ground, of which 211.5g was defatted and extracted. The yield was 17.6g of DCM extract and upon cleaning only 3.9g resulted and was loaded onto Sephadex LH-20. HPLC fractionation resulted in four triterpenoids.

Terminalia stulhmanii (TST)

This was the only species of the genus *Terminalia* studied in this work and also the only report on contituents of the stem bark reported here. 751.6g of the dry stem bark yielded only 826.6mg of DCM extract. This gave 632.4mg of HP-20 –cleaned extract which was loaded onto a Sephadex LH-20 column. Four compounds were isolated of which only two were sufficiently pure and are reported here.

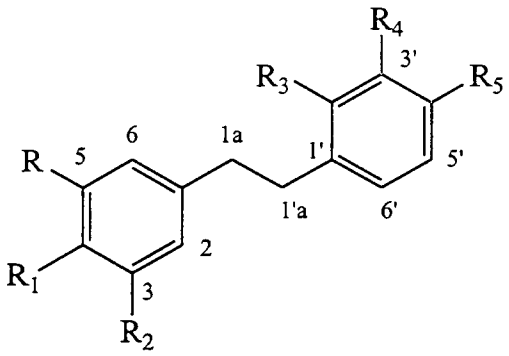
5.1.2 PHENOLIC COMPOUNDS ISOLATED

These compounds are presented here in different structural classes which are established to rationalise their biosynthetic and chemical relationships and to better understand the commonality of their chemistry and spectroscopic character.

5.1.2.1. STILBENOIDS

The term 'stilbenoids' will be used as a phytochemical classification that embraces the bibenzyl, stilbenes (or styrenes) and phenanthrene derivatives. The stilbenoids reported here are the bibenzyls (also known as dihydrostilbenes (Hart, 1981)). These synonyms will be used interchangeably here with 'bibenzyl' being the preferred term as it has been more widely employed in the literature. The stilbenoids are diphenylethanes in their general chemical structure, solid at room temperature with molecular weights between 200-300 and melting points in the range 150-300°C (Hart, 1981). They possess two aromatic rings which are connected by an ethane bridge. One or both of the rings are highly oxygenated with hydroxyl and methoxyl substituents.

Five bibenzylic compounds were isolated from two of the species investigated, four from *C. apiculatum* and a single one from *C. collinum*. They were identified as 4'-hydroxy-3,4,5-trimethoxybibenzyl (coded B14-6), 3-hydroxy-4',5-dimethoxybibenzyl (B14-7), 3,4'-dihydroxy-4,5-dimethoxybibenzyl (B27-16A), 4,4'-dihydroxy-3,5-dimethoxybibenzyl (B27-16B) and 4'-hydroxy-3',3,4,5-tetramethoxybibenzyl (D3-9). See Fig. **5.01.** below. This appears to be the first report of bibenzyls in *C. collinum.*



Compound	R	R_1	R_2	R_3	R_4	R_5
B14-h6	OCH_3	OCH_3	OCH_3	H	H	OH
B14-h7	H	OCH_3	H	OH	H	OCH_3
B27-16A	OCH_3	OCH_3	OH	H	H	OH
B27-16B	OCH_3	OH	OCH_3	H	H	OH
D3-9	OCH_3	OCH_3	OCH_3	H	OCH_3	OH

Fig. 5.01. Chemical structures of bibenzyls isolated in this study.

General characterisation of isolated bibenzyls

IR spectral data which were common to all the compounds indicated aromatic rings (*ca.* 3000, 1630-1595cm^{-1}), with multiple substitution (*ca.* 900-720 cm^{-1}) and hydroxyl (*ca.* 1040-30 cm^{-1}), methylene and methoxyl groups (*ca.*2940-20 cm^{-1}) (Williams and Fleming, 1995)

The ^{1}H NMR (Table **5.01**.) shows peaks (δ_H *ca* 2.72 – 2.82) which are typical of the methylene of aryl-methylene (Ar-CH_2) (Kemp, 1986). The integration is for four protons (i.e. two methylenes which are each connected to a different benzene ring). The general splitting pattern is multiplet in nature (2-5Hz), but in B27-16 and B14-6 these peaks show up as singlets, which is accidental since they show cross-peaks in ^{1}H-^{1}H COSY implying that these methylenes are coupled to each other. The conclusion drawn is that they form an ethylenic bridge between two phenyl rings.

The coupling pattern of this bridge deserves some consideration. The AA'BB' system resulting from this coupling is typical of these compounds (Bai *et al*, 1993). Initially, it would appear to suggest some restriction in rotation, possibly due to the steric effects of the benzenoid C-2 and C-6 substituents (Bai *et al*,1996). Another possible explanation involves diastereoisomeric conformation (Fessenden and Fessenden, 1994). This is illustrated in Fig. **5.02** which is an end-on Newman projection of the bond between the two aryl-methylenes. This shows free rotation about the linking ethane moiety giving rise to different conformations according to the dihedral angle (Mislow, 1966) i.e. the staggered conformation, in which bond angle, θ is at its maximum 60°; the eclipsed, when θ is equal to zero, and the skewed, when θ lies between 0° and 60°. It was thought that these conformations resulted in the environment of the two methylene units being different leading to the four protons "perceiving" one another as being magnetically different and hence "splitting" each other.

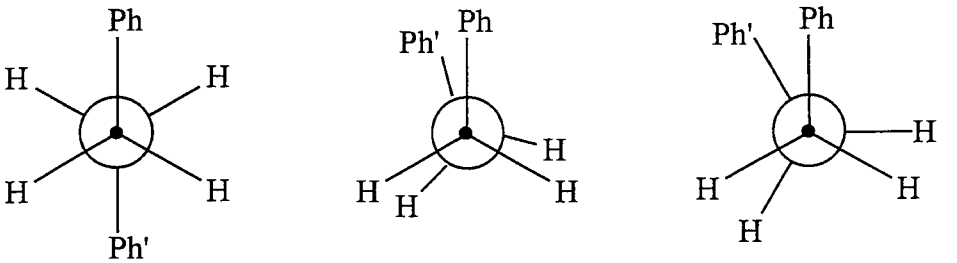


Fig. 5.02 Newman Projection illustrating diastereotopic effects which may possibly result in the complex AB quartet seen in ^{1}H NMR of bibenzylic compounds.

This explanation was not found to be entirely true mainly because of the plane of symmetry within the molecule. This is, in fact, an example of the AA'BB' splitting pattern arising due to the interaction of a pair of equivalent protons which have a variable relationship with each proton of another equivalent pair.

The occurrence of methoxyl substitution is obvious from the existence of O-methyls at characteristic δ_H values (*ca* 3.58-3.70). The number differed with each compound, and positions of attachment to the benzene rings were deduced from HMBC. The J-modulated (or PENDANT, in some instances) ^{13}C NMR and the HMQC were used to confirm the positions of the carbons. The methylene carbons invariably show up well resolved (δ_C *ca* 30-40), and the methoxyl carbons occur around δ_C *ca* 55 - 62 depending on the *ortho* substituents which may influence their spatial orientation (Pelter and *et al*,1976). In general, when the methoxyl is out of plane, it tends to resonate at lower field.

Despite the necessity for NMR data, the mainstay of the elucidation of bibenzylic structures is mass spectrometry as the first step towards solving the structure. The MS fragmentation of the bibenzyls and stilbenes produces few fragments and is diagnostic of the presence of two phenyl rings and also the substitution pattern (Wenkert and Gottlieb, 1976). Invariably two intense peaks appear which signal the cleavage of the benzylic linkage with the loss of keten (2 molecules) and the formation of stable tropylium moieties, one of which is the base peak (Letcher *et al*, 1972). See Fig.**5.03**. Once the substituents have been established, HMBC is then used to place them at their correct location around the aromatic rings.

Identification of B14-7

B14-7 (51.4mg) was isolated as a peach amorphous powder with t_R 15min on HPLC, mp. 165°C.

NMR spectra showed a complex multiplet (δ_H 2.73 – 2.83) of four protons indicating two aryl methylenes linked together as is typical in bibenzylic moieties, two clean singlets of methoxyl groups δ_H 3.75 (δ_C 56.0) and δ_H 3.7 1 (δ_C 55.9) as well as seven aromatic protons. Two doublets appear at δ_H 6.80 (2H, d,J=3, 8.5Hz) , δ_C 130.8 and δ_H 7.05, (2H,d, J=3, 8.5) δ_C 115.1 forming an AA'BB' splitting pattern characteristic of a *para*-disubstituted benzene ring. The remaining three protons are at δ_H 6.25, δ_C 109.5 (1H, J=3.5), δ_H 6.22 δ_C 107.0 (1H, J=2) and δ_H 6.21 δ_C 100.0 (J<5, poorly resolved).

HREIMS yielded the molecular ion [M^+] at *m/z* 258.12 (32%) corresponding to $C_{16}H_{18}O_3$ and the methyltropylium base peak [M-$C_8H_9O_2$]$^+$ at *m/z* 121.06, a very small peak *m/z* 137 (5%) [M-C_8H_9O]$^+$ as well as another peak at *m/z* 77 (8%) [M-$C_{10}H_{12}O_3$]$^+$. The two small peaks are due to the other hydroxymethoxytropylium, which is likely to lose the hydroxyl group and give the base peak, and the benzylic ion as illustrated. This fragmentation pattern is diagnostic of bibenzyls (Letcher *et al*, 1972). See Fig. **5.03**.

Table 5.01. ^{1}H NMR (500MHz) spectral data of the bibenzyls

Position	B14-6	B14-7	B27-16A	B27-16B	D3-9
1a	2.66(s)	2.74(m)	2.73(m)	2.75(s)	2.80(m)
2	6.26(s)	6.22(s)	6.33(d, 2)	6.35(s)	6.42 (s)
3	-	-	-	-	-
4	-	6.21(s)	-	-	-
5	-	-	-	-	-
6	6.26(s)	6.25(s)	6.27(d,2)	6.35(d, 8.5)	6.42 (s)
1'a	2.65(s)	2.81(m)	2.78(m)	2.75(s)	2.77 (m)
2'	6.82(d, 8,3)	7.05(d, 8.5,3)	6.97 (d,9,3)	6.93(d,8.5)	6.60
3'	6.54(d, 8,3)	6.80(d, 8.5,3)	6.71 (d, 9,3)	6.66 (d,8.5)	6.82 (d,8)
4'	6.54(d, 8,3)	6.80(d, 8.5,3)	-	-	-
5'	-	-	6.71 (d, 9,3)	6.66 (d, 8.5)	-
6'	6.82(d, 8,3)	7.05(d, 8.5,3)	6.97(d,9,3)	6.93(d, 8.5)	6.66 (d,2)
3-OCH_3	3.62(s)	-	-	3.74(s)	3.76 (s)
4-OCH_3	3.58(s)	-	3.75(s)	-	3.74 (s)
5-OCH_3	3.62(s)	3.70(s)	3.74(s)	3.74 (s)	3.76 (s)
3'-OCH_3	-	-	-	-	-
4'-OCH_3	-	3.74(s)	-	-	-
5'-OCH_3	-	-	-	-	3.82 (s)

All data obtained in CD_3OD. Coupling constant ***J*** in Hz is in parentheses.

Table 5.02. ^{13}C NMR (125MHz) spectral data of isolated bibenzyls

Position	B14-6	B14-7	B27-16A	B27-16B	D3-9
1a	40.1	39.9	39.9	38.4	39.7
1	139.9	145.9	139.8	138.3	139.9
2	107.4	109.5	110.6	105.8	107.4
3	154.6	159.7	151.6	147.9	154.5
4	137.6	100.3	136.2	133.0	137.5
5	154.6	162.6	154.6	147.9	154.5
6	107.4	107.0	105.8	105.8	107.4
1a'	38.6	38.3	38.6	37.3	38.7
1'	134.2	135.6	134.4	133.5	135.0
2'	130.2	130.8	130.9	129.3	120.6
3'	116.4	115.1	116.4	114.9	113.4
4'	156.9	159.7	156.8	155.3	147.7
5'	116.4	115.1	116.4	114.9	147.7
6'	130.2	130.8	130.8	129.5	117.2
3-OCH_3	57.6	-	-	59.9	57.0
4-OCH_3	61.5	-	57.1	-	61.7
5-OCH_3	57.6	55.9	61.4	59.9	57.0
3'-OCH_3	-	-	-	-	57.1
4'-OCH_3	-	56.0	-	-	-
5'-OCH_3	-	-	-	-	-

All data obtained in CD_3OD.

The occurrence of the base peak at *m/z* 121 indicates that one of the aryl rings has a methoxyl substituent, the other by implication has a hydroxyl group in addition. Thus this ring will have four protons while the other one will have the remaining three. The two doublets seen in ^1H-domain are suggestive of symmetry in ring B. The HMBC shows strong correlation between the two-proton multiplet δ_H 7.05 and the aryl-methylene carbon at δ_C 38.3. Conversely, the methylene protons at δ_H 2.81 (H-1'a) correlate to the carbon of these protons at δ_C 130.8 (C-2'/6'). H-2'/6' also correlate with the carbon at δ_C

159.7 (C-4') and demonstrate ^{2}J coupling to the carbon at δ_C115.1 (C-3'/5'). The two protons (H-3/5) which form a multiplet at δ_H 6.80 show ^{3}J correlation to the quaternary carbon at δ_C 135.6. Thus ring B has a *para*-methoxy-substituent. The protons which are *ortho* to the oxygenated substituent are known to move upfield (Pelter *et al*, 1976); in this case they resonate at δ_H 6.80 (δ_C 115.1).

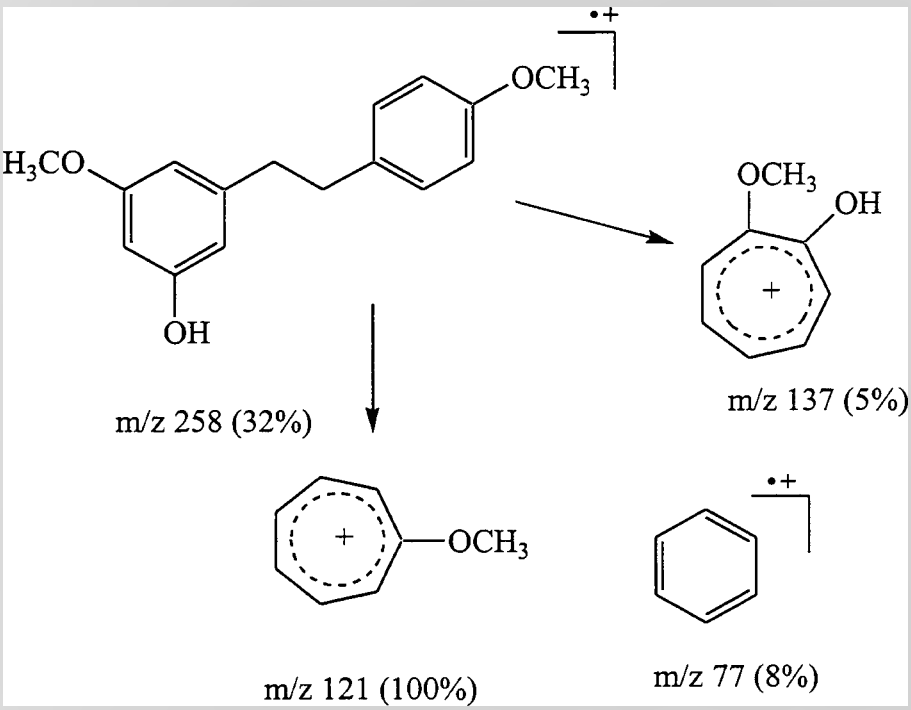


Fig.5.03. The fragmentation pattern of typical bibenzylic derivatives as exemplified by B14-7.

From MS, Ring A is known to possess a hydroxyl and a methoxyl substituent. Two of the aromatic protons are attached to carbons which appear very close in the ^{13}C –domain, resonating at δ_C 107.0 (δ_H 6.22) and δ_C 109.5 (δ_H 6.25). Their protons show HMBC

correlations to each other's carbon, to the methine at δ_C 100.3 (δ_H 6.21) and also, more importantly, with C-1a. The H-1a correlated to the methine carbon at δ_C 107.04 and δ_C 109.5. NMR shows no *ortho* coupling in this ring but 2,4,6 *meta*-coupling instead, with the methine at δ_C 100.3 (δ_H 6.21) sandwiched between two oxygenated substituents which explains why it is relatively shielded. ^{2}J coupling places the methoxyl substituent in position C-5 (δ_C 162.6). This is because H-6 (δ_H 6.22, δ_C 107.0) and H-4 (δ_H 6.21, δ_C 100.0) couple to this position in HMBC. Position C-3 (δ_C 159.7) is hydroxylated and shows ^{2}J correlation to H-2 (δ_H 6.25 δ_C 109.5) and H-4. This ring is thus typical of resorcinol substitution commonly resulting from the biosynthetic pathway of stilbenoids (Hart, 1981).

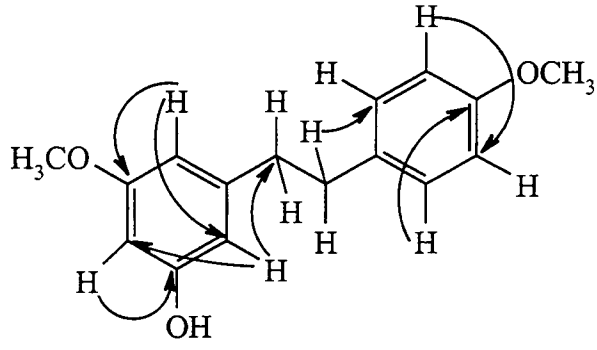


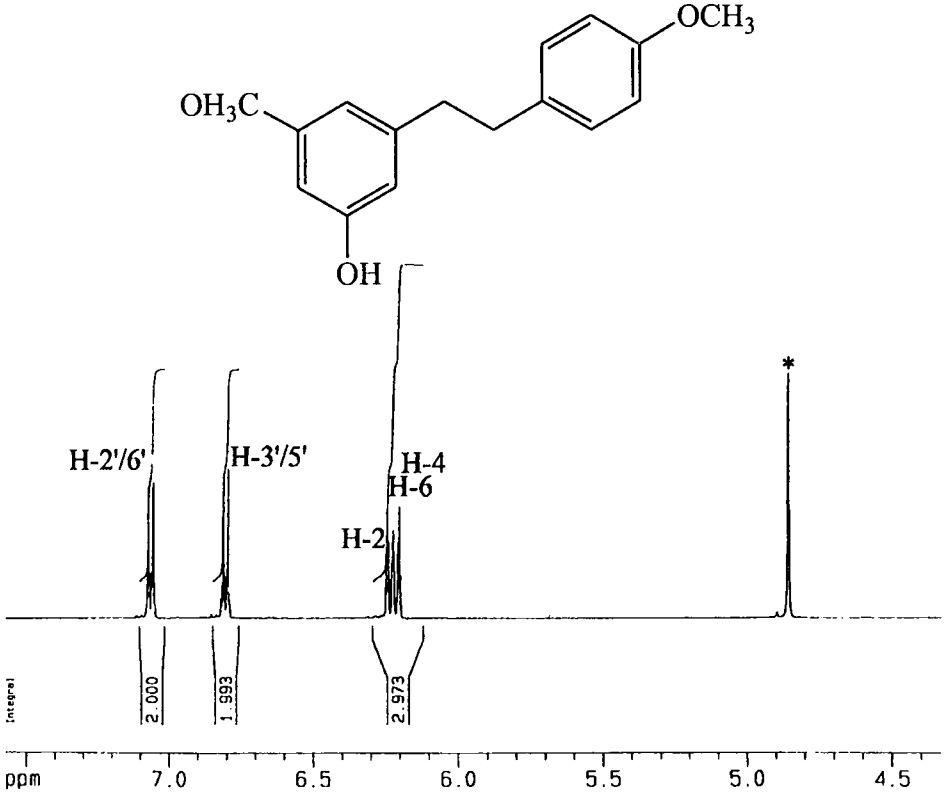
Fig.5.04. Significant HMBC correlations of B14-7

Table 5.03. HMBC correlations for B14-7 (in CD_3OD)

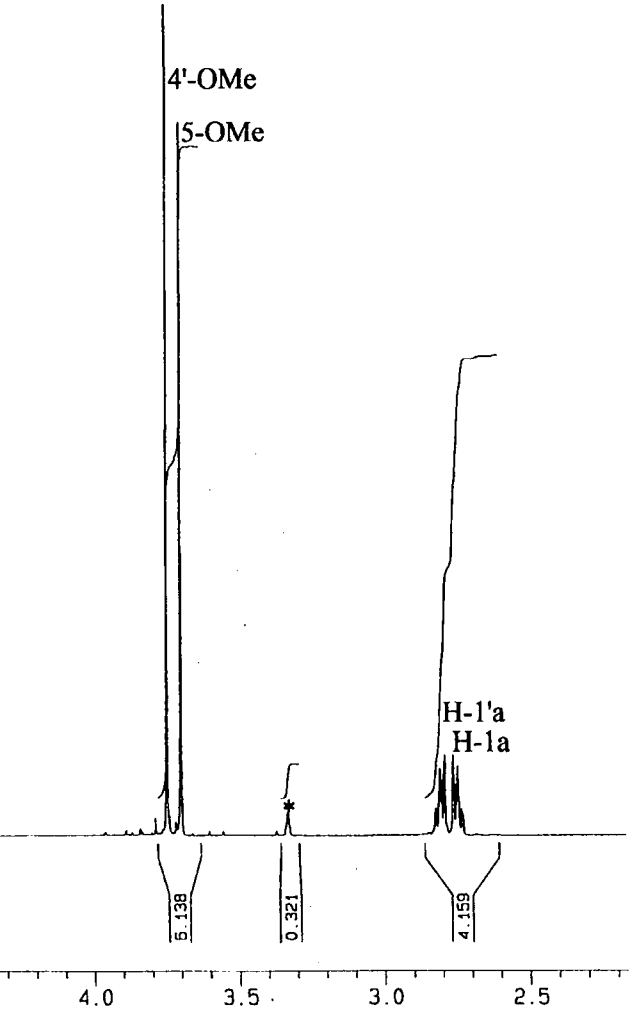
Position (δ)	^1J	^2J	^3J
H-1a (2.74)	39.9	-	135.5(C-1')
H-2 (6.25)	109.5	159.7 (C-3)	107.0 (C-6), 100.3(C-4), 39.9 (C-1a)
H-6 (6.22)	107.0	162.6 (C-5)	109.5 (C-2), 100.3 (C-4), 39.9 (C-1a)
H-1'a (2.81)	38.3	135.6 (C-1')	130.8 (C-2'/6'), 145.9 (C-1)
H-2'/6' (7.05)	130.8	115.1 (C-3'/5')	159.7 (C-4')
H-3'/5' (6.80)	115.1	159.7 (C-4')	135.6 (C-1'), 115.1 (C-5'/3')
5-OCH_3 (3.70)	55.9	-	162.6 (C-5')
4'-OCH_3 (3.75)	56.0	-	159.7 (C-4')

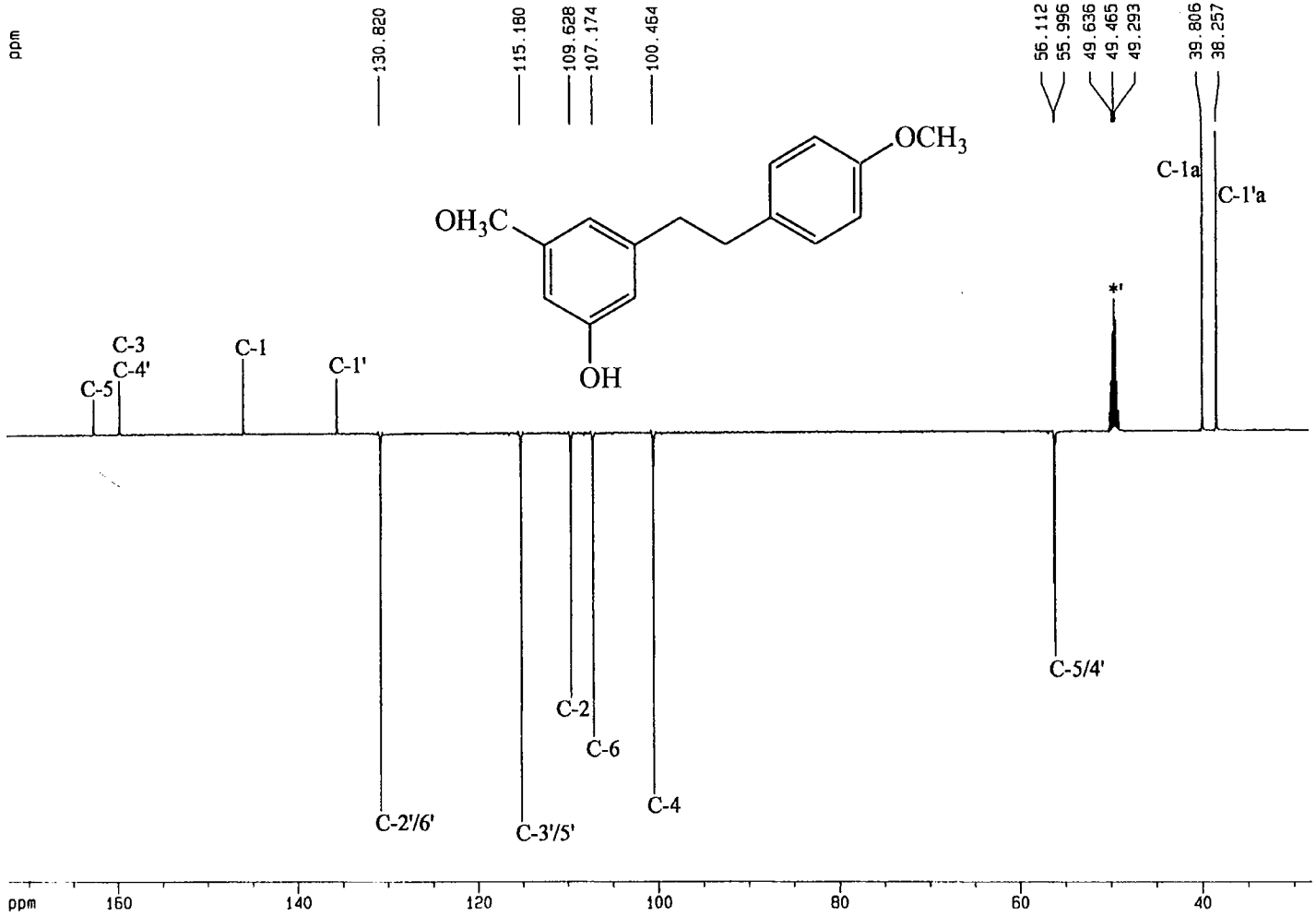
All data obtained in CD_3OD

Comparison of selected NMR data with the literature confirmed the coupling pattern and chemical shifts (Majumder et al, 1999). B14-7 was characterized as 3-hydroxy-4',5-dimethoxybibenzyl (IUPAC name- 5-hydroxy-3, 4'-dimethoxybibenzyl) with a calculated molecular weight of *m/z* 258.1261 compared to the measured weight of *m/z* 258.126. This is the first report of this compound. It is hereby given the trivial name bibernardine.

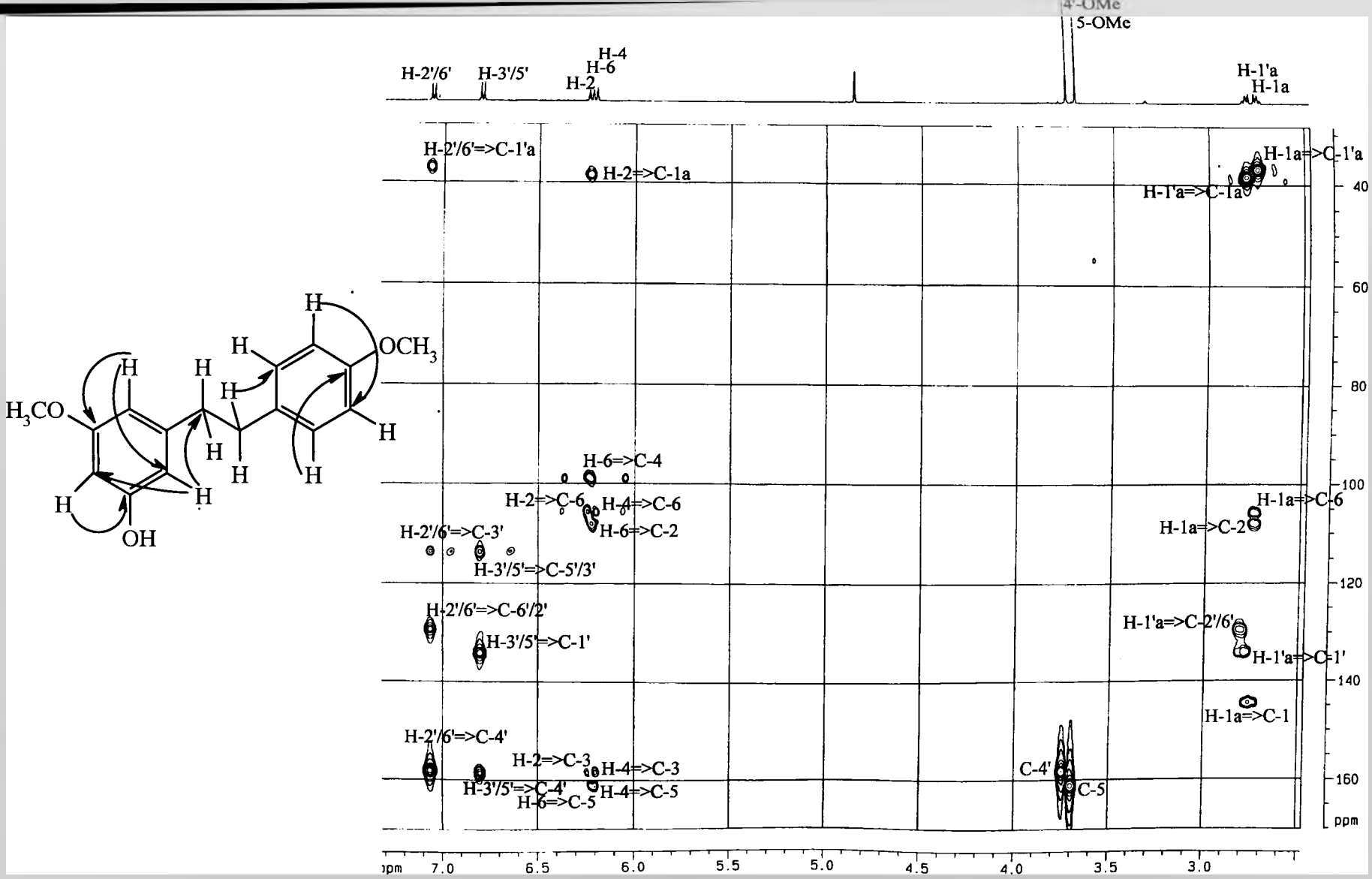


Spectrum 5.01. ^{1}H NMR spectrum (500MHz, CD_3OD*) of B14-7


4'-OMe
5-OMe
H-1'a
H-1a
5.138
0.321
4.159
4.0
3.5
3.0
2.5



Spectrum 5.02. ^{13}C (PENDANT) NMR Spectrum (125MHz, CD_3OD*) of B14-7



Spectrum 5.03. HMBC spectrum (500MHz, CD_3OD*) of B14-7

Identification of B14 –6

B14-6 was isolated as a peach amorphous solid t_R 14.5min by HPLC, mp 110°C. IR (NaCl ν_{max}) showed the presence of methoxyl (*ca.* 2840 cm^{-1}), aromatic methines (*ca.* 1520, 1460 and 830 cm^{-1}) and no carbonyl absorption (*ca.* 1725-1590 cm^{-1}) or conjugated aromatic systems (*ca.* 625 cm^{-1}) (Williams and Fleming, 1995).

The NMR data showed the presence of three aromatic methoxyl groups, two of which are equivalent at δ_H 3.63 (s, 6H), δ_C 57.1 and δ_H 3.59 (s, 3H), δ_C 61.5; six aromatic protons at δ_H 6.26 (s, 2H), δ_C 167., δ_H 6.82 (d, J=3.5, 2H), δ_C 130.2, δ_H 6.54 (d, J=4.5,poorly-resolved, 2H), δ_C 116.4. There are also four methylene protons resonating in ^1H NMR at δ_H 2.65 but resolve to two peaks at δ_C 40.1 and 38.6ppm in the ^{13}C-domain. These are the aryl-methylene protons which are joined into an ethylene bridge which is typical of bibenzyls (Majumder *et al*, 1997; Majumder and Ghosal, 1994).

The distribution of the substituents on the aryl rings was established by examining the HREIMS fragments, which were typical of bibenzyls (Letcher *et al*, 1972). The molecular formula was $C_{17}H_{20}O_4$ from the molecular ion M^+ *m/z* 288 (25%). Other prominent peaks appeared at *m/z* 181 (base peak) $[M\text{-}C_7H_7O]^+$ and *m/z* 107 $[M\text{-}C_{10}H_{13}O_3]^+$. Thus B14-6 was shown to give trimethoxytropylium and hydroxytropylium.

The exact location of the substituents was determined by the chemical shifts, the splitting pattern and HMBC correlations. The two-proton doublet at δ_H 6.82 and δ_H 6.54 exhibited

the typical AA'BB' splitting pattern of a para-disubstituted phenyl ring (Majumder *et al*, 1999) and also showed ^{2}J and ^{3}J coupling to C-4 (δ_C 156.9, so that they were assigned H-6/2 and H-3/5 respectively. H-3/5 are slightly shielded because they are *ortho* to the C-4 hydroxyl (as implied from mass spectral data).

The remaining two aromatic protons resonate as a singlet (δ_H 6.26, δ_C 107.4) and therefore are equivalent, and they are attached to a trioxygenated ring. They show ^{3}J coupling to the methylene carbon (δ_C 40.1) and as well as to quaternary carbon at δ_C 154.6 (weak) and δ_C 137.6 (strong) which implies that this ring has a pyrogallol substitution pattern. One methoxyl group is at δ_C 61.5, sandwiched by two others which are upfield at δ_C 57.6. This group is characteristically out of plane due to steric factors. The carbon to which it is attached shows an upfield shift because it is *ortho* to two oxygenated carbons (Miyase and Ueno, 1991).

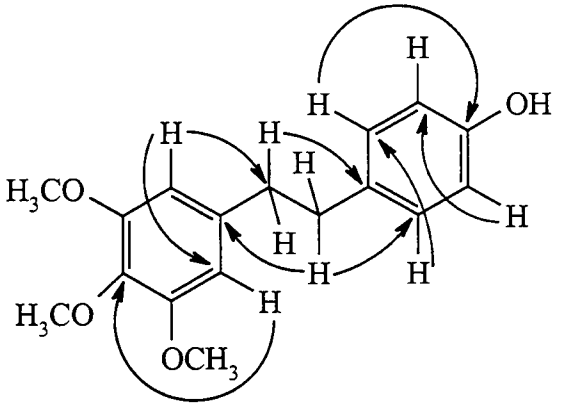


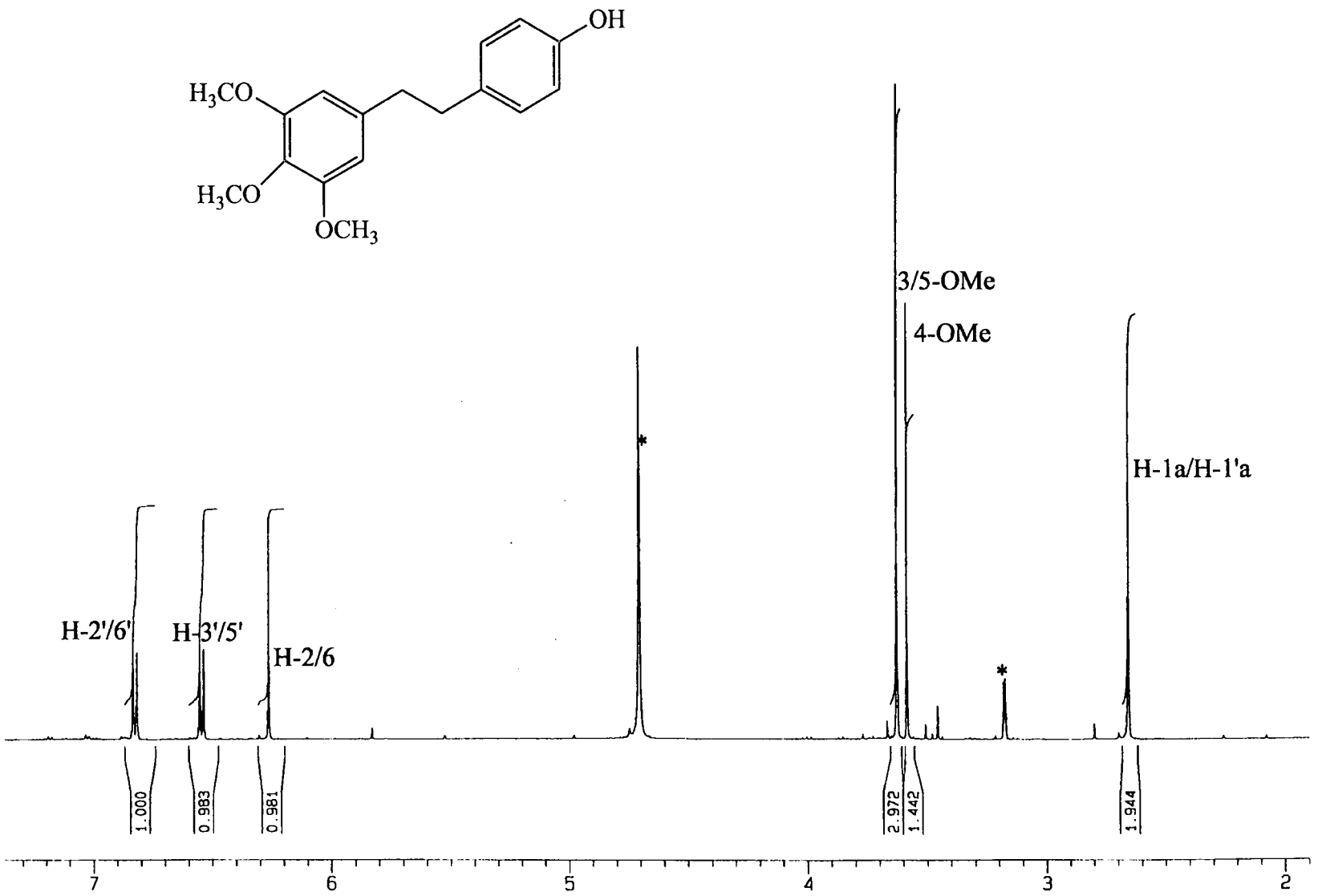
Fig.5.05. Significant HMBC correlations of B14-6

Table.5.04. HMBC correlations for B14-6 (in CD_3OD)

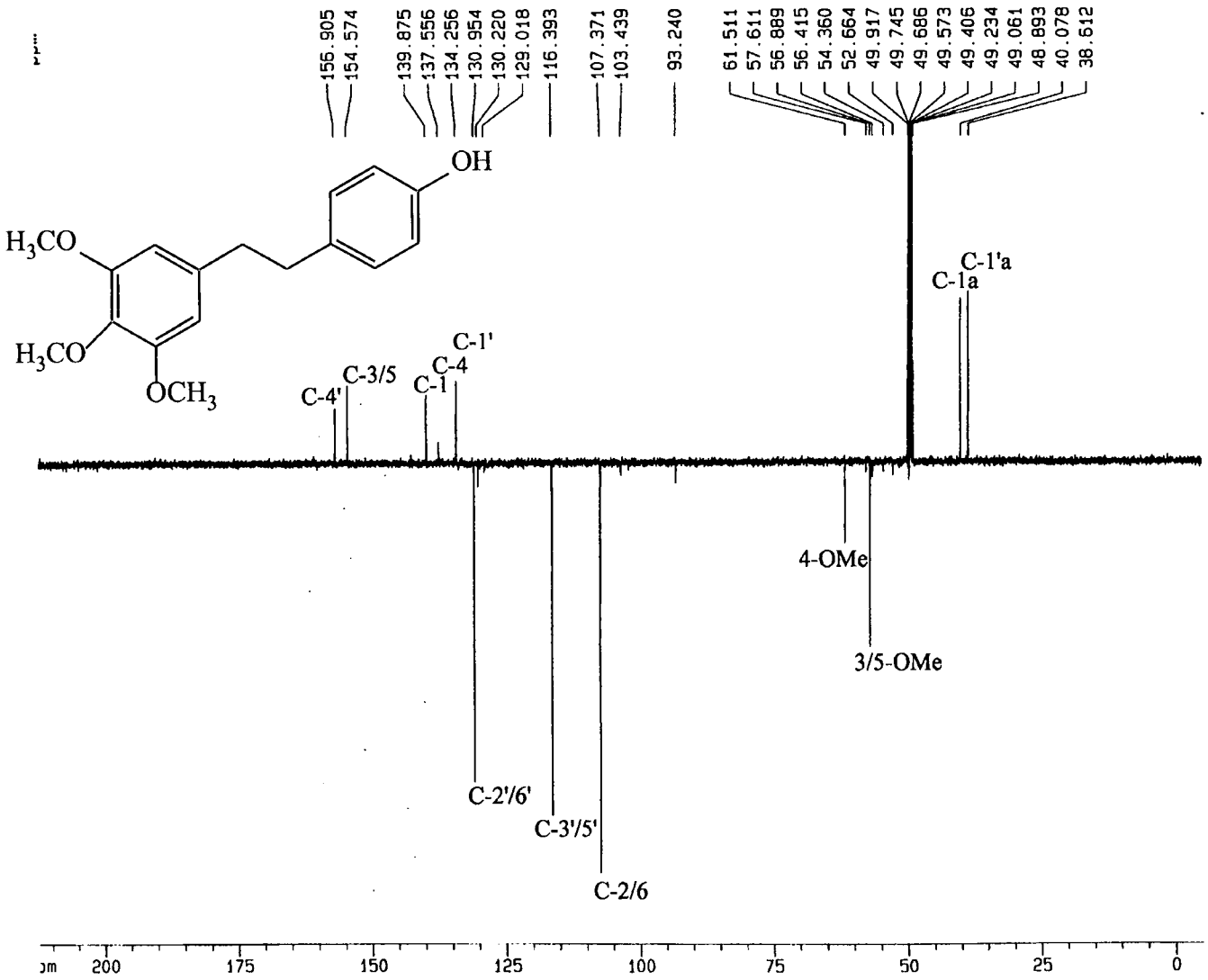
Position (δ)	^1J	^2J	^3J
H-1a (2.63)	40.1	139.9 (C-1)	107.4 (C-2/6), 134.3 (C-1')
H-2/6 (6.26)	107.4	154.6 (C-3/5)	137.6 (C-4), 107.4 (C-6/2)
H-1'a (2.65)	38.6	134.3 (C-1')	139.9 (C-1), 130.2 (C-2'/6')
H-2'/6' (6.82)	130.2	116.4(C-3'/5')	156.9(C-4'), 130.2 (C-6'/2'), 38.6 (C-1'a)
H-3'/5' (6.54)	116.4	156.9 (C-4')	134.3 (C-1'), 116.4 (C-5'/3')
3/5- OCH_3 (3.62)	57.6	-	154.6 (C-5/3)
4- OCH_3 (3.58)	61.5	-	137.6 (C-4)

All data obtained in CD_3OD.

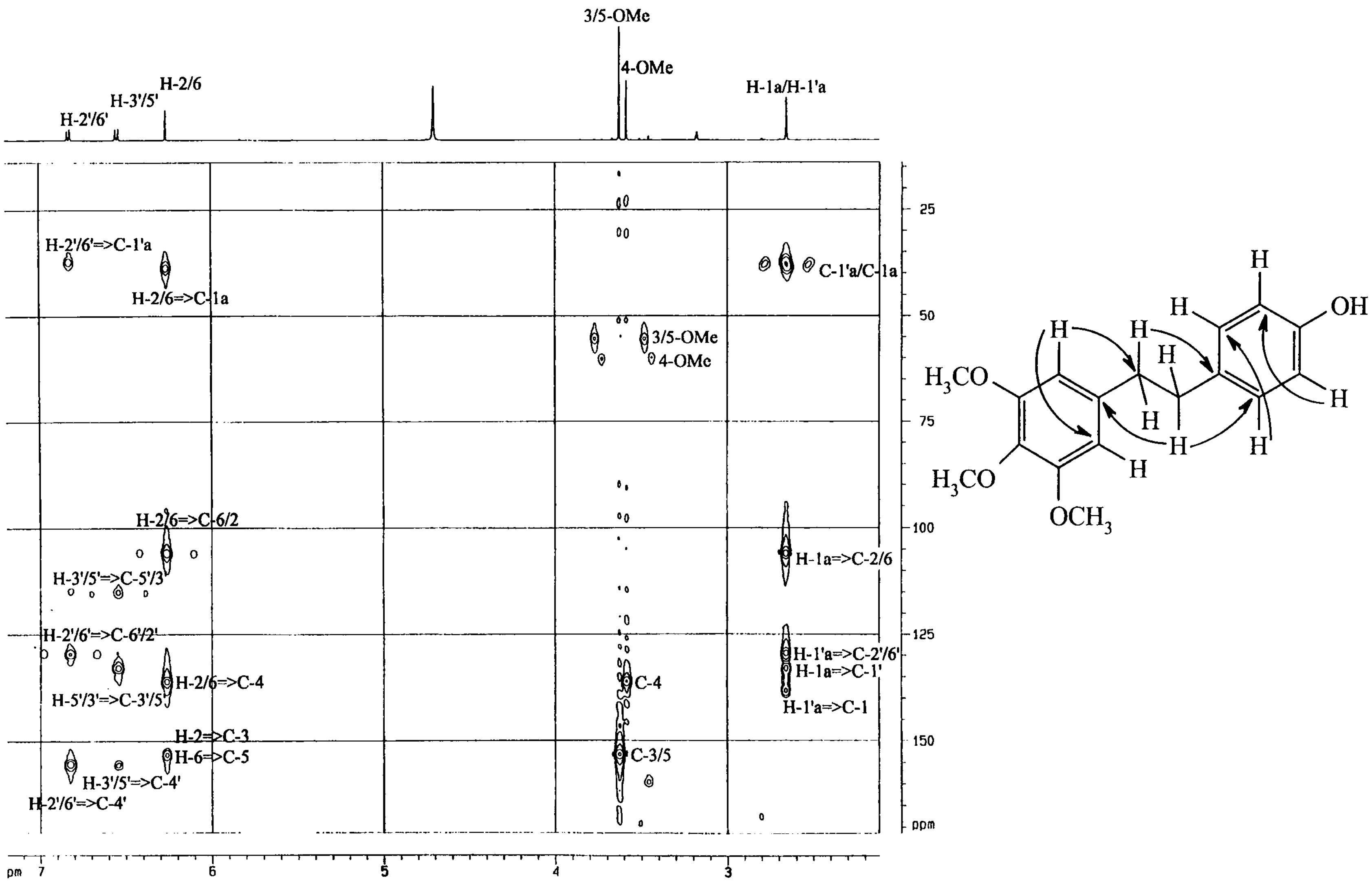
BID14-6 was characterised as 4'-hydroxy-3,4,5-trimethoxybibenzyl or alternatively 3',4',5'-trimethoxy-bibenzyl-4-ol. It has been isolated previously from the heartwood of the related species, *C. psidiodes* by Letcher and Nhamo (1972) who confirmed that structure by synthesis and acetylation. This is the first time its presence is reported in the leaves of *C. apiculatum* and also the first report, as far as could be ascertained, of its ^{13}C and 2-D NMR data.



Spectrum 5.04. . ^{1}H NMR spectrum (500MHz, CD_3OD*) of B14-6



Spectrum 5.05.^{13}C (PENDANT) NMR Spectrum (125MHz, CD_3OD*) of B14-6



Spectrum 5.06. HMBC Spectrum (500MHz, CD_3OD*) of B14-6

Identification of B27-16

B27-16 was found to be a mixture of two co-eluting compounds t_R 9.2min. They were separated by re-crystallization to give B27-16A, a peach coloured amorphous solid, 7.8mg, mp. 125°C and B27-16B, a peach coloured crystalline solid, 2.9mg, mp. 131°C. These two compounds had similar IR (NaCl ν_{max}) profiles showing the presence of hydroxyl (*ca* 3400/3380 cm^{-1}), aromatic rings (*ca.* 1600, 1515, 1460, 830 cm^{-1}) and *ortho*, *meta* and *para* substitution on the rings (*ca.* 860-730 cm^{-1}). Their elucidation is considered separately below

Characterisation of B27-16A

B27-16A showed a four proton complex multiplet δ_H 2.71-2.80, typical of the bibenzylic ethylene bridge; two singlets due to methoxyl groups δ_H 3.75 (δ_C 57.1) and δ_H 3.74 (δ_C 61.4); six aromatic protons resonating as two singlets 6.27 (δ_C 105.8) and 6.33 (δ_C 110.6) and two doublets δ_H 6.97 (d, J=8.5Hz, 2H), δ_C 130.8 and δ_H 6.71 (d, J=8.5Hz, 2H), δ_C 116.35.

HREIMS showed the molecular ion M^+ *m/z* 274, with 8 double bond equivalents (DBE) and the molecular formula $C_{16}H_{18}O_4$. The fragmentation pattern showed M^+ *m/z* 274 (28.5%), $[M-C_7H_7O]^+$ *m/z* 167 (base peak) and $[M-C_9H_{11}O_3]^+$ *m/z* 107 (12.6%). This is typical of cleavage into the hydroxy-dimethoxytropylium and hydroxytropylium ions which is diagnostic (Malan and Swinny, 1993).

The one benzene unit, ring B is *para*-hydroxylated as can be seen from the presence of the AA'BB' splitting pattern in both compounds. HMBC shows that the two-proton doublet at δ_H 6.97 correlated to the hydroxylated carbon δ_C 156.8 and also to the methylene carbon δ_C 38.60, whose protons have ^3J coupling to the corresponding carbon at δ_C 130.8. The other two-proton doublet, located slightly upfield at δ_H 6.71 (δ_C 116.4) shows HMBC correlation to the quaternary carbon at δ_C 134.4. These two protons must bear the hydroxy substituent between them which explains why they show some shielding in both the ^1H- and ^{13}C-domains. Further these four protons are coupled in the ^1H -^1H COSY. Thus this ring shows the presence of protons in the 2,3,5,6 positions, with C-4 bearing a hydroxyl group.

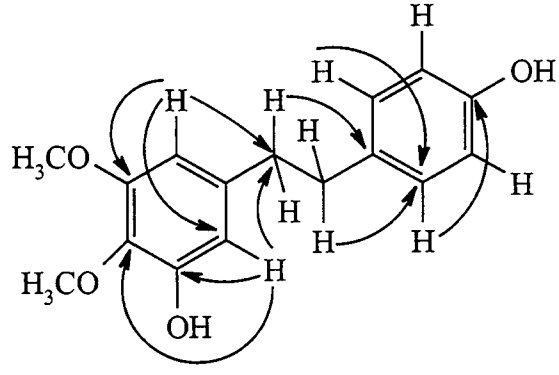


Fig.5.06. Significant HMBC correlations of B27-16A

The ring designated A, shows two singlets and three distinct quaternary resonances. The HMBC shows the proton at δ_H 6.27 (δ_C 105.8) correlating to C-1a (δ_C 39.9), and to the methine carbon at δ_C 110.6 as well as a quaternary carbon at δ_C 136.2, while the proton at

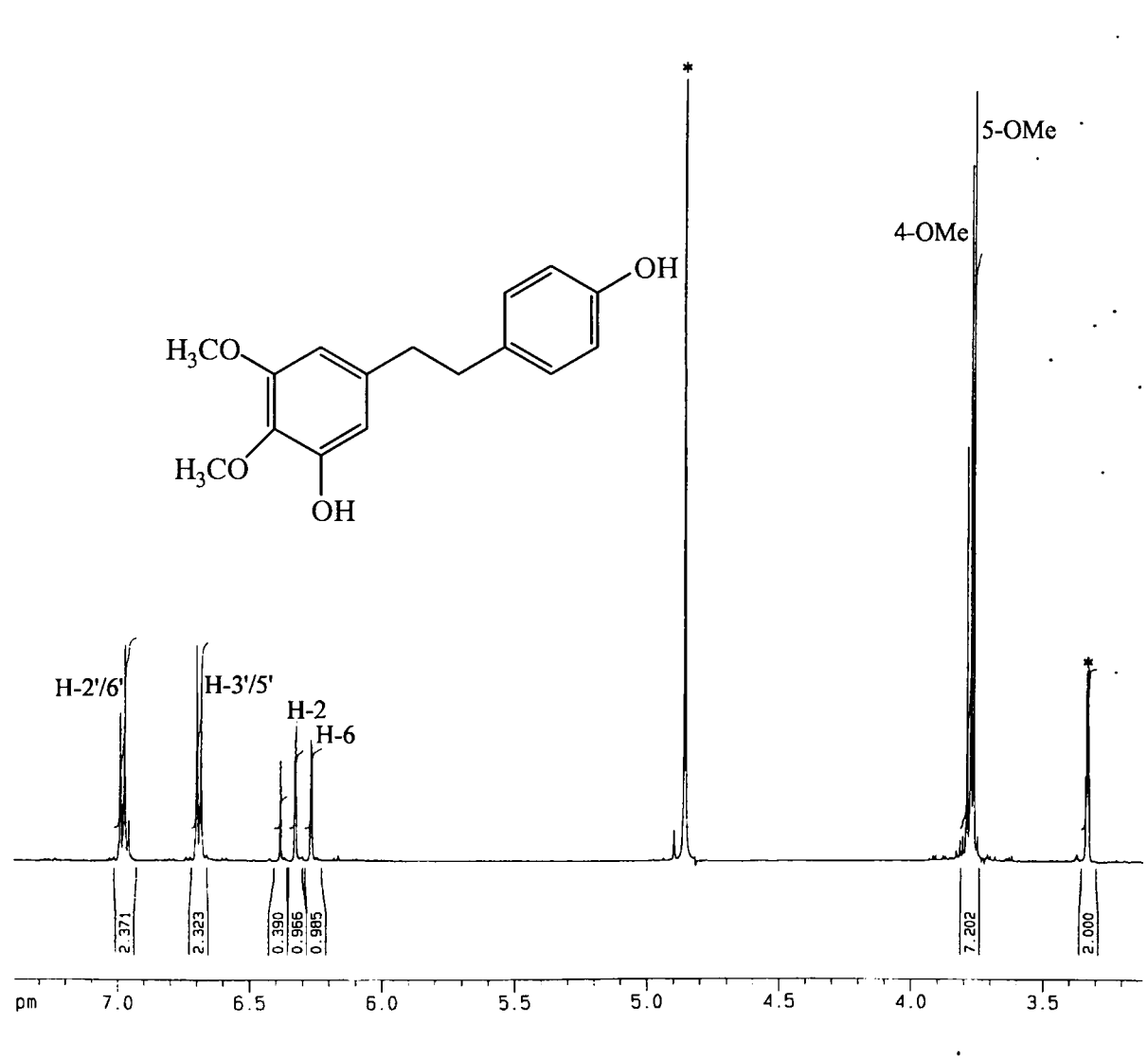
δ_H 6.33 also correlates to C-1a, and the same quaternary at δ_C 136.2. There is ^2J coupling of these protons to δ_C 154.6 and δ_C 151.6 respectively. See Fig. 5.06 and Table 5.0

Table 5.05. HMBC correlations for B27-16A (in CD_3OD)

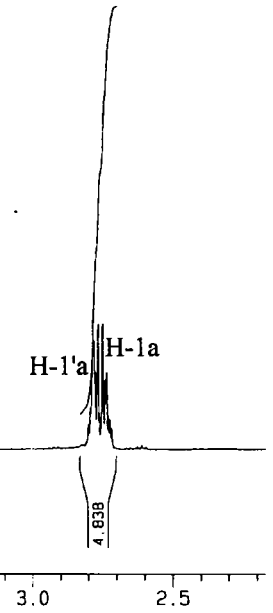
Position (δ)	^1J	^2J	^3J
H-1a (2.73)	39.9	139.8 (C-1), 38.6 (C-1'a)	110.6 (C-2), 105.8 (C-6), 134.4 (C-1')
H-2 (6.33)	110.6 (C-2)	151.6 (C-3)	136.2 (C-4), 105.8 (C-6), 39.9 (C-1'a)
H-6 (6.27)	105.8	139.9 (C-1), 154.6 (C-5)	110.6 (C-2), 136.2 (C-4), 39.9 (C-1'a)
H-1'a (2.78)	38.6	39.9 (C-1a), 134.4 (C-1')	139.8 (C-1), 130.9 (C-2'), 130.8 (C-6')
H-2' (6.97)	130.9	116.4 (C-3')	156.8 (C-4'), 130.8 (C-6'), 38.6 (C-1'a)
H-3'/5' (6.71)	116.4	156.8 (C-4'), 130.9 (C-2'/6')	116.4 (C-5'/3'), 134.4 (C-1')
H-6' (6.97)	130.8	116.4 (C-3')	156.8 (C-4'), 130.9 (C-2') 38.6 (C-1'a)
4-OCH_3 (3.75)	57.1	-	136.2 (C-4)
5-OCH_3 (3.74)	61.4	-	154.6 (C-5)

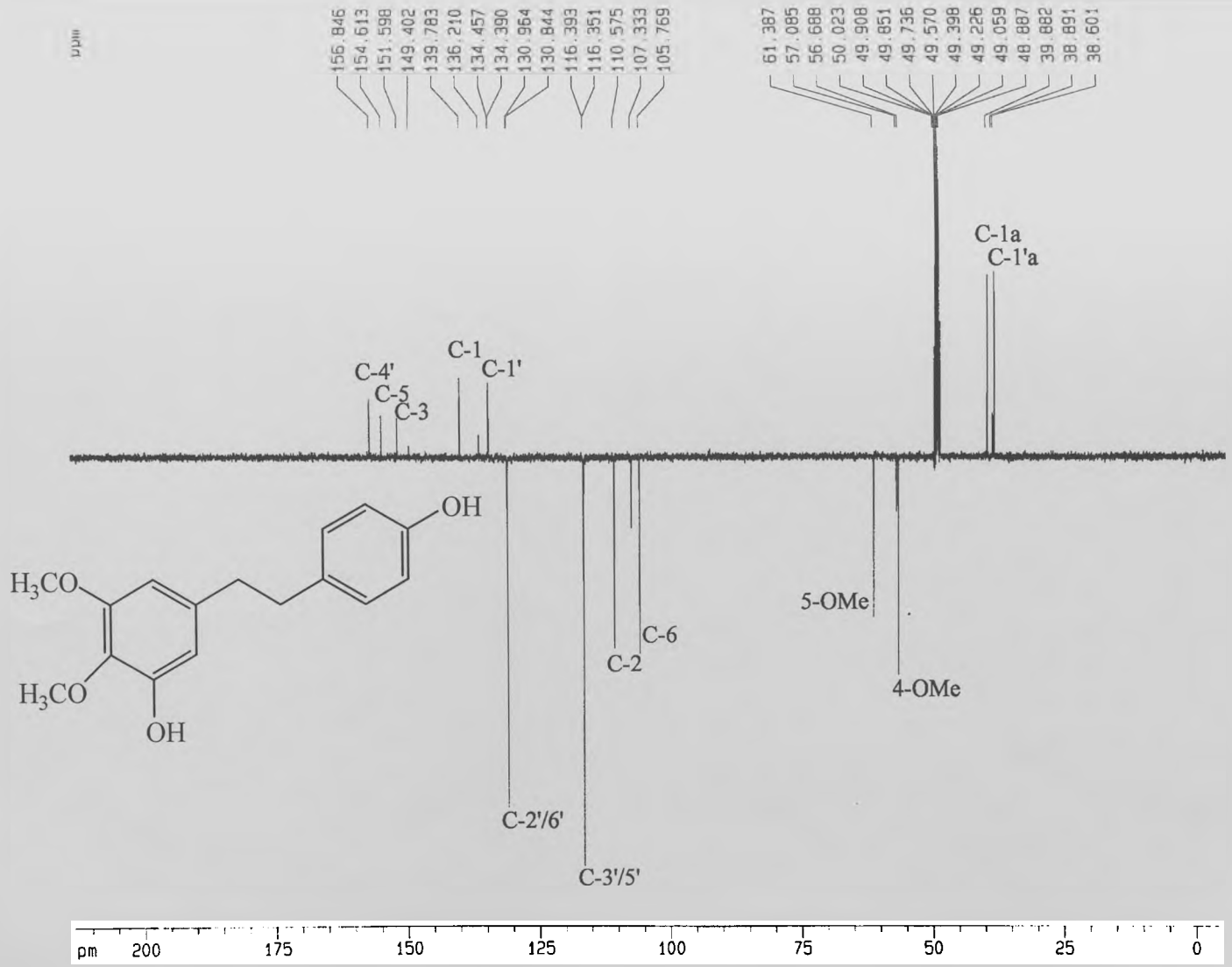
All data obtained in CD_3OD.

B27-16A was characterized as 3,4'-dihydroxy-4,5-dimethoxybibenzyl (IUPAC name-4',5-dihydroxy-3,4-dimethoxybibenzyl). It has been previously isolated from the heartwoods of *C. molle* (Letcher *et al*, 1972) and *C. apiculatum* (Malan and Swinny, 1993).



Spectrum 5.07. . ^{1}H NMR spectrum (500MHz, CD_3OD*) of B27-16A


H-1'a
H-1a
4.838
3.0
2.5



Spectrum 5.08.^{13}C (PENDANT) NMR Spectrum (125MHz, CD_3OD*) of B27-16A

Characterisation of B27-16B

B27-16B is uncannily similar in its NMR spectra to that of B27-16A. To all intents and purposes, it would appear that B27-16A and 16B are isomers, if not identical. This may explain why they co-elute in HPLC.

HREIMS showed the molecular ion M^+ *m/z* 274, and the molecular formula $C_{16}H_{18}O_4$ with two significant fragments at *m/z* 167 (100%) $[M-C_7H_7O]^+$ and $[M-C_9H_{11}O_3]^+$ *m/z* 107 (18%).

The substitution pattern in ring B is similar to that of B27-16A. There are two-proton doublets at δ_H 6.93 and δ_H 6.66 (J=8.5). The former correlates in HMBC to C-1'a (δ_C 37.3), and C-4' (δ_C 155.3), while the latter correlates with C-1' (δ_C 133.5). This places these protons in positions H-2'/ 6' and H-3'/ 5' respectively.

The appearance of a singlet at δ_H 6.35 integrating for two protons in B27-16B, whereas B27-16A has two signals at δ_H 6.27 and 6.33 is a significant distinguishing feature. This implies that ring A has a symmetrical arrangement with two identical protons and two identical quaternary carbons at δ_C 147.9.

In HMBC, the equivalent protons at δ_H 6.35 (H-2/6), correlate strongly to C-1a (δ_C 38.4) and C-4 (δ_C 133.0). They also show ^2J coupling, presumably to the identical carbons at δ_C 147.9 (C-3/5).

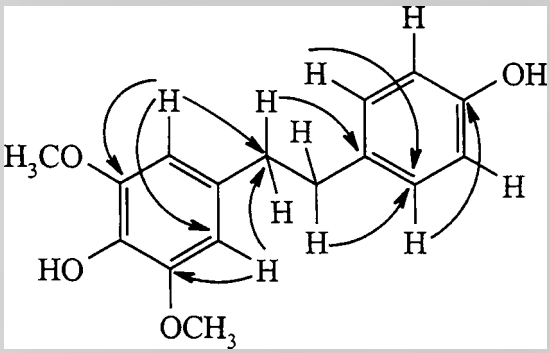


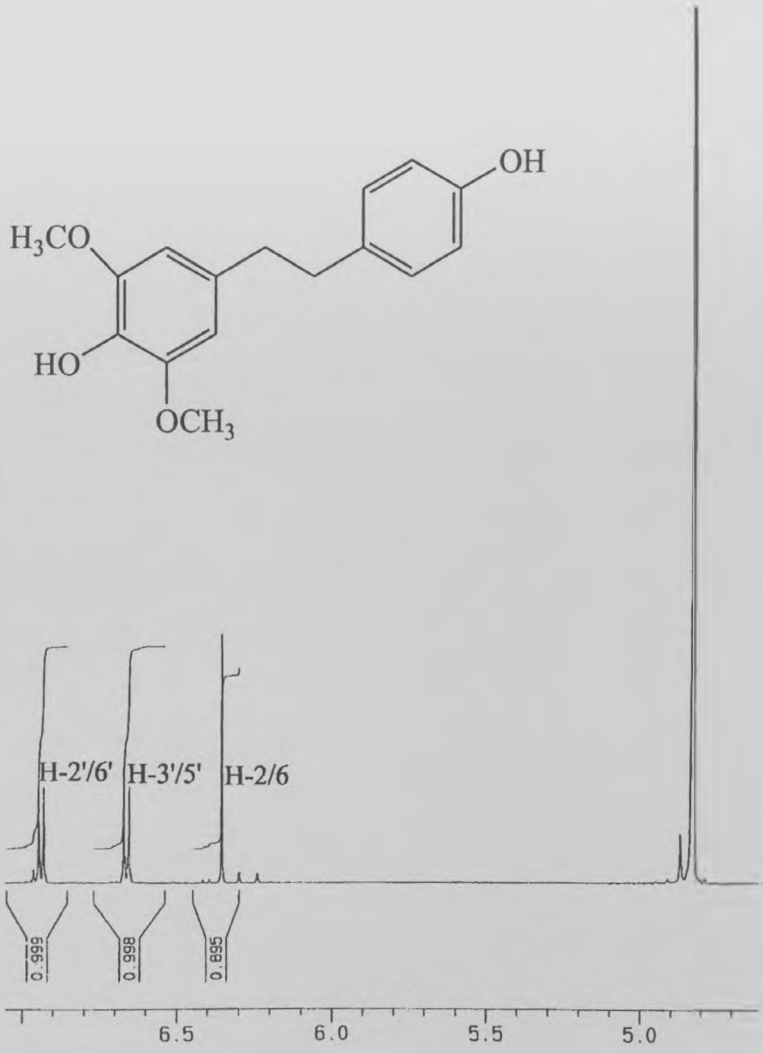
Fig.5.07. Significant HMBC correlations of B27-16B

Table 5.06. HMBC correlations for B27-16B (in CD_3OD)

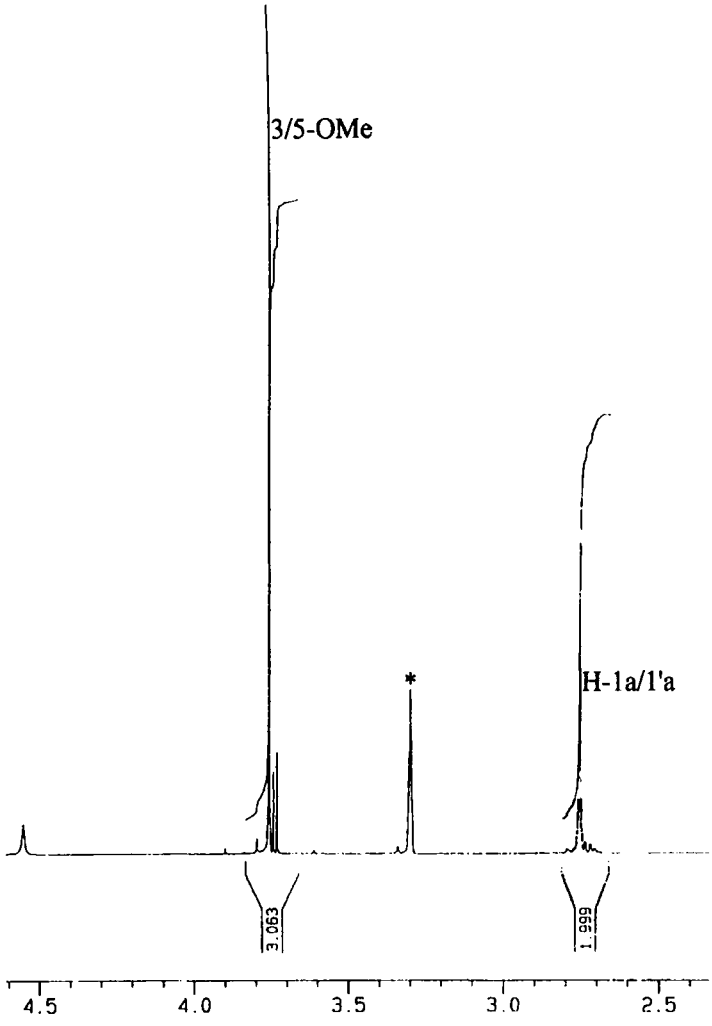
Position (δ)	^1J	^2J	^3J
H-1a (2.75)	38.4	37.3 (C-1'a)	133.5 (C-1'), 105.8 (C-2/6)
H-2/6 (6.35)	105.8	147.9(C-3/5)	133.0 (C-4)
H-1'a (2.75)	37.3	38.4(C-1a) 133.5(C-1')	129.3 (C-2'/6')
H-2'/6' (6.93)	129.3	114.9(C-3'/5')	37.3 (C-1'a), 129.3 (C-6'/2'), 155.3 (C-4')
H-3'/5' (6.66)	114.9	155.3 (C-4')	114.9 (C-5'/3'), 133.5 (C-1')
3-OCH_3 (3.74)	59.9	-	147.9(C-3)
5-OCH_3 (3.74)	59.9	-	147.9(C-5)

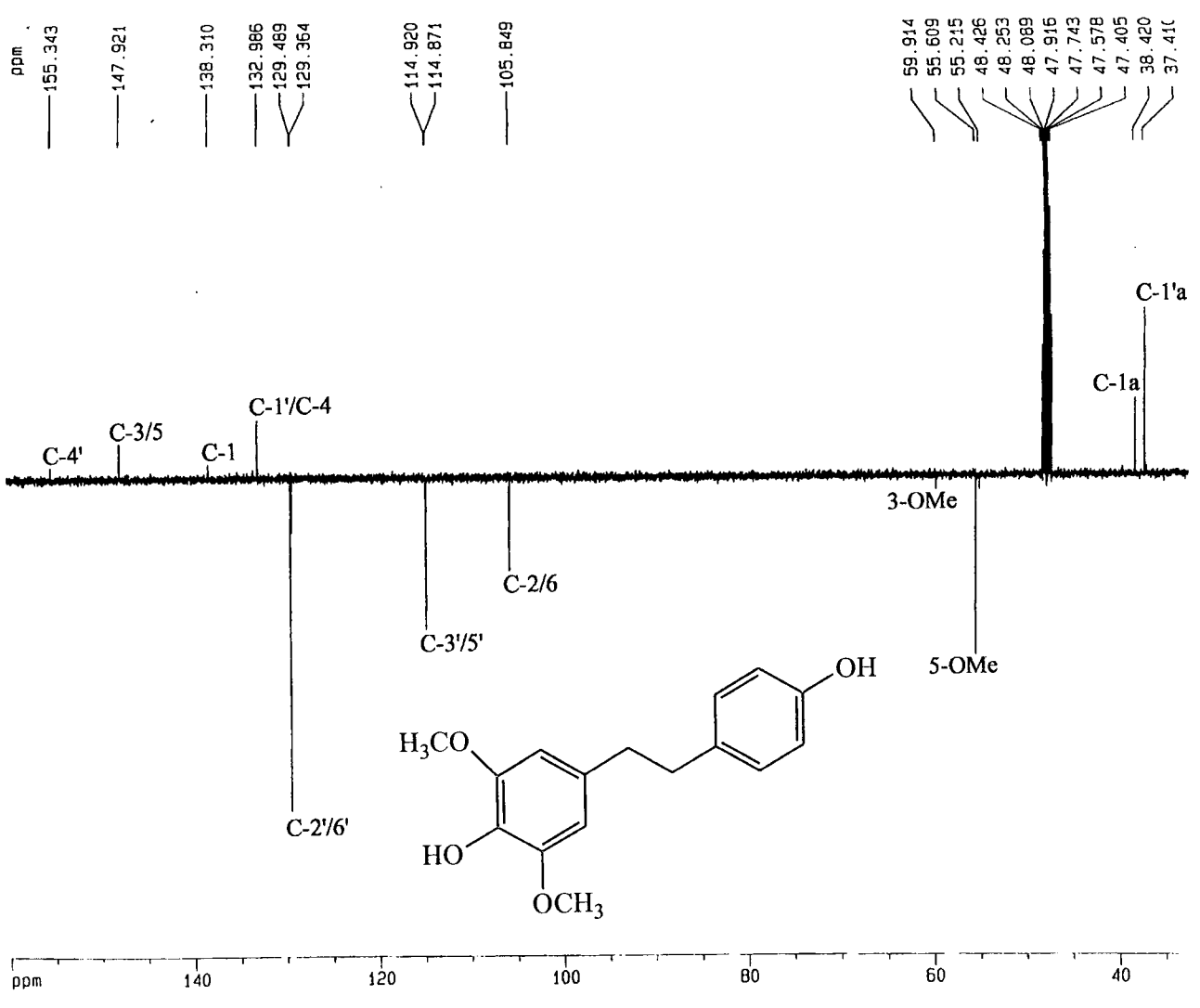
All data obtained in CD_3OD.

BID27-16B was thus characterized as 4,4'-dihydroxy-3, 5-dimethoxybibenzyl. It has been isolated from the heartwood of *C. psidiodes* (Letcher *et al*, 1972) and by Malan and Swinny (1993) from the heartwood of *C. apiculatum*.



Spectrum 5.09: ^{1}H NMR (500MHz, CD_3OD*) of B27-16B


3/5-OMe
*
H-1a/1'a
3.063
1.999
4.5
4.0
3.5
3.0
2.5



Spectrum 5.10: ^{13}C (PENDANT) NMR (125MHz, CD_3OD*) of B27-16B

At the risk of being pedantic, the differences between B27-16A and B27-16B are briefly re-examined. From the NMR data, ring B is identical in both compounds, while ring A shows subtle differences. It shows pyrogallol substitution in both, but B27-16A has 3-hydroxy-4, 5-dimethoxy moiety (I), while B27-16B, being symmetrical, has the 4-hydroxy-3, 5-dimethoxyl pattern (II). See Fig. **5.08.**



(I) (II)

Fig.5.08. The substitution pattern of ring A in B27-16A (I) and B27-16B (II).

This is the first time that detailed NMR data are being given. This would also appear to be the first report of the presence of these compounds in the leaves of *C. apiculatum*, which may suggest that they are phytoalexins (Hart, 1981) apart from being constitutive anti-fungals. It would appear to mean that the leaves may have been under stress or fungal invasion but it was not possible to establish whether this happened *in loco* or after harvesting. See Section **6.1.1**

Identification of D3-9

D3-9 (10mg) was isolated as a yellow amorphous solid, t_R 9min, by HPLC, mp. 169°C. The IR was typical of that of bibenzyls as seen previously.

NMR spectra showed a four-proton complex multiplet δ_H 2.76-2.81 (δ_C 38.7, 39.7), four O-methyl groups, two of which were equivalent at δ_H 3.74 (s, 3H), δ_C 61.7, δ_H 3.76 (s, 6H), δ_C 57.0 and δ_H 3.82 (s, 3H), δ_C 57.1 and 5 aromatic protons located at δ_H 6.42 (s, 2H), δ_C 107.4, δ_H 6.60 (d,J poorly resolved, 1H), δ_C 120.6, δ_H 6.66 (s, 1H), δ_C 117.2, and δ_H 6.82 (d, J poorly resolved, 1H), δ_C 113.4.

Table 5.07. ^{1}H NMR (500MHz) and ^{13}C NMR (125MHz) Spectra

Position	δ_H	δ_C
1a	2.80 (m)	39.7
1	-	139.9
2/6	6.42 (s)	107.4
3/5	-	154.5
4	-	137.5
1'a	2.77 (m)	38.7
1'	-	135.0
2'	6.66 (s)	117.2
3'/4'	-	147.7
5'	6.82 (d)	113.4
6'	6.60 (d)	120.6
3/5-OCH_3	3.76 (s)	57.0
4-OCH_3	3.74 (s)	61.7
4'-OCH_3	3.82 (s)	57.1

All data collected in CD_3OD.

HRESIMS showed principally three fragments - the molecular ion, M^+ *m/z* 318 corresponding to the molecular formula of $C_{18}H_{22}O_5$, the base peak at *m/z* 181, due to the trimethoxytropylium ion $[M-C_8H_9O_2]^+$, and the hydroxymethoxytropylium ion at *m/z* 137 $[M-C_{10}H_{13}O_3]^+$.

From the NMR data, which showed symmetry in one of the rings, it was concluded that ring A had a pyrogallol arrangement while ring B had a catechol-type arrangement with oxygenation at C-3 and C-4. Comparison with similar structures showed good concordance (Majumder *et al*, 1999; Junega *et al*, 1987; Leong *et al*, 1997). Because of the ambivalence of the signals around δ_C 147.7, two ring B structures are possible – the 4-hydroxy-5-methoxy (I) or the 3-hydroxy-4-methoxy (II). See Fig.**5.09**.

Table 5.08. HMBC correlations for D3-9

Position	^1J	^2J	^3J
H-1a (2.80)	39.7	38.7 (C-1'a)	107.4 (C-2/6), 135.0 (C-1')
H-2/6 (6.42)	107.4	154.5(C-3/5)	137.5 (C-4), 107.4 (C-6/2)
H-1'a (2.77)	38.7	39.7(C-1a)	139.9 (C-1), 120.6 (C-6'), 117.2 (C-2')
H-2' (6.66)	117.2	147.7 (C-3')	147.7 (C-4'), 120.6 (C-2'), 38.7 (C-1'a)
H-5' (6.82)	113.4	147.7 (C-4')	135.0 (C-1'), 147.7 (C-3')
H-6' (6.60)	120.6	-	117.2 (C-2'), 147.7 (C-4'), 38.7 (C-1'a)
3/5-OCH_3 (3.76)	57.0	-	154.5 (C-5/3)
4-OCH_3 (3.74)	61.7	-	137.5 (C-4)
4'-OCH_3 (3.82)	57.1	-	147.7 (C-4')

All data collected in CD_3OD

HMBC was not useful in differentiating the two structures because H-2', H-5' and H-6' all showed equally strong correlations to a carbon at around 147ppm which may either be a hydroxyl or a methoxyl substituent. It is not possible to say based on that data whether these correlations are two-or three bond. In all cases H-2' (δ_H 6.66) and H-6' (δ_H 6.60) correlated to C-4' at δ_C 147.7. Further H-6' might also be showing ^2J correlations to C-5' also at δ_C 147.7, while H-3' showed both ^2J and ^3J to C-4' and C-5' respectively. It is thus impossible to say where the methoxyl group is located between C-4' and C-5'. By considering the greater shielding effect of methoxyl substituent on *ortho*-located groups, a solution is finally reached (Pelter *et al*, 1976). The relatively more shielded frequency of C-3' (δ_C 113.4) compared to that of C-6' (δ_C 120.6) implies that the former is neighbouring a methoxy-bearing carbon, making structure II more logical in this case than I.

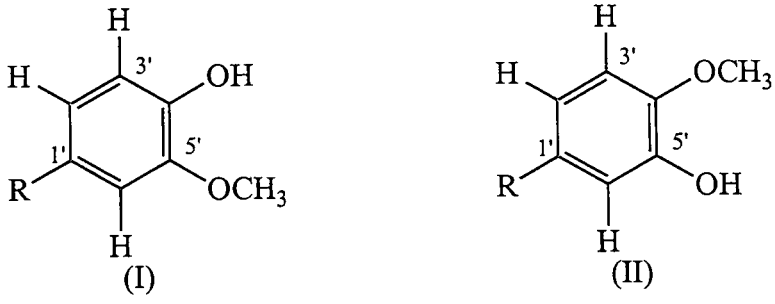


Fig.5.09. This diagram shows the two possible arrangements I and II for the A ring

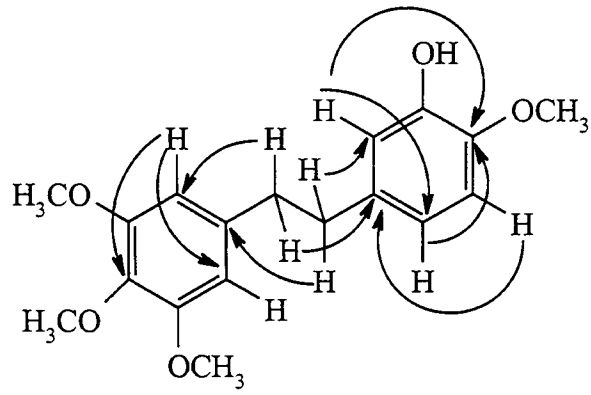
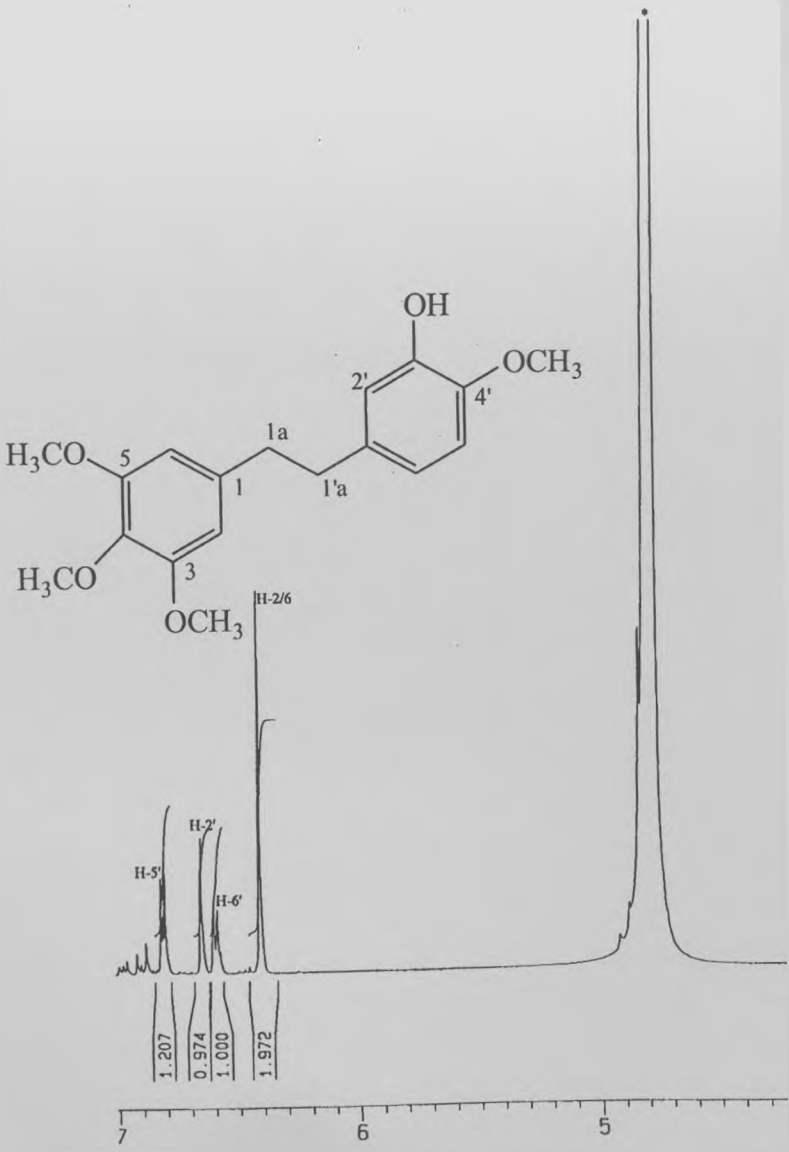
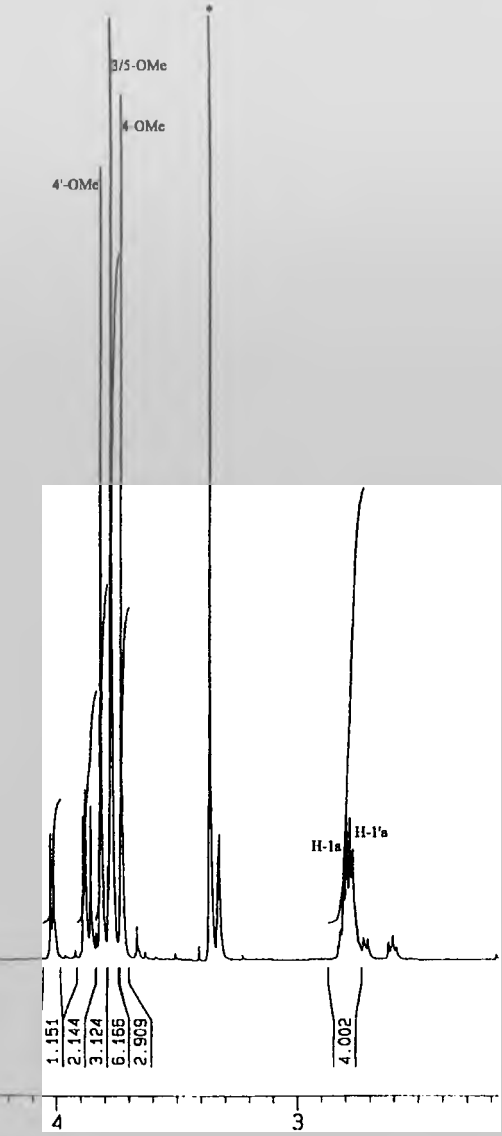


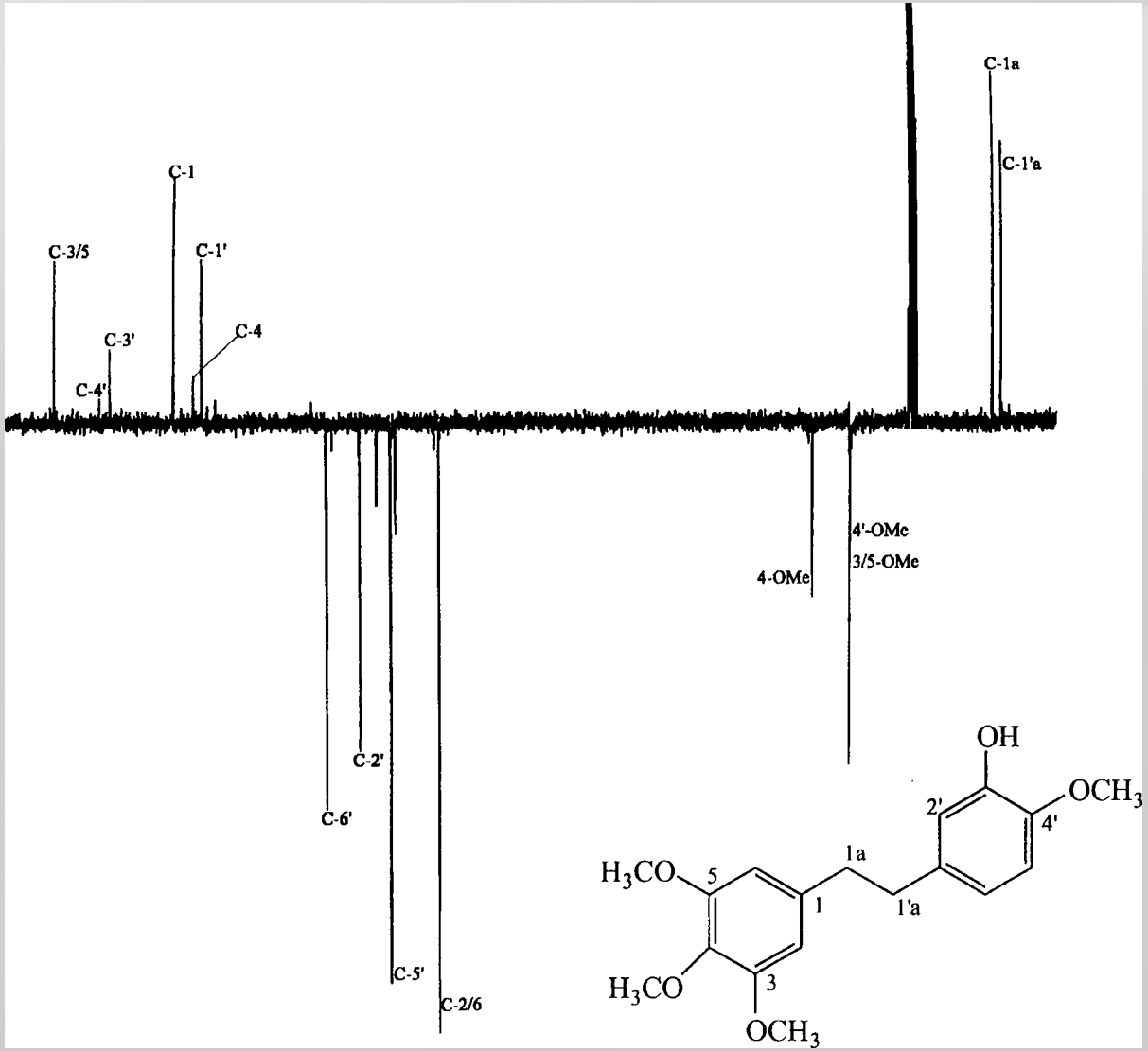
Fig.5.10. Significant HMBC correlations seen in D3-9

D3-9 was identified to be 3'-hydroxy-3, 4,4', 5-tetramethoxybibenzyl (IUPAC name 5'-hydroxy-3,4,4',5-tetramethoxybibenzyl). This has been previously isolated from *C. caffrum* (Pettit *et al*, 1988) and from *Eria carinanta* (Majumder *et al*, 1984). This is the first report of the presence of this compound in the leaves of *C. collinum*.



Spectrum 5.11: ^{1}H NMR (500MHz, CD_3OD*) of D3-9


3/5-OMe
4-OMe
4'-OMe
H-1a
H-1'a
1.151
2.144
3.124
6.166
2.909
4.002
4
3



Spectrum 5.12: ^{13}C (PENDANT) NMR (125MHz, CD_3OD) of D3-9

Characterisation of DA-h4

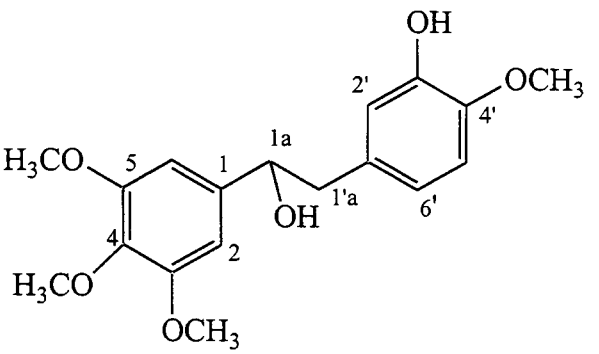


Fig.5.11. Structure of DA-h4

DA-h4 (10.9mg) was isolated as an off-white crystalline solid by HPLC, t_R 5.4min, mp. 138°C. FTIR showed the presence of hydrogen bonded hydroxyl (*ca.*3440 broad, 2920 cm^{-1}), hydroxyl (*ca.*1330, 1275 cm^{-1}), methoxyl (*ca.*2840 cm^{-1}), benzene ring (*ca.*1500 cm^{-1}) which is multiply-substituted (*ca.*900, 835, 760 cm^{-1}) (Williams and Fleming, 1995).

The NMR spectra were, in many respects, similar to those seen for the bibenzyls above. However there were important differences. Four methoxyl groups were located at δ_H 3.75 (s, 3H), δ_C 61.5; δ_H 3.78 (s, 6H), δ_C 56.9 and δ_H 3.82 (s, 3H), δ_C 56.9; and four aromatic protons were found to resonate at δ_H 6.54 (poorly-resolved, 2H), δ_C 105.0; δ_H 6.54 (poorly-resolved, 1H), δ_C 122.3; δ_H 6.60 (s, 1H) δ_C118.2 and δ_H 6.80 (d, J=8, 1H). The major difference comes in the appearance of a triplet at δ_H 4.75 (J=2, 6.5) δ_C76.0, two doublets of doublets at δ_H 2.95 δ_C 46.7 (J=6.5-7) and δ_H 2.80 δ_C 46.7 (J=6.5-7). Thus

there is no complex multiplet typical of the ethane bridge in bibenzylic compounds, but rather an oxymethine as seen at δ_C 76.0 and a methylene at δ_C 46.7.

Table 5.09. ^{1}H NMR (500MHz) and ^{13}C NMR (125MHz) spectral data of DA-h4

Position	δ_H	δ_C
1a	4.75 (t, 7Hz)	76.0
2/6	6.54 (s)	105.0
3	-	154.5
4	-	137.0
5	-	154.5
1a'	2.80/2.95 (q, 7Hz)	46.7
1'	-	133.1
2'	6.60	118.2
3'	-	147.8
4'	-	147.9
5'	6.80 (d, 8Hz)	113.0
6'	6.54 (d, 8Hz)	122.3
3-OCH_3	3.78 (s)	56.9
4-OCH_3	3.75 (s)	61.5
5-OCH_3	3.78 (s)	56.9
4'-OCH_3	3.82 (s)	56.9

Obtained in CD_3OD. Coupling constant, ***J*** in Hz in parentheses.

HRESIMS yielded an exceedingly small peak *m/z* 334 from which the molecular formula was given, putatively as $C_{18}H_{22}O_6$. The three more prominent peaks at *m/z* 197 (50%), 169 (55%) and 138 (100%) were more telling as they suggested the presence of derivatives of the typical tropylium ions suspected i.e. hydroxy-trimethoxytropylium ($C_{10}H_{13}O_4$) (*m/z* 197), hydroxy-dimethoxytropylium ($C_9H_{11}O_3$) (*m/z* 167) and

hydroxymethoxytropylium ($C_8H_9O_2$) (*m/z* 137). These fragments serve to confirm that the M^+ ion is indeed $C_{18}H_{22}O_6$, and also the substituents of the two rings. See Fig. **5.12.**

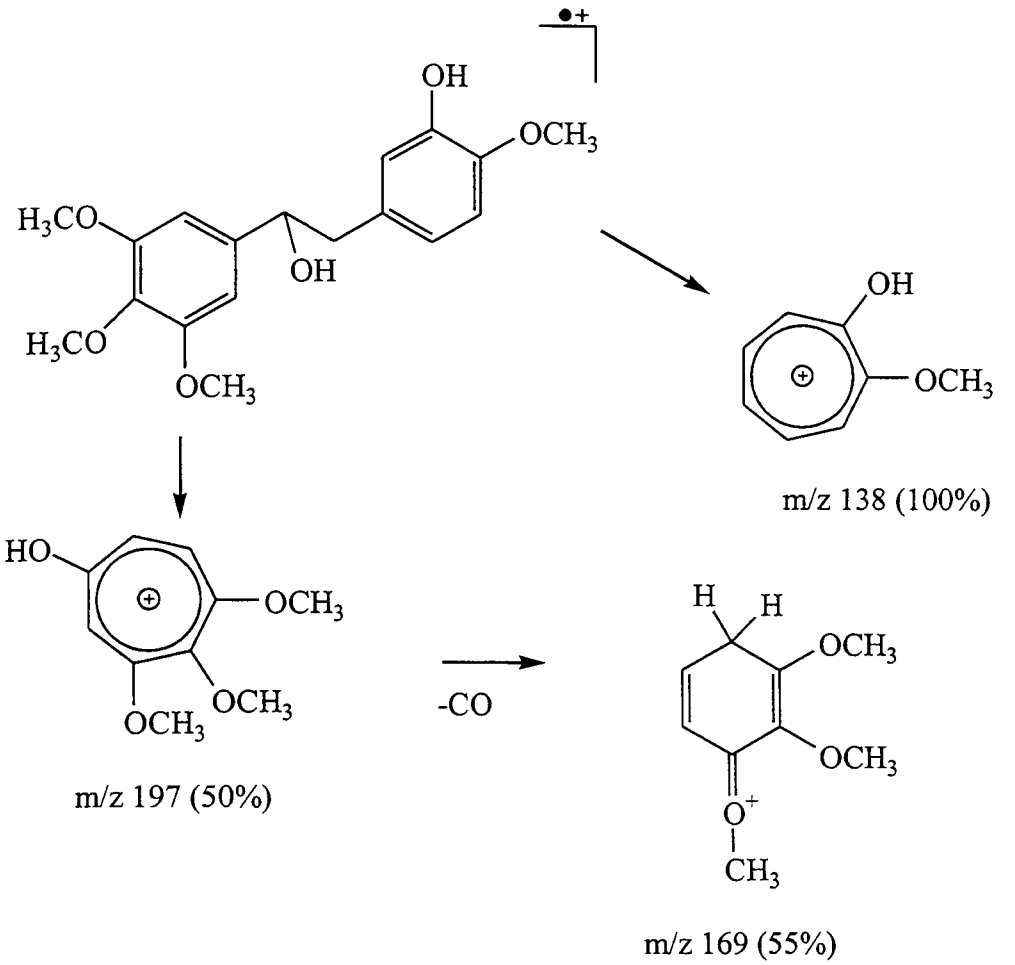


Fig.5.12. Mass spectral fragmentation of DA-h4

HMBC showed correlation between the oxy-methine proton δ_H 4.75 and the methylene carbon δ_C 46.7, a weak correlation to the quaternary carbon at δ_C 142.3 and ^3J to the equivalent aromatic methines at δ_C 105. This would imply that these are the 2,6 protons in a symmetrical ring system, which has 3,4,5 trimethoxy-substituents as implied from the MS. This explains why they are both shielded since they are *ortho* to oxygenated groups.

The methylene protons δ_H 2.80 and δ_H 2.95 show HMBC correlations to the methine carbon δ_C 76.0, which is a neighbour since it shows coupling in ^1H-^1H COSY, and also to the aromatic methines at δ_C 118.2 and δ_C 122.3. Their protons, in turn couple to the methylene carbon δ_C 46.7, to each other's carbons and also to the same oxygenated quaternary carbon at δ_C 147.8. The protons resonating at δ_H 6.60 and 6.54 and described earlier as showing ^3J coupling to C-1'a (δ_C 46.7) are designated H-2' and H-6' and may, in fact, be interchangeable. However the proton at δ_H 6.54 δ_C 122.3 is a doublet and thus shows AB coupling to the proton at δ_H 6.80 δ_C 113.0. This proton shows coupling to C-1' at δ_C 133.1 and also to a carbon at around δ_C 147. The ^{13}C-spectrum shows two quaternary carbon signals around δ_C 147, one of which must belong to the methoxyl group and the other to a hydroxyl, as implied by the MS fragment *m/z* 137. This ambiguity was resolved by applying the same reasoning as applied with the analogous compound, D3-9 above i.e. locating the methoxyl substituent next to the more shielded carbon, which in this case is C-5'.

Thus, DA-h4 was characterised as 1a, 3'-dihydroxy-3, 4,4', 5-tetramethoxybibenzyl (5-[2-hydroxy-2- (3,4,5-trimethoxy-phenyl)-ethyl]-2-methoxy-phenol) based on the foregoing and comparison with the literature. It has been previously isolated from the leaves, branches and fruit of *C. caffrum* and given the trivial and chemical name combretastatin (Pettit *et al*, 1982). More recently, it has been isolated from the leaves and branches of *C. erythrophyllum* and *C. kraussi* (Brookes *et al*, 1999). This is the

prototypical compound of the combretastatins. This is the first report of the presence of combretastatin in *C. collinum*.

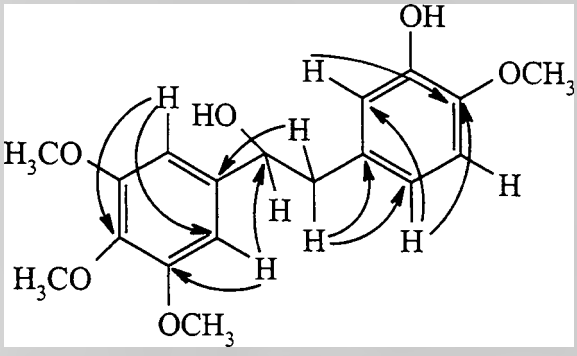
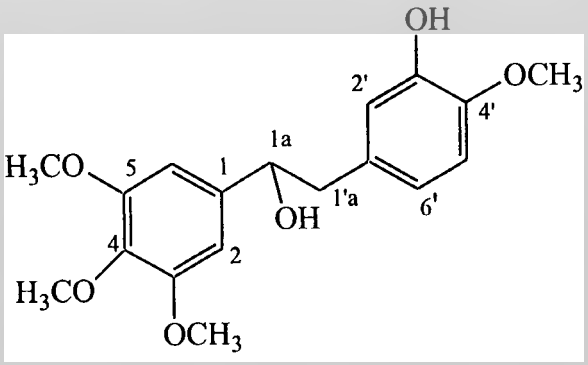


Fig.**5.13.** Significant HMBC correlations of DA-h4

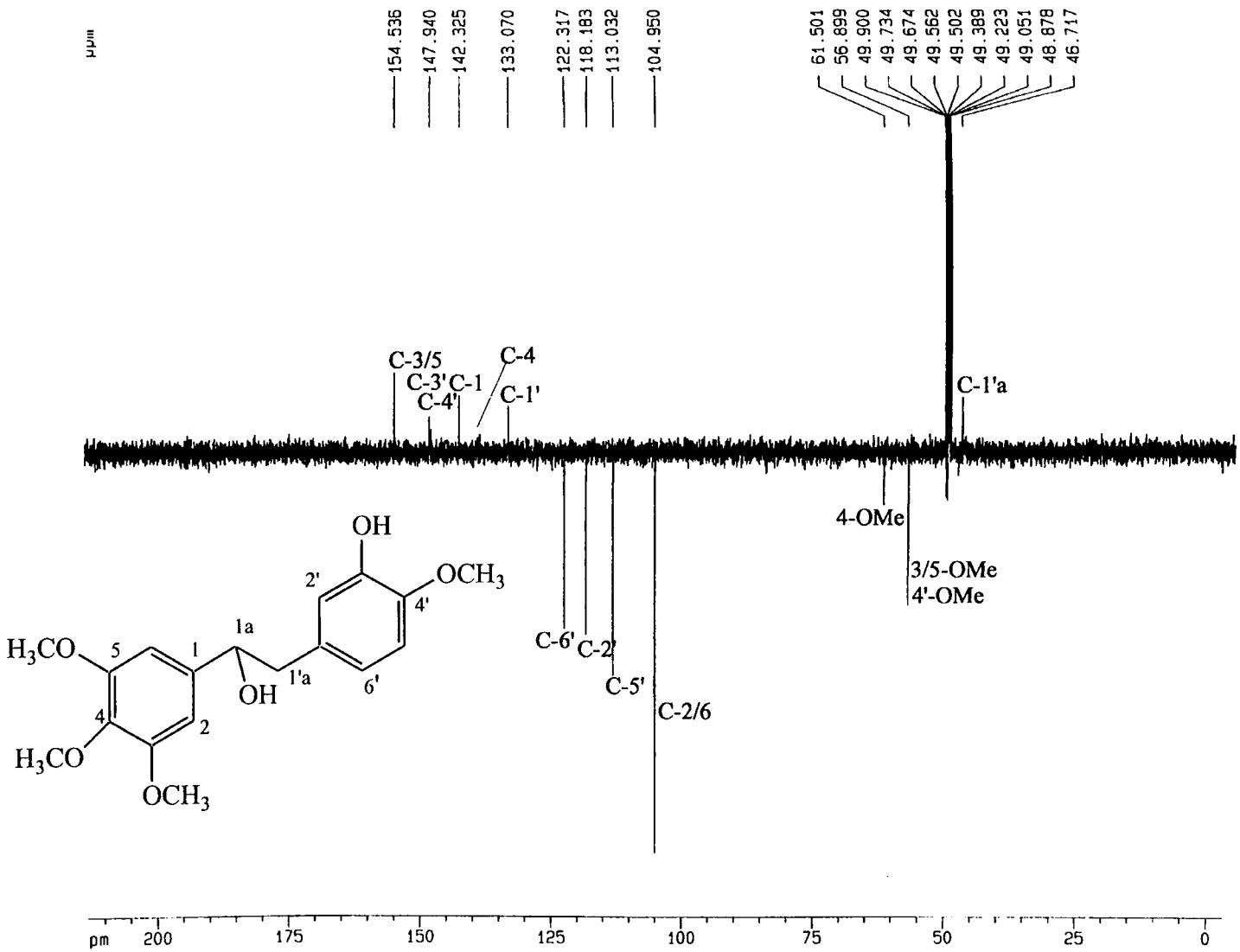
Table 5.10. Significant HMBC correlations of DA-h4

Position (δ)	δ_C	^2J	^3J
H-1a (4.75)	76.0	46.7 (C-1'a), 142.3 (C-1)	105.0 (C-2/6),
H-2/6 (6.54)	105.0	154.5(C-3/5)	137.0 (C-4), 76.0 (C-1a)
H-1'a (2.80/2.95)	46.7	76.0 (C-1a), 133.1 (C-1')	142.3 (C-1),122.3 (C-6'), 118.2 (C-2')
H-2' (6.60)	118.2	147.8 (C-3')	147.9 (C-4'),122.3 (C-6'), 46.7 (C-1'a)
H-5' (6.80)	113.0	147.9 (C-4')	133.1 (C-1'), 147.8 (C-3')
H-6' (6.54)	122.3	-	147.9 (C-4'), 46.7 (C-1'a)
3/5-OCH_3 (3.78)	56.9	-	154.5 (C-5/3)
4-OCH_3 (3.75)	61.5	-	137.0 (C-4)
4'-OCH_3 (3.82)	56.9	-	147.9 (C-4')

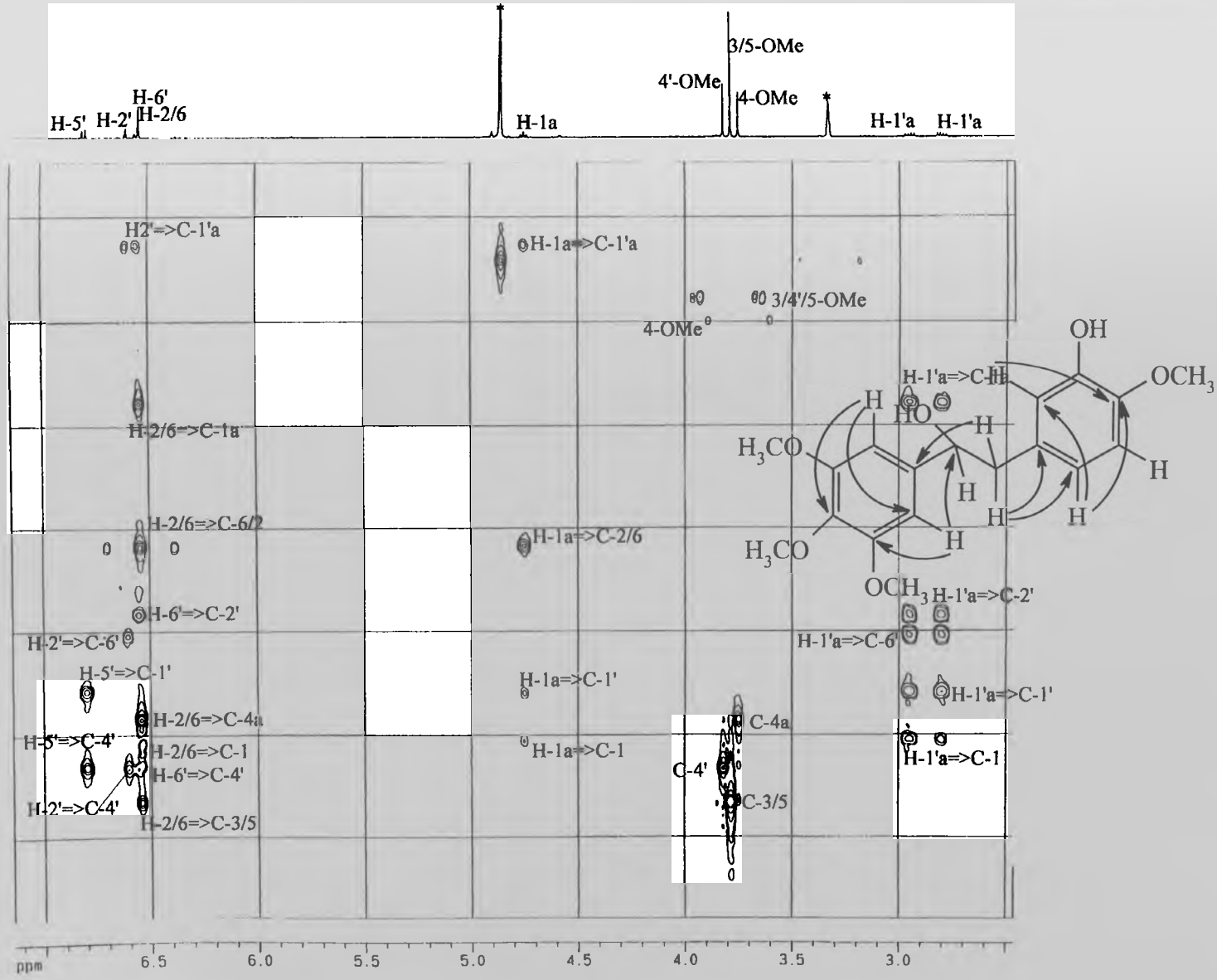
Data obtained in CD_3OD



Spectrum 5.13 : ^{1}H NMR (500MHz, CD_3OD*) of DA-h4



Spectrum 5.14 : ^{13}C (PENDANT) NMR (125MHz, CD_3OD*) of DA-h4



Spectrum 5.15: HMBC correlations of DA-h4

Summary of physical properties and spectral data of isolated stilbenes.

The compounds are named according to IUPAC rules on nomenclature

B14-7 (5-Hydroxy-4', 3-dimethoxybibenzyl)

Peach amorphous solid. IR ν_{max} film cm^{-1} 3400, 2995, 2840, 1610, 1590, 1460,760. Found $[M^+]$ 258.126 ($C_{16}H_{18}O_3$ requires 258.126). HREIMS *m/z* (rel. int. %): 258 (33), 137 (5), 121 (100), 77 (8). ^1H and ^{13}C NMR Tables **5.01** and **5.02**, and Spectra **5.01-03**.

B14-6 (4'-Hydroxy-3, 4, 5-trimethoxybibenzyl)

Peach amorphous solid. IR ν_{max} film cm^{-1} 3400, 2940, 1590, 1512,760. Found $[M^-]$ 288.14 ($C_{17}H_{20}O_4$ requires 288.136). HREIMS *m/z* (rel. int. %): 288 (25), 181 (100), 107 (9). ^1H and ^{13}C NMR: Tables **5.01** and **5.02**, and Spectra **5.04-06**.

B27-16A (5,4'-Dihydroxy-4, 3-dimethoxybibenzyl)

Peach amorphous solid. IR ν_{max} film cm^{-1}3380, 2930 1600, 1520, 1360, 1095, 830. Found $[M^+]$ 274.121 ($C_{16}H_{18}O_4$ requires 274.121). HREIMS *m/z* (rel. int. %): 274 (33), 167 (100), 107 (18). ^1H and ^{13}C NMR Tables **5.01** and **5.02**, and Spectra **5.07** and **5.08**.

B27-16B (4,4'-Dihydroxy-4, 5-dimethoxybibenzyl)

Peach crystalline solid. IR ν_{max} film cm^{-1}3400, 2940, 2840, 1610, 1515, 1460, 1100, 830. Found $[M^+]$ 274.121 ($C_{16}H_{18}O_4$ requires 274.121). HREIMS *m/z* (rel. int. %): 274 (49), 167 (100), 107 (15). ^1H and ^{13}C NMR Tables **5.01** and **5.02**, and Spectra **5.09** and **5.10**.

D3-9 (3'-Hydroxy-3, 4, 4', 5-tetramethoxybibenzyl)

Yellow amorphous solid. Found $[M^+]$ 318.147 ($C_{18}H_{22}O_5$ requires 318.147). HRESIMS *m/z* (rel. int. %): 318 (5), 181 (100), 137 (30). ^1H and ^{13}C NMR Tables **5.08**, and **5.11-12**

DA-h4 (1a, 3'-Dihydroxy-3, 4, 4', 5-tetramethoxybibenzyl) (combretastatin)

Off-white crystalline solid. IR ν_{max} film cm^{-1} .3440, 2920, 1330, 1275, 2840, 1500, 900, 835, 760. Found $[M^+]$ 334.0. ($C_{18}H_{22}O_6$ requires 334.14). HRESIMS *m/z* (rel. int. %): 197 (50), 169 (55), 138 (100). ^1H and ^{13}C NMR Tables **5.09** and **5.10**, and Spectrum **5.13-15**.

5.1.2.2. PHENANTHRENES AND THEIR DERIVATIVES

Phenanthrenes are phenolic compounds in which the basic skeleton is a unit of three fused rings at least two of which are aromatic. Phenanthrenes and their derivatives are widely distributed in the families Orchidaceae, Combretaceae, Dioscoreaceae and Horaceae (Miyase and Ueno, 1991). Their glycosides, however have been found only confined to the genus *Epimedium* (Berberidaceae).

In this study four phenanthrenes were isolated from the fruit of *Combretum hereroense,* and one from *C. collinum.* Four of these have been isolated previously and one is reported here for the first time. This would appear to the first report of phenanthrenes in *C. collinum.*

The phenanthrenes isolated were characterized as 9,10-dihydro-3, 5-dimethoxy-2,7-phenanthrenediol (CHE-h7), 3,5,7-trimethoxy-2,6-phenanthrenediol (CHE-h10), 5,7-dimethoxy-2,3-phenathrenediol (CHE-h12) , 2,4-dimethoxy-6, 7, 8-phenanthrenetriol (CHE-h17) and 9, 10-dihydro-3, 6, 7-trimethoxy-2,5-phenanthrenediol (DA-h9).

General characterisation of phenanthrenes

Phenanthrenes will usually occur simply as oxygenated or prenylated derivatives or as 9,10-dihydrophenanthrenes. The latter show a four- proton complex signal around δ_H 2.52 also characteristic of the bibenzyls (as discussed in Section **5.1.2**), and which is diagnostic for the 9,10 methylene protons (Juneja *et al*, 1987).

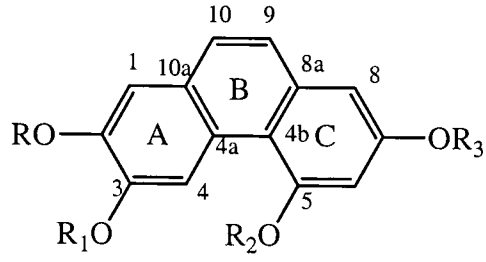


Fig 5.14. General structure of phenanthrenes isolated from natural sources.

In most cases, phenanthrenes are polyoxygenated and the IR spectra show hydroxyl (3500, 1410-1260 cm^{-1}), and benzenoids which are variously substituted (1600, 1500, 900-680 cm^{-1}), and the absence of carbonyl (1640-1590 cm^{-1}) or ketone carbonyl (1700-1680 cm^{-1}) (Williams and Fleming 1995; Letcher *et al*, 1971, 1972, 1973).

The ^{1}H-NMR shows aromatic protons but the highly deshielded location of the H-4 or H-5 proton (δ_H 7.50 – 9.80) is significant and usually points to 4,6 disubstitution in the 5-unsubstituted phenanthrenes (Letcher and Wong, 1978; Majumder *et al*, 1996). The carbon of an aromatic methoxyl group having at least one *ortho* hydrogen atom normally resonates at *ca.* δ_C 55-57 (Wenkert *et al*, 1976). The exact substitution pattern in phenanthrenes can only usually be established by application of NOESY (and sometimes by HMBC) (Singh and Pettit, 1989), and even then, as seen in this study, this may still result in equivocal structures. Some deviation from overall planarity due to strong steric interaction has been shown (Pettit *et al*, 1987) and this may influence the chemical shifts in NMR spectroscopy.

carbon monoxide (M-43) interchangeably (Letcher and Nhamo, 1971). The 9,10-dihydrophenanthrenes show consecutive losses of keten fragments to yield the base peak (Malan and Swinny, 1993).

Characterisation of CHE-h7

CHE-h7 (6.2mg) was isolated by HPLC, t_R=12.5min as a dark brown amorphous solid, m.p.125°C. IR showed the presence of hydroxyl (broad 3380, 1330, 1280 cm^{-1}), benzenoid which are variously substituted (1610, 1510, 875, 840, 805, 750 cm^{-1}), and no ketone (1700- 1680 cm^{-1}).

NMR showed a complex multiplet at δ_H 2.60 and 2.65 typical of the 1,2 diarylethane arrangement (Juneja *et al*, 1987). Two methoxyl groups resonate at δ_H 3.87 (δ_C 56.5 and 57.1) and four aromatic protons – a *meta*-coupling pair at δ_H 6.33 (d, J=2Hz, 1H) and δ_H 6.43 (d,J= 2.5Hz, 1H), and two singlets at δ_H 6.66 and δ_H 7.87. This last singlet is typically deshielded, resonating at even lower field in pyridine, and is typical of the H-4 or H-5 proton (Majumder and Kar, 1987). In pyridine-*d*5 the signals appear to move *en masse* slightly downfield and there is a broad peak between δ_H 11.50 and 12ppm which is typical of phenolic hydroxyl protons which do not appear in methanol-*d*4 due to exchange (Williams and Fleming, 1995).

HRFABMS showed the M^+-H peak at *m/z* 271 (60%), the molecular formula being $C_{16}H_{16}O_4$ requiring *m/z* 272. Other prominent peaks were seen at *m/z* 240 (55%) $[M-CH_3OH]^+$, 225 (37%) $[M-C_2H_7O]^+$, 223 (41%) $[M-C_2H_9O]^+$, 209 (94%) $[M-C_3H_{11}O]^+$, 176 (54%) $[M-C_3H_{11}O_3]^+$ and the base peak at *m/z* 144 $[M-C_{10}H_7]^+$.

Having constructed the partial structure with 1-D NMR spectra and HMQC, ^{1}H-^{1}H NOESY was used to locate the exact positions of the substituents on the suggested structure. See Spectrum **5.18**. It showed that the benzylic methylene δ_H 2.60 (δ_C 30.6) interacts in space with the proton at δ_H 6.66 (δ_C 115.7), while the other methylene interacts with the proton at δ_H 6.33 (δ_C 109.0). This places protons at C-1 and C-8, respectively.

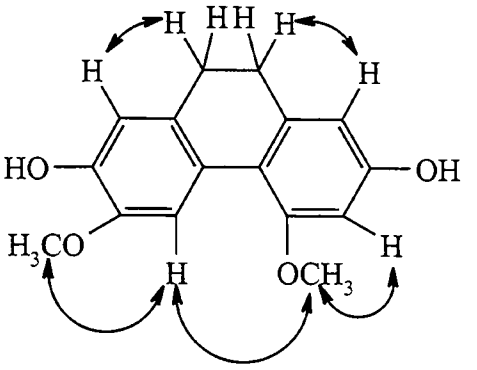


Fig.5.15. NOESY correlations seen in CHE-h7

The other *meta*-coupled proton at δ_H 6.43 (δ_C 99.9) shows NOE with the O-methyl group at δ_H 3.91 (δ_C 56.5) which also shows a weak interaction with the deshielded proton at δ_H 7.87 (δ_C 114.1). See Fig **5.15**. This deshielded proton in turn interacts with the methoxyl at δ_H 3.91 (δ_C 57.1). For this to happen the methyls must be in different rings, with one *ortho* to the proton in question (δ_H 7.87) which is typically found as H-4 or H-5 (Letcher and Nhamo, 1972). The other methoxyl group is therefore attached to C-5 (or C-4 depending on the above-mentioned) and neighbouring the proton at δ_H 6.43. In fact this proton (H-6) is attached to a shielded carbon at δ_C 99.9 precisely because it is *ortho* to two oxygenated substituents. Once

the methoxyl substituents are fixed, the other oxygenated positions are taken up by hydroxyl groups implied from the mass spectral data and the broad peak in PMR spectra.

Thus the proposed structure is 9,10-dihydro-3,5-dimethoxy-2,7-hydroxyphenathrenediol and ^{2}J and ^{3}J correlations confirm it as the definitive arrangement of CHE-h7. The methylene protons show coupling to each other from the ^{1}H-^{1}H COSY as well as HMBC, see Fig **5.16.**

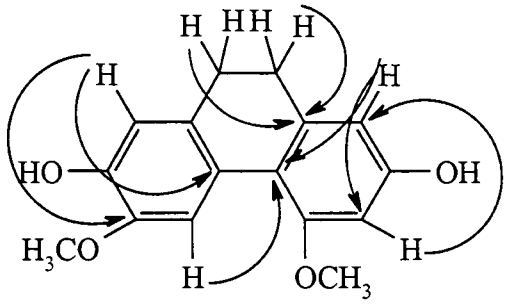


Fig.5.16. Significant HMBC correlations for CHE-h7

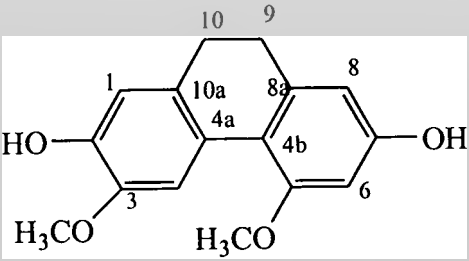
In addition to correlating to each other's neighbouring quaternary carbons C-8a and C-10a, H-1 at δ_H 6.66 shows strong correlations to C-3 (δ_C 147.1), C-4a (δ_C 126.6) and to the upfield methylene at δ_C 30.6 (C-10). H-4 (δ_H 7.87) shows correlations to C-2 (δ_C 145.7), C-3 (δ_C 147.1), C-8a (δ_C 132.7) and C-4b (δ_C 117.3) while H-6 is coupled to C-4b as well as C-8, whose proton at δ_H 6.33 couples in turn to C-6 and also to its carbinol neighbour C-7 (δ_C 158.0) and to C-4b.

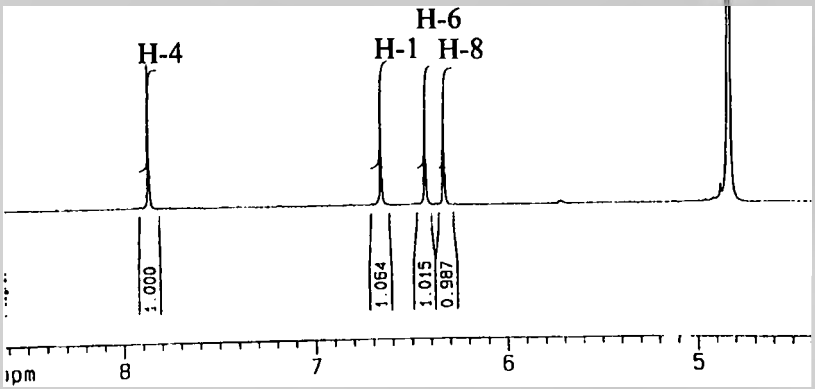
Table 5.11. ^{1}H NMR (500MHz), ^{13}C NMR (125MHz) spectral data and significant HMBC correlations of CHE-h7

Position	δ_H	δ_C	^{2}J	^{3}J
1	6.66 (s)	115.7		30.6 (C-10),126.6 (C-4a), 147.1 (C-3)
2	-	145.7		-
3	-	147.1		-
4	7.87 (s)	114.1	147.1 (C-3),	145.7 (C-10), 132.7 (C-10a), 126.6 (C-4a) 117.3 (C-4b)
4a	-	126.6	-	-
4b	-	117.3	-	-
5	-	159.4	-	-
6	6.43 (d, J=2.5)	99.9		117.3 (C-4b), 109.0 (C-8)
7	-	158.0	-	-
8	6.33 (d, J=2.5)	109.0	158.0 (C-7)	99.9 (C-6), 117.3 (C-4b)
8a	-	142.4	-	-
9	2.65 (m)	32.4	142.4 (C-8a), 30.6 (C-10)	132.7 (C-10a)
10	2.60 (m)	30.6	132.7 (C-10a), 32.4 (C-9)	142.4 (C-8a)
10a	-	132.7	-	-
3-OCH_3	3.91 (s)	57.1	-	147.1 (C-3)
5-OCH_3	3.91 (s)	56.5	-	159.4 (C-5)

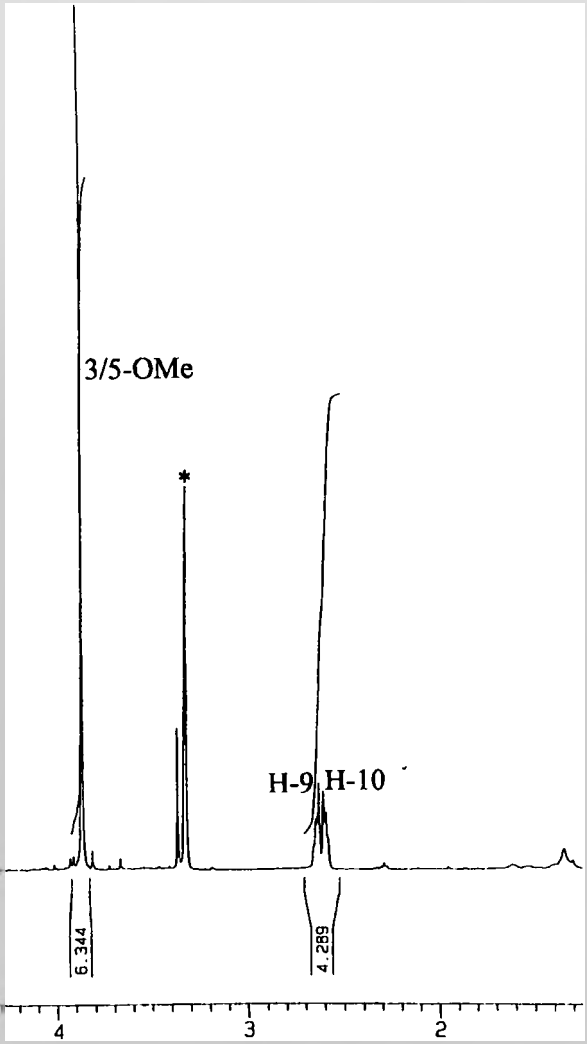
Data obtained in C_5D_5N

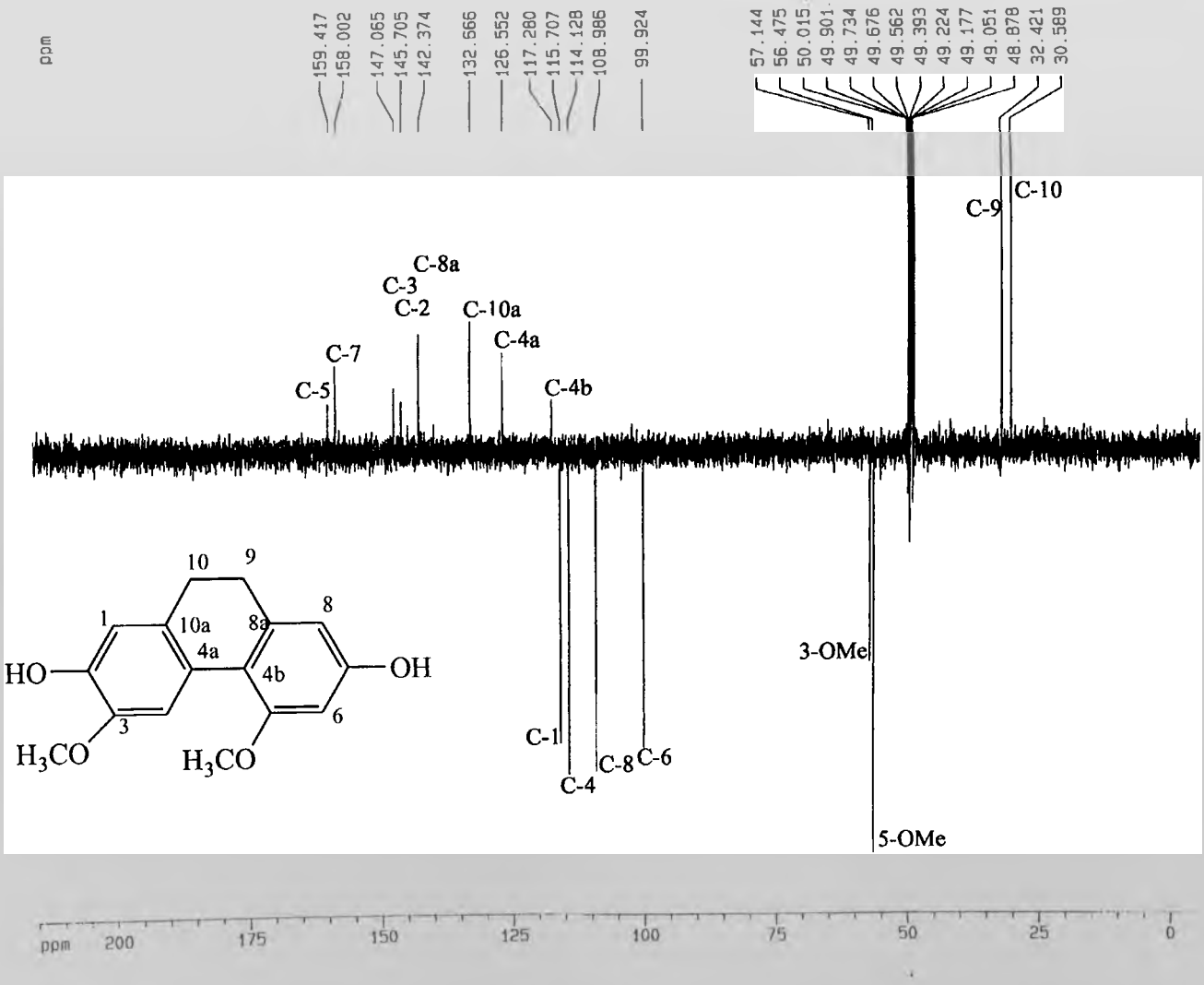
CHE-h7, also named 6-O-methyl coelonin, has been previously isolated from *Cymbidium aloifolium* (Juneja *et al*, 1987), *Agrostophyllum callosum* (Majumder *et al,* 1995) and *Bulbophyllum vaginatum* (Leong *et al*, 1997), all species of Orchidaceae. This is the first report of its isolation from Combretaceae.



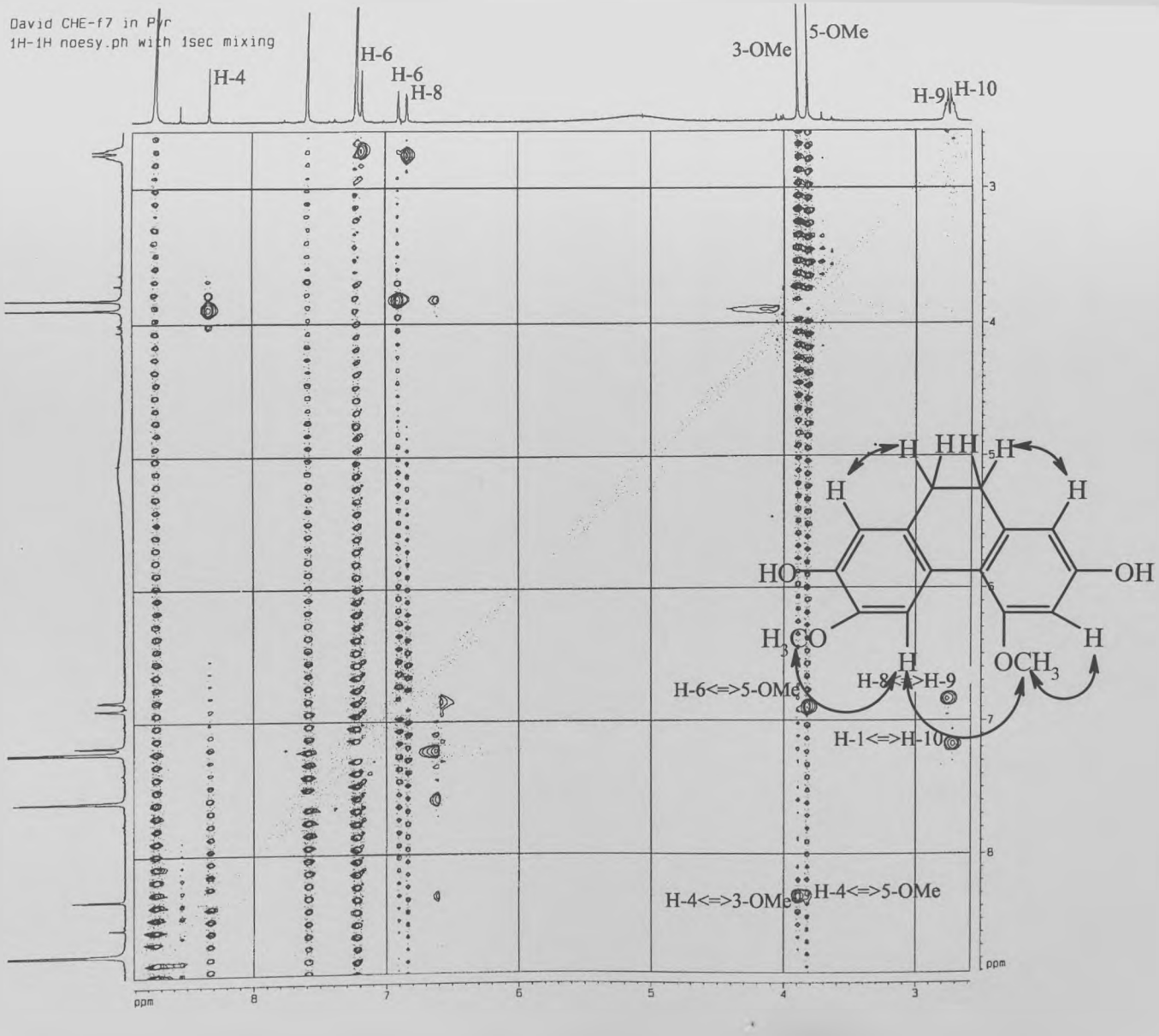


Spectrum 5.16: ^{1}H NMR (500MHz, CD_3OD) of CHE-7


3/5-OMe
*
H-9, H-10
6.344
4.289
4
3
2



Spectrum 5.17: ^{13}C (J-modulated) NMR (125MHz, CD_3OD*) of CHE-7



Spectrum 5.18:^{1}H-^{1}H NOESY correlations of CHE-7

Characterisation of DA-9

DA-9 (2.1mg) was isolated from the leaves of *C. collinum* as a brownish-red amorphous solid, t_R =11.5min, mp. 201°C. IR showed the presence of hydroxyl (*ca.* 4337, 1362 cm^{-1}), methoxyl (2937 cm^{-1}) and aromaticity (1612, 1453, 952, 755cm^{-1}) and was similar to that of CHE-7 above. HREIMS gave the molecular ion, M^+ at *m/z* 302.12 (100%) which corresponds to the molecular formula, $C_{17}H_{18}O_5$. The other significant peaks are due to the discreet loss of small fragments, a phenomenon typical of phenanthrenes (Letcher and Nhamo, 1972). They appear at *m/z* 285 (70%) $[M\text{-}OH]^+$, 269 (25%) $[M\text{-}CH_3OH]^+$, 255 (95%) $[M\text{-}C_2H_5OH]^+$, 241 (25%) $[M\text{-}C_3H_7OH]^+$ and 227 (27%) $[M\text{-}C_4H_9OH]^+$

The PMR spectrum is typical of phenanthrenes with a downfield proton at δ_H 8.81, typical of H-4 or H-5. The hydroxyl groups appear as a broad peak at *ca* δ_H 11.0 while further upfield there is a singlet at δ_H 6.57, three methoxyl groups at δ_H 3.92 (δ_C 56.8), δ_H 3.91 (δ_C 61.0) which is probably out of plane and thus downfield in the ^{13}C-domain, and at δ_H 3.83 (δ_C 56.3). There is a multiplet which integrates for four protons at δ_H 2.84-2.75 (δ_C 31.7 and 30.2) typical of the AA'BB' complex of 9,10 dihydrophenanthrenes.

The ^{13}C NMR is almost superimposable on that of CHE-h7, except that the compound in this case has three instead of two methoxyl substituents. The spectrum showed 17 signals – two methylene carbons at *ca* δ_C 30 typical of C-9 and C-10, three methoxyl carbons, and three methines at δ_C 104.4, 116.5, 117.3. The rest of the signals are due to the quaternary carbons and lie between δ_C 117 and 152.

A tentative structure was proposed based on the NMR data and comparison with CHE-7. See Fig. **5.17** below.

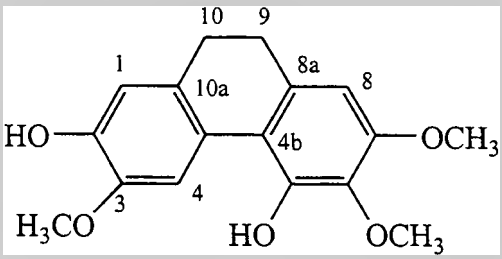


Fig.5.17. Proposed structure of DA-h9.

From HMBC, it would appear that there is a proton under the pyridine peak at δ_H 7.22, attached to the carbon resonating at δ_C 116.5. This proton shows strong correlations to C-10 (δ_C 30.2) and also to two quaternary carbons at δ_C 146.9 and 149.5. The singlet at δ_H 6.57 couples to C-9 (δ_C 31.7). Other significant correlations are shown in Table **5.12.**

^1H-^1H NOESY spectra showed the correlation of H-1 (δ_H 7.22) to H-10, and H-8 (δ_H 6.57) to H-9. This proton (H-8) also correlates to the methoxyl at δ_H 3.83 which is attached to C-7 (δ_C 151.8). One other methoxyl (δ_H 3.92) also has nOe correlation to H-4 (δ_H 8.81). This is placed at C-3.

Table 5.12. ^{1}H NMR (400MHz), ^{13}C NMR (100MHz) spectral data and significant HMBC correlations of DA-h9

Position	δ_H	δ_C	^{2}J	^{3}J
1	7.22 (s)	116.5	-	30.2 (C-10),126.0 (C-4a), 147.2 (C-3)
2	-	146.9		-
3	-	147.2		-
4	8.11 (s)	114.3	147.2 (C-3), 126.90 (C-4a)	146.0 (C-2), 132.7 (C-10a)
4a	-	126.0	-	-
4b	-	134.9	-	-
5	-	149.5	-	-
6	-	137.0	-	-
7	-	151.8	-	-
8	6.57(s)	104.4	151.8 (C-7) 117.3 (C-8a)	137.0 (C-6), 134.9 (C-4b)
8a	-	117.3	-	-
9	2.79 (m)	31.7	117.3 (C-8a), 30.2 (C-10)	134.9 (C-4b), 104.4 (C-8)
10	2.79 (m)	30.2	131.9 (C-10a) 31.7 (C-9)	116.5 (C-1), 126.0 (C-4a)
10a	-	131.9	-	-
3-OCH_3	3.92 (s)	56.8	-	147.2 (C-3)
6-OCH_3	3.91 (s)	61.0	-	137.0 (C-6)
7-OCH_3	3.83 (s)	56.3	-	151.8 (C-7)

Obtained in C_5D_5N

DA-h7 was characterised as 9,10-dihydro-3, 6, 7-trimethoxy-2,5-phenanthrenediol. This has been isolated from the heartwood of *C. apiculatum* and *C. psidiodes* previously (Letcher *et al*, 1972). This is the first report of phenanthrenes in *C. collinum*. There is no NMR data in the literature with which to compare.

Identification of CHE-h10

CHE-h10 (5mg) was isolated by HPLC, t_R =14.5min. It is a brown amorphous solid, mp. 120°C. IR was similar to that of CHE-h7 showing hydroxyl (3370, 1390, 1350 cm^{-1}), aromatic methines 1600, 1470, 930, 840 cm^{-1}) with *meta*-substitution (880, 860, 840 cm^{-1}) and *para* / *ortho*-disubstitution (750 cm^{-1}) and methoxyl groups (2840 cm^{-1}).

Obtaining the NMR data in pyridine-*d*5 was found to be much better than in methanol-*d*4 resulting in better resolution especially in the aromatic region and allowed for the visualization of hydroxyls as a broad peak below 11ppm. The NMR spectra showed the presence of three methoxyl groups at δ_H 3.86 (δ_C 61.9), δ_H 3.86 (δ_C 61.2) and δ_H 3.99 (δ_C 56.7) and five aromatic protons at δ_H 7.29 (s, 1H), δ_H 7.63 (d, J=8.8, 1H), δ_H 7.74 (d, J=8.7, 1H), δ_H 7.81 (s,1H) and at δ_H 9.60 (s,1H). The presence of the highly deshielded proton at δ_H 9.60 as well as what appears to be an AB quartet in the aromatic region implies that the structure of the compound in question is probably a phenanthrene. HRESIMS yielded the molecular ion, M^+, which was also the base peak, at *m/z* 300 which implied a molecular formula of $C_{17}H_{16}O_5$. Other prominent peaks were at *m/z* 285 (39%) $[M\text{-}CH_3]^+$, 257 (20%) $[M\text{-}COCH]^+$, 242 (37%) $[M\text{-}COC_2H_6]^+$and 149 (7%), a tropylium.

The ^1H-^1H NOESY spectrum led to the proposal of at least three isomeric structures – 3,5,8-trimethoxy-2, 7-phenanthrenediol (I), 3,5,7-trimethoxy-2,8-phenanthrenediol (II) and 3,5,7-trimethoxy-2,6-phenanthrenediol (III). See Fig.**5.18.**

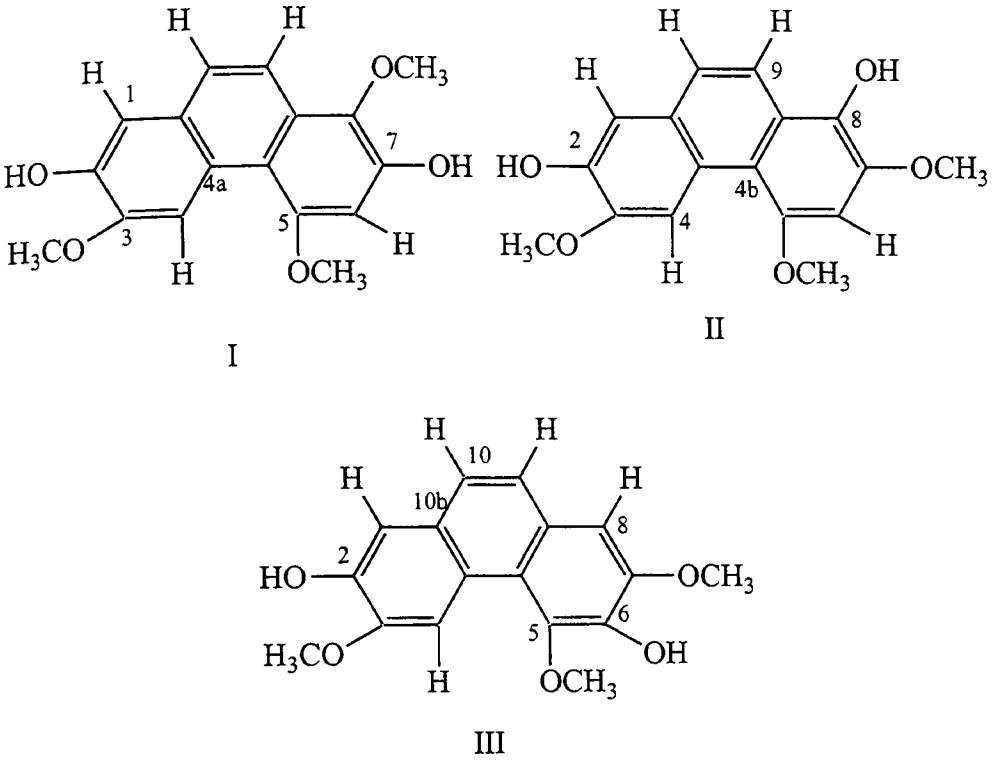


Fig.5.18. The possible structures of CHE-h10 constructed on the basis of observed NOESY interactions.

These all satisfy the NOESY correlations of H-10 (δ_H 7.64) to H-1 (δ_H 7.30), and of H-4 (δ_H 9.62) to the methoxyl groups attached to C-3 and C-5 and appearing at δ_H 4.00 and δ_H 3.88 respectively in the proton domain. Spatial interactions in ring C are more tenuous because the poor resolution in the methoxyl signals (δ_H 3.87-400ppm) makes it difficult to ascertain the nOe interactions. There is, however, no interaction between H-9 and the substituent at C-8 as would be expected for I and II. No obvious nOe correlations were seen between H-9 and H-8 to give credence to III.

The solution to this problem lay with a J-modulated ^{13}C spectrum and HMBC. It has been observed in this study, that the carbon spectra of the phenanthrenes isolated were of poor quality and resolution. This would be expected because of the prevalence of quaternary carbon atoms which are virtually isolated from the protons which aid their relaxation (Croasum and Carlson, 1994). To circumvent the problem, J-mod ^{13} C NMR was obtained over 18 hours (i.e. 8000 scans) with relaxation period, d1, increased to 7sec. This produced a better spectrum which revealed the existence of methines at δ_C 106.2, 112.6, 113.4, 124.4 and 126.9, and more significantly, the absence of a signal at *ca.* δ_C 100ppm. This absent signal is usually indicative of a methine carbon which is *ortho* to two oxygenated neighbours (Juneja *et al*, 1987; Majumder and Kar, 1987). The evidence suggesting the absence of a proton in position 6 effectively discounted I and II.

Thus, CHE-h10 can only be 3,5,7-trimethoxy-2, 6-phenanthrenediol. This is supported by HMBC data which show ^3J coupling between H-10 (δ_H 7.64) and C-1 (δ_C 106.2) and C-4a (δ_C 119.4), between H-1 (δ_H 7.30) and C-3 (δ_C 143.0) and C-4a, and between H-4 (δ_H 9.62) and C-2 (δ_C 147.5), C-4a and C-4b (δ_C 124.8). The proposed arrangement in ring C is confirmed by the HMBC coupling of H-8 to C-7 (δ_C 148.4), C-6 (δ_C 152.2) and C-9 (δ_C 124.4). H-9 in turn couples to C-8 (δ_C 113.4).

The foregoing is a good example to show the limitations of NOESY and its importance when used in conjunction with the other NMR methods.

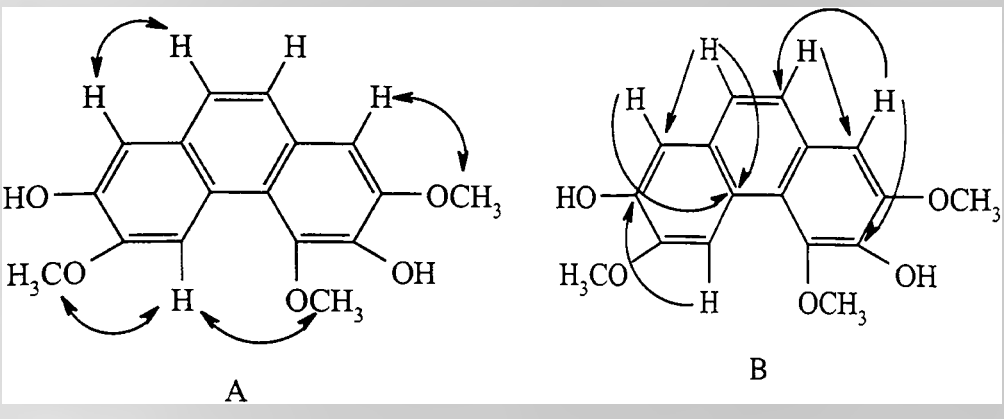


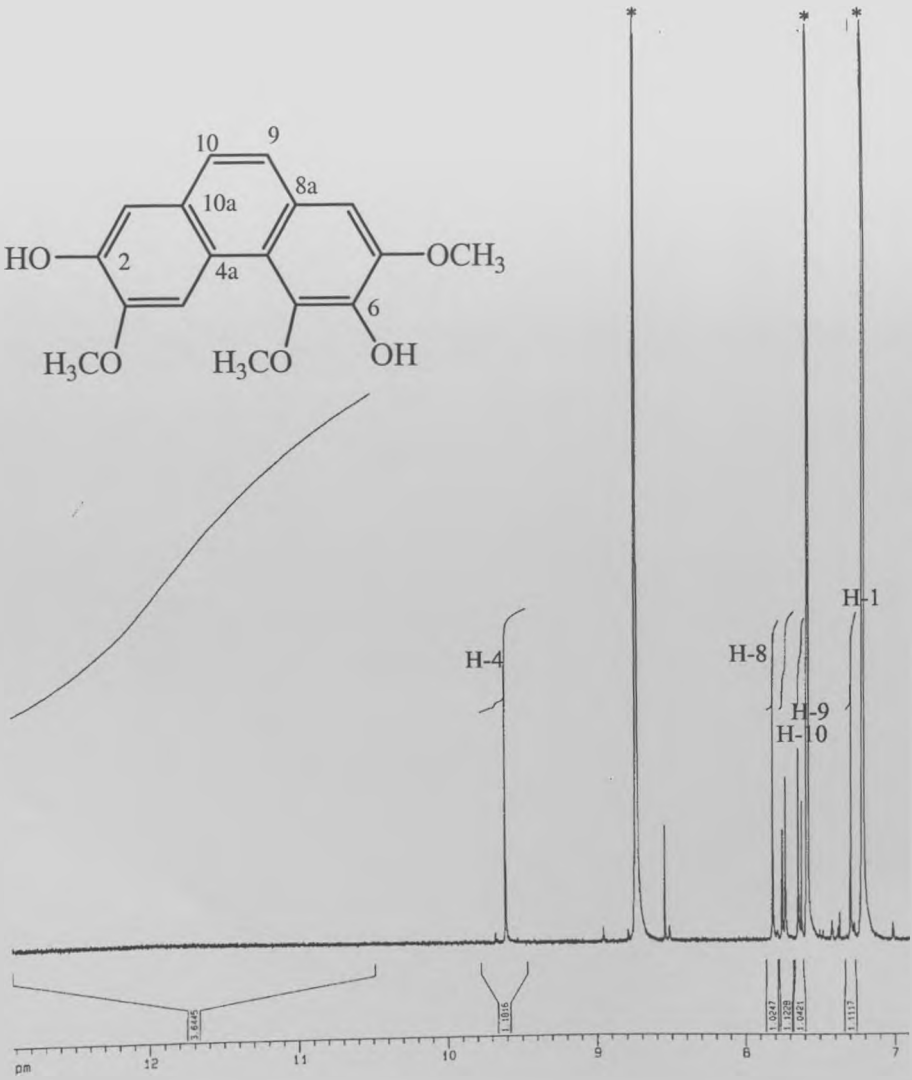
Fig. 5.19. Significant NOESY interaction (A) and HMBC correlations (B) for CHE-h10

Table 5.13. ^{1}H (500MHz), ^{13}C (125 MHz) and HMBC NMR spectral data for CHE-h10

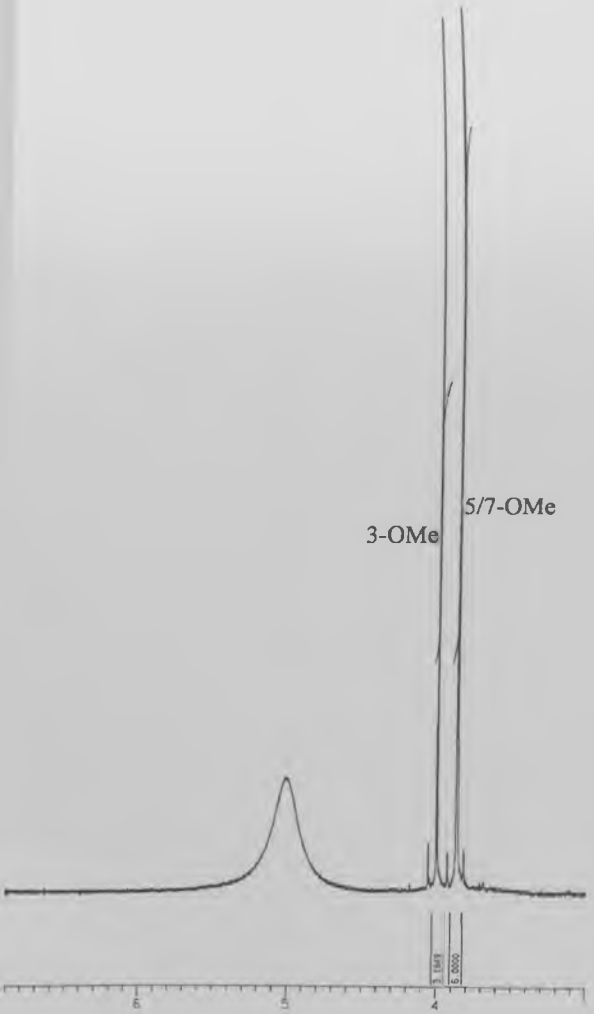
Position	δ_H	δ_C	2J	3J
1	7.30 (s)	106.2	-	119.4 (C-4a), 143.0 (C-3)
2	-	148.4	-	-
3	-	143.0	-	-
4	9.62 (s)	112.6	119.4 (C-4a)	147.5 (C-2), 124.8 (C-4b)
4a	-	119.4	-	-
4b	-	124.8	-	-
5	-	152.2	-	-
6	-	152.2	-	-
7	-	147.5	-	-
8	7.82	113.4	148.4 (C-7)	152.2 (C-6), 124.4 (C-9)
8a	-	128.0	-	-
9	7.74 (ABq, J=8.7)	124.4	-	113.4 (C-8)
10	7.64 (Abq, J=8.7)	126.9	-	106.2 (C-1), 119.4 (C-4a)
10a	-	130.0	-	-
7-OCH_3	3.87	61.2	148.4	-
3-OCH_3	4.00	55.8	143.0	-
5-OCH_3	3.88	60.3	152.2	-

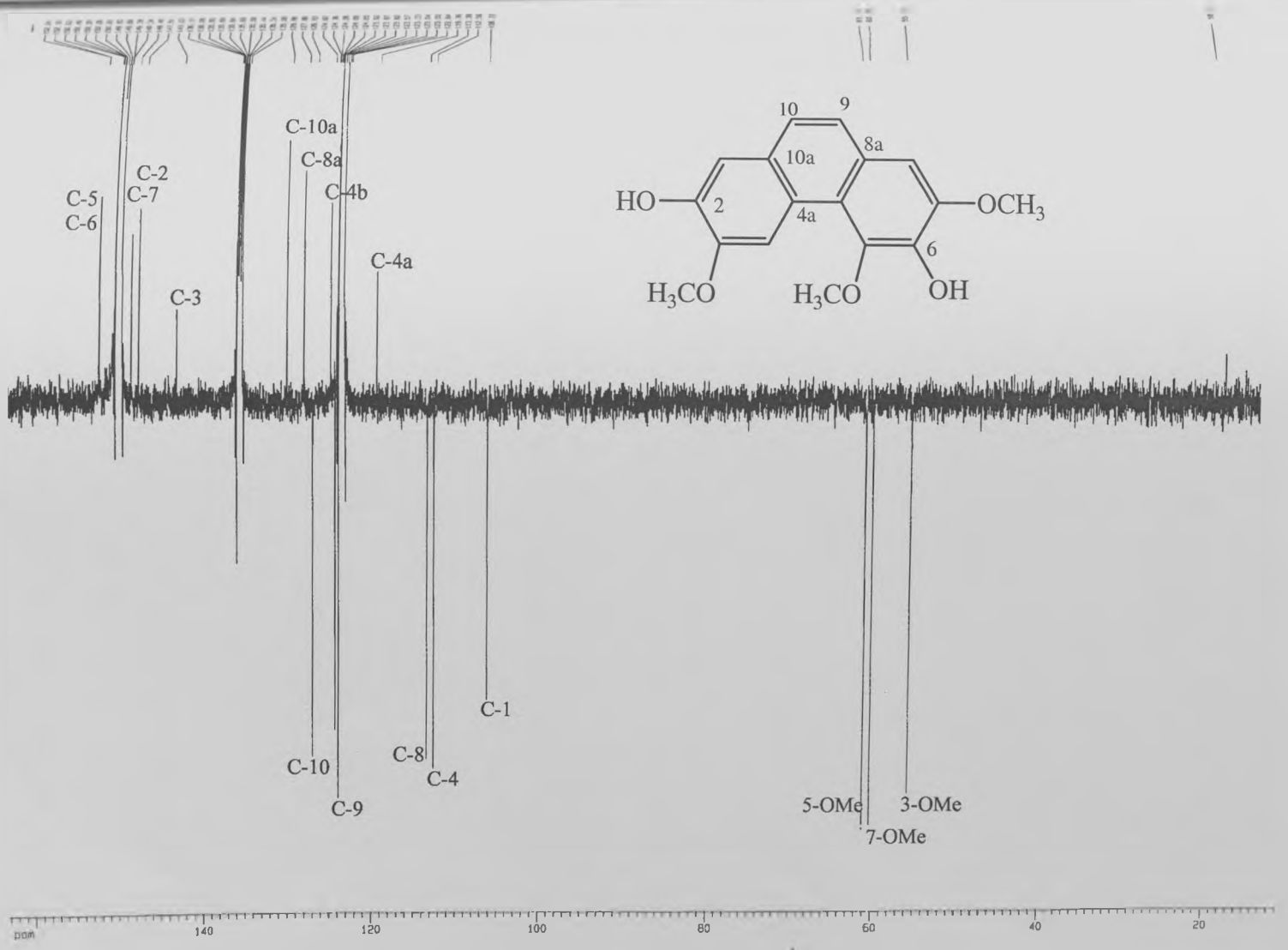
Obtained in C_5D_5N

The interaction between H-9 and neighbours attached to C-10 and C-8 does not occur probably because of some loss of overall planarity due to steric interactions as postulated by Pettit and his co-workers (1989) or the spatial orientation of the atoms. It may also be that n.O.es do exist between these moieties but different "mixing times" need to be tried to enable them to show up.

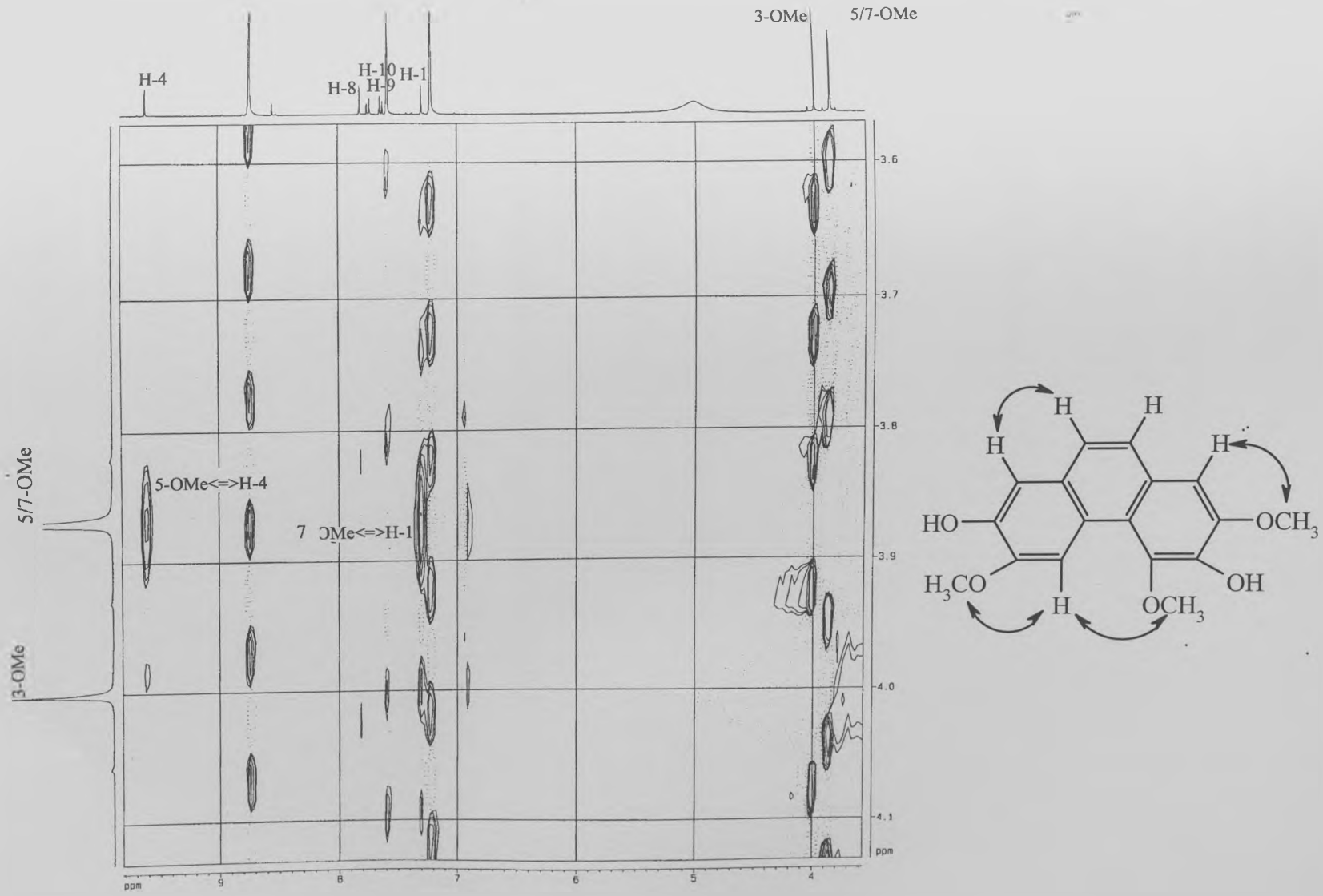


Spectrum 19: ^{1}H NMR (500MHz, C_5D_5N*) of CHE-h10


3-OMe
5/7-OMe
6
5
4



Spectrum 20:^{13} C (PENDANT) NMR (125MHz, C_5D_5N*) of CHE-h10



Spectrum 5.21:^{1}H-^{1}H NOESY (400MHz, C_5D_5N*) of CHE-h10

Identification of CHE-12

CHE-12 was isolated by HPLC, t_R=15.5min, as a deep brown amorphous solid (7.6mg). FTIR indicated the presence of hydroxyl (3380, 2750, 1390, 1360 cm^{-1}), aromatic ring (1610, 1475, 940, 840 cm^{-1}) multiply-substituted (900-680 cm^{-1}) i.e. 880, 830, 760, 720 and 680 cm^{-1}, and methoxyl (2840, 1360 cm^{-1}).

NMR indicated the presence of two methoxyl singlets at δ_H 3.85 and 3.72 as well as six aromatic protons – two *meta*-coupled at δ_H 6.90 (d, J=2.4Hz, 1H) and δ_H 7.13 (d, J=2.4Hz, 1H), and AB quartet at δ_H 7.67 (d, J=9Hz, 1H) and δ_H 7.80 (d, 9Hz, 1H), a singlet at δ_H 7.83 and another at δ_H 9.69 which is diagnostic of the H-5 or H-4 proton in phenanthrenes (Letcher and Wong, 1978; Majumder *et al*, 1996; Takasugi *et al*, 1987).

HREIMS gave the base peak and molecular ion, M^+, *m/z* 270 which corresponded to the molecular formula of $C_{16}H_{14}O_4$, with other significant fragments at *m/z* 255 (19%) $[M-CH_3]^+$, 237 (10%) $[M-CH_5O]^+$, 227 (15%) $[M-COCH_3]^+$, 212 (14%) $[M-COC_2H_6]^+$, 184 (8%) $[M-C_3H_6O_2]^+$, a tropylium at *m/z* 149 (28%) $[C_9H_9O_2]^+$ and *m/z* 77 (66%) $[C_6H_5]^+$. See Fig **5.20.**

^1H-^1H NOESY was used to find out the substitution pattern on the putative tetraoxygenated phenanthrene. This showed spatial interaction between the methoxyl at δ_H 3.72 (C-5) and the protons at C-4, δ_H 9.69 and C-6, δ_H 6.90.

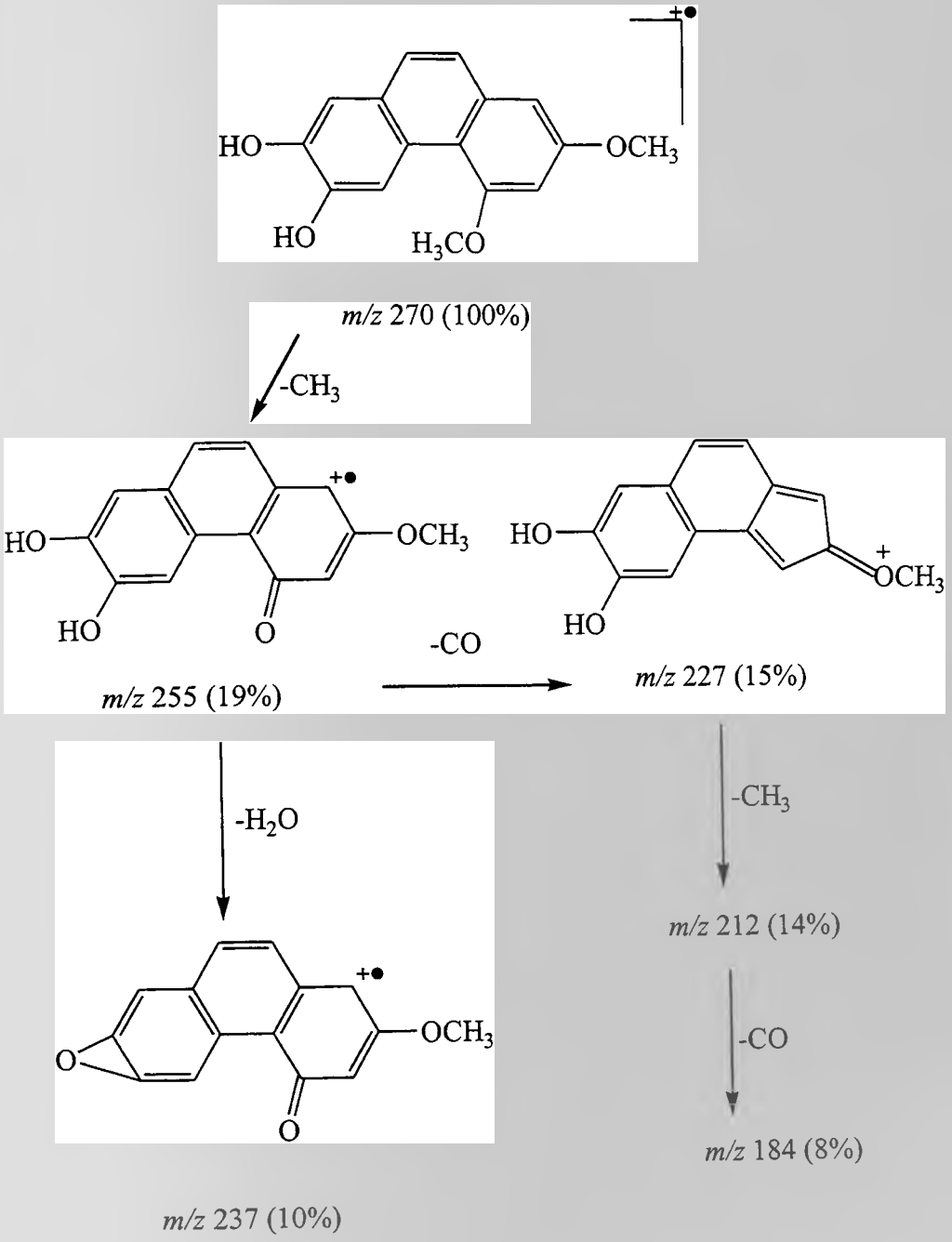


Fig.5.20. Suggested fragmentation pattern typical of phenanthrenes which is exemplified by CHE-12.

The latter shows NOESY correlations to the methoxyl at δ_H 3.85 which is coupling to the neighbouring H-8 proton which in turn shows through-space coupling to H-9 at δ_H 7.67.See Fig. **5.21.**

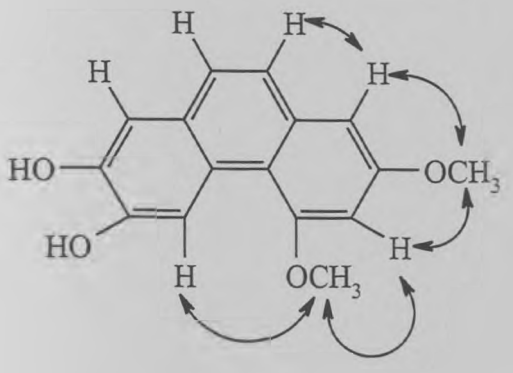


Fig.5.21. Significant NOE interactions of CHE-h12

CHE-14 was identified as 5,7-dimethoxy-2,3-phenanthrenediol. It has been previously isolated from the heartwood of this species, *Combretum hereroense* (Letcher and Nhamo, 1973) and also from the chinese yam, *Dioscorea batatas*, kept in a moist chamber at 15°C for one day then inoculated with *Pseudomonas cichorii* and incubated at 15°C for three days (Takasugi *et al*, 1987). In the latter, the objective was to induce its synthesis via the phytoalexin metabolic pathway.

This is the first report of the presence of this phenanthrene in the fruit of *C. hereroense* which may point to its possible anti-feedant properties.

Table 5.14. ^{1}H (400MHz) and ^{13}C (100MHz) NMR spectra for CHE-12

Position	δ_H	δ_C
1	7.83	113.8
2	-	148.1
3	-	146.9
4	9.69	113.2
4a	-	123.8
4b	-	116.2
5	-	159.7
6	6.90 (d, J=2.4)	99.4
7	-	157.8
8	7.13 (d, J=2.4)	102.1
8a	-	125.6
9	7.67 (d, J=9)	124.5
10	7.80 (d, J=9)	128.1
10a	-	127.8
5-OCH_3	3.72	55.3
7-OCH_3	3.85	55.4

Obtained in C_5D_5N

Characterisation of CHE-17

CHE-17 (6.6mg) a brown solid, was isolated by HPLC, t_R = 21 min, mp. 199-202°C. The IR spectrum was typical of substituted phenanthrenes and similar to that seen for the other compounds isolated, above. It showed the presence of hydroxyl (*ca* 3400, 1360 cm^{-1}), benzenoid (1610, 1465, 940, 840, 750 cm^{-1}) and methoxyl (2940, 2870 cm^{-1}).

The 1D-NMR was quite similar to that seen for the other phenanthrenes. The aromatic region showed the characteristically deshielded proton at δ_H 9.75 (δ_C113.5) which is diagnostic of non-substitution at either position 4 or 5.

The two *ortho* coupled protons at δ_H 7.51 and 7.79 (J=9Hz) are the 9 and 10 protons respectively. In addition, and as with CHE-12, there is a *meta*-coupled pair resonating at δ_H 7.05 and 6.94 (J = 2.5Hz). The two signals upfield are due to the two methoxyl groups and they appeared as singlets at δ_H 3.79 and 3.72.

Comparison of the spectra with CHE-12 and the literature allowed one of the *meta*-coupled protons resonating at δ_H 6.94 (δ_C100.0) to be placed *ortho* to two oxygenated positions C-2 and C-4. This proton is relatively more shielded than its partner which is placed at position 8. When all the protons have been accounted for and placed as H-9, H-10, H-6 and H-8, the remaining proton is confirmed as being at position 4. All the other positions are carrying oxygenated substituents. See Fig.**5.22.**

HMBC showed both 2-bond and 3-bond correlations. H-10 (δ_H 7.79) correlates to C-1 (δ_C 135.7), C-10a (δ_C 128.0) and C-4a (δ_C 120.5) while its partner H-9 (δ_H7.51) showing HMBC coupling to C-10a, C-8 (δ_C 102.2) and C-4b (δ_C 117.0). The H-4 proton correlates to its neighbours C-3 (δ_C 148.4) and C-4a and strongly to C-2 (δ_C 146.0), C-10a and C-4b. The H-6 and H-8 have common coupling to C-7 (δ_C 158.2) and C-4b and also couple to each other's carbon.

Thus the 2,4,6,7,8-pentaoxygenated phenanthrene was constructed from the 1-D NMR data and from HMBC, but the actual substitution pattern could only be confirmed by NOESY. See Fig. **5.23**. This shows that H-9 (7.51) spatially interacts with H-10, as

expected, in addition to H-8 (7.05). This proton, in turn, has n.O.e interaction with a methoxyl at 3.79. The proton at 6.94 (H-6) appears to interact with both methoxyls, but the stronger interaction is with the methoxyl at 3.72. This type of preferential interaction was seen with the other phenanthrenes in this study especially CHE-12, and may arise due to the non-planar orientation of the substituent concerned. The methoxyl at 3.72 also, as expected, interacts with the proton at H-4 (9.75). The implication of placing the methyls at C-5 and C-7 on ring C, is that the substituents in ring A are all hydroxyl.

Table 5.15. ^{1}H NMR (400MHz), ^{13}C NMR (100MHz) spectral data and significant HMBC correlations of CHE-h17

Position	δ_H	δ_C	^{2}J	^{3}J
1	-	135.7	-	-
2	-	146.0	-	-
3	-	148.4	-	-
4	9.75 (s)	113.5	148.4 (C-3) 120.5 (C-4a)	146.0 (C-2), 128.0 (C-10a), 117.0 (C-4b)
4a	-	120.5	-	-
4b	-	117.0	-	-
5	-	160.4	-	-
6	6.94 (d, J=2.5)	100.0	160.4 (C-5), 158.2 (C-7)	102.2 (C-8), 117.0 (C-4b)
7	-	158.2	-	-
8	7.05 (d, J=2.5)	102.2	158.2 (C-7)	117.0 (C-4b), 100.0 (C-6)
8a	-	126.6	-	-
9	7.51 (d, J=9)	124.9	-	128.0 (C-10a), 117.0 (C-4b), 102.2 (C-8)
10	7.79 (d, J=9)	127.3	128.0 (C-10a)	135.7 (C-1), 120.5 (C-4a)
10a	-	128.0	-	-
5-OCH_3	3.72 (s)	55.7	-	160.4 (C-5)
7-OCH_3	3.79 (s)	56.0	-	158.2 (C-7)

Data obtained in C_5D_5N. In parentheses is the coupling constant, J, in Hz.

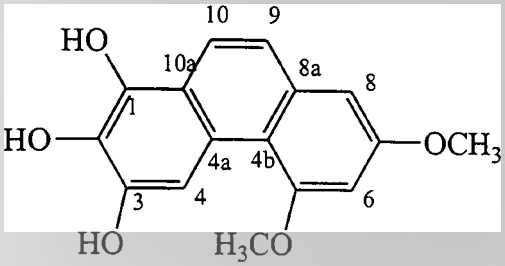
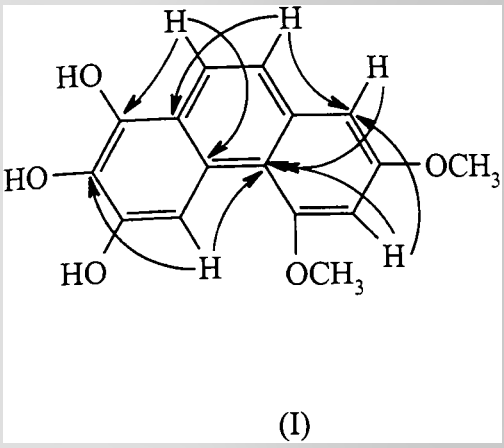
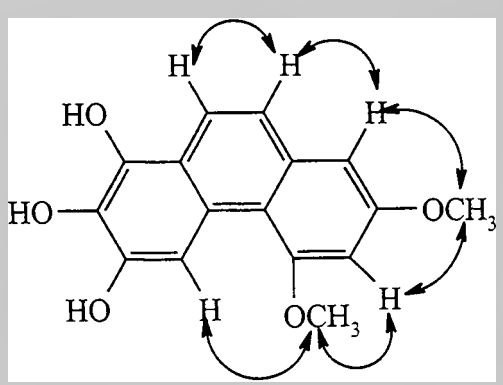


Fig.5.22. Proposed structure of CHE-17

Based on the NMR correlations, 5,7-dimethoxy-1, 2, 3-phenanthrenetriol was proposed as the structure of CHE-17. It would be expected to have a molecular ion at *m/z* 286 from the molecular formula $C_{16}H_{14}O_5$. HREIMS showed an insignificantly small peak at this mass number. The base peak was, in fact, at *m/z* 270 which corresponds to the loss of a hydroxyl moiety from the putative molecular ion. Considering that this compound is trihydroxylated in addition to dimethoxy-substitution, then it would be highly unstable and unlikely to yield the molecular ion in mass spectrometry. The other fragments were as expected from phenanthrenes (Letcher and Nhamo, 1972). They are due to the gradual loss of small stable compounds as illustrated for CHE-12. Some of the important peaks appear at *m/z* 255 (25%) $[M\text{-}CH_3O]^+$, 227 (30%) $[M\text{-}C_8H_6]^+$, 167 (116%) $[M\text{-}C_8H_7O]^-$ and 149 (34%) $[M\text{-}C_8H_9O_2]^+$.



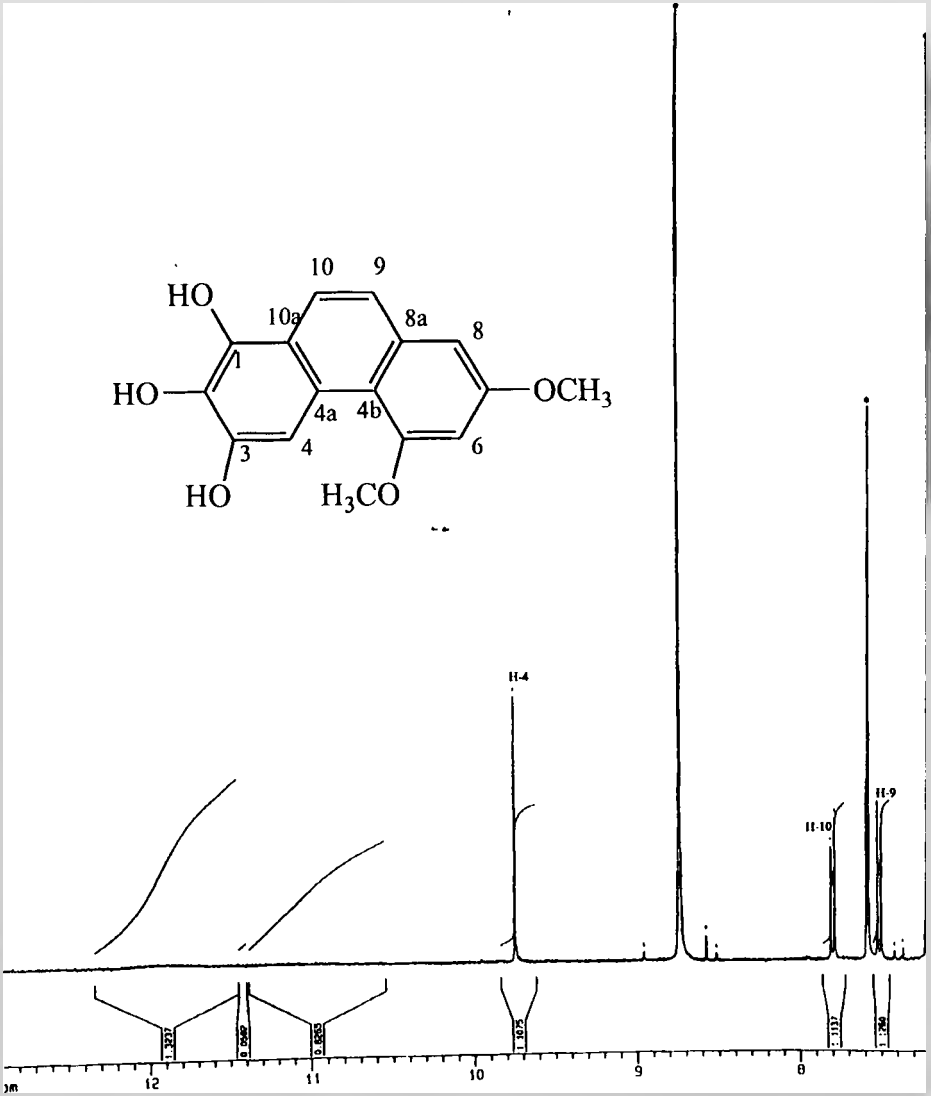
(I)



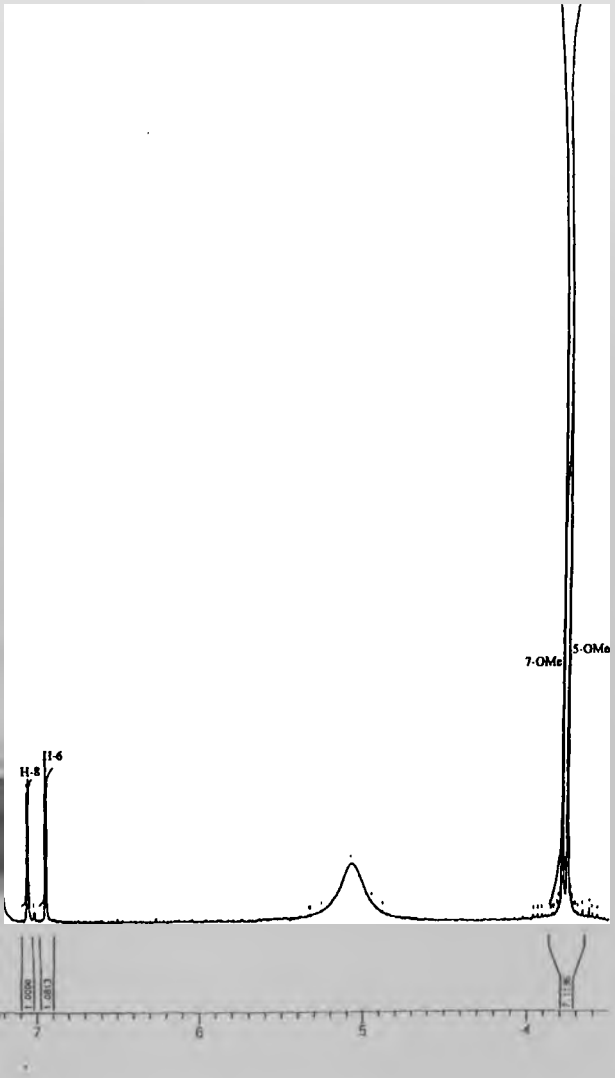
(II)

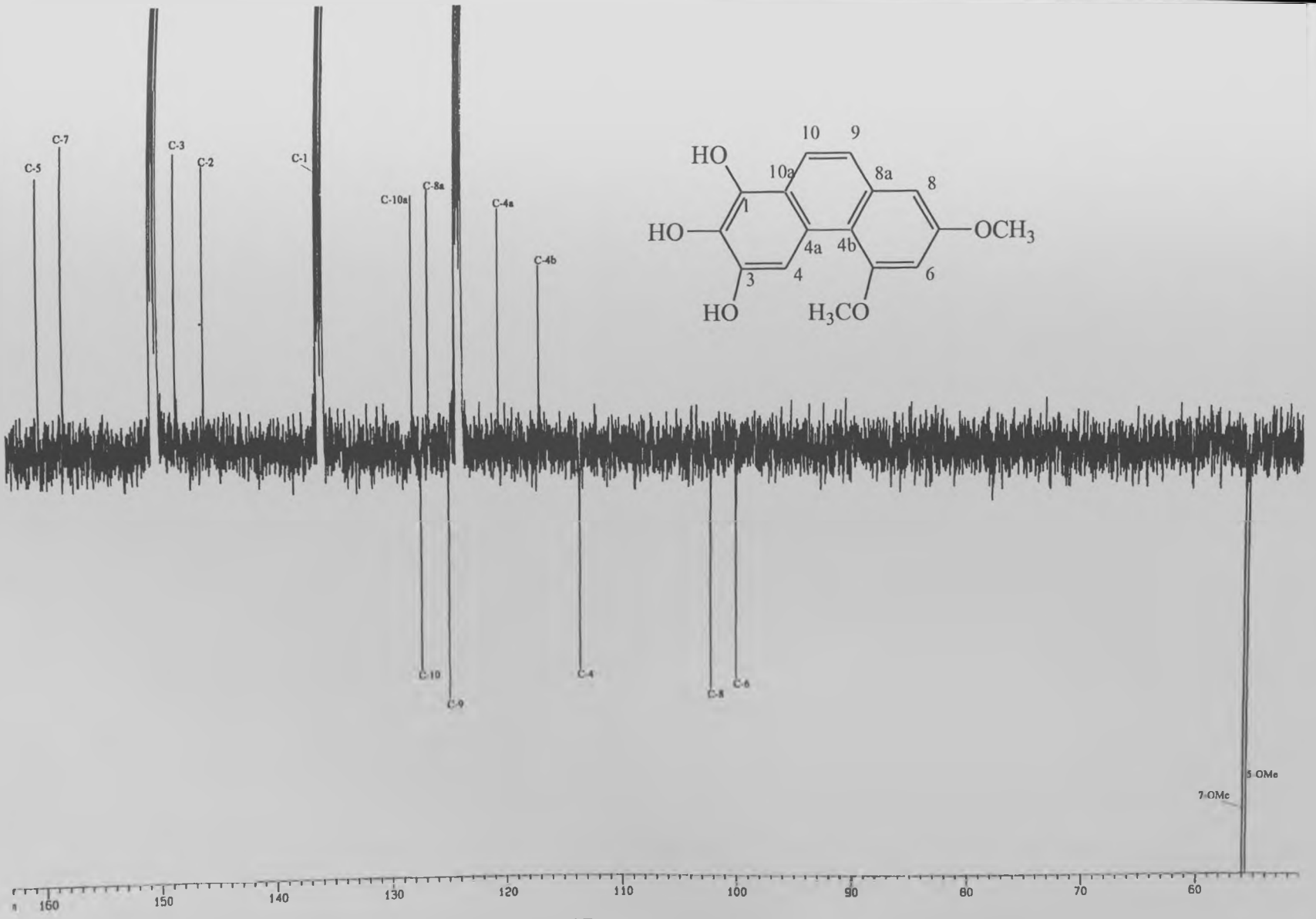
Fig.5.23. Significant HMBC (I) and NOESY (II) correlations in CHE-17.

The foregoing can then be used to justify the proposed structure. CHE-17 is reported here for the first time and is given the trivial name heroranol.

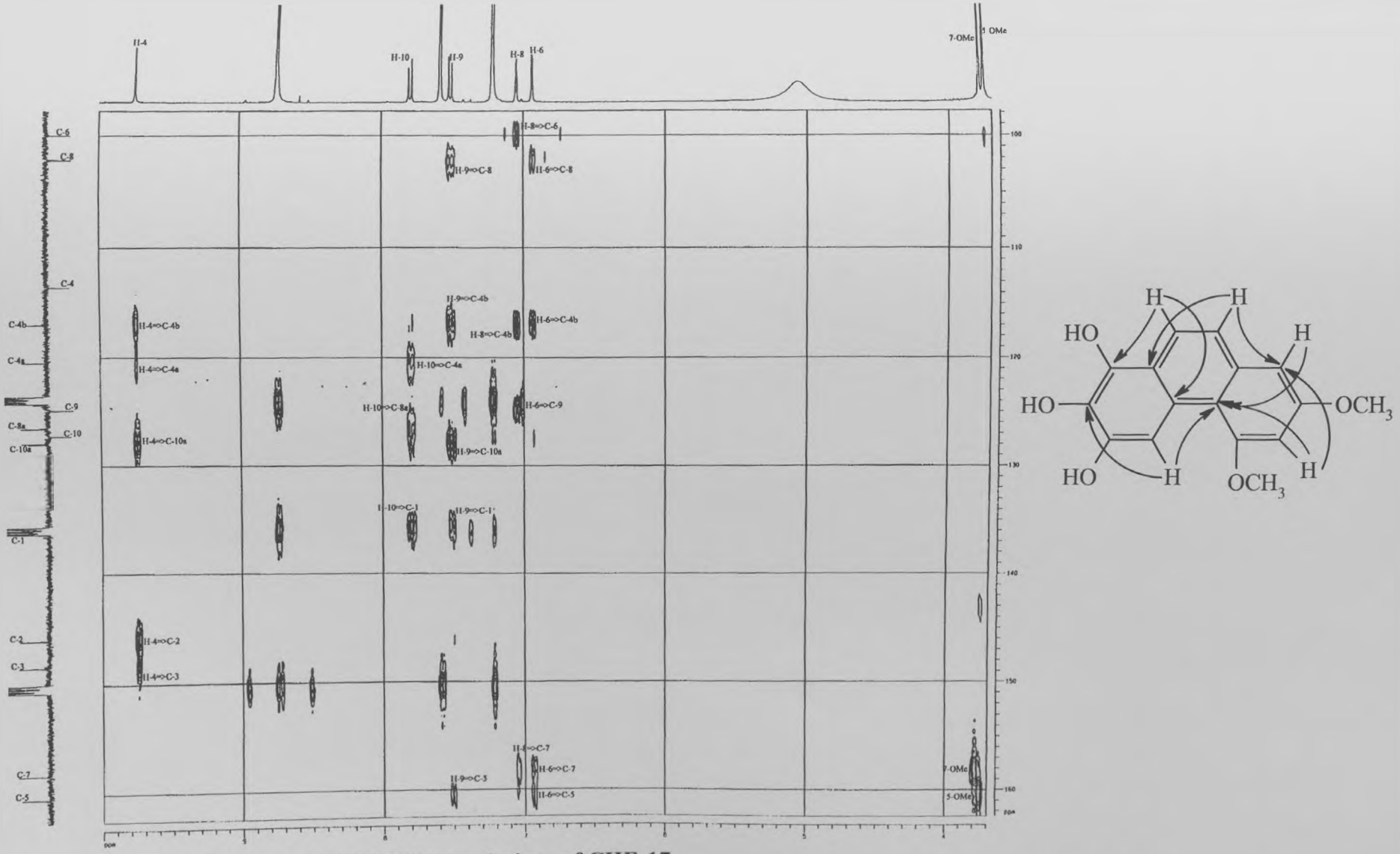


Spectrum 5.22: ^{1}H NMR (400MHz, C_5D_5N) of CHE-17


H-8
H-6
7-OMe
5-OMe
1.000
1.083
7.116
7
6
5
4



Spectrum 5.23: ^{13}C NMR (100MHz, C_5D_5N) of CHE-17.



Spectrum 5.24:HMBC correlations of CHE-17

Properties and spectral data of isolated phenanthrene derivatives

CHE-7 (9,10-Dihydro-3,5-dimethoxy-2,7-phenanthrenediol)

Dark brown amorphous solid. IR ν_{max} film cm^{-1} 3380, 2900, 2840, 1610, 1500, 840,750. Found [M-H] 271.101 ($C_{16}H_{16}O_4$ requires 272.105). HRFABMS *m/z* (rel. int. %): 271 (60), 240 (55), 225 (37), 223 (41), 209 (94), 176 (54), 144 (100).^{1}H and ^{13}C NMR: Table **5.11**. and Spectra **5.16 –18.**

DA-h9 (9,10-Dihydro-2,3,6-trimethoxy-4,7-phenanthrenediol)

Reddish-brown amorphous solid. IR ν_{max} film cm^{-1} 3437, 3018, 1362, 1294, 2903, 2838, 1680, 1612, 1499, 952, 875, 755, 668. Found [M^+] 302.12 ($C_{16}H_{16}O_4$ requires 302.1154). HREIMS *m/z* (rel. int. %): 302.12 (100%), 285 (70), 269 (25), 255 (95), 241 (25), 241 (25), 227 (27).^{1}H and ^{13}C NMR: Table **5.12**.

CHE-10 (2,4,6-Trimethoxy-3,7-phenanthrenediol)

Brown amorphous solid. IR ν_{max} film cm^{-1} 3370 (OH), 2940, 2840 (OCH_3), 1600, 1470, 1390, 1350, 840,750. Found [M^+] 300.099 ($C_{17}H_{16}O_5$ requires 300.0998). HRESIMS *m/z* (rel. int. %): 300 (100), 285 (39), 257 (20), 242 (37), 171 (17), 149 (7). ^{1}H and ^{13}C NMR: Table **5.13**.and Spectra **5.19-20**.

CHE-12 (2,4-Dimethoxy-6,7-phenanthrenediol)

Brown amorphous solid. IR ν_{max} film cm^{-1} 3380 (OH), 2750 (OCH_3), 1610,1475,1390, 940, 840, 720, 680. Found [M^+] 270.096 ($C_{16}H_{14}O_4$ requires 270.089). HREIMS *m/z* (rel. int. %): 270 (100), 255 (19), 237 (10), 227 (15), 212 (14), 184 (8), 149 (28), 84 (95) 77 (66). ^{1}H and ^{13}C NMR: Table **5.14**.

CHE-17 (2,4-Dimethoxy-6,7,8-phenanthrenetriol)

Brown amorphous solid. IR ν_{max} film cm^{-1} 3400 (OH), 2865 (OCH_3), 1610,1460,1360,1280, 1210, 940, 840, 750. Found [M-OH] 270.0892 ($C_{16}H_{14}O_5$ requires 286.0). HREIMS *m/z* (rel. int. %): 270 (100), 255 (25), 237 (10), 227 (30), 212 (15), 184 (23), 167 (16), 149 (34), 84 (90). ^{1}H and ^{13}C NMR: Table **5.15**.and Spectra **5.22-24**.

5.1.2.3. CHALCONOIDS

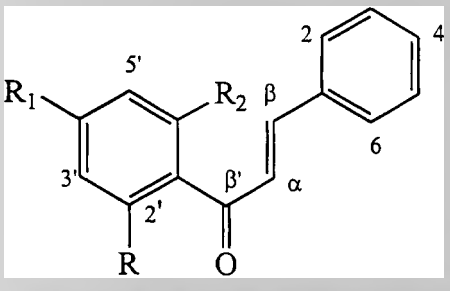
The chalconoids possess a 1,3-diarylprop-2-en-1-one framework with an olefinic bond and a ketonic group (Agrawal and Bansai, 1989). The carbon atoms, all being of sp^2 hybrid character resonate between δ_C 90 and 195ppm depending on the aromatic substitution. When there is a hydroxyl substituent at C-2' the carbonyl signal is shifted downfield by 3-5ppm due to intramolecular hydrogen bonding. The presence of substituents e.g. OMe at both C-2' and C-6' positions results in the loss of coplanarity between the aromatic ring A and the keto group resulting in the latter occurring at a relatively deshielded position around δ_C 191.5ppm. See Fig. **5.23.**

C-β resonates at low field (δ_C 136-147ppm) due to the electron withdrawing benzoyl group attached to the neighbouring carbon atom. This position is dependent on the substitution on C-2, shifting upfield in the presence of an oxygen bearing moiety. The other ethylenic carbon, C-α absorbs at δ_C 116-129ppm.

In this study two simple chalcones were isolated from *C. apiculatum* (B69-13 and B27-8) and one similar to B69-13 and designated AO-18 from *C. albopunctatum*. They were characterized as 2', 4'-dihydroxy-6'-methoxychalcone and 4'-hydroxy-2', 6'-dimethoxychalcone respectively. In addition, three chalcone dimers were also isolated from *C. albopunctatum* (AO-66 and AO-68) and *C. apiculatum* (B69-12). B69-12 and AO-66 are identical, and were characterised as 4-hydroxy-2, 6-dimethoxychalcone dimer while AO-68 was identified as 2,4,4a-trihydroxy-2a,6,6a-trimethoxychalcone dimer.

5.1.2.3.1 SIMPLE CHALCONES

The leaves of *C. apiculatum* afforded two simple chalcones, while another one was isolated from the leaf and fruit extract of *C. albopunctatum*.



Compound	R	R_1	R_2
B69-13	OCH_3	OH	OH
B27-8	OCH_3	OH	OCH_3
AO-18	OCH_3	OH	OH

Fig.5.23. The chalcones isolated in this study. B69-13 and and AO-18 are identical.

General characterisation of isolated chalconoids

The IR spectra showed a broad peak at *ca* 3280cm^{-1} due to a hydroxyl function, aryl ether (*ca* 2845 cm^{-1}) and aromatic rings (*ca* 3010, 1650, 1595 cm^{-1}) with *para*-disubstitution (*ca* 820 cm^{-1}) and *mono*-substitution (*ca* 690 cm^{-1}). The presence of α,β-unsaturated carbonyl was implied by a peak at *ca* 1595 cm^{-1} (Williams and Fleming, 1995). HREIMS and NMR spectra of AO-18, B69-13 and B27-8 were generally similar and implied that the basic skeleton was identical in all of them.

Table 5.16. ^{1}H NMR (500MHz) spectral data of isolated chalcones

Position	B69-13	B27-8	AO-18
β	7.77 (d, 15.5)	7.36 (d, 16.5)	7.73 (d, 15.5)
α	7.99 (d, 15.5)	6.97 (d, 16)	8.01 (d, 15.5)
3'	6.01 (d, 2)	6.22 (s)	6.04 (d, 2)
5'	6.09 (d, 2)	6.22 (s)	6.11 (d, 2)
2/6	7.71 (m, 6.5, 1.0)	7.58 (m, 3.5)	7.73 (m, 1.6, 6.5)
3/5	7.51 (m, 2.5, 1.5)	7.41(m, 3.5)	7.51 (m, 1, 1.4)
4	7.51 (m, 2.5, 1.5)	7.40 (m, 3.5)	7.47 (m, 1,5)
2'-OMe	4.02 (s)	3.75 (s)	4.03 (s)
6'-OMe	-	3.75 (s)	-

All data obtained in CD_3OD. Coupling constant, ***J*** in Hz is in parentheses.

Table 5.17. ^{13}C NMR (125MHz) spectral data of the chalcones.

Position	B69-13*	B27-8	AO-18*
β	143.2	146.3	143.2
α	128.6	130.4	129.4
β'	194.2	197.5	194.0
1'	106.9	111.5	106.0
2'	165.2	160.9	165.0
3'	97.5	93.4	97.6
4'	169.1	162.9	169.0
5'	93.1	93.3	93.0
6'	167.4	160.9	168.0
1	137.3	136.6	137.0
2/6	129.6/129.3	129.8	129.7
3/5	130.4	130.4	130.4
4	131.5	131.9	131.5
2'-OMe	56.8	56.6	56.8
6'-OMe	-	56.6	-

*Alpinetin chalcone (cardomonin) was isolated from both *C. apiculatum* (B69-13) and also from *C. albopunctatum* (AO-18).

^1H NMR showed the presence of a mono-substituted aromatic ring as implied by a complex doublet and two complex, sometimes indistinguishable, triplets integrating for five protons below δ_H 6.90. The olefinic set of doublets with a coupling constant, J=14-16Hz is typical of simple chalcones, while the H-3'/5' (similar to the H-6/8) of the flavones and flavanones appear between δ_H 5.9 and 6.3ppm. See section **5.1.2.5** and **5.1.2.6.** The signals which occur upfield are more distinguishing features as they show the substitution pattern.

Characterisation of B69-13/ AO-18

This was a yellow crystalline solid with melting point 168-170°C. B69-13 was isolated from *C. apiculatum* by HPLC t_R= 19min, while AO-18 (4.8mg) crystallized from a Sephadex LH-20 fractionation (eluent 100% TCM) of *C. albopunctatum*. The discussion below concentrates on the data obtained for AO-18, but should equally apply to B69-13.

HREIMS showed the molecular ion, M^+ at *m/z* 270 implying the molecular formula to be $C_{16}H_{14}O_4$. The base peak appeared at *m/z* 193 $[M-C_2H_5O_3]^+$. Other prominent peaks were at 183 (61%) $[M-C_4H_2O]^+$, 167 (56%) $[M-C_8H_7]^+$, 112 (92%) $[M-C_{11}H_{10}O]^+$, and 103 (49%) $[C_8H_7]^+$. See Fig.**5.24.** The fragmentation pattern is indicative of a chalcone with two hydroxyl and one methoxyl substituents and a mono-substituted B ring (Wollenweber and Siegler, 1982; Nascimento and Mors, 1972).

The ^{1}H NMR of AO-18 showed a methoxyl group at δ_H 4.02ppm and two doublets at δ_H 6.04 (J=2Hz) and δ_H 6.11 (J=2Hz). These are typical of the 3'/5' protons on the chalcone ring and appear characteristically reversed in the ^{13}C spectrum with C-3' downfield at δ_C 97.6ppm and C-5' slightly upfield at δ_C 93.0. The multiplets between δ_H 7.4 and 7.8ppm are due to the mono-substituted ring system which show three different resonances due to the protons attached to C-2/6(δ_C129.7/ δ_H *ca.*7.73), C-3/5 (δ_C130.4/ δ_H *ca* 7.51) and C-4 (δ_C131.5/ δ_H *ca.*7.47) which couple to give complex doublet, triplet, triplet respectively. The last two are usually merged and the carbon resonances only distinguishable from the HMQC. The olefinic protons are *trans* to each other as the coupling constant is large (J=15.5Hz) and appear appreciably deshielded as C-β (δ_C143.2/ δ_H 7.73) and C-α(δ_C129.4/ δ_H 8.01). The carbonyl signal is found at δ_C 194ppm. The presence of hydrogen bonding would move this further downfield. In this case there is the possibility of intramolecular hydrogen bonding but the ability of the phenyl substituent to rotate about the C-β'-C-1 bond makes this improbable.

HMBC showed correlations of H-3' (δ_H 6.04) to C-5' (δ_C 93.0) and also to the quaternary carbon at δ_C 106.0 (C-1'). H-5' shows this coupling as well, and also to C-3' (δ_C 97.6). H-α (δ_H 8.01) shows ^{2}J coupling to the carbonyl at δ_C 194.0 and ^{3}J coupling to C-1. H-β also couples to the carbonyl and to the C-2/6 at δ_C 129.7.

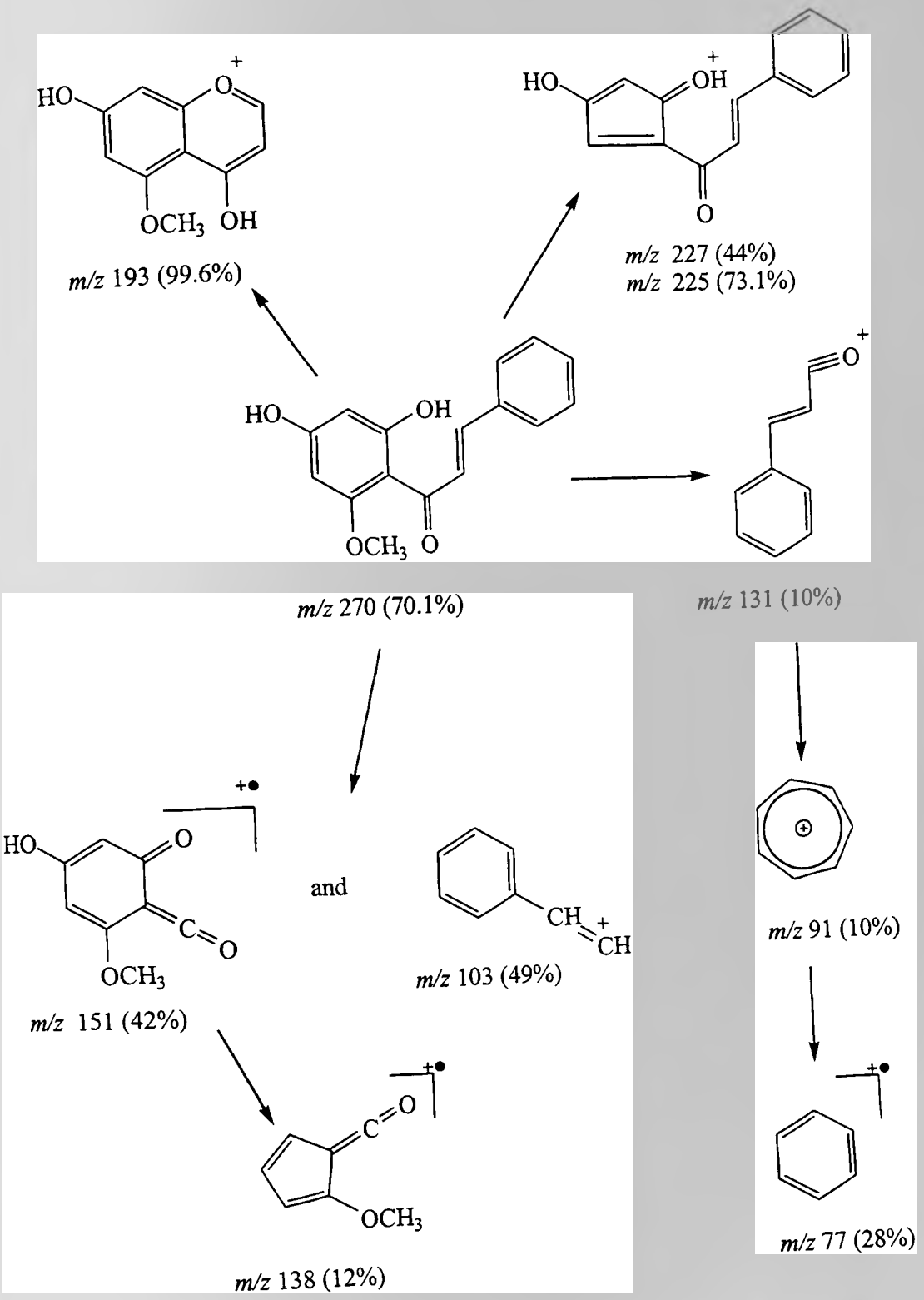


Fig.5.24. Typical fragmentation pattern in chalcones as exemplified by B69-13.

The HMBC coupling in the mono-substituted B ring is obvious and straightforward. In general with chalcones and the flavonoids isolated in this study, the carbon resonances were arranged in reverse order of the proton signals in this ring i.e. C-2/6 were upfield slightly, followed in most cases by C-4 and then C-3/5. All of them appear tightly bunched between δ_C 128-131ppm.

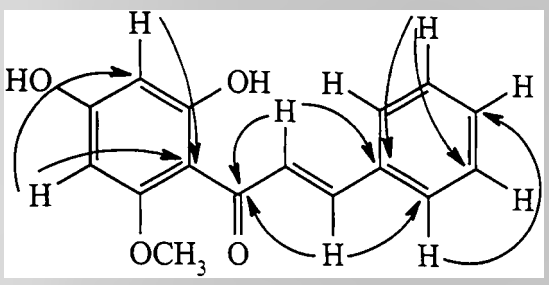


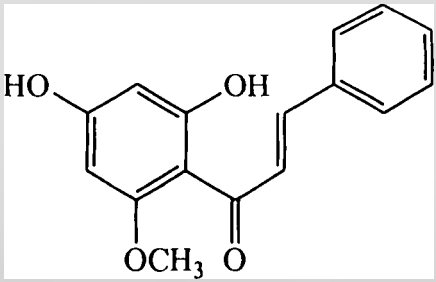
Fig. 5.25. Significant HMBC correlations in AO-18.

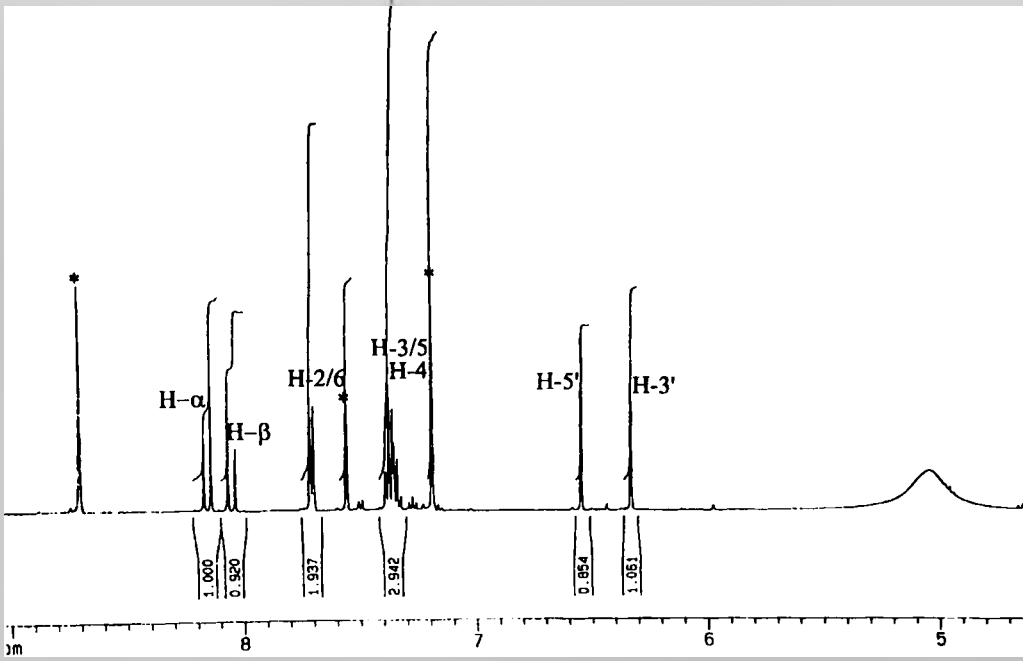
Table 5.18. HMBC correlations for AO-18 (in CD_3OD)

Position (δ)	^1J	^2J	^3J
H-β (7.73)	143.2	-	129.7 (C-2/6), 194.0 (C-β')
H-α (8.01)	129.4	194.0 (C-β')	136.0 (C-1)
H-3' (6.04)	97.6	-	93.0 (C-5'), 106.0 (C-1')
H-5' (6.11)	93.0	-	97.6 (C-5'), 106.0 (C-1')
H-2/6 (7.73)	129.7	-	143.2 (C-β), 131.5 (C-4)
H-3/5 (7.51)	130.4	131.5 (C-4)	136.0 (C-1), 130.4 (C-5/3)
H-4 (7.47)	131.5	130.4 (C-3/5)	1.29.7 (C-2/6)

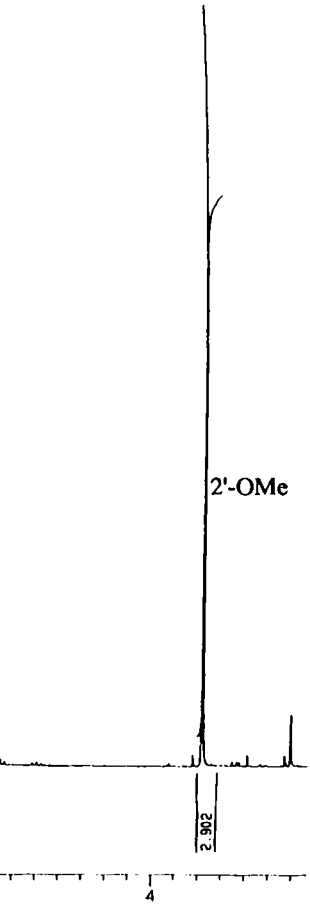
AO-18 (and B69-13) was identified as 2',4' dihydroxy-6'-methoxychalcone. This is the chalcone of alpinetin (which was also isolated in this study) and is known as cardamomin. It has been previously isolated from *Boesebergia pandura, Pityrogramma*

chrysophylla, and species of *Alpinia, Piper and Populus* (Itokawa *et al*, 1981). The NMR data were considerably similar to those in the literature (Agrawal, 1989). Despite its common occurrence, this would appear to be the first report of its presence in Combretaceae.





Spectrum 5.25: ^{1}H NMR (500MHz, C_5D_5N*) of B69-13


2'-OMe
2.902
4

Identification of B27-8

B27-8 (11.8mg) was isolated by HPLC from *C. apiculatum,* t_R 6min. It was a yellow/brown crystalline solid mp. 85-87°C. IR spectra showed the presence of hydroxyl (3280 cm^{-1}), methoxyl (2840, 1130 cm^{-1}) and aromatic rings (900-800 cm^{-1}).

HREIMS showed a similar fragmentation pattern to AO-18 above. The molecular ion M^+ was at *m/z* 284.1 corresponding to $C_{17}H_{16}O_4$, the base peak at 256 $[M-CO]^+$ and other prominent peaks at *m/z* 181 (17%) $[M-C_7H_3O]^+$, 166 (23%) $[M-C_8H_6O]^+$, 103 (23%) $[M-C_9H_9O_4]^+$, and 91(30%) due to the seven-membered tropylium ion, $[C_7H_7]^+$ (Nascimento and Mors, 1972).

The ^1H- and ^{13}C-NMR spectra was almost superimposable on that of B69-13 above except for a singlet at δ_H 3.75 δ_C 56.6 integrating to six protons implying that this compound has one more methoxyl group compared to B69-13. The H-3'/5' signals show the same chemical shift at δ_H 6.22

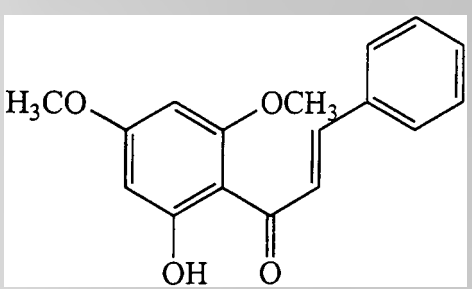


Fig.**5.26.** The structure initially proposed for B27-8 (flavokawain A).

Based on this information, the structure initially suggested was 2'-hydroxy-4', 6'-dimethoxychalcone which is also known as flavokawain A and has previously been isolated from *Piper methysticum*, *Alpinia speciosa*, *Myrica pensylvania* and *Pinus* species (Itokawa *et al*,1981; Wollenweber *et al*, 1981) as well as more recently from *Goniothalamas gardneri* (Seidel, 1999).

A closer inspection of the ^{13}C NMR, however revealed the fact that the methoxyls resonate at δ_H 3.75 (δ_C 56.6) and they are attached to carbons which resonate at δ_C 160.9. C-3'/5' both show up at δ_C 93.4 which implied that they were identical. Further, the literature data of flavokawain A, when compared with the data in this study appeared to be significantly at variance. The A ring shows a significant shift upfield for oxygenated carbons of 4-6ppm, while the rest of the carbons shift downfield by anything between 2 to 5ppm. Because of the similarities in the chemical shifts of C-3'/5' protons NOESY was considered inappropriate as it would give no further insight into the problem.

The sample, initially run in methanol-*d*4 was then run in pyridine-*d*5 to see if the resonances at positions 3'/5' would separate. Such a separation was not seen, confirming the magnetic equivalence of the protons and carbon at these positions. This was indicative of a symmetrical arrangement of the substituents around ring A.

Thus, B27-8 is, in fact, 4'-hydroxy-2', 6'-dimethoxychalcone. HMBC correlation supported this structure – showing H-3'/H-5' both coupling to the quaternary C-1' at 111.5 and to the carbinol at δ_C 162.9. It can then be rationalized that these C-3'/C-5' both

occur at upfield resonances because they are each *ortho* to a methoxyl (Pelter *et al.*, 1976). The rest of the ^{2}J and ^{3}J correlations are as shown in Table **5.21.**

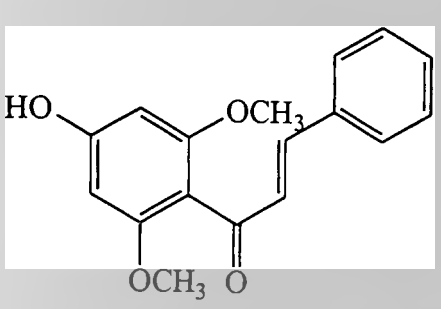


Fig.**5.27.** The structure of B27-8

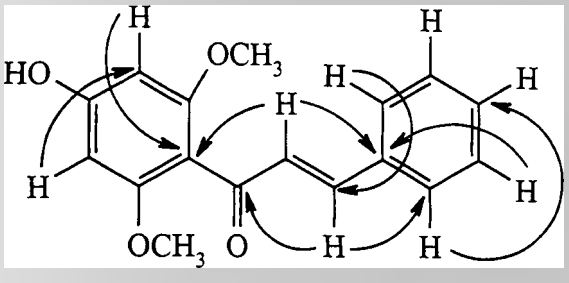


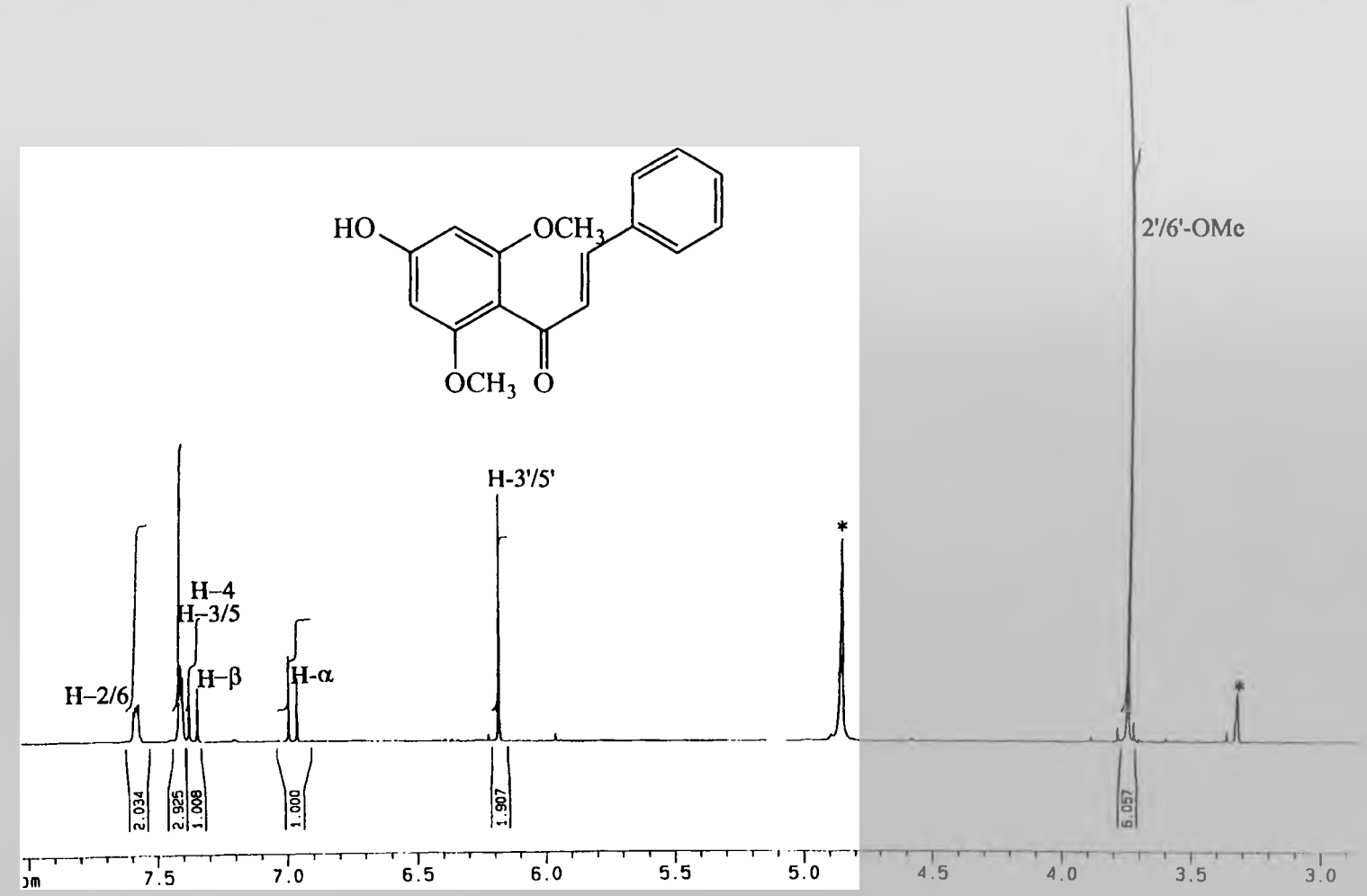
Fig.**5.28.** Signifcant HMBC correlation for B27-8.

Table.5.19. HMBC correlations for B27-8 (in CD_3OD)

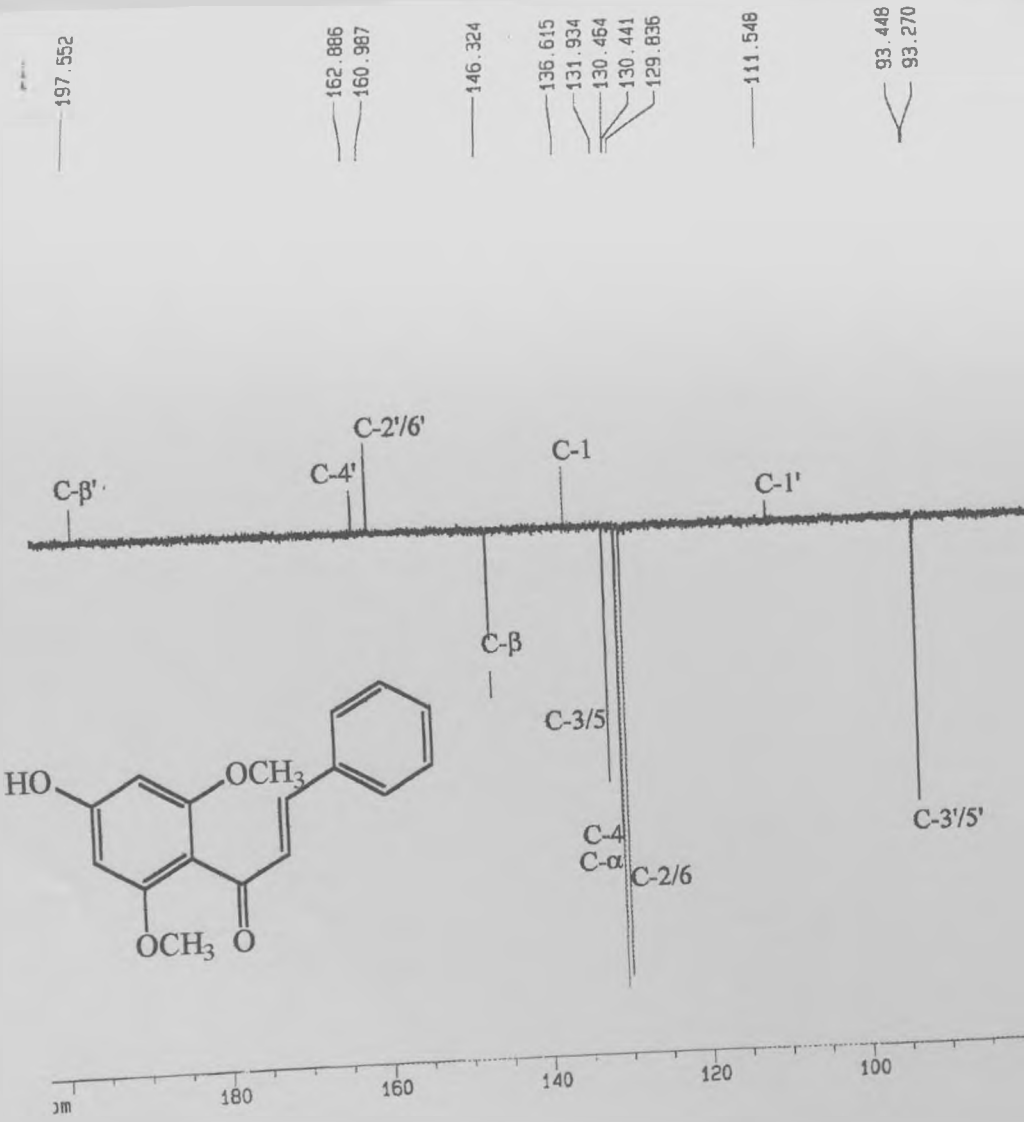
Position (δ)	^{1}J	^{2}J	^{3}J
H-β (7.36)	146.3	130.5 (C-α), 136.6 (C-1)	129.8 (C-2/6), 197.6 (C-β')
H-α (6.97)	130.5	197.6 (C-β')	111.5 (C-1'), 136.6 (C-1)
H-3' (6.22)	93.4	162.9 (C-4')	111.5 (C-1'), 93.4 (C-5')
H-5' (6.22)	93.4	162.88 (C-4')	93.4 (C-3'), 111.5 (C-1')
2'/6'-OMe (3.75)	56.6	-	160.9 (C-2'/6')

This compound has only been recently shown to be naturally-occurring in *Woodsia scopulina* (Economides and Adam, 1998). It was not possible to compare NMR data as

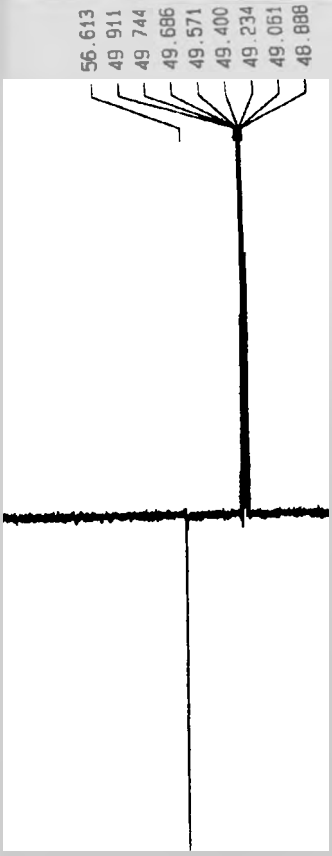
these were not given in that study. Previously it was only known to be a synthetic compound. Its presence in *C. apiculatum* is reported here for the first time with full NMR data.



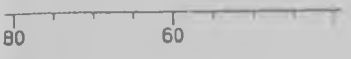
Spectrum 5.26: ^{1}H NMR (500MHz, CD_3OD*) of B27-8

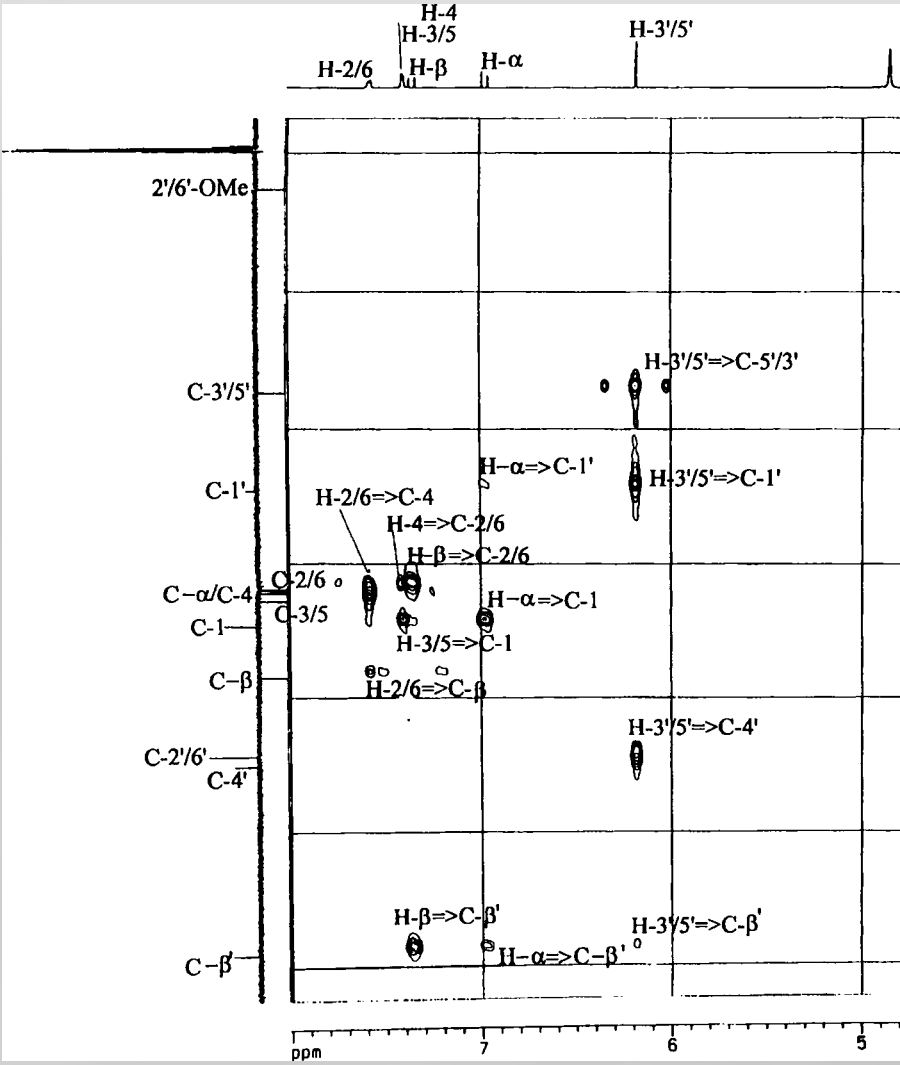


Spectrum 5.27 : ^{13}C (PENDANT) NMR (125MHz, CD_3OD*) of B27-8

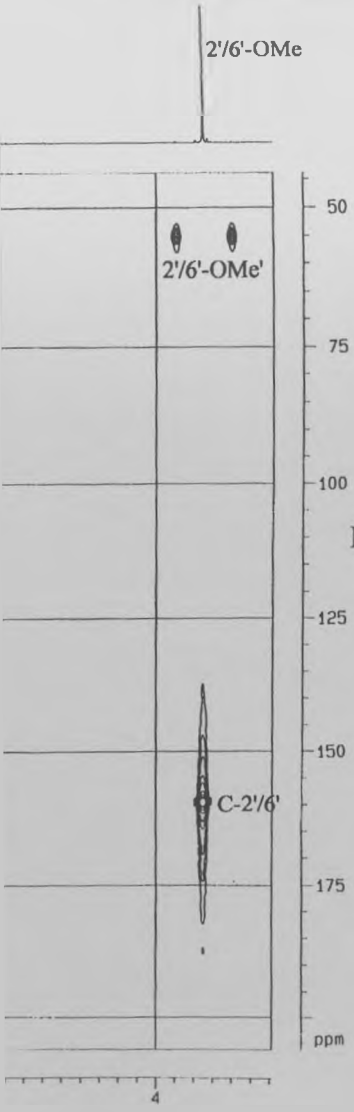


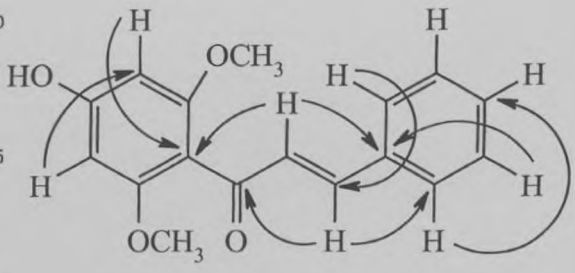
2'/6'-OMe





Spectrum 5.28 : HMBC correlations of B27-8

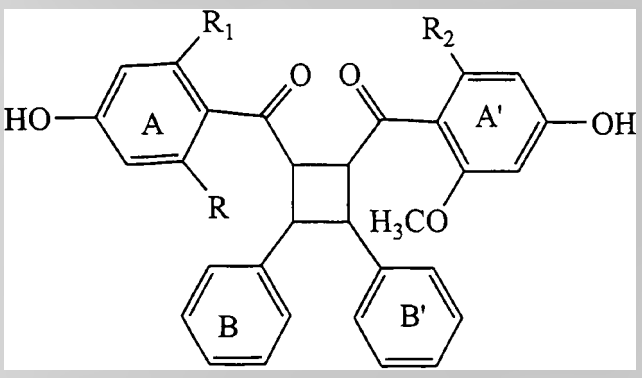

2'/6'-OMe
2'/6'-OMe'
50
75
100
125
150
175
ppm
C-2'/6'
4


H
OCH3
HO
H
H
H
H
H
H
H
OCH3
O
H
H

5.1.2.3.2. CHALCONE DIMERS

Three chalcone dimers were isolated in this study – two from *C. albopunctatum* (AO-66 and AO-68) and one from *C. apiculatum* (B69-12). B69-12 and AO-66 are identical, and characterised as 4-hydroxy-2,6-dimethoxychalcone dimer while AO-68 was identified as 2,4,4a-trihydroxy-2a,6,6a-trimethoxychalcone dimer.

The IR spectra were similar for both dimers, showing the presence of hydroxyl (*ca.* 3295, 1340, 1130 cm^{-1}), methoxyl (*ca* 2845, 1130 cm^{-1}), α,β-unsaturated carbonyl (*ca.* 1590 cm^{-1}), benzenoid (*ca.* 815, 750, 715 cm^{-1}) (Williams and Fleming, 1995).



Compound	R	R_1	R_2
AO-68	OH	OCH_3	OCH_3
AO-66	OCH_3	OCH_3	OCH_3
B69-12	OCH_3	OCH_3	OCH_3

Fig.5.29. Chalcone dimers isolated from *C. albopunctatum* (AO) and *C. apiculatum* (B69)

Chalcone dimers may be either α-truxillic (head-to-tail) or β-truxinic (head-to-head) and may be distinguished from one another by melting point differences if they are known. The former has a higher melting point while the latter is the low-melting isomer (Montaudo and Caccamese, 1973).

In this study, HMBC and ^{1}H-^{1}H NOESY were used, with limited success to gain insight into the substitution pattern and relative stereochemistry of the compounds isolated. Because of the equivocal conclusions reached with NMR data in this case, it fell to X-ray crystallography, "the ultimate arbiter" to give a definitive crystal structure.

Identification of AOD-68

AOD-68 (5.6 mg) was isolated by HPLC, t_R=7.35. The ^{1}H NMR shows three methoxyl groups, one at δ_H 3.26 δ_C 56.8, and two which appear to be equivalent at δ_H 3.53 δ_C 57.4. Four sets of well-resolved triplets appear between *ca.* δ_H 4.20 (δ_C 45) and δ_H 5.40 (δ_C 55), each one due to a single proton and with a coupling constant of 9Hz. Theses signals are all due to methine protons. The aromatic protons are located at δ_H 6.11, (J=2, δ_C 94.1), δ_H 6.48, (J=2, δ_C 98.8), a singlet of two protons at δ_H 6.38, δ_C 95.0 and a complex pattern of merging chemical shifts *ca.* δ_H 7.20-7.70 (δ_C 130). Having tried several solvents, there appears to be better resolution of this area in pyridine which shows what appears to be a complex set of doublet, triplet, triplet (from left to right) which pattern is reminiscent of the unsubstituted ring in the flavonoids (Agrawal, 1989). This integrates to at least nine

protons. A small singlet appears at δ_H 13.7 which is characteristic of hydroxyl protons (Williams and Fleming, 1995; Kemp, 1986).

HRFABMS gave the molecular ion, M^+, which is also the base peak at 555.200 (M+1) corresponding to a molecular formula of C_{33} $H_{30}O_8$. There were also prominent peaks at 401.1 (20.7%) $[M-C_8H_9O_3]^+$, 347.1 (21.3%) $[M-C_{11}H_{11}O_4]^+$, 285.1 (99.9%) $[M-C_{13}H_{13}O_6]^+$ 277.2 (29.9%) $[M-C_{17}H_9O_4]^+$, 271.1 (21.6%) $[M-C_{17}H_{15}O_7]^+$, 246.1 (24.8%) $[M-C_{19}H_{16}O_4]^+$ and 207.1 (30.3%) $[M-C_{19}H_{21}O_6]^+$. Smaller fragments at 181, 167 and 136 are probably due to substituted tropylium ions as seen with the stilbenes in Section **5.1.2.1.** and arise from the major benzenoid fragments.

The connectivities of this seemingly large molecule were worked out, in part, from ^1H-^1H COSY. It was found that the most downfield triplet, designated H-1 at δ_H 5.31 δ_C 55.1 is connected to its neighbour, H-2, at δ_H 4.81 δ_C 55.6 which in turn is connected to its upfield neighbour, H-3,at δ_H 4.50 δ_C 48.4 and then to the methine at,H-4 δ_H 4.22 δ_C 50.5 which in turn is connected to the most downfield proton, H-1. This connection also applies the other way i.e. H-1 to H-4 to H-3 to H-2 then back to H-1. Thus, these protons in fact are constituted into a cyclobutane ring. The chemical shifts are in total agreement with those of similar structures in the literature (Montaudo *et al,* 1974; Toda *et al*, 1998).

A cursory look at the HMBC shows that apart from ^2J and ^3J coupling within the cyclobutane, the protons in the ring all show ^2J coupling to one another in addition to

strong three-bond correlations. This is further evidence to support the fact that these protons have no geminal coupling i.e. they are methines, and none of them is magnetically equivalent to any other.

The arrangement of the aromatic protons is characteristic of the flavonoid-type compounds; in fact, it is uncannily similar – with the protons at δ_H 5.77, δ_C 92.2, δ_H 5.87, δ_C 97.1 being *meta*-coupled as the H-6 and H-8 protons in the flavone ring. The singlet at δ_H 5.48 δ_C 93.2 integrates for two protons which should be another set of H-6, 8 protons. In this case they are equivalent and are located on another ring. While the resonances for the carbons are identical to those of the other flavonoids isolated in this study (and also diagnostic of C-6 and C-8), the proton signals are relatively more shielded than they normally are in the corresponding monomers. In the chalcones isolated in this study, for instance, C-6 resonates at *ca.* δ_C 97ppm and C-8 at *ca.* 93ppm with their corresponding protons showing at δ_H 6.04 and 6.11 respectively in AO-18. In B27-8, these methines are identical and resonate at δ_H 6.22 δ_C 93.4. That these positions are upfield in the dimer may be due to steric factors and spatial interactions between the rings as well as intramolecular and intermolecular hydrogen bonding.

These factors may also explain why the carbonyl signals are highly deshielded and appear at *ca.* δ_C 204-205. The complex multiplet represents at least two unsubstituted aromatic rings as implied by the integration and the splitting pattern similar to those seen with the other flavonoids.

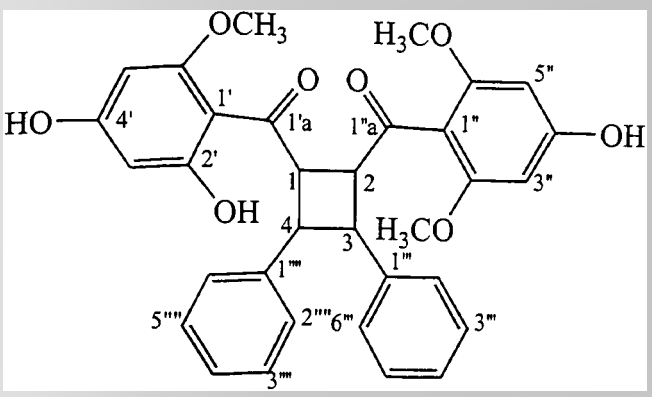


Fig.5.30. Proposed structure of AO-68

The NMR and particularly the FABMS points to a dimer based on two different flavonoid monomeric units. Based on this observation, two structures were proposed for AOD-68 – one head-to-head (I) and the other head-to-tail (II), with two similar unsubstituted benzene rings, a symmetrically substituted aromatic ring (hence two equivalent protons) and a fourth ring with non-equivalent protons. See Fig.**5.31.**

The truxillic configuration is eliminated by HMBC as the correlations seen between the protons in the cyclobutane ring and the phenyl attachments are not possible in this arrangement. The area of interest involves the correlations of the cyclobutyl protons. ^{2}J correlations are seen from H-1 to C-2 and C-4, and from the other protons to their neighbouring carbons in the ring. However it is the correlations from H-3 and H-4 to the carbonyl carbons which are significant. Both H-3 at δ_H 4.22 (δ_C 50.5) and H-4 at δ_H 4.50 (δ_C 48.4) are placed directly next to the benzene rings because they show ^{2}J coupling to C-1"" (145.7) and C-1"' (145.5) as well as ^{3}J to C-2/6"' (129.0) and C-2/6"" (129.9).

Table.5.20. ^{1}H (500MHz), ^{13}C (125MHz) NMR and HMBC spectral data for AO-68

Position	δ_H	δ_C	2J	3J
1	5.31 (t, J=9)	55.1	55.6 (C-2), 50.5 (C-4)	204.0 (C-1'a)
2	4.81 (t, J=9)	55.6	55.1 (C-1), 48.4 (C-3)	205.4 (C-1"a), 145.7 (C-1"')
3	4.22 (t, J=9)	50.5	55.1 (C-1), 48.4 (C-4)	205.4 (C-1"a) 129.9 (C-2/6"')
4	4.50 (t, J=9)	48.4	55.6 (C-2), 50.5 (C-3), 145.7 (C-1"'), 145.5 (C-1"")	204.0 (C-1'a) 129.0 (C-2""/6"")
1'a	-	204.0	-	-
1'	-	108.0	-	-
2'	-	169.1	-	-
3'	6.48 (d, J=2)	98.8	-	94.1 (C-5'), 108.0 (C-1')
4'	-	170.2	-	-
5'	6.11 (d, J=2)	94.1	-	108.0 (C-1'), 98.8 (C-3')
6'	-	165.0	-	-
6'-OCH_3	3.26 (s)	56.8	-	165.0 (C-6')
1"a	-	205.4	-	-
1"	-	114.0	-	-
2"/6"	-	161.3	-	-
3"/5"	6.38 (s)	95.0	161.3 (C-2"), 164.3 (C-4")	114.0 (C-1") 95.0 (C-5")
4"	-	164.3	-	-
2/6"-OCH_3	3.53 (s)	57.4	-	161.3 (C-2/6")
1"'	-	145.7	-	-
2/6"'	7.54 (m)	129.9	130.8 (C-3/5"')	129.5 (C-4"'), 50.5 (C-3)
3/5"'	7.31 (m)	130.8	-	145.7 (C-1"') 130.8 (C-5/3"')
4"'	7.21 (m)	129.5	130.8 (C-3/5"')	129.9 (C-2/6"')
1""	-	145.5	-	-
2/6""	7.58 (m)	129.0	130.8 (C-3/5"")	48.4 (C-4) 128.8 (C-4"")
3/5"'	7.31 (m)	130.8	129.0 (C-2/6"")	145.5 (C-1"")
4""	7.21 (m)	128.8	130.8 (C-3/5"")	129.0 (C-2/6"")

Data obtained in C_5D_5N

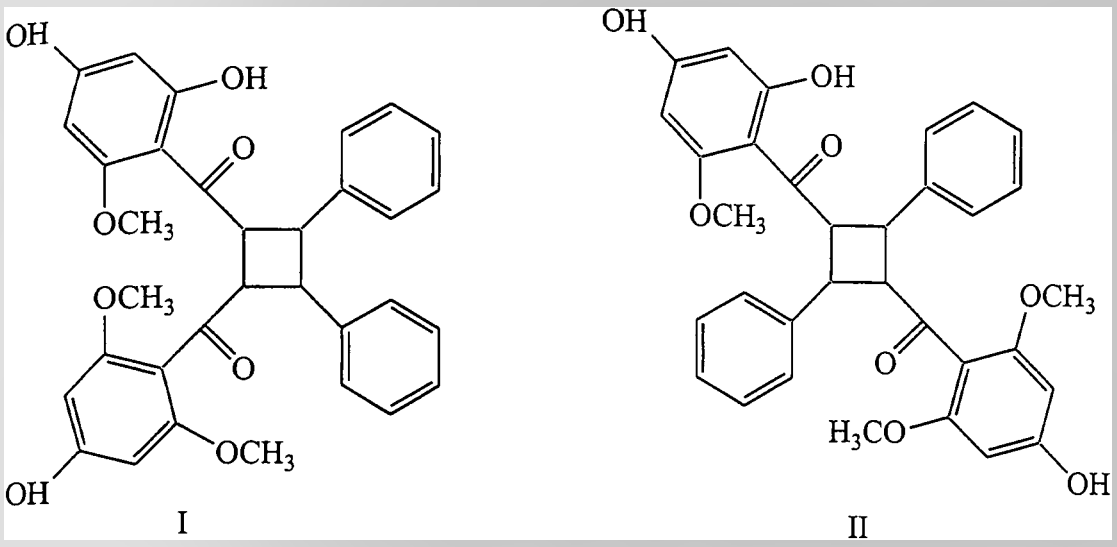


Fig.5.31. Two possible configurations of AO-68; the truxinic (I) and the truxillic (II)

The two cyclobutyl protons show coupling to separate carbonyl signals: H-3 correlating with C-1'a (δ_C 204) and H-4 with C-1"a (δ_C 205.4). H-1 correlates to C-1'a, which is on the other monomer, while H-2 correlates to C-1"a. These HMBC correlations would appear possible only in the truxinic (I) and not the truxillic (II) configuration.

Having said that, however, and because of the closeness of the signals and poor resolution in chemical shifts of these protons, ambiguities remain. There was an expectation that NOESY would help to solve the problem of orientation as well as establish the relative stereochemistry. It showed the compactness of this structure by showing that the methyl groups not only interact with neighbouring protons, but also

protons on the cyclobutane ring. For example, H-1 at 5.31 shows ^{1}H-^{1}H NOESY interaction with the methoxyl group attached to C-6''''. However this is a small nOe interaction. The important interactions are between H-1 and H-3 as well as H-2 and H-4. These mean that the structure is as illustrated in part in Fig.**5.32.**

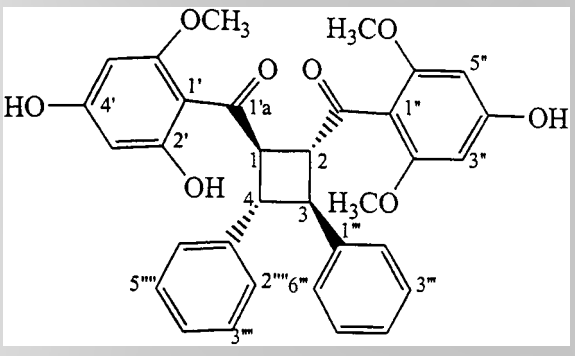
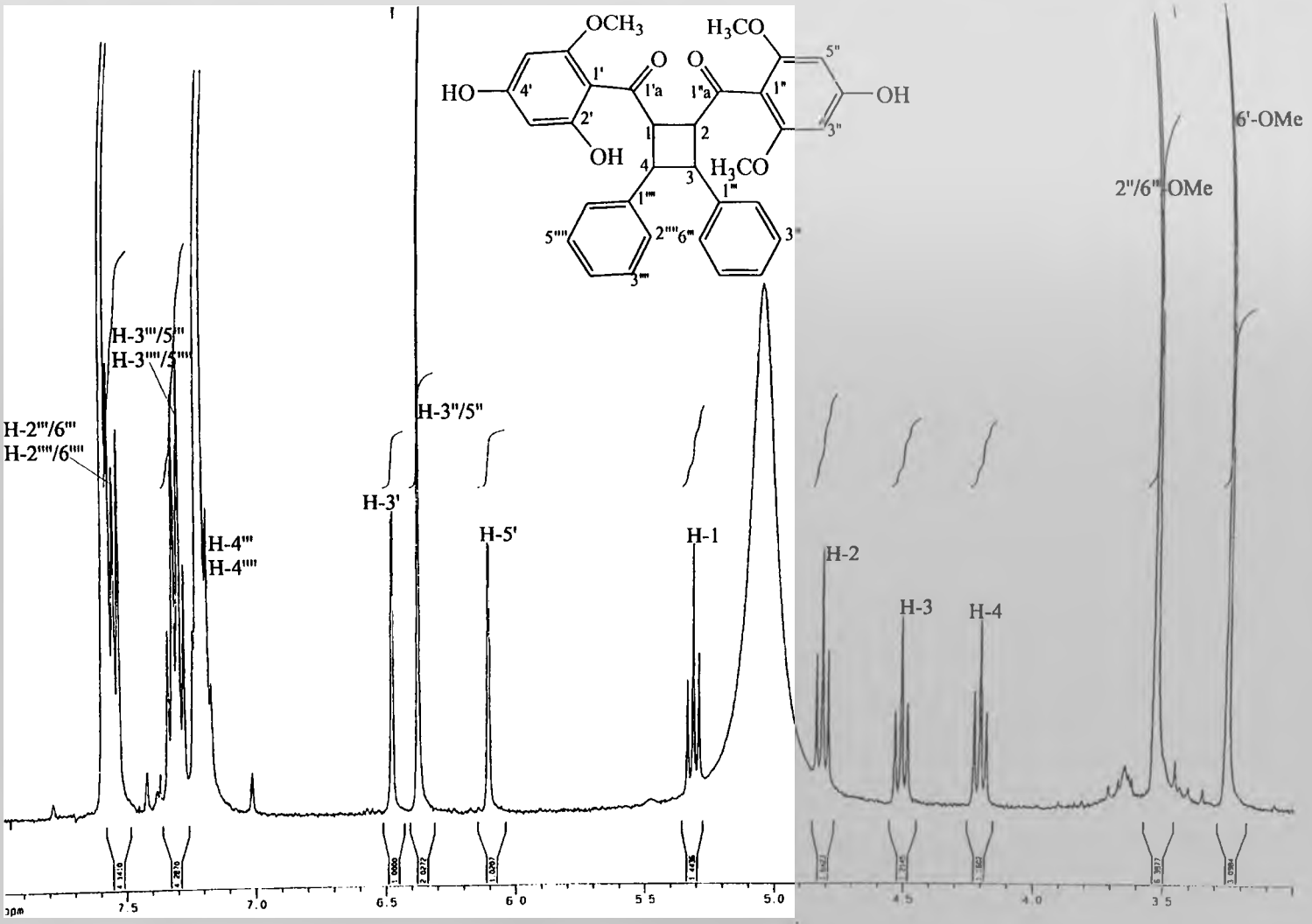


Fig.5.32. Conformation around the cyclobutane ring suggested by NOESY interactions

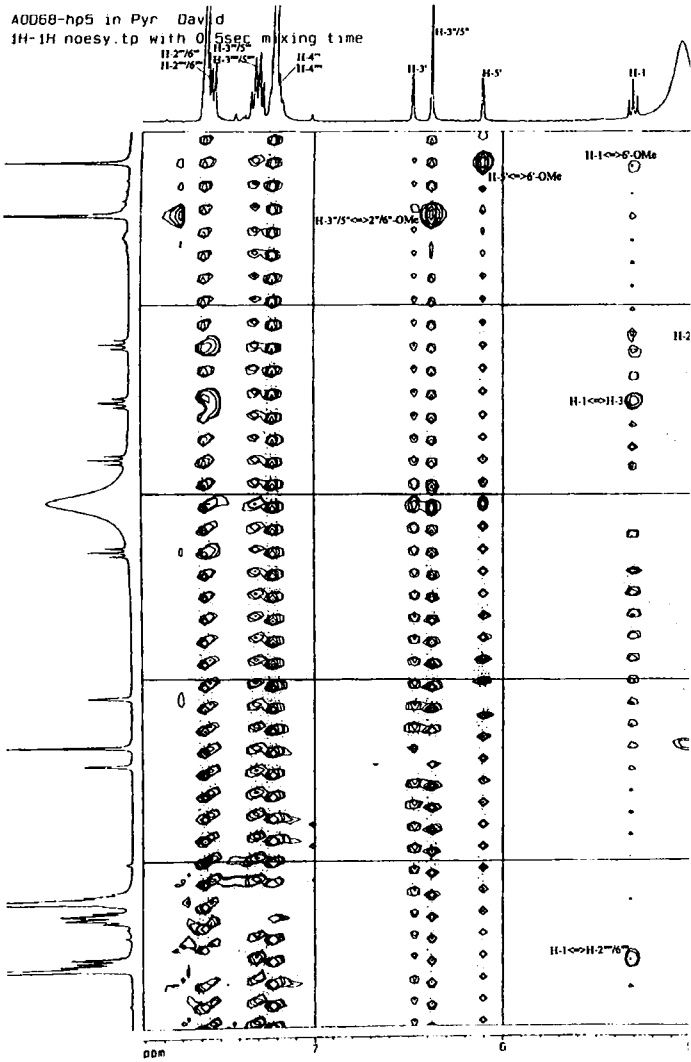
From the foregoing, AO-68 was putatively identified as *rel*-1β-(2,4-dihydroxy-6-methoxy) benzoyl-*rel* -2α-(4-hydroxy-2,6-dimethoxy) benzoyl- *rel*-(3β,4α)-diphenyl cyclobutane (or 2,4,4a-trihydroxy-2a,6,6a-trimethoxy chalcone dimer). The Beilstein Index has called it (2,4-dihydroxy-6-methoxy-phenyl)-[2-(4-hydroxy-2,6-dimethoxy-benzoyl)-3,4-diphenyl-cyclobutyl]-methanone. The former is based on the IUPAC naming system as used by Cacamesse *et al*, 1979.

This compound is reported here for the first time. It is debatable whether it is a metabolic product in the Combretaceae or results from exposure to sunlight after harvesting, or both. It is interesting to note that the monomeric units which make up this dimer were

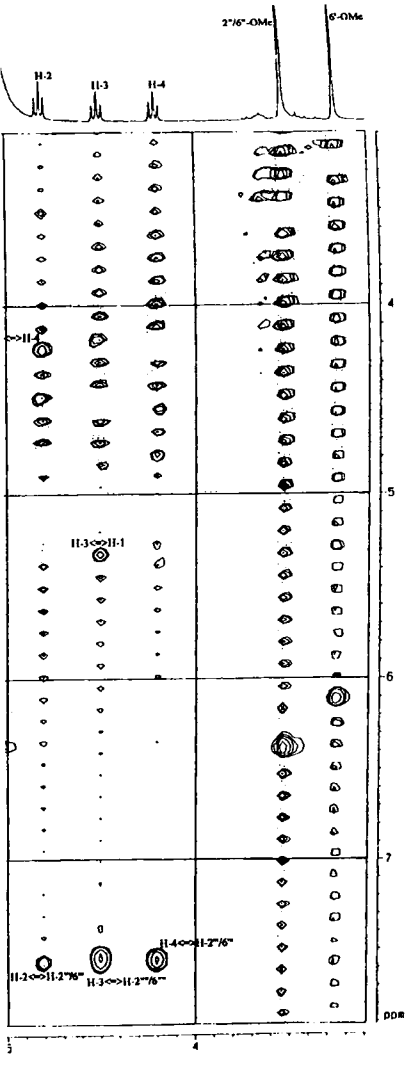
also isolated in this study and identified as 4', 6'-dihydroxy-2'-methoxychalcone (cardamonin) (B69-13/ AO-18) and 4'-hydroxy-2', 6'-dimethoxychalcone (B27-8). X-ray crystallography had not been performed at the time of writing, however there is sufficient NMR data to support the structure proposed above.

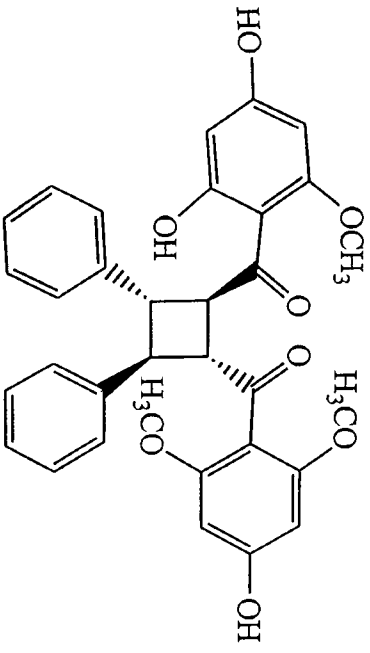


Spectrum 5.29: ^{1}H NMR (500MHz, C_5D_5N*) of AO-68



Spectrum 5.30: ^{1}H-^{1}H NOESY spectrum of AO-68.


H-2
H-3
H-4
2"/6"-OMe
6'-OMe
H-3<=>H-1
H-4<=>H-2"/6"
H-2<=>H-2"/6"
H-3<=>H-2"/6"
4
5
6
7
ppm


OH
HO
OCH3
O
O
H3CO
H3CO
HO

Characterisation of B69-12/AO-66

B69-12 was isolated by HPLC as a yellow amorphous solid, t_R=17min from the leaves of *C. apiculatum*. AO-66, on the other hand was isolated from a column of Sephadex LH-20 eluted with 50% MeOH/TCM from an extract of a mixture of fruit and leaves from *C. albopunctatum*. They were found to be identical by comparison of the carbon and proton NMR spectra. The discussion that follows concerns the elucidation of the structure using data acquired for the former.

The 1D NMR of B69-12 showed two methoxyl groups at δ_H 3.44 δ_C 55.9, two inverted multiplets (nicknamed "batman ears") at δ_H 4.39 δ_C 46.4 and δ_H 4.57 δ_C 55.2, a singlet at δ_H 6.31 integrating for two protons, and a complex set of multiplets at *ca.* δ_H 7.25 – 7.30 (δ_C 127-130) typical of the mono-substituted ring in phenolics.

A first look at this data was suggestive of a flavonoid of some sort but the inverted multiplet was inexplicable. Initially it was thought to be due to solubility problems or machine error, so different solvents were used on a different machine. It could not be ignored when it appeared again in acetone-*d*6 and methanol-*d*4 in addition to pyridine-*d*5, which was the first solvent used.

The appearance of an inverted multiplet ("batman ears") between δ_H 3.5 and 4.5ppm in ^{1}H NMR is indicative of restricted rotation. This assertion is made in retrospection as it was hitherto thought to arise from some other idiosyncrasies!

HRFABMS gave M+1 at *m/z* 569.2175 which requires the molecular ion to be $C_{34}H_{32}O_8$. Prominent peaks were also seen at *m/z* 506 (6.5%) $[M-C_2H_6O_2]^+$, 415 (20%) $[M-C_{12}H_8]^+$, 284 (54%) $[M-C_{17}H_{16}O_4]^+$, 181 (100%) $[M-C_{25}H_{23}O_4]^+$and 166 (62%) $[M-C_8H_6O_4]^+$. This suggested that B69-12 was a large molecule, and if it was a flavonoid, then it was dimeric in form.

The ^1H-^1H COSY shows the protons causing the multiplets at δ_H 4.57 and 4.39ppm to be coupling to each other, and HMBC shows the correlation of equivalent partners i.e. each proton attached to the carbon at δ_C 46.4 and δ_C 55.2 shows up in the HMBC as a line of three spots, one in the centre (showing coupling to a proton of equivalent chemical shift) in addition to two spots on its left and right (commonly called satellites). This pattern appears to suggest that the protons are arranged into a cyclobutane ring. They show strong ^3J correlations to the carbonyl and aromatic carbons at δ_C 202.9 and δ_C 144.4ppm respectively.

The oxymethyl functional groups are inseparable in the proton and carbon domain at δ_H 3.44ppm and δ_C 55.9ppm. They are attached to the carbons resonating at δ_C 159.9ppm. The conclusion is that these are two magnetically equivalent moieties both attached to equivalent carbons resonating at δ_C 159.9. These carbons were designated C-2' and C-6'. They show strong correlations to the protons placed on C-3' and C-5' which are also indistinguishable from each other and appear at δ_H 6.32. These protons are reminiscent of the protons in chalcones attached to C-3' and C-5', which normally show up as separate signals in the carbon domain between δ_C 94 and 100ppm (Agrawal, 1989). In this case they are relatively shielded, upfield and equivalent.

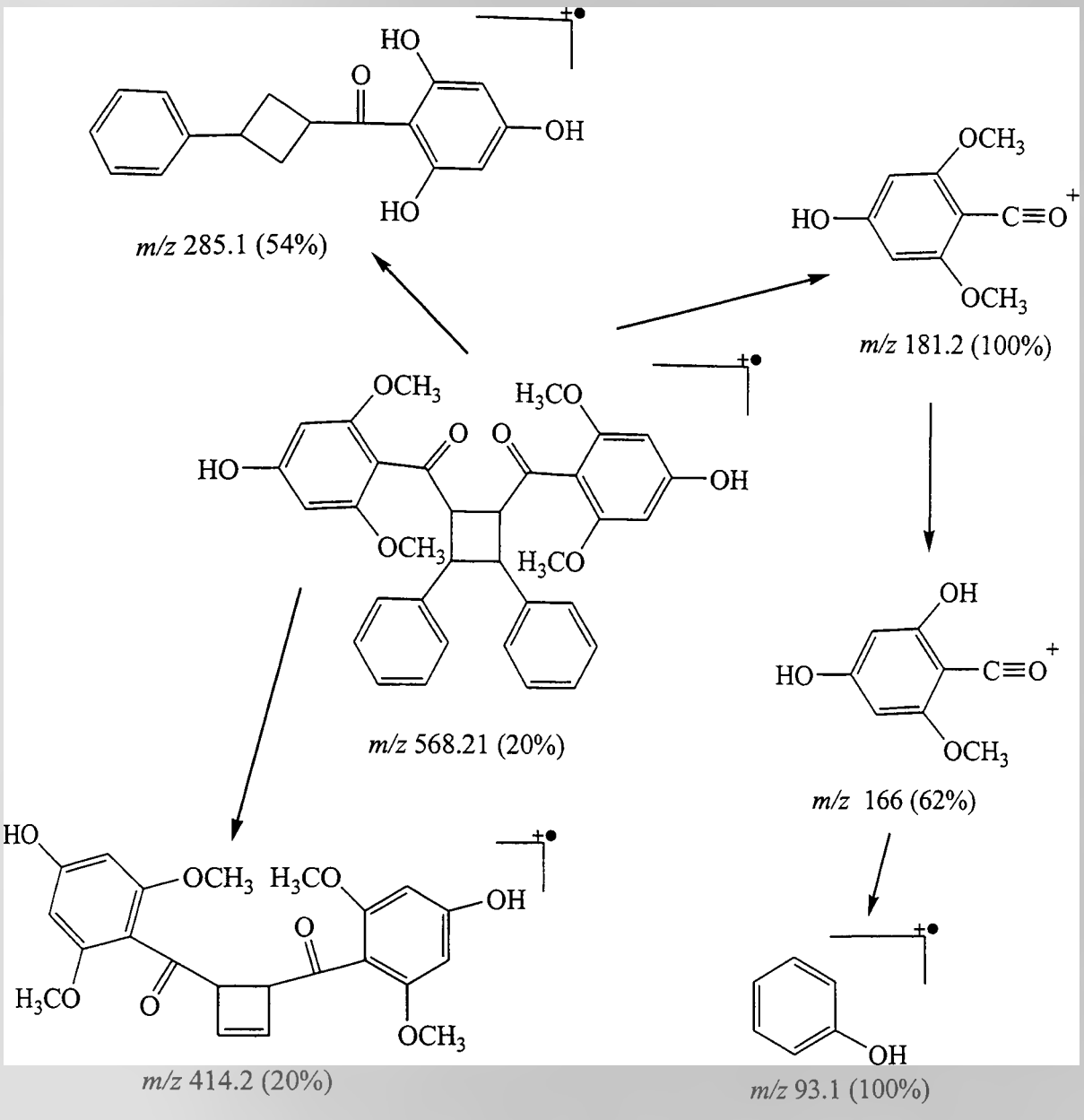


Fig.5.33. Suggested fragmentation pattern for B69-12.

All this is evidence to suggest that the oxygenated ring has a phloroglucinol (rather than a pyrogallol) substitution pattern. Further, this compound is symmetrical based on the NMR and MS data.

Two possible structures were proposed showing the head-to-head (α-truxinic) or head-to-tail (β-truxillic) conformations. They could, however, not be differentiated from each other either by NOESY or HMBC. See Fig. **5.34.**

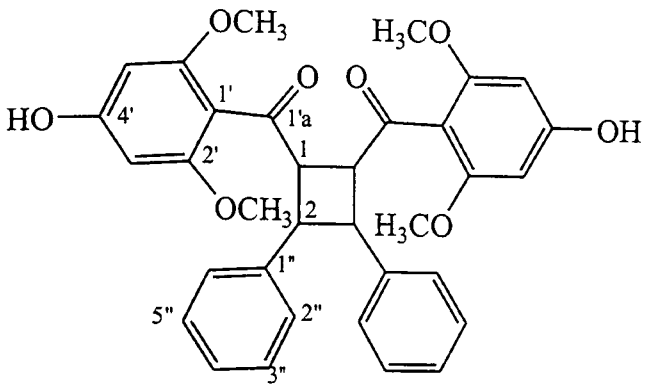


Fig. 5.34. One of two proposed structures of B69-12

NOESY was only able to show the spatial proximity of certain parts of the compound. It showed that the proton attached to C-1 at δ_C 55.2ppm was on the same side as the methoxyl attached to C-2'/6'and also that there is n.O.e between that proton (or its equivalent) and H-2 on the cyclobutane ring. However, the fact that this is (on the face of it) a symmetrical dimer means that the NOESY interpretation can be confusing. HMBC is no more useful as the correlations tend to be equivocal.

It was then left to X-ray crystallography to determine the relative configuration.
A single crystal of B69-12 was obtained by slow evaporation of a methanol-ethanol solution. Diffraction data were collected at 150 K on a Kappa CCD single crystal diffractometer using MoK_α radiation.

Table.5.21. ^{1}H (400MHz), and ^{13}C (100MHz) NMR spectral data for AO-66

Position	δ_H	δ_C
1	4.60 (m*)	55.5
2	4.42 (m*)	46.6
1'a	-	203.5
1'	-	112.0
2'/6'	-	160.1
3'/5'	6.34(s)	93.5
4'	-	163.1
2/6'-OCH_3	3.44	56.1
1"	-	144.7
2"/6"	7.52 (m)	128.6
3"/5"	7.28 (m)	129.4
4"	7.20 (m)	129.6

Data obtained in C_5D_5N. * inverted multiplet.

Table.5.22. ^{1}H (400MHz), ^{13}C (100MHz) NMR and HMBC spectral data for B69-12

Position	δ_H	δ_C	^{2}J	^{3}J
1	4.57 (m*)	55.2	46.4 (C-3), 202.9 (C-1'a)	144.4 (C-1")
2	4.39 (m*)	46.4	55.2 (C-1), 144.4 (C-1")	202.9 (C-1'a), 128.3 (C-2/6")
1'a	-	202.9	-	-
1'	-	112.3	-	-
2'/6'	-	159.9	-	-
3'/5'	6.32 (s)	93.1	159.9 (C-2'/6'), 162.6 (C-4')	112.2 (C-1')
4'	-	162.6	-	-
2/6'-OCH_3	3.44	55.9	-	159.9 (C-2'/6')
1"	-	144.4	-	-
2"/6"	7.51 (m)	128.3	129.3 (C-3"/5")	127.4 (C-4"), 128.3 (C-6"/2")
3"/5"	7.28 (m)	129.3	127.4 (C-4")	129.3 (C-5"/3"), 144.4 (C-1")
4"	7.19 (m)	127.4	-	128.3 (C-3"/5")

Data obtained in C_5D_5N. * inverted multiplet.

The structure was solved by direct methods using the program SIR-97 (Altomare *et al.*, 1997) with atomic positions and displacement parameters refined using SHELXL-97 (Sheldrick, 1997). 5271 unique observations were used in the final refinement (401 parameters) yielding a final R-factor of 0.0507.

Refinement of $|F|^2$ was carried out against all reflections. The weighted R-factor, wR, and goodness of fit, S, are based on $|F|^2$ whilst the conventional R-factor, R, is based on |F|, with |F| set to zero for negative $|F|^2$. The threshold expression of $|F|^2 > 2\sigma(|F|^2)$ is used only for calculating R-factors and is not relevant to the choice of reflections for refinement. R-factors based on $|F|^2$ are statistically about twice as large as those based on |F|, and R-factors based on all data are even larger. Selected crystal data and refinement details are listed in Table **5.23.**

The molecule crystallises as an ethanol solvate monohydrate in the triclinic space group P$\bar{1}$ with two molecules in the unit cell (a = 9.5521(3), b = 11.3163(3), c = 15.9182(6), α = 77.579(1), β = 72.926(1), γ = 74.768(1)°). Fractional co-ordinates and equivalent isotropic displacement parameters (U_{eq}) for all non-hydrogen atoms are presented in Table **5.24.** The ethanol molecules are disordered over two positions in the unit cell. Anisotropic displacement parameters (U_{ij}), H-atoms fractional co-ordinates and atomic displacement parameters are listed in Appendix **1.1** and **1.2.** All covalent bond lengths (Appendix **1.3**) and angles are in good agreement with expected values (Allen *et al*, 1999).

There are three unique intermolecular hydrogen bonds in the crystal structure (see Table **5.25.**) In addition, five unique weak electrostatic interactions of type C-H...O are identified (Table **5.26.**).

Table 5.23.Crystal Data and Details of the Structure Determination

Crystal Data	
Formula	$2(C_{34}H_{32}O_8)$, 2(), 2()
Formula Weight	1159.60
Crystal System	triclinic
Space group	*P* Ì (No. 2)
a, b, c [Å]	9.5521(3), 11.3163(3), 15.9182(6)
α, β, γ [°]	77.579(1), 72.926(1), 74.768(1)
V [Å^3]	1569.20(9)
Z	2
D_{calc} [g/cm^3]	1.227
Mu(MoKα) [/mm]	0.088
F(000)	611
Crystal Size [mm]	0.06 x 0.10 x 0.20
Data Collection	
Temperature (K)	150
Radiation [Å]	MoKα 0.71073
θ Min-Max [°]	3.3, 24.6
hkl (min.:max.)	-11: 11 ; -13: 13 ; -18: 18
Total, Unique Data, R_{int}	9909, 5271, 0.044
Observed data [I > 2.0σ(I)]	3457
Refinement	
N_{ref}, N_{par}	5271, 401
R, wR, S	0.0507, 0.1140, 1.05
max. and av. Shift/Error	0.00, 0.00
min. and max. residual density [e/Å^3]	-0.19, 0.34

Table 5.24. Final Co-ordinates and Equivalent Isotropic Displacement Parameters of the non-Hydrogen atoms

Atom	x	y	z	$U_{eq}/Å^2$
O1	1.06962(18)	0.46617(14)	0.69411(10)	0.0301(6)
O2	1.05139(18)	0.66522(14)	0.77918(11)	0.0353(6)
O3	0.62946(18)	0.99248(14)	0.73282(12)	0.0380(6)
O4	0.66938(17)	0.58043(13)	0.68904(10)	0.0284(6)
O5	0.79049(18)	0.11292(14)	0.79209(10)	0.0319(6)
O6	0.55842(17)	0.26336(14)	0.72425(10)	0.0307(6)
O7	0.71692(19)	0.41419(17)	0.41595(11)	0.0420(7)
O8	1.06990(18)	0.24521(16)	0.59552(11)	0.0382(6)
C1	0.8634(3)	0.39570(19)	0.79715(14)	0.0229(7)
C2	0.9335(3)	0.25970(19)	0.78271(15)	0.0232(7)
C3	0.9352(3)	0.2224(2)	0.88134(15)	0.0247(8)
C4	0.9070(3)	0.36184(19)	0.88951(14)	0.0247(8)
C5	0.9387(3)	0.4945(2)	0.73570(15)	0.0237(8)
C6	0.8570(2)	0.62547(19)	0.73525(14)	0.0231(8)
C7	0.9183(3)	0.7124(2)	0.75645(15)	0.0263(8)
C8	0.8451(3)	0.8349(2)	0.75652(15)	0.0284(8)
C9	0.7076(3)	0.8734(2)	0.73460(15)	0.0286(8)
C10	0.6441(3)	0.7907(2)	0.71210(15)	0.0267(8)
C11	0.7189(3)	0.6681(2)	0.71332(14)	0.0230(8)
C12	1.1253(3)	0.7495(2)	0.79571(18)	0.0406(10)
C13	0.5325(3)	0.6185(2)	0.66131(17)	0.0317(9)
C14	0.8442(3)	0.2004(2)	0.74648(15)	0.0241(8)
C15	0.8137(3)	0.25555(19)	0.65838(15)	0.0243(8)
C16	0.6664(3)	0.28428(19)	0.64796(15)	0.0248(8)
C17	0.6355(3)	0.3370(2)	0.56699(15)	0.0289(8)
C18	0.7524(3)	0.3617(2)	0.49474(16)	0.0296(9)
C19	0.8994(3)	0.3344(2)	0.50149(15)	0.0296(8)
C20	0.9287(3)	0.2813(2)	0.58298(16)	0.0276(8)
C21	0.4093(3)	0.2753(3)	0.71757(18)	0.0440(10)
C22	1.1896(3)	0.2827(3)	0.52472(18)	0.0550(13)
C23	1.0727(3)	0.1378(2)	0.90481(15)	0.0259(8)
C24	1.1208(3)	0.1509(2)	0.97549(16)	0.0356(9)
C25	1.2487(3)	0.0742(2)	0.99649(18)	0.0435(10)
C26	1.3307(3)	-0.0175(2)	0.94673(19)	0.0438(10)
C27	1.2852(3)	-0.0330(2)	0.87622(19)	0.0425(10)
C28	1.1571(3)	0.0439(2)	0.85514(17)	0.0352(9)
C29	0.7957(3)	0.4164(2)	0.96746(15)	0.0245(8)
C30	0.6742(3)	0.3652(2)	1.01802(16)	0.0309(8)
C31	0.5716(3)	0.4195(2)	1.08811(16)	0.0334(9)
C32	0.5886(3)	0.5257(2)	1.10941(16)	0.0348(9)
C33	0.7071(3)	0.5786(2)	1.05947(17)	0.0402(10)
C34	0.8098(3)	0.5252(2)	0.98914(16)	0.0342(9)
*O1S	0.6771(18)	0.0732(13)	0.4290(10)	0.111(5)
*C1S	0.821(3)	0.060(2)	0.4316(18)	0.123(8)
*C2S	0.889(2)	0.008(2)	0.5011(16)	0.108(7)
*O1W	0.5135(14)	0.9201(11)	0.4100(8)	0.086(4)

U_{eq} = 1/3 of the trace of the orthogonalized U Tensor

* Atoms are disordered, i.e. atom sites have a Site Occupancy Factor (S.O.F.) less than 1.0

Table 5.25. Hydrogen Bonds (Angstrom, Deg)

D-H...A	D-H	H...A	D...A	D-H...A	SymmetryOperator
O3-H3...O5	0.8399	1.9244	2.761(2)	173.48	x,1+y,z
O3-H3...O6	0.8399	2.5320	2.942(2)	111.24	x,1+y,z
O7-H7...O1	0.8402	1.8912	2.719(2)	168.07	2-x,1-y,1-z

Table 5.26. Weak C-H..O intermolecular contacts (Angstrom, Deg)

C-H...O	C-H	H...O	D...O	C-H...O	SymmetryOperator
C1-H1...O4	0.9994	2.4634	3.009(3)	113.82	x,y,z
C2-H2...O1	1.0009	2.4460	2.871(3)	104.86	x,y,z
C2-H2...O8	1.0009	2.3147	2.902(3)	116.42	x,y,z
C8-H8...O5	0.9500	2.4845	3.197(3)	131.80	x,1+y,z
C13-H13B...O7	0.9802	2.5355	3.115(4)	117.77	1-x,1-y,1-z

The data provided vital structural information on the molecular and crystal structure of B69-12 in the solid state. The crystallographic data confirmed the structure to be a dimer, comprising two identical monomers (as predicted from NMR data) which are head to head rather than head to tail. See Fig **5.34** and **5.35**. The cyclobutane ring shows a degree of puckering (see Fig **5.35**), which reduces the strain in the ring-system by minimising the extent to which adjacent substituents are eclipsed.

B69-12 was identified as *rel* (1α,2β)-di-(4-hydroxy-2,6-dimethoxy)benzoyl-*rel* (3α,4β)-diphenyl cyclobutane (or 4-hydroxy-2,6-dimethoxychalcone dimer). It is a dimer of identical monomers which were also isolated in this study from *C. apiculatum* and characterised as 4'-hydroxy-2',6'-dimethoxychalcone (B27-8). This is the first report of this compound in both the leaves (and/or fruit) of *C. albopunctatum* (AO-66) and the leaves of *C. apiculatum* (B69-12).

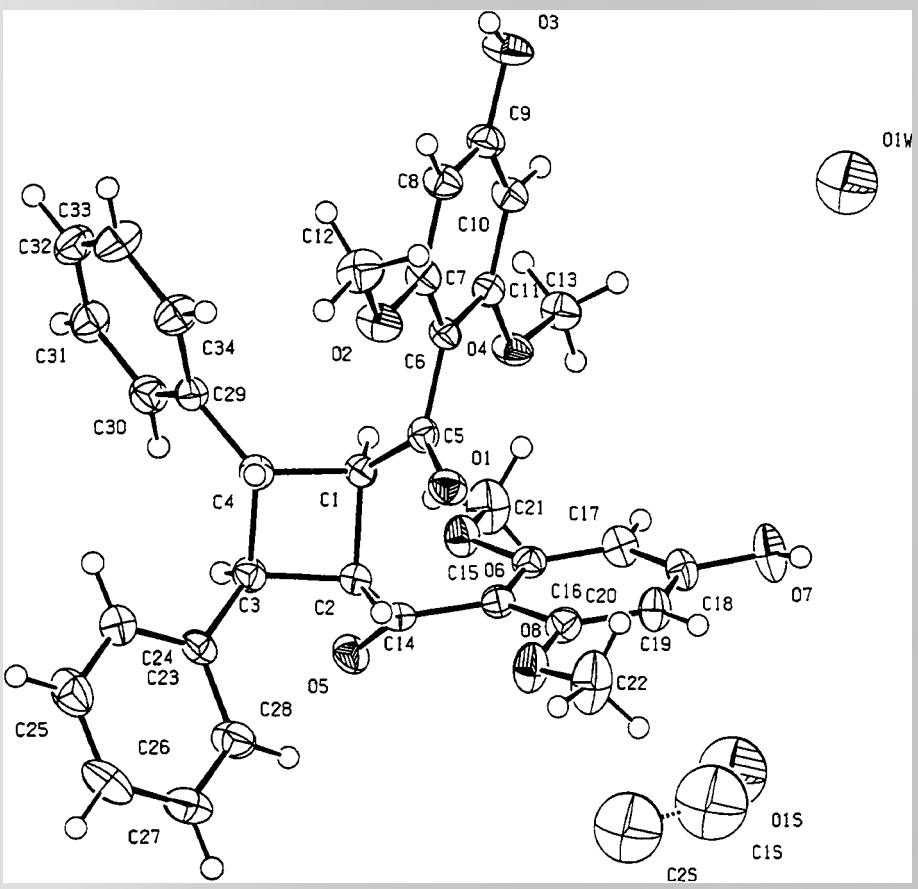


Fig.5.34. ORTEP plot of B69-12 ethanol solvate. Thermal ellipsoids are drawn at 50% probability.

It is uncertain whether this compound as well as AO-68 are products of biosynthesis in the Combretaceae. They have been shown to result from photodimerization in previous studies (Montaudo *et al*,1974; Toda, 1998). Certainly the isolation of the corresponding monomeric units seems to lend credence to this theory and does not, at the same time, disprove the possibility of biosynthetic origin.

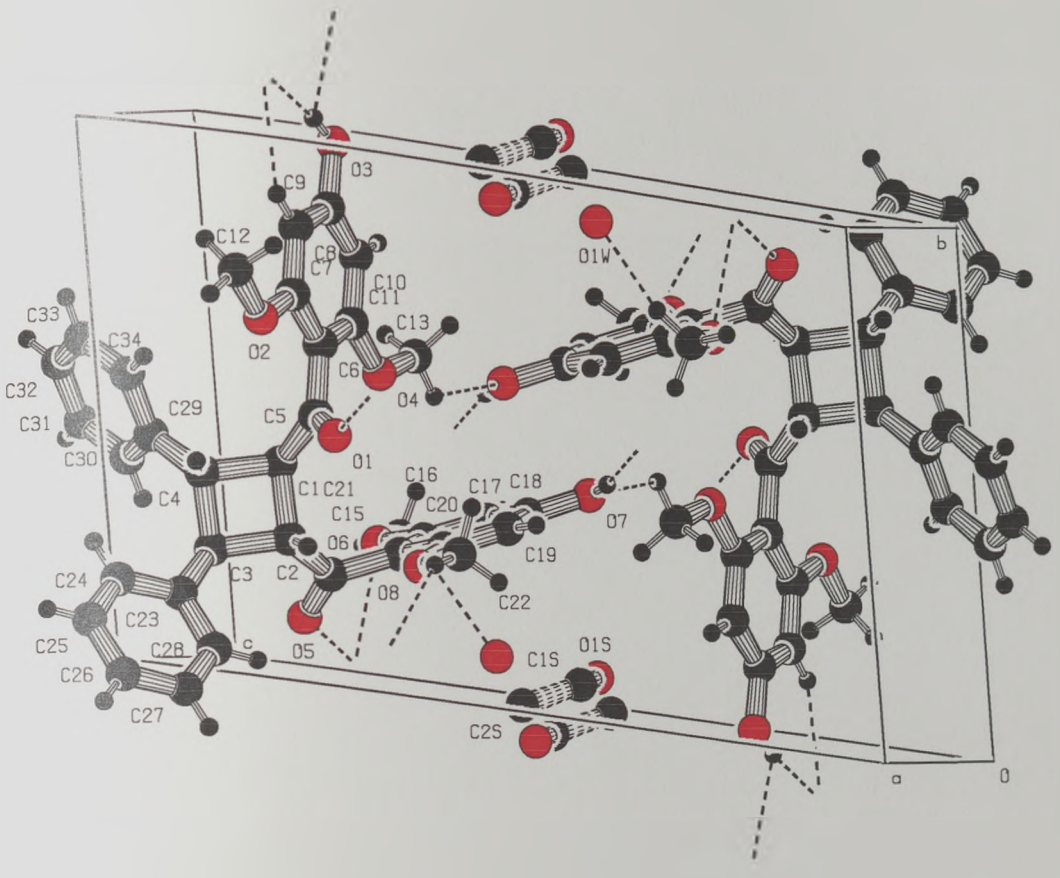
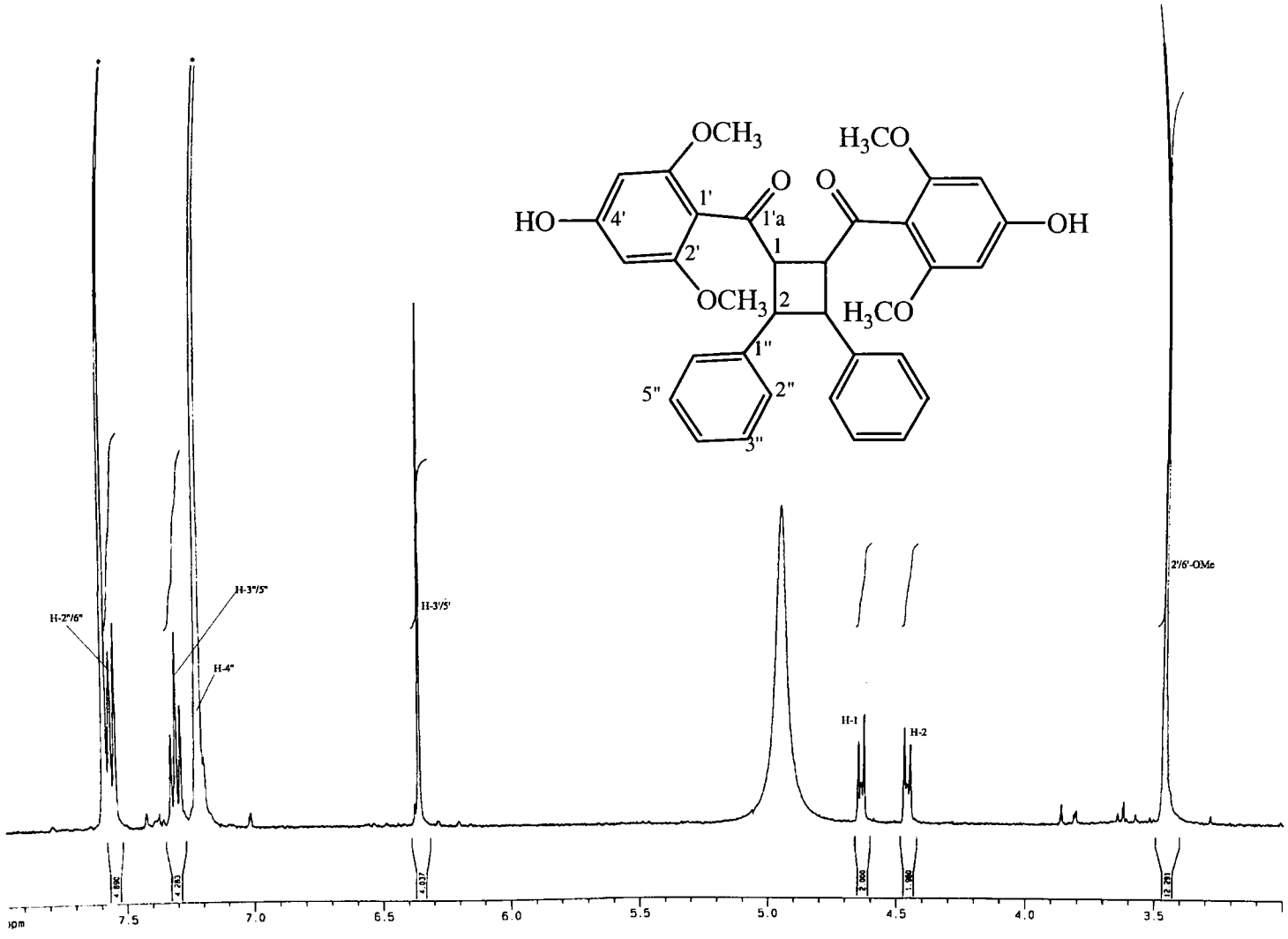
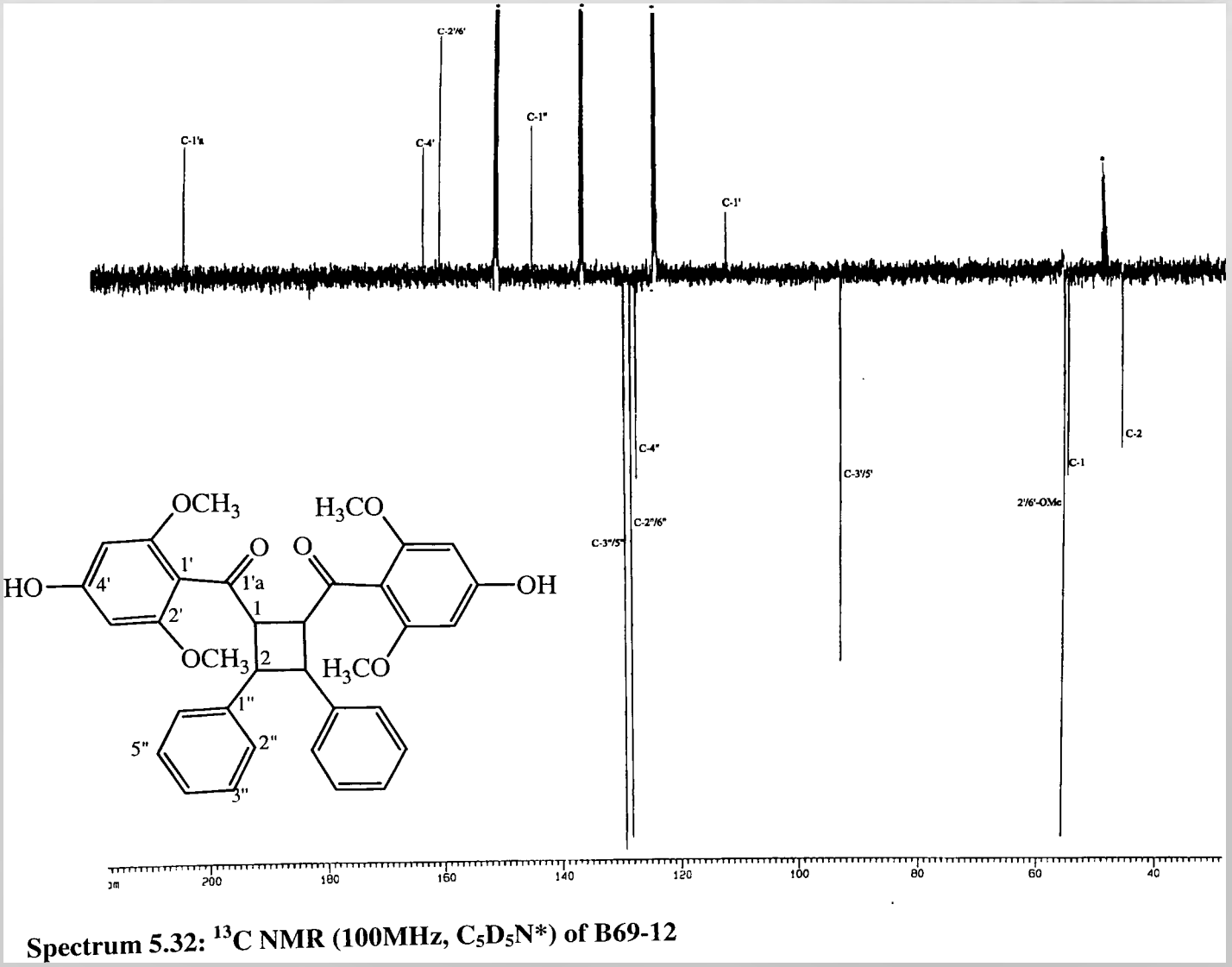




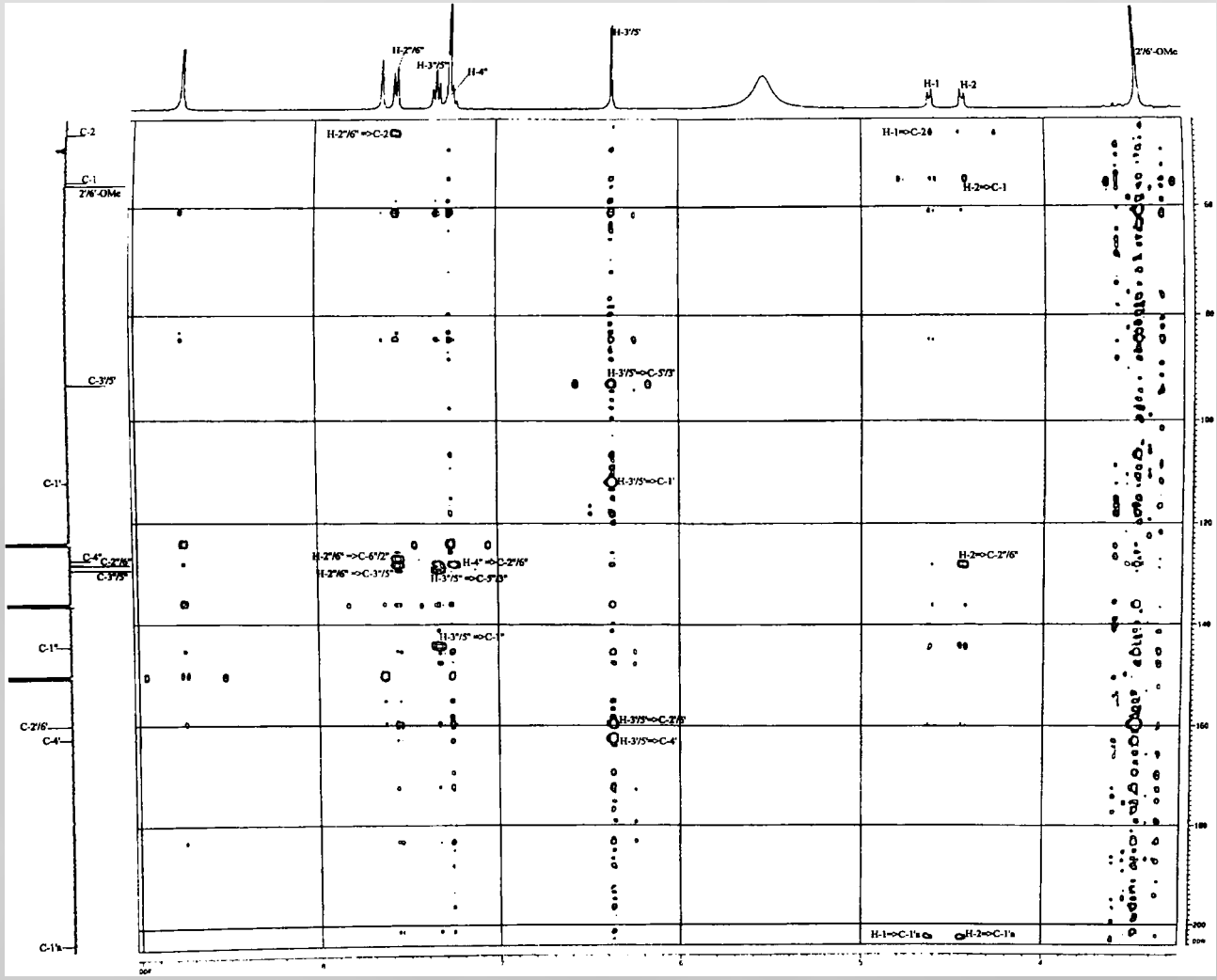
Fig.5.35. Packing diagram of ethanol solvate (top). Edge-on view of the cyclobutane ring showing relative orientation of benzoyl (R and R') and benzyl (R1 and R1') moieties.



Spectrum 5.31: ^{1}H NMR (400MHz, C_5D_5N*) of B69-12



Spectrum 5.32: ^{13}C NMR (100MHz, C_5D_5N*) of B69-12



Spectrum 5.33: HMBC correlations observed in B69-12

Properties and spectral data of isolated chalcones

AOD-18/ B69-13 (4', 6'-Dihydroxy-2'-methoxychalcone) (Cardamonin)

Yellow crystalline solid. IR ν_{max} film cm^{-1} 3130, 2925, 111625, 1450, 1340, 1210, 1115, 970, 745. Found [M^+] 270.089 ($C_{16}H_{14}O_4$ requires 270.089). HREIMS *m/z* (rel. int. %): 270 (70), 256 (82), 226 (44), 191 (100), 189 (54), 166 (44), 151 (42), 131 (10), 124 (28), 77 (28). ^1H and ^{13}C NMR: Table **5.16**.

B27-8 (4'-Hydroxy-2',6'-dimethoxychalcone)

Yellowish-brown crystalline solid. IR ν_{max} film cm^{-1} 3280, 3010, 2940, 2840, 1595, 1430, 1280, 1080. Found [M^+] 284.105. ($C_{17}H_{16}O_4$ requires 284.105). HREIMS *m/z* (rel. int. %): 284 (32), 256 (100), 181 (17), 166 (23), 103 (23). ^1H and ^{13}C NMR: Table **5.16**.

AO-68 (*rel*-1β-(2,4-Dihydroxy-6-methoxy) benzoyl-*rel*-2α-(4-hydroxy-2,6-dimethoxy) benzoyl-*rel* (3β,4α)-diphenyl cyclobutane)

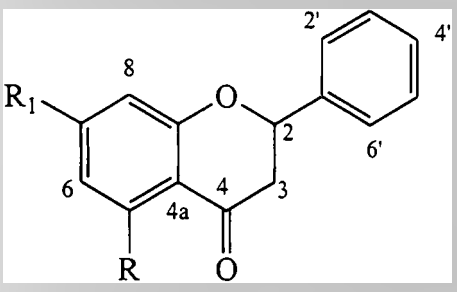
Yellowish amorphous solid. IR ν_{max} film cm^{-1} 3290, 3010, 2940, 2840,1616, 1466, 1429, 1235, 1168, 1131, 698. Found [M+1] 555.20016 (C_{33} $H_{30}O_8$ requires 554.20). HRFABMS *m/z* (rel. int. %): 401.1 (20.7), 347.1 (21.3), 285.1 (99.9), 277.2 (29.9), 271.1 (21.6), 246.1 (24.8), 207.1 (30.3). ^1H and ^{13}C NMR: Table **5.20**, and Spectrum **5.29** and **5.30.**

B69-12/ AO-66 (*rel* (1α,2β)-Di-(4-hydroxy-2,6-dimethoxy)benzoyl-*rel* (3α,4β)-diphenyl cyclobutane)

White crystalline solid. IR ν_{max} film cm^{-1} 3292, 3012, 2940, 2844, 1591, 1472, 1429, 1340, 1213, 1132, 1080, 996, 813, 754, 714.Found [M+1] 569.2175 ($C_{34}H_{32}O_8$ requires 568.21) HRFABMS *m/z* (rel. int. %) 506 (6.5), 415 (20), 284 (54), 181 (100), 166 (62). ^{1}H and ^{13}C NMR: Table **5.21**.,Spectra **5.31 - 5.33**

5.1.2.4. FLAVANONES

Three flavanones were isolated in this study from *C. apiculatum*, and two similar ones from *C. albopunctatum*. They were identified as 5,7-dihydroxyflavanone (coded B69-10B/ AO-15), 7-hydroxy-5-methoxyflavanone (B69-4B/AO-14) and 5,7-dimethoxyflavanone (B69-3).



Compound	R	R_1
B69-10B*	OH	OH
B69-4B#	OCH_3	OH
B69-3	OCH_3	OCH_3
AO-14#	OCH_3	OH
AO-15*	OH	OH

*B69-10B and AO-15 are identical as are #B69-4B and AO-14.

Fig.5.36. Flavanones isolated from *C. apiculatum* (BID) and *C. albopunctatum* (AOD) in this study.

General characterisation of flavanones

IR spectral data suggested the presence of aromatic rings (*ca* 3034, 1605 cm^{-1}) with monosubstitution (*ca* 765 cm^{-1}) and *para*-substitution (*ca* 830 cm^{-1}) as well as a carbonyl group conjugated with aromatic rings (*ca* 1630 cm^{-1}) (Williams and Fleming, 1995).

The ^{1}H NMR shows an ABX system with protons around *ca.* δ_H 2.5 and 3.5 and 5.5ppm. The heterocyclic C ring has three resonances – an oxymethine (C-2) resonating between δ_C71.3-80.3, an aliphatic methylene (C-3) absorbing between δ_C 39.5-46.4 and the carbonyl (C-4) in the range δ_C 186.4-198.5 (Agrawal *et al*, 1989). Ring B was found to be monosubstituted in all but one case. Typically it exhibits three different resonances arising from 5 protons, two sets of equivalent protons (C-2', C-6' and C-3', C-5'), and C-4'. They all occur between δ_C 125-132. In the proton domain, C-2'/6' appears at the lowest field and as a complex doublet, followed upfield by the C-3'/5' pair which appears as a complex triplet and a smaller triplet (which is not usually well resolved) due to the C-4' proton. The absence of substitution on the B ring is often confirmed by the presence in MS of *m/z*196 $[C_9H_8O_5]^+$ and 104 $[C_8H_8]^+$ (Tian-Shung Wu *et al*, 1981).

The C-6 and C-8 protons in ring A are important diagnostic assignments in 5,7-disubstituted flavonoids (Wagner *et al*, 1976). In ^{1}H NMR they occur very close together (sometimes as a singlet) between δ_H 5.9-6.5ppm, with H-6 usually slightly upfield relative to H-8. In ^{13}C-NMR, the order is reversed and the C-6 and C-8 methines appear

anywhere between δ_C 92 and 113ppm (Agrawal *et al*, 1989) depending on neighbouring groups. In this case, the C-6 signal always shows up at lower field than C-8.

It has been suggested that in aromatic rings, the carbon attached to methoxy groups is deshielded by *ca.*30ppm, whereas the carbon atoms *ortho* and *para* to the group are shielded by *ca.* 15.5 and 9 ppm (Pelter *et al*, 1976; Kingsbury and Looker, 1975). These rules appear to hold true on analysis of the flavanones as well as the other phenolic metabolites isolated in this study.

Table. 5.27. ^{1}H NMR (500MHz) spectral data of flavanones isolated from *C. apiculatum*.

Position	B69-10B	B69-4B	B69-3#
2	5.47 (dd, 2.5, 12.5)	5.44 (dd, 3,9.5)	5.50 (dd, 3,9.8)
3	3.11 (q, 4, 13)	3.09 (q, 4,13)	3.16 (dd, 3.6,12.7)
	2.79 (dd, 3, 14.5)	2.75 (dd, 3,13.5)	2.93(dd, 3,13.2)
6	5.92 (d, 2)	6.07 (d, 2.5)	6.48 (d, 2)
8	5.96 (d, 2)	6.12 (d, 2.5)	6.53 (d,2.2)
2'/6'	7.46 (m, 7.5)	7.50 (d, 7.5)	7.57 (m)
3'/5'	7.43 (m, 7)	7.43 (m, 1.5, 6)	7.39 (m, 1.4,6)
4'	7.38 (m, 7)	7.37 (m, 2.5, 6)	7.33 (m, 1.4,7.3)
5-OCH_3	-	3.85 (s)	3.80 (s)
7-OCH_3	-	-	3.80 (s)

All data obtained in CD_3OD except # which was obtained in C_5D_5N

Coupling constant ***J*** in Hz is in parentheses.

Table. 5.28. ^{1}H NMR (500MHz) spectral data of flavanones isolated from *C. albopunctatum*.

Position	AO-14	AO-15
2	5.42 (dd, 3,10)	5.43 (dd, 1.6, 9.7)
3a	2.99 (q, 4, 12.5)	3.06 (q, 4, 13)
3b	2.73 (dd, 3, 13)	2.75 (dd, 1.6, 14)
6	6.04 (s)	5.90 (d, 2)
8	6.09 (s)	6.00 (d, 2)
2'/6'	7.47 (m. 7.5)	7.48 (m, 1.2, 7)
3'/5'	7.40 (m, 1.5,6.5)	7.40 (m, 1.2, 7)
4'	7.34 (m, 1.5,5)	7.25 (m, 1.2, 7)

All data obtained in CD_3OD

Table 5.29. ^{13}C NMR (125MHz) spectral data of isolated flavanones

Position	B69-10B	B69-4B	B69-3#	AO-14	AO-15
2	80.8	80.6	not done	80.6	not done
3	44.6	46.8		46.8	
4	197.7	192.1		187.4	
4a	103.8	103.0		104.6	
5	165.9	165.0		164.1	
6	97.6	97.6		97.6	
7	168.9	168.0		167.0	
8	96.7	94.8		94.8	
9	165.0	164.7		162.2	
1'	140.8	140.0		139.2	
2'/6'	127.7/127.6	127.6		127.6	
3'/5'	130.0	130.1		130.0	
4'	130.1	129.6		129.9	
5-OCH_3	-	56.6		56.6	

All data obtained in CD_3OD

Identification of B69-4B/AO-14

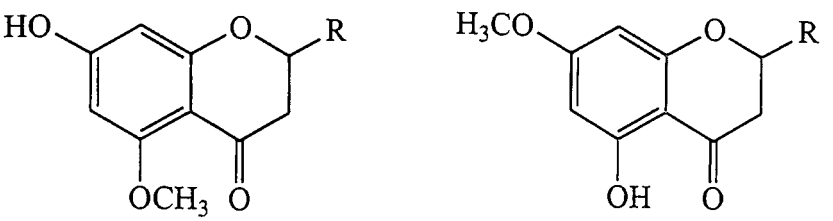
B69-4B (2.1mg) was isolated by HPLC fractionation of *C. apiculatum*, t_R 5.93min. It crystallized out of solution as cream-coloured needles. It was shown by NMR data to be identical to AO-14 from *C. albopunctatum.* The isolation of the latter is described here in greater depth.

AO-14 crystallized from a Sephadex LH-20 fraction of 100% trichloromethane (TCM) elution. From the NMR, which showed three coupling protons at δ_H 5.42 (dd, J =3, 10), 2.99 (dd, J=4, 12.5) and 2.72 (dd, J = 3, 13), it was thought that the basic structure was 2-phenylchromanone with a methoxyl and hydroxyl substituent. The C-6 and C-8 methines were diagnostic of 5,7-oxygenation. IR confirmed the presence of aromatic rings (*ca.* 3030, 1605 cm^{-1}) with *para*-disubstitution (*ca.* 830 cm^{-1}) and carbonyl conjugated to an aromatic system (*ca.* 1630 cm^{-1}) as well as a methoxyl function (*ca.* 2850 cm^{-1}).

In the Mass Spectra, HREIMS gave the molecular ion, M^+ at *m/z* 270 corresponding to $C_{16}H_{14}O_4$. The base peak was seen at *m/z* 256 $[M\text{-}CH_3]^+$, and there were other prominent peaks at *m/z* 179 (70%) $[M\text{-}C_4H_{11}O_2]^+$, 152 (70%) $[M\text{-}C_9H_{10}]^+$ and 124 (55%) $[M\text{-}C_{10}H_{10}]^+$. The fragmentation is typical of flavonoids in general as illustrated in Fig.**5.40**.

Two structures were then suggested to be possible – 5-methoxy-7-hydroxyflavanone (I) or the 5-hydroxy-7-methoxy (II) isomer. The former (I) was proposed as the more plausible structure based on circumstantial evidence. Agrawal *et al* (1989) suggest that

C-4 resonates at a position which is dependant on the electron movements at C-5. Thus in 5-hydroxylated flavanones, C-4 is appreciably deshielded at δ_C 195.6-197.3 as in B69-10B (where C-4 is at δ_C197.7) whereas when there is no interactive group (e.g. OMe or OAc), C-4 absorbs at higher field as in this case.



(I) (II)

Fig.5.37. Two possible substitution patterns in AO-14.

The reason for this shift is because of intramolecular hydrogen bonding in one case and not the other. A Gradient nuclear Overhauser Effect (GOESY) experiment was also carried out to provide evidence. It was found that the methoxy substituent interacted with the proton at δ_H 6.04 (i.e. H-6) rather than that at δ_H 6.09 (H-8).

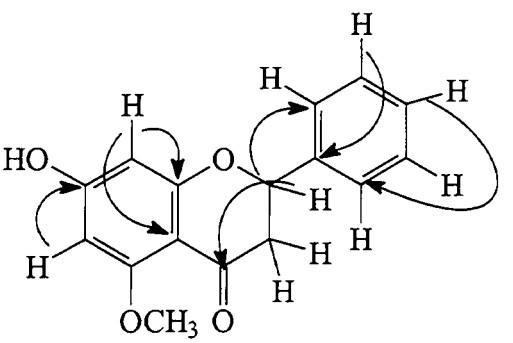
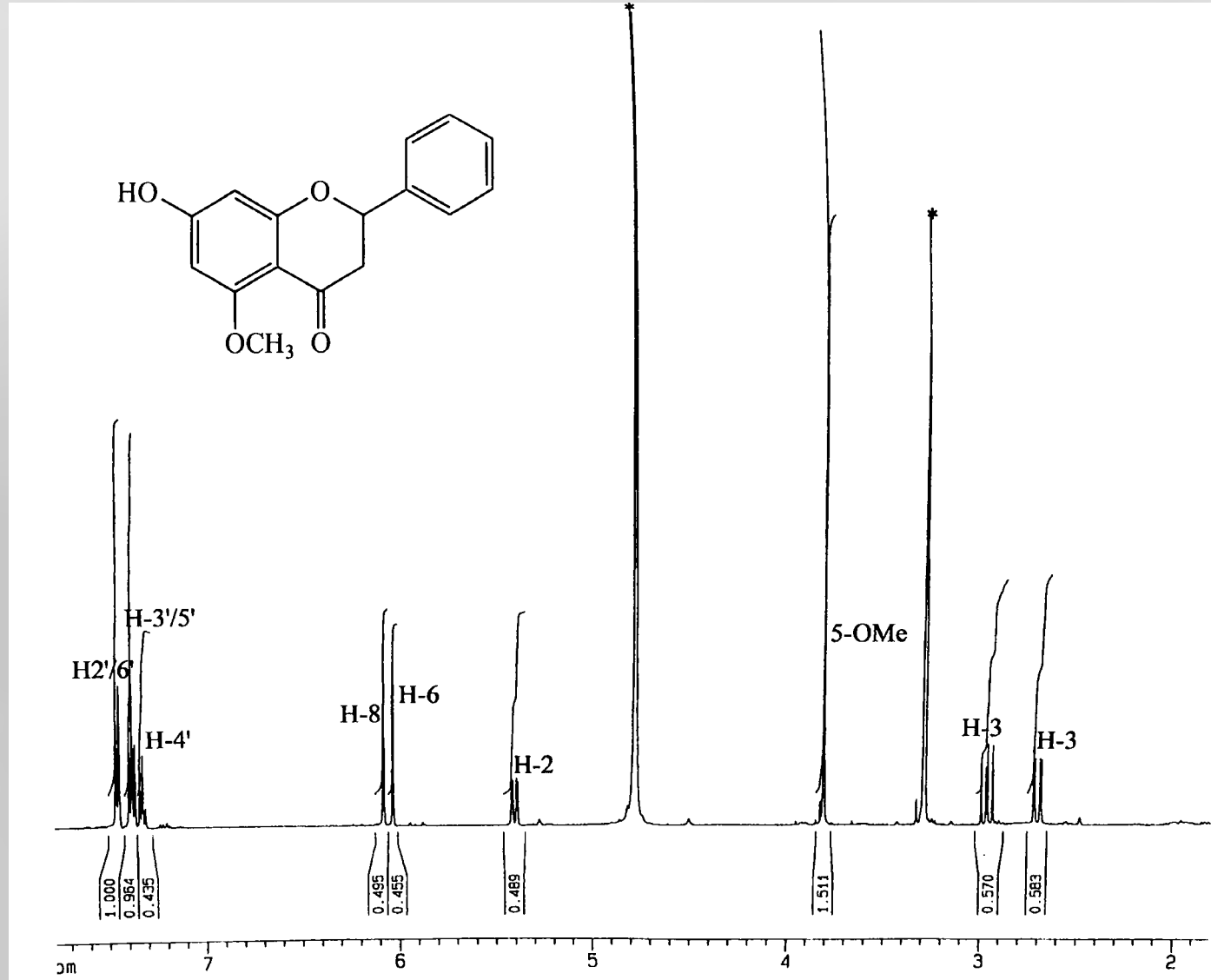


Fig.5.38. Significant HMBC correlations in AO-14

Table 5.30. HMBC correlations for AO-14 (in CD_3OD)

Position (δ)	^1J	^2J	^3J
H-2 (5.42)	80.6	-	187.4 (C-4)
H-3 (2.99)	46.8	187.4 (C-4)	139.2 (C-1')
(2.73)	46.8	187.4 (C-4)	139.2 (C-1')
H-6 (6.04)	97.6	167 (C-7)	104.6 (C-4a), 94.8 (C-8)
H-8 (6.09)	94.8	167.0 (C-7)	104.6 (C-4a), 97.6 (C-6)
H-2'/6' (7.47)	127.6	130.1 (C-3'/5') 127.6 (C-2'/6')	80.6 (C-2), 129.9 (C-4)
H-3'/5' (7.40)	130.1	-	130.1 (C-5'/3'), 139.2 (C-1')
H-4' (7.34)	129.9	130.1 (C-3'/5')	127.6 (C-2'/6')

AO-14/B69-4B was identified as alpinetin (7-hydroxy-5-methoxyflavanone) which has been previously isolated from *Chrysothamnus nauseosus, Helichrysum herbaceum*, *Dalea scandens*, *Alnus, Alpinia*, *Kaempferia, Eucalyptus* and *Piper* species (Burke and Nair, 1986). This is the first report of alpinetin in Combretaceae. It is interesting that its corresponding chalcone (A0-18/B69-13) was isolated from both *C. apiculatum* and *C. albopuncatum.* This raises the possibility of one or other of these compounds not being an authentic metabolite, but rather resulting from the extraction process (Wollenweber, 1981).



Spectrum 5.34 : ^{1}H NMR (500MHz, CD_3OD*) of B69-4B

Characterisation of B69-3

B69-3 was isolated by HPLC, t_R=5min. Its spectroscopic profile was similar to that of B69-4. The ^{1}H-NMR spectrum showed the same ABX system as seen in alpinetin with the only difference being the presence of two oxygen-bearing methyls at δ_H 3.80. Thus it was determined to be 5,7-dimethoxyflavone, also known as chrysin dimethyl ether. This has been previously isolated from *Boesenbergia pandurata*, *Aniba riparia* and *Eucalyptus* species (Itokawa, 1981).

Characterisation of B69-10B

A cream-coloured crystalline solid isolated by HPLC, t_R = 13min, mp 210-215°C was identified as 5,7-dihydroxyflavanone (pinocembrin). It differed from alpinetin (AO-14 above) in its lack of a methoxyl substituent in the NMR spectra. The IR showed the presence of hydroxyl (3235, 1340 cm-1), aromatic rings (*ca.* 3035, 1605 cm^{-1}) with *para*-disubstitution (*ca.* 830 cm^{-1}) and carbonyl conjugated to an aromatic system (*ca.* 1630 cm^{-1}). Significantly, the non-appearance of a peak at *ca.* 2850 cm^{-1} confirms the absence of methoxyl as suggested by the proton NMR spectrum.

HREIMS showed the molecular ion, M^+ at *m/z* 256 corresponding to the molecular formula, $C_{15}H_{12}O_4$ with other fragments at *m/z* 179 (63%) $[M-C_6H_5]^+$, 151 (40%) $[M-C_8H_9]^+$, 124 (41%) $[M-C_9H_7O]^+$, 104 (18%) $[C_8H_8]^+$ and its rearranged derivative, a tropylium, at *m/z* 91 $[C_7H_7]^+$. These are typical fragments of the flavanones (Siedel, 1999; Agrawal, 1989; Tian-Shung Wu *et al*, 1981).

The fragmentation envisaged for B69-10B typifies all the flavanones isolated in this study. The methoxylated compounds appear to lose these substituents to give the base peak at *m/z* 256 ($C_{15}H_{12}O_4$) which is the molecular formula of B69-10B, and in the main the ions obtained are similar to those of the other flavanones isolated here. See Fig **5.40**.

The C-4 resonance is relatively more downfield at δ_C 197.7 compared to that in alpinetin (AO-14) which is found at δ_C187.4. Intramolecular hydrogen bonding between the hydroxyl group attached to C-5 and the ketone at C-4 is instrumental for this type of shift (Agrawal, 1989). The presence of hydrogen bonding (both intramolecular and intermolecular) would also explain the longer retention time of 13 minutes compared to that of AO-14 (*ca.* 6 min) (Wollenweber and Mann, 1985).

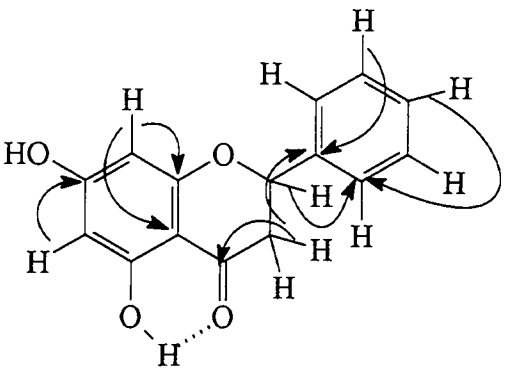


Fig.5.39. Significant HMBC correlations in B69-10B.

The HMBC correlations were in line with the suggested structure showing H-6 (δ_H 5.92) correlating to C-8 (δ_C 96.7) and C-4a (δ_C 103.8) while H-8 (δ_H 5.96) correlates to C-6 (δ_C 97.6) and also C-4a. The protons attached to C-3 show ^2J coupling to C-4 (δ_C 197.7) and

^3J to C-1' (δ_C 140.8) while H-2 correlates to C-2/6 (δ_C 127.6). The correlations seen in ring B are common to the chalcones isolated in this study as shown earlier.

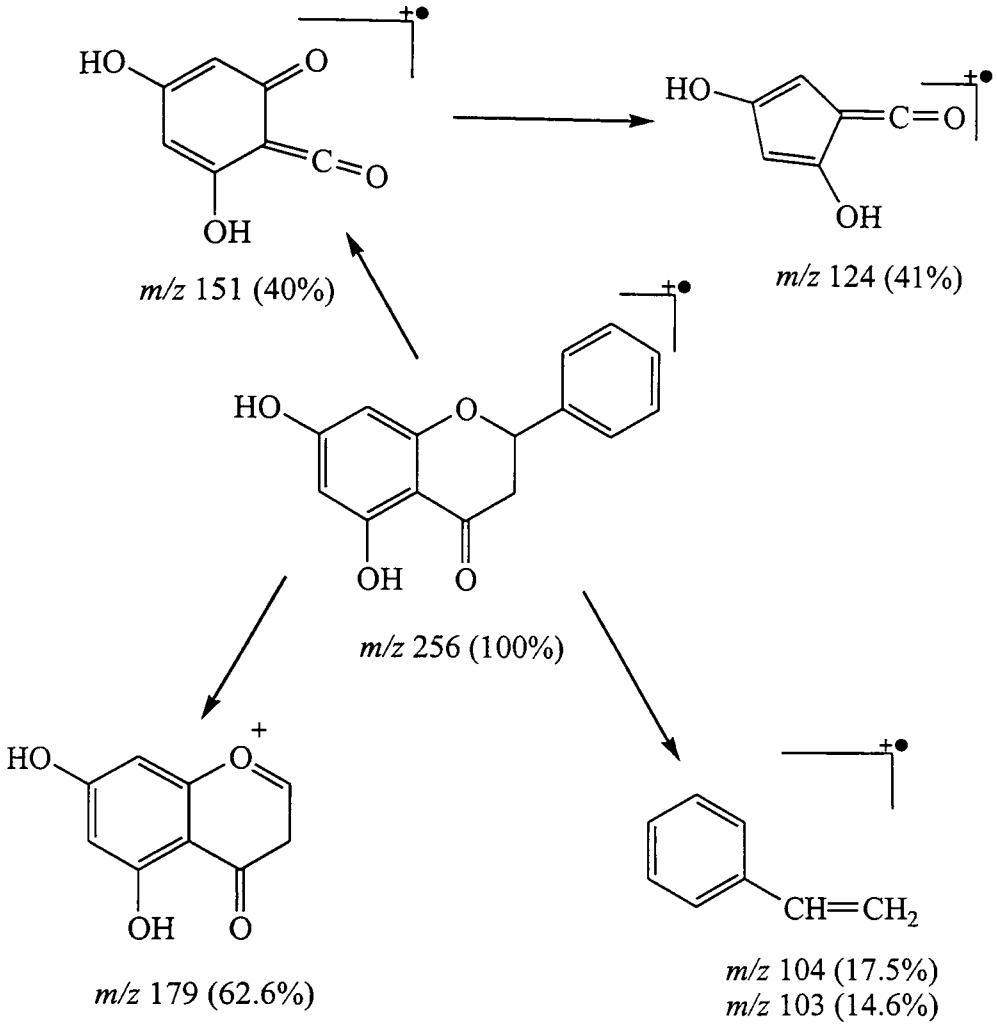


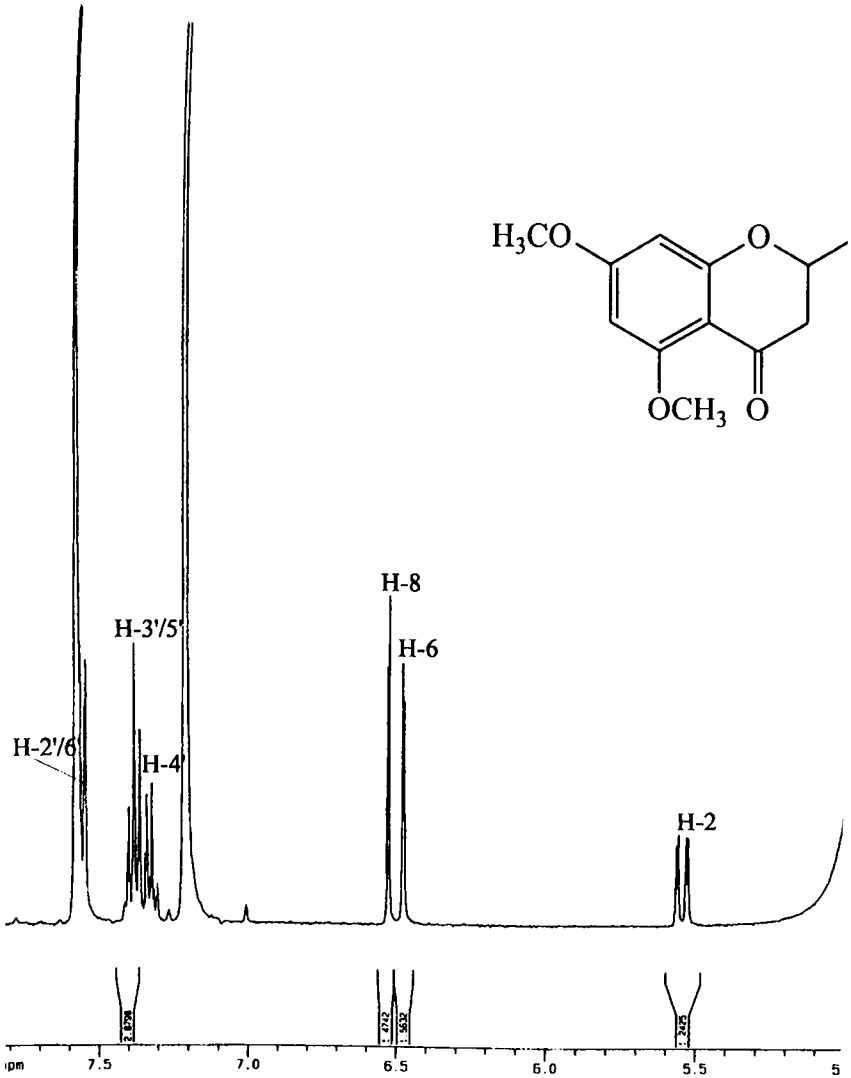
Fig. 5.40. The suggested fragmentation pattern of B69-10B which is typical of flavanones

B69-10B was identified as 5,7-dihydroxyflavanone. This is a commonly occurring flavanone, also called pinocembrin. It has been isolated from a variety of sources including the *Alpinia*, *Alnus, Eucalyptus*, *Pinus*, and *Populus* species (Itokawa, 1981).

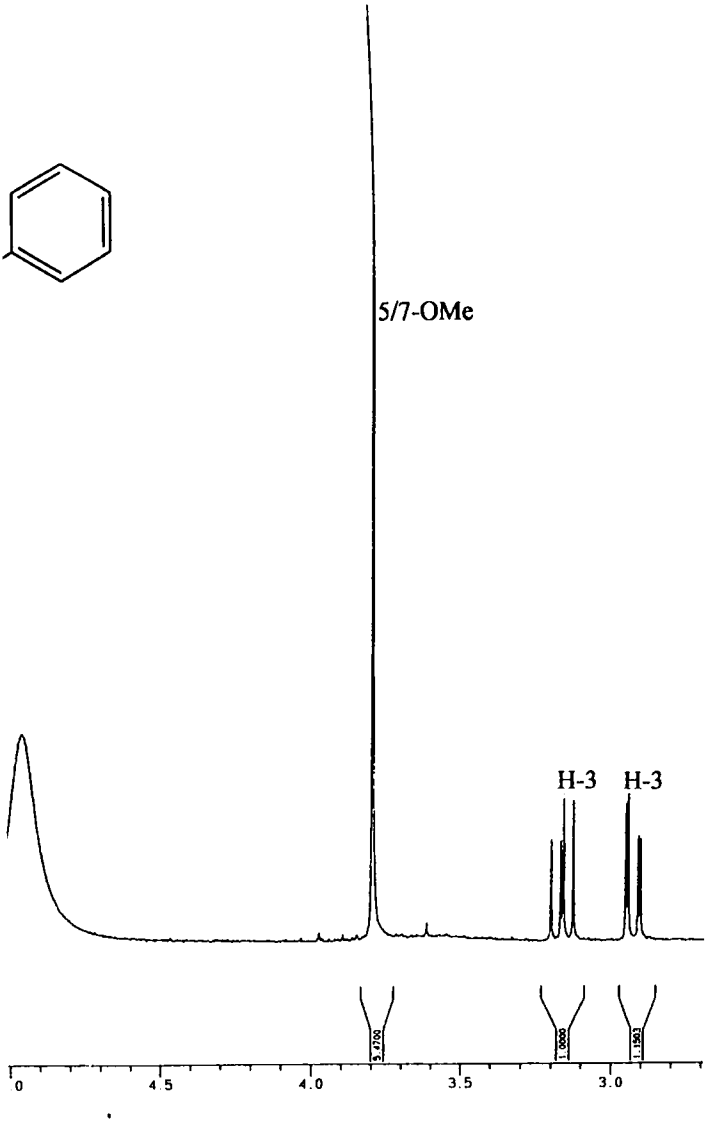
Table 5.31. HMBC correlations for B69-10B (in CD_3OD)

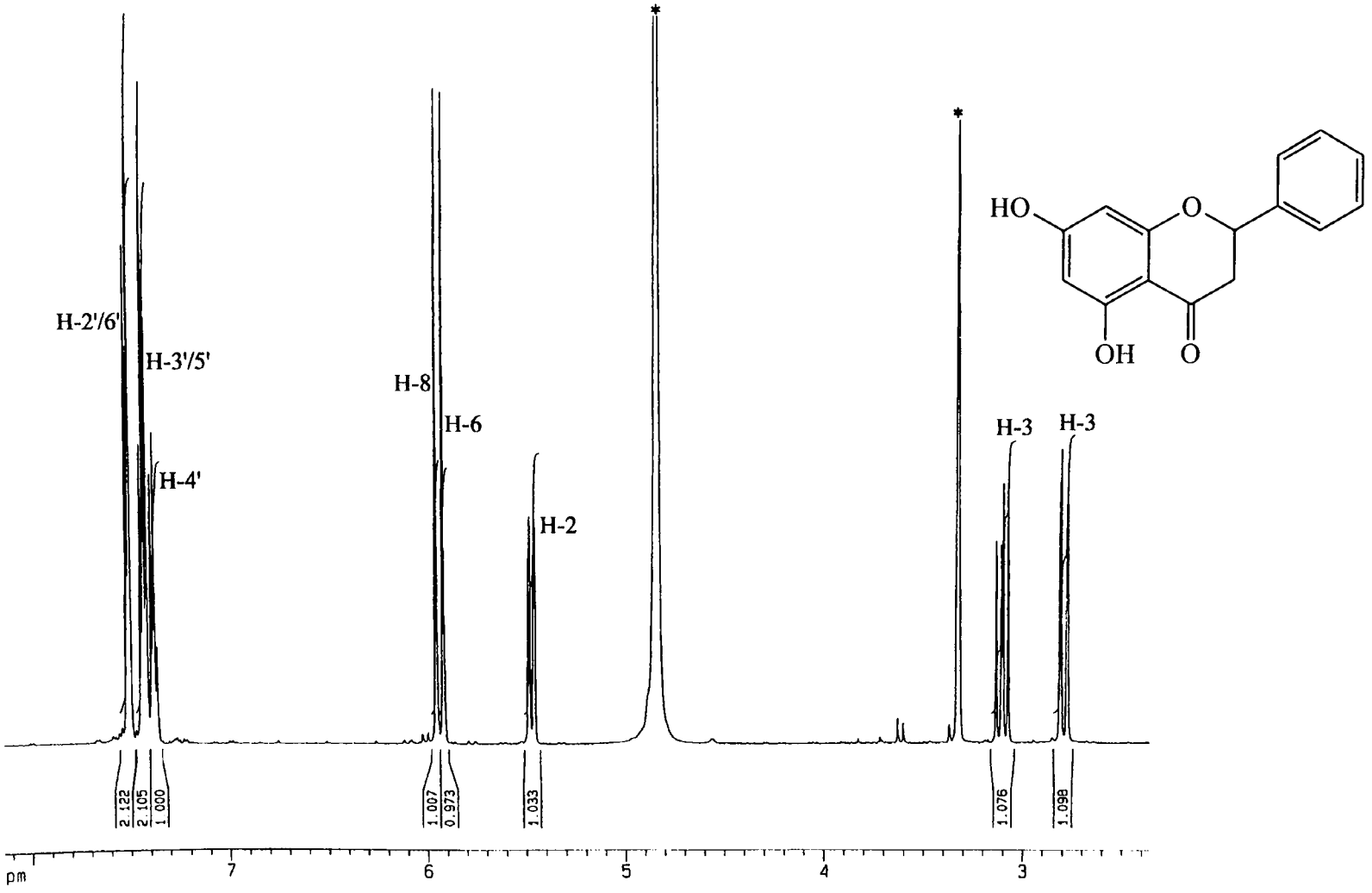
Position (δ_H)	δ_C	^2J	^3J
H-2 (5.47)	80.8	-	127.6 (C-2'/6')
H-3 (3.11)	44.6	80.8 (C-2), 197.7 (C-4)	140.8 (C-1')
(2.79)	44.6	197.7 (C-4)	-
H-6 (5.92)	97.6	165.9 (C-5), 168.9 (C-7)	103.8 (C-4a), 96.7 (C-8)
H-8 (5.96)	96.7	165.9 (C-9), 168.9 (C-7)	103.8 (C-4a), 97.6 (C-6)
H-2' (7.46)	127.7	-	80.2 (C-2), 127.6 (C-6')
H-3'/5' (7.43)	130.0	127.7 (C-2'), 127.6 (C-6'), 130.1 (C-4')	140.8 (C-1')
H-4' (7.38)	130.1	-	127.6 (C-6'), 127.7 (C-2')
H-6' (7.46)	127.6	-	80.8 (C-2)

This is the first report citing the presence of pinocembrin in both *C. apiculatum* and *C. albopunctatum*, and possibly in the African Combretaceae as a family.

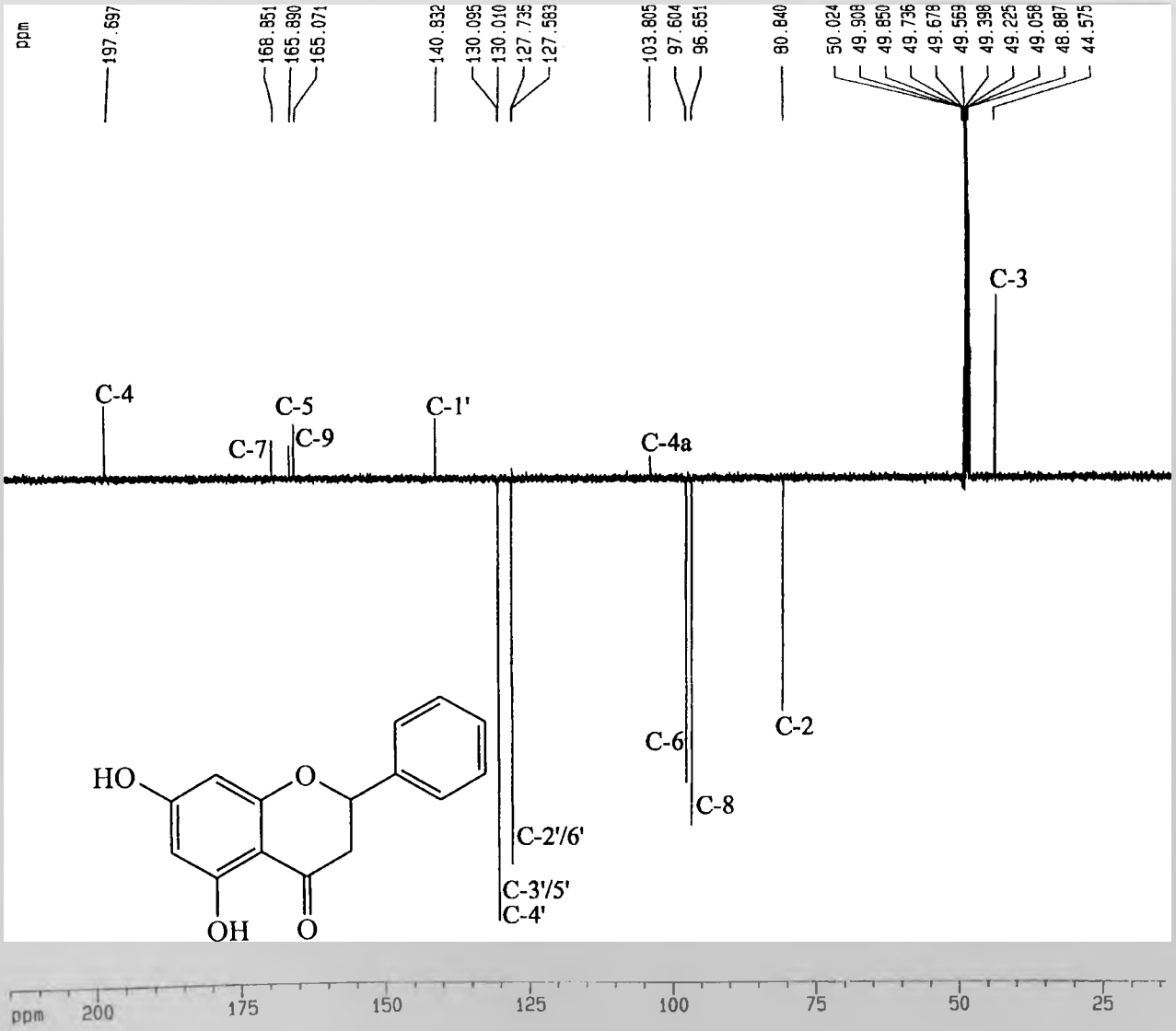


Spectrum 5.35: ^{1}H NMR (500MHz, C_5D_5N) of B69-3.


5/7-OMe
H-3
H-3
5.4700
1.0000
1.1503
0
4.5
4.0
3.5
3.0



Spectrum 5.36 : ^{1}H NMR (500MHz, CD_3OD*) of B69-10B



Spectrum 5.37: ^{13}C (PENDANT) NMR (125MHz, CD_3OD*) of B69-10B

Summary of the physical properties and spectral data of flavanones isolated

AO-14/ B69-4B (5-Hydroxy-7-methoxyflavanone (Alpinetin)

White crystalline solid. IR ν_{max} film cm^{-1} 3030, 1605 cm^{-1} (OH), 830 cm^{-1} (aryl), 1630 cm^{-1} 2850 cm^{-1} (OCH_3). Found [M^+] 270.089 ($C_{16}H_{14}O_4$ requires 270.089). HREIMS *m/z* (rel. int. %): 270 (13), 256 (100), 255 (57), 179 (70), 152 (71), 124 (50). ^1H and ^{13}C NMR: Tables **5.27 - 5.29**, and Spectrum **5.34**.

B69-3 (5,7-Dimethoxyflavanone)

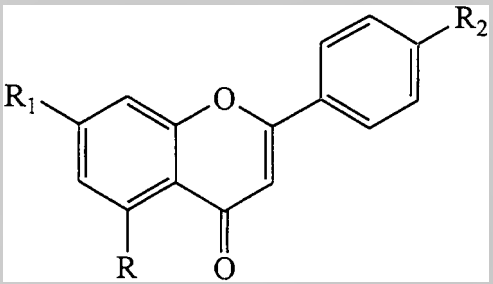
Pale yellow crystalline solid). Found [M^+] 284.2 ($C_{17}H_{16}O_4$ requires 284.105). HREIMS *m/z* (rel. int. %):284 (0.3), 270 (56), 193 (25), 166 (100), 149 (9), 138 (52), 104 (66). ^1H and ^{13}C NMR: Tables **5.27 - 5.29**, and Spectrum **5.35**.

AO-15/ B69-10B (5,7-Dihydroxyflavanone) (Pinocembrin)

Cream crystalline solid. IR ν_{max} film cm^{-1} 3235, 3070, 1630, 1600, 1495, 1275, 1160, 1090, 830, 765, 700. Found [M^+] 256.074 ($C_{15}H_{12}O_4$ requires 256.0736). HREIMS *m/z* (rel. int. %): 256 (100), 255 (44), 179 (63), 124 (41), 104 (18). ^1H and ^{13}C NMR: Tables **5.27 - 5.29**, and **5.36** & **5.31**.

5.1.2.5. FLAVONES

Two flavones were isolated, one, 5,7-dihydroxyflavone (chrysin), designated B69-10 from *C. apiculatum* and the other 5,7,4'-trihydroxyflavone (apigenin) (CIM-65) from *C. imberbe.*



Compound	R	R_1	R_2
B69-10[#]	OH	OH	H
CIM-65[*]	OH	OH	OH

Fig.5.41. Flavones isolated from [#]*C. apiculatum* and [*]*C. imberbe.*

Table 5.32. ^{1}H NMR (500MHz) spectral data of isolated flavones

Position	B69-10	CIM-65
3	6.97 (s)	6.89 (s)
6	6.75 (d, 2)	6.74 (d,2)
8	6.82 (d, 2)	6.81 (d,2)
2'/6'	7.91 (m)	7.92 (m)
3'/5'	7.47 (m, 1.5-3)	7.17 (m)
4'	7.42 (m, 1-5)	-

All data obtained in C_5D_5N

Table 5.33. ^{13}C NMR (125MHz) spectral data of isolated flavones

Position	B69-10	CIM-65
2	164.7	163.8
3	107.0	104.1
4	183.6	181.8
4a	106.0	104.1
5	164.0	161.1
6	101.1	100.0
7	167.1	164.1
8	95.8	94.9
9	159.4	157.3
1'	132.8	121.3
2'/6'	127.6	128.9
3'/5'	132.7	116.9
4'	130.7	161.5

All data obtained in C_5D_5N

Characterisation of B69-10

B69-10 (18.2mg) was isolated as a yellow/ off-white amorphous solid by HPLC, t_R = 11.7min, mp 260°C. It was the major compound of a mixture with B69-10B, a flavanone, discussed earlier. It was purified by recrystallization from a mixture of trichloromethane and methanol.

The IR spectrum showed the presence of hydroxyl (3440, 1350, 1100 cm^{-1}) with intramolecular hydrogen bonding (3010, 2930 cm^{-1}) and aromaticity (840, 790 cm^{-1}) and the absence of methoxyl functions (2850-2810, 1360-1355 cm^{-1}).

The ^1H NMR showed signals between δ_H 6.5 and 8ppm only, indicating the absence of methoxyl and aliphatic groups. It showed *meta*-coupling protons at δ_H 6.75 (J=2Hz)

(δ_C 101.1) and δ_H 6.82 (J=2Hz) (δ_C 95.8) which are typical of the C-6 and C-8 in the A-ring of flavonoids. The singlet at δ_H 6.97 δ_C 107.0 was the olefinic proton at C-3 while the complex signal at δ_H 7.40-7.49 and at *ca.* δ_H 7.91 δ_C 127.58 was due to signals from the mono-substituted aromatic ring. These signals are generally quite similar to those of the flavanones and simple chalcones except for the appearance of an olefinic singlet at δ_H 6.97 which is diagnostic of flavones (Agrawal, 1989).

In HMBC, ^2J and ^3J correlations are seen. See Table **5.34**. The *meta*-coupling protons at δ_H 6.75 and 6.82 correlate to each other's carbon and to the quaternary carbon at δ_C 106.0. This confirms their positions as H-6 and H-8 respectively. They also show a weak correlation to a common carbinol at δ_C 167.1, presumably C-7, while H-6 only shows correlation to another carbinol at δ_C 164.0. This evidence supports the conclusion that ring A is 2,4-dihydroxylated.

The olefinic proton at δ_H 6.97 shows strong correlation to C-4a (δ_C 106.0) and also to another quaternary at δ_C 132.8 which in turn has heteronuclear correlations from H-3/5 protons. The coupling in the B ring is obvious from the splitting pattern which recurs in all but one of the flavonoids isolated in this study. This is the complex doublet, triplet triplet arrangement from left to right which is typical of a mono-substituted aromatic ring and whose vicinal coupling is seen from ^{1}H-^{1}H COSY and can be confirmed by HMBC.

Table 5.34. HMBC correlations for B69-10

Position (δ_H)	δ_C	^2J	^3J
H-3 (6.97)	107.0	183.6 (C-4) 164.7 (C-2)	132.8 (C-1'), 106.0 (C-4a)
H-6 (6.75)	101.1	167.1 (C-7), 164.0 (C-5)	106.0 (C-4a), 95.8 (C-8)
H-8 (6.82)	95.8	159.4 (C-9), 167.1 (C-7)	106.0 (C-4a), 101.1 (C-6)
H-2'/6' (7.91)	127.6	132.7 (C-5'/3')	164.7 (C-2), 127.6 (C-6'/2') 130.7 (C-4')
H-3'/5' (7.47)	132.7	130.7 (C-4'), 127.6 (C-6'/2')	132.7 (C-5'/3'), 132.8 (C-1')
H-4' (7.42)	130.7	-	127.6 (C-2'/6')

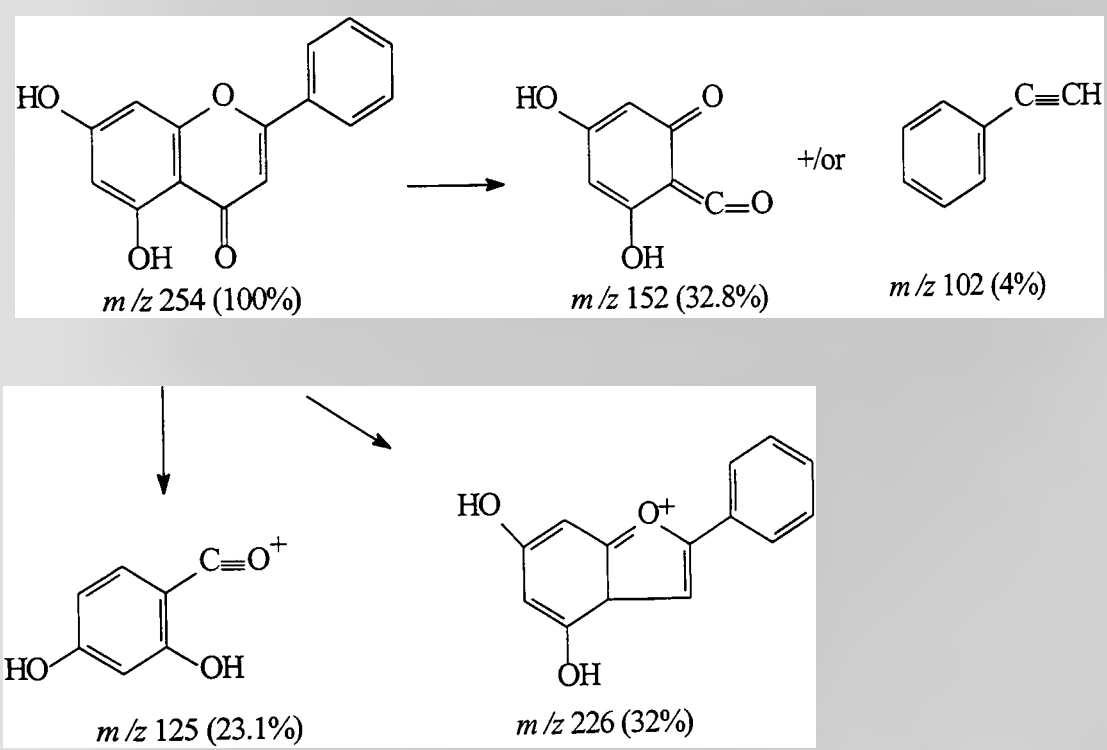
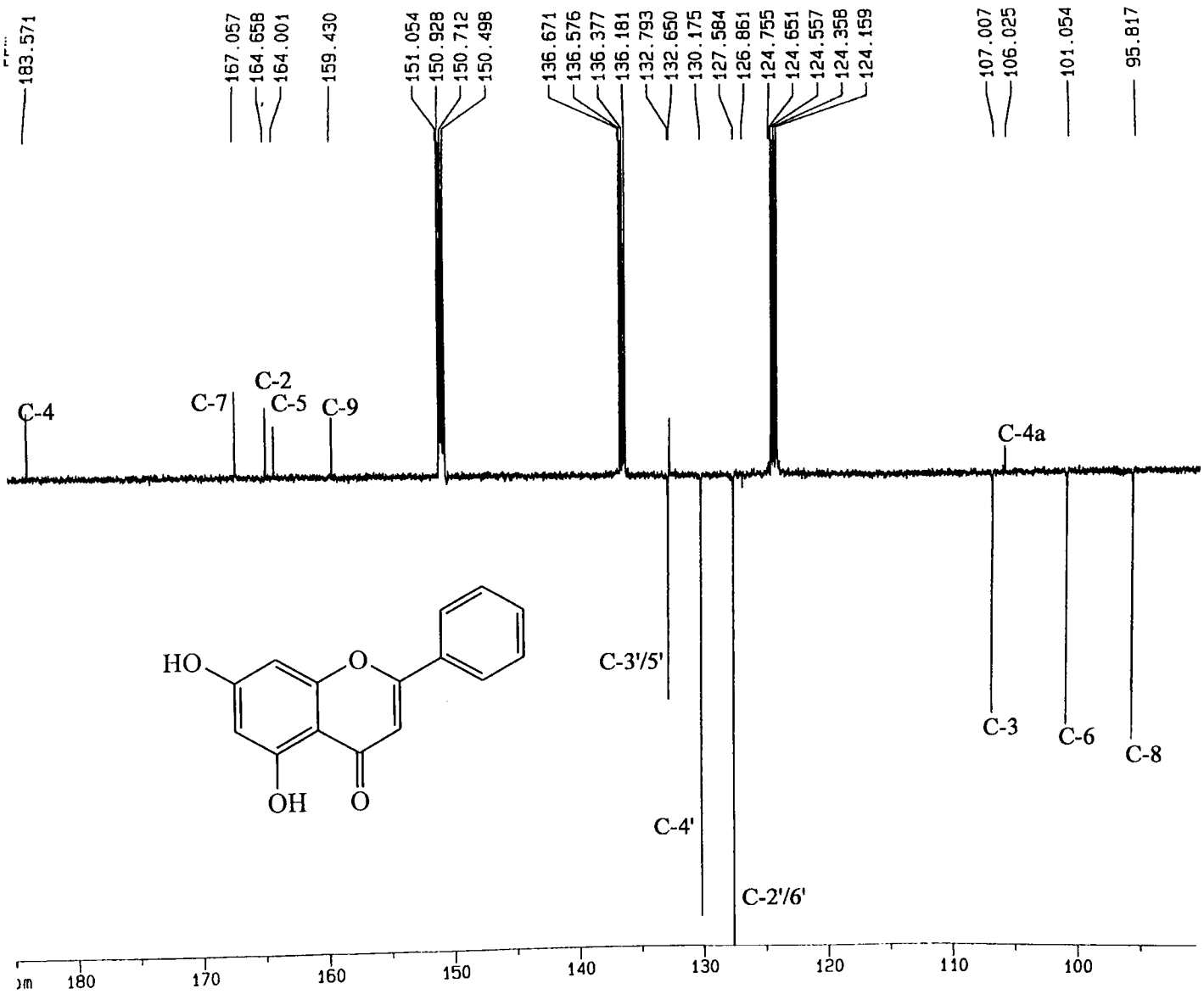


Fig.5.42. The EIMS fragmentation of flavones as exemplified by B69-10.

HREIMS showed the base peak and molecular ion, M^+ to be at *m/z* 254 giving the molecular formula as $C_{15}H_{10}O_4$ and other prominent peaks at *m/z* 226 (32%) $[M\text{-}CO]^+$, 152 (32.8%) $[M\text{-}C_8H_6O_4]^+$, and 102 (4%) $[M\text{-}C_7H_4O_4]^+$. This is typical of flavones; see Fig.**5.42**.

BID69-10 was identified as 5,7-dihydroxyflavone which is commonly known as chrysin and has been isolated from *Ulnus sieboldiana*, *Flourensia resinosa*, *Oroxylum indicum*, and *Pinus* and *Scutellaria* species (Huneck et al, 1986, Agrawal and Schneider, 1983). This is the first time its presence is reported in the Combretaceae. Its methylated dihydroflavone analogue (chrysin dimethyl ether) has also been isolated in this plant in this study. See B69-3 discussed above and Section **5.1.2.4**.



Spectrum 5.38: ^{13}C (PENDANT) NMR (125MHz, C_5D_5N*) of B69-10 ·

Characterisation of CIM-65

CIM-65 (10.5mg) crystallized from a fraction of Sephadex LH-20 (100 % TCM). It was a peach amorphous solid. HREIMS gave the molecular ion, M^+ at *m/z* 270 which corresponds to $C_{15}H_{10}O_5$ as the molecular formula. Other fragments were characteristic of flavones - *m/z* 153 (25%) $[C_7H_5O_4]^+$, *m/z* 192 (23%) $[M\text{-}C_6H_6]^+$ and *m/z* 91 (64%) $[C_7H_7]^+$. By comparison of the ^{13}C-NMR spectrum with the literature, CIM-65 was identified as 4', 5, 7-trihydroxyflavone (apigenin) which is an almost ubiquitous flavone (Agrawal, 1989). It is likely that it has been isolated from Combretaceae before, but not reported in the literature at hand.

Summary of properties and spectral data for flavones isolated.

B69-10 (5,7-Dihydroxyflavone) (Chrysin)

Yellow/ off-white flaky (amorphous) solid. IR ν_{max} film cm^{-1} 3440, 1350, 1100, 3010, 2930, 840, 790. Found $[M^+]$ 254.057. ($C_{15}H_{10}O_4$ requires 254.058). HREIMS *m/z* (rel. int. %): 254 (100), 226 (32), 217 (16), 152 (33), 124 (23). ^{1}H and ^{13}C NMR: Tables **5.32.** and **5.33.**, and Spectrum **5.38.**

CIM-65 (5,7,4'-Trihydroxyflavone) (Apigenin)

Creamy amorphous solid. Found $[M^+]$ 270.05 ($C_{15}H_{10}O_5$ requires 270.053). HREIMS *m/z* (rel. int. %): 270 (100), 242 (18), 192 (22), 153 (27), 107 (36), 75 (70). ^{1}H and ^{13}C NMR: Tables **5.32.** and **5.33.**

5.1.3. TERPENOIDS ISOLATED

Preliminary analysis of NMR data showed that the isoprenoids from both genera, *Combretum* and *Terminalia* were triterpenoid-type compounds. From previous work, tetracyclic and pentacyclic triterpenes have been isolated from related species of Combretaceae. See section **3.1.5.4.**

FTIR showed the presence, invariably, of hydroxyl (*ca.* 3440 cm^{-1}), olefinic (*ca.* 1650 cm^{-1}), methyl (*ca.* 1460 cm^{-1}) and geminal dimethyl (*ca.* 1380 cm^{-1}), and carboxylic (*ca.* 1680 cm^{-1}) or its esterified form (*ca.* 1730 cm^{-1}). It was also significant to note the absence of aromatic (*ca.* 3030, 1600, 1500, 920, 830 cm^{-1}), methoxyl (*ca.* 2850 – 2810 cm^{-1}) and cyclopropyl (*ca.* 2955 cm^{-1}) (Williams and Fleming, 1995; Mahajan *et al*, 1995; Ganzera *et al*, 1998).

Mass Spectrometry has become the most important tool for the elucidation of the skeleton and location of double bonds in pentacyclic triterpenoids, and the high mass peaks are diagnostic (Ogunkoya, 1981). Djerassi *et al* (1962) have established the retro-Diels-Alder fragmentation pattern which was seen in this study, and was a handy tool for the complete elucidation of the isolated compounds with the aid of biogenetic assumptions and previous work.

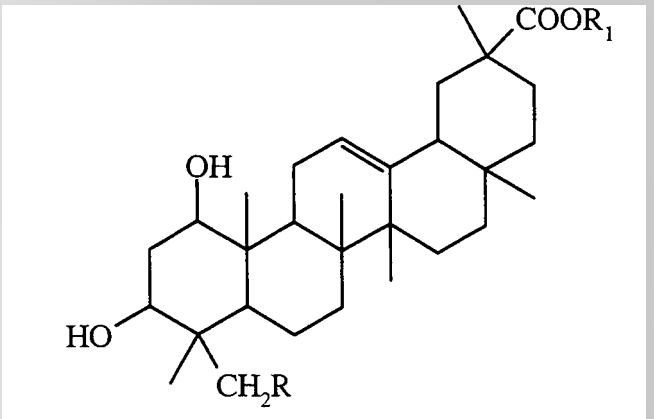
In general, elucidation was dependent on interpretation of a combination of NMR spectra to define the partial structure which was then compared with model known compounds as

proposed by Mahato and Sen (1997). The ^{1}H NMR spectra of the triterpenes are diagnostic and unique showing the typical forest of methyl singlets between δ_H 0.8 and 1.5ppm as well as overlapping methylenes and methines. The chemical shifts of C-12, C-13, C-17, C-18 and C-28 are of diagnostic value and useful for determining the stereochemistry of ring D/E junction.

In the case of the glycosides, the anomeric protons are evident in the region δ_H 4.3 – 5.9ppm, while the methyl doublets appear for 6-deoxy sugar residues at δ_H 1.1-1.3ppm (Agrawal, 1992). The elucidation of these sugar units is systematically approached by way of using the "structural reporter group" i.e. a group with signals outside the bulk region, generally resonating between δ_H 3-4.2ppm. This is then used to establish the through-bond connectivity to its immediate neighbours and to determine the nature and number of the spin systems corresponding to individual sugar regions. ^{1}H-^{1}H COSY was employed extensively in this study to unravel the structure and connectivities of the sugar moieties. The majority of sugar units have stable hexopyranose ring conformations and can be identified by the size of $^3J_{H\text{-}H}$. This is greater than 6Hz for anti-periplanar orientation of the vicinal ring protons and small (< 5Hz) for their *gauche* orientation.

Six 1,3-hydroxylated pentacyclic olean-12-ene triterpenoids were isolated in this study by preparative HPLC as described in section **4.1.2**. One of them, CIM-31, was isolated in a large quantity and from various other fractions which may imply that this is the major constituent in the leaves of *Combretum imberbe*. It has been previously isolated from the

same plant species by Rogers and Subramony (1988). In this study it was found to be the aglycone common to all the other variously-derivatized glycosidic compounds. See Fig.**5.43.**



Compound	R	R_1
CIM-31	H	H
CIM-10	4-Ac-O-Rh	H
CIM-11	3,4-Ac-O-Rh	H
CIM-9	3,4-Ac-O-Rh	Rh
TST-8	4-Ac-O-Rh	Rh
TST-11	4-Ac-O-Rh	H

Fig.5.43. 1,3-hydroxylated pentacyclic triterpenoids isolated in this study

Four of the compounds were isolated from *C. imberbe*, and two of them, designated CIM-9 and CIM-11 are reported here for the first time. The other two compounds were isolated from *Terminalia stulhmanii* and these, interestingly, possess the aglycone typical

of the triterpenes of *C. imberbe* i.e. CIM-31. This is the first report of this seemingly important biogenetic link and also the discovery of a new compound, a bidesmosidic triterpene designated TST-8.

Characterisation of CIM-31

CIM-31 (70mg) crystallized as a white-to-colourless solid from a Sephadex LH-20 fraction after elution with 100% trichloromethane (TCM). It would appear to be a major constituent in *C. imberbe* as it was also isolated from a different fraction by RP HPLC with retention time, t_R, 8.4min, mp. 282°C.

The NMR spectra were suggestive of a triterpenoid-type compound with a carboxylic function, a trisubstituted double bond and an AB system. IR showed peaks at *ca* 3440cm^{-1} (hydroxyl), *ca* 1695cm^{-1} (carboxylic), *ca* 1650cm^{-1} (olefinic), *ca* 1470cm^{-1} (methyl), and *ca* 1380cm^{-1} (geminal dimethyl) (Biessels *et al*, 1974; Mahajan *et al*, 1995). This pattern was seen in all the other compounds isolated from this plant suggesting a similarity in the chemical structure of the aglycone unit modified by various substituents.

LR-FABMS showed the pseudomolecular ion, (M+1) to be at *m/z* 473 (3%), and the molecular formula of $C_{30}H_{48}O_4$ was deduced from this. Other prominent peaks were seen at *m/z* 453 (19%) $[M\text{-}H_20]^+$, *m/z* 436 (6%) $[M\text{-}2H_2O]^+$, *m/z* 248 (10%) $[M\text{-}C_{14}H_{24}O_2]^+$, the base peak at *m/z* 188.9 (100%) $[M\text{-}C_{16}H_{27}O_4]^+$, *m/z* 130 (45%) $[C_{10}H_{10}]^+$ and m/z 93 (74%) $[C_7H_9]^+$. See Fig. **5.44.**These fragments were strongly suggestive of a

hydroxylated Δ^{12}-pentacyclic triterpene. There is initial loss of water, then a Wagner-Meerwein rearrangement with subsequent loss of another water molecule (Geissman, 1959). The rearranged fragment then typically undergoes retro-Diels Alder fragmentation (Djerassi *et al,* 1962; Karliner and Djerassi, 1966). This view was reinforced by the appearance in the ^{13}C NMR spectrum of six quaternary carbon atoms as well as signals at around δ_C123 and 145ppm which are characteristic of the Δ^{12}-oleanene skeleton (Doddrell, 1974; Amoros and Girre, 1987).

The analysis of 1D and 2D-NMR spectra revealed the nature of the compound to a great extent, and where signals were not clear there was recourse to previous work on similar compounds (Bowden, 1975; Houghton and Lian, 1986;Rogers and Subramony, 1988; Rogers, 1989).

Seven tertiary methyl proton signals (δ_H 0.97-1.45) were seen in the NMR spectra. Two of these were geminally coupled as indicated by the HMBC. These were the protons attached to C-23 (δ_H 0.97, δ_C 28.5) and C-24 (δ_H 1.10, δ_C 17.3), and C-30 (δ_H 1.45 δ_C 20.1) coupled to the carbonyl (C-29).

The hydroxy-methine protons were placed at C-1 and C-3 because they show HMBC correlations with tertiary methyls at C-25 (δ_C 16.5) and C-23 (δ_C 28.5), C-24 (δ_C 17.3) respectively as well as a correlation which is common to both with C-2 (δ_C 35.8).

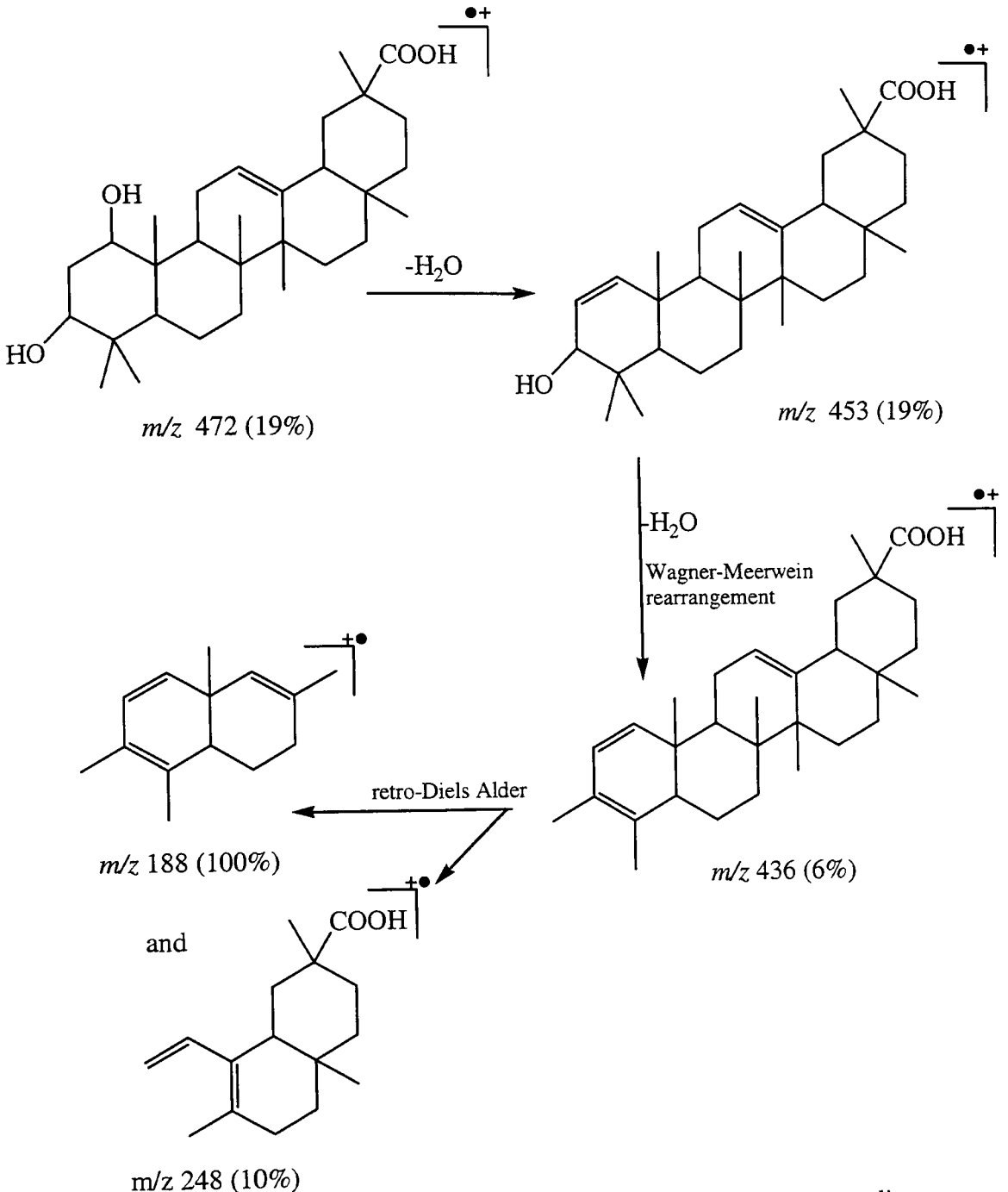


Fig. 5.44. The suggested fragmentation pattern of CIM-31 typical of pentacyclic triterpenoids isolated in this study

The H-1 proton forms a double doublet at δ_H 4.35 (J=4.5/7Hz) as it has a large axial and a small equatorial coupling to the H-2 protons which appear at δ_H 2.19ppm and 2.23ppm. H-3 on the other hand appears as a broad singlet at δ_H 3.86 and must therefore be equatorial relative to H-2. Using the methyls as starting points, HMBC correlations were shown as illustrated in Fig.**5.45.** These are rudimentary but important in confirming the suspected structure.

The alkenic proton δ_H 5.36 was found to be a broad singlet and shown by HMQC to be attached to the carbon at δ_C 123.3 (C-12). It is not coupled strongly to the neighbouring C-11 methylene which splits H-9 δ_H 2.89 (δ_C 38.3) into a quartet J=5Hz. This is again due to the fact that they have an axial-equatorial relationship to this proton whereas the Karplus dihedral angle to H-12 is quite big (possibly around 90°) making the coupling constant small (Williams and Fleming, 1995).

One of the methylene protons at C-19 (δ_H 2.50, δ_C 41.6) appears as a clean triplet J=13.5 while the other one is an indistinguishable multiplet at δ_H 1.71. See Spectra **5.39**. These show ^3J coupling to the carboxylic acid C-29 (δ_C 181.4) and C-30 (δ_C 20.1). The other protons are difficult to distinguish because they overlap, but ^1H-^1H COSY, HMQC and HMBC were able to build a detailed partial structure which was suggestive of a 1,3-dihydroxy-12-oleanen-29- oic acid.

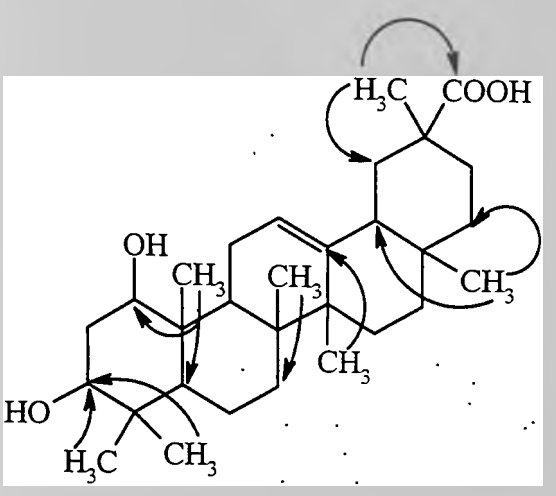


Fig.5.45. Significant HMBC correlations of CIM-31 confirming the olean-12-ene framework.

Table.5.35. HMBC correlations for Methyl groups in CIM-31

Position	δ_H	δ_C	^2J	^3J
23	0.97 (s)	28.5	40.0 (C-4)	72.7 (C-3), 48.4 (C-5), 17.4 (C-24)
24	1.10 (s)	17.3	40.0 (C-4)	72.7 (C-3), 48.4 (C-5), 28.5 (C-23)
25	1.32 (s)	16.5	42.5 (C-10)	38.3 (C-9), 71.8 (C-1), 48.4 (C-5)
26	1.07 (s)	16.7	39.7 (C-8)	32.8 (C-7), 41.5 (C-14)
27	1.31 (s)	26.2	41.5 (C-14)	39.7 (C-8), 144.5 (C-13)
28	1.32 (s)	28.9	32.8 (C-22)	27.4 (C-16), 46.7 (C-18), 36.6 (C-22)
30	1.45 (s)	20.1	42.8 (C-20)	41.6 (C-19), 29.8 (C-21), 81.4 (C-29)

Comparison of the experimental data with the literature showed CIM-31 to correspond to 1α,3β-dihydroxyolean-12-en-29-oic acid. See Fig.**5.46**.

Table 5.36.^{13}C (125MHz) and ^{1}H (500MHz) NMR spectra data for CIM-31

Position	δ_C	δ_H
1	71.8	3.86 brs
2	35.8	2.19, 2.23 ax, eq
3	72.7	4.35, dd, J=4.5, 7
4	40.0	-
5	48.4	1.75
6	18.8	1.72, 1.35
7	32.8	1.60, 1.36
8	39.7	-
9	38.3	2.88 (q, J=6.5)
10	42.5	-
11	23.7	2.02, 2.31
12	123.3	5.36 brs
13	144.5	-
14	41.5	-
15	26.6	1.85, 1.05
16	27.4	0.87 (q, ill-res), 2.13 (d, J=3.7)
17	32.8	-
18	46.7	2.16
19	41.6	1.71, 2.50 (t, J=13.5)
20	42.8	-
21	29.8	2.20, 1.72
22	36.6	1.53, 1.35
23	28.5	0.97 (s)
24	17.3	1.10 (s)
25	16.5	1.32 (s)
26	16.7	1.07 (s)
27	26.2	1.31 (s)
28	28.9	1.32 (s)
29	181.4	-
30	20.1	1.45 (s)

All data obtained in C_5D_5N

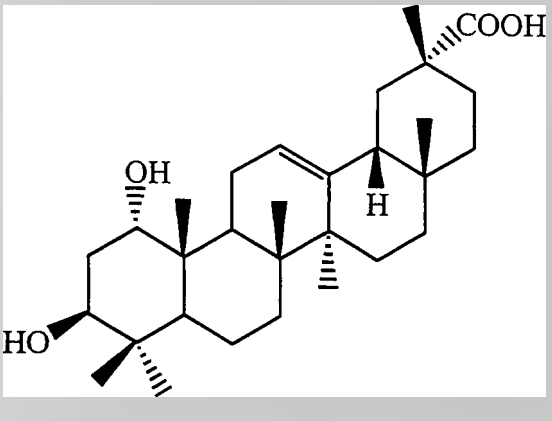


Fig.5.46. The proposed structure of CIM-31.

From NOESY, the relative stereochemistry of the OH group at C-1 was determined as α and axial with a cross-peak between H-1 and H_3-25 while the configuration of C-3 hydroxyl was assigned β and equatorial on the basis of a NOESY correlation between H-3/5 and H-3/ -23 as well as on chemotaxonomic grounds (Nick *et al*, 1995; Rogers and Subramony, 1988). There were other interactions between H_3-27/H-9, H-19/ H_3-27, H-18/ H_3-30 and H_3-28/H-18. Using a molecular modelling kit, a ball and stick model was constructed based on information from NOESY and it provides a good illustration of the chair orientation of the five rings as well as the spatial arrangement around them. See Fig.**5.47**.

Two isomers of this compound have been previously isolated – hydroxy-imberbic acid from this same plant species by Rogers and Subramony (1988) and dillenic acid E from *Dillenia papuana* (Dilleniaceae). The former has the carboxylic acid in the α,

equatorial position. Comparison of the ^{13}C NMR data from these two studies and those obtained here led to the conclusion that CIM-31 is hydroxyimberbic acid.

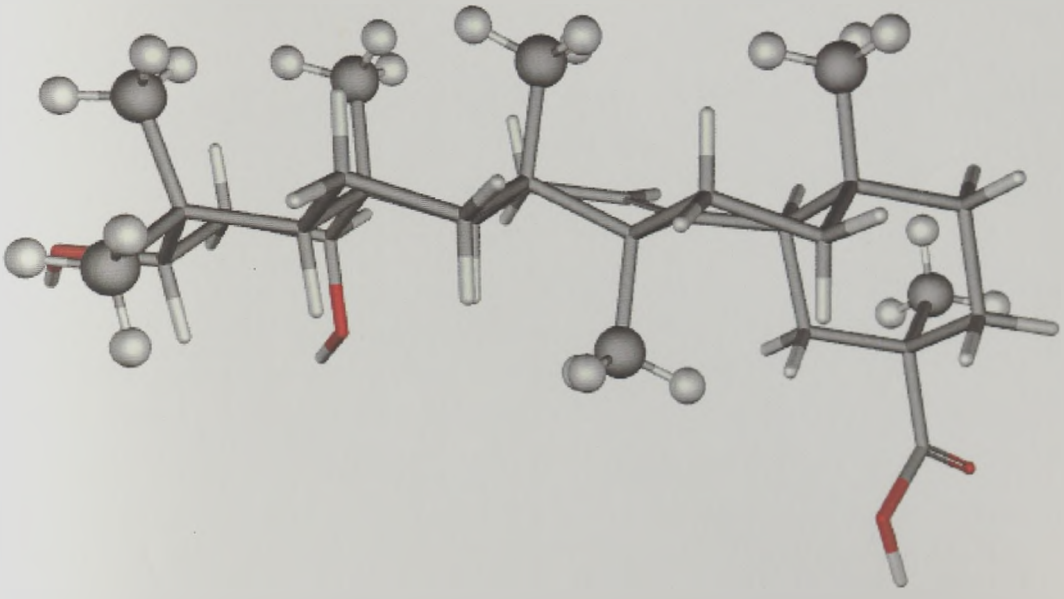
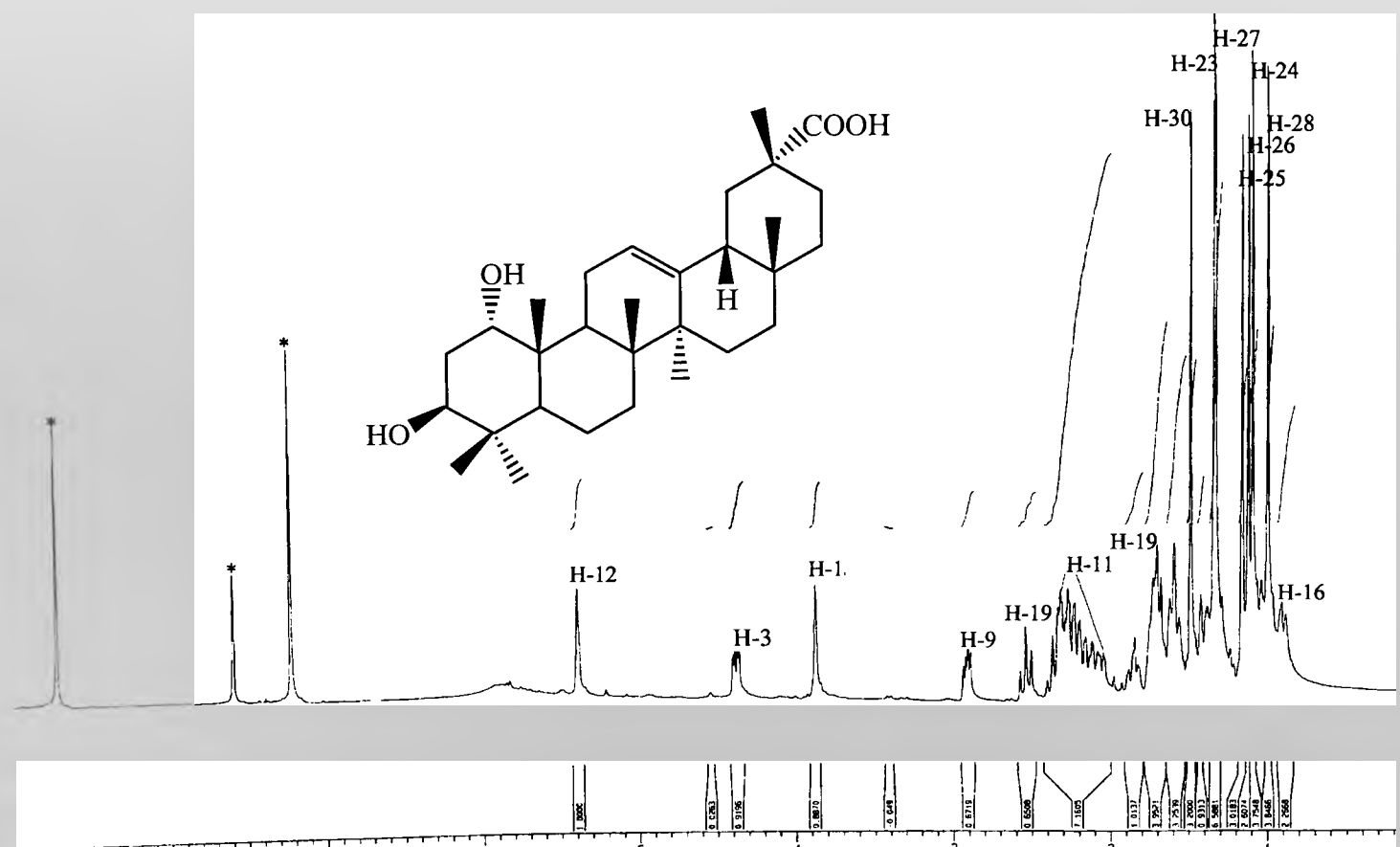
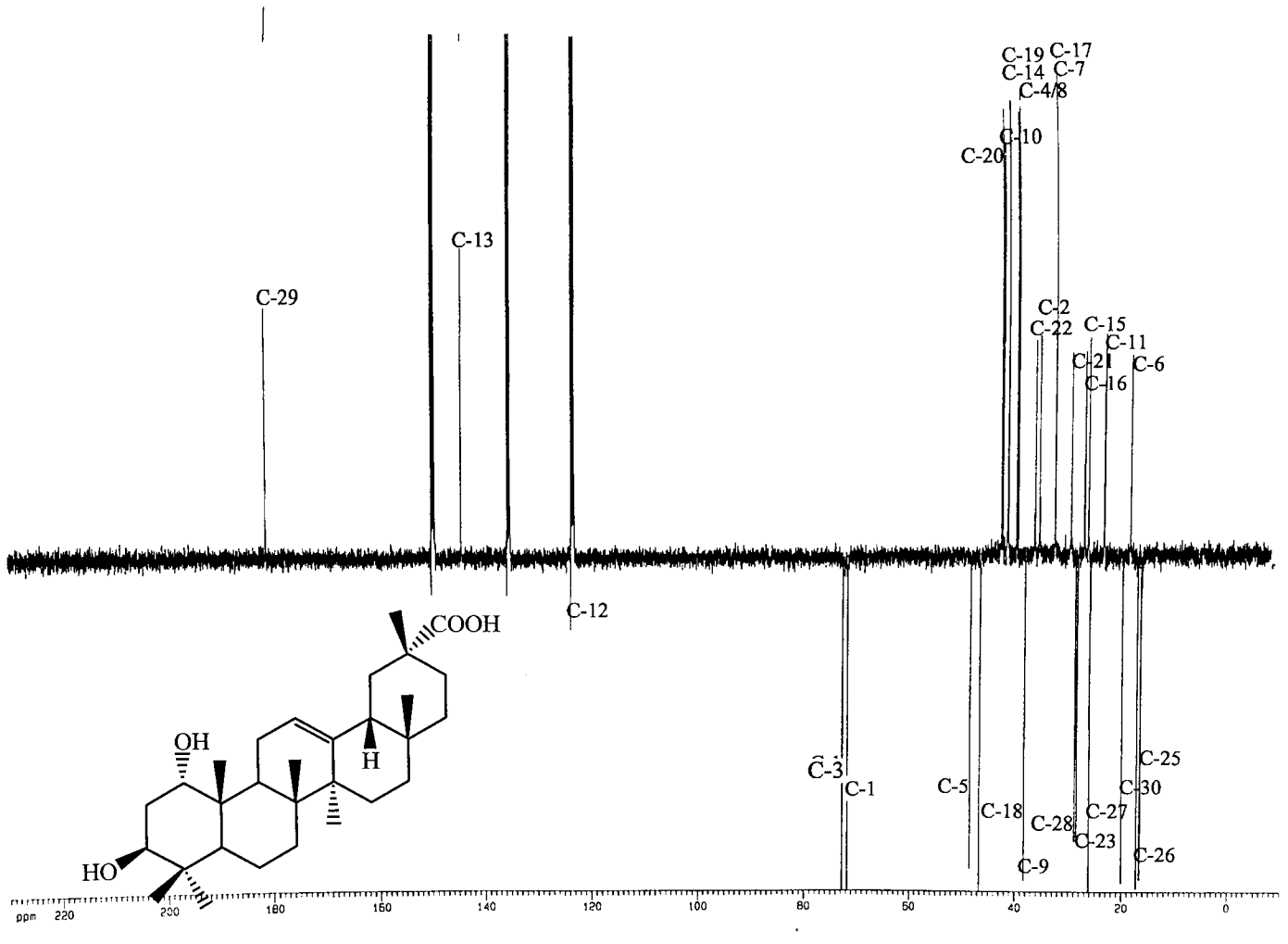


Fig.5.47. A ball and stick model of CIM-31 showing its 3-D structure.

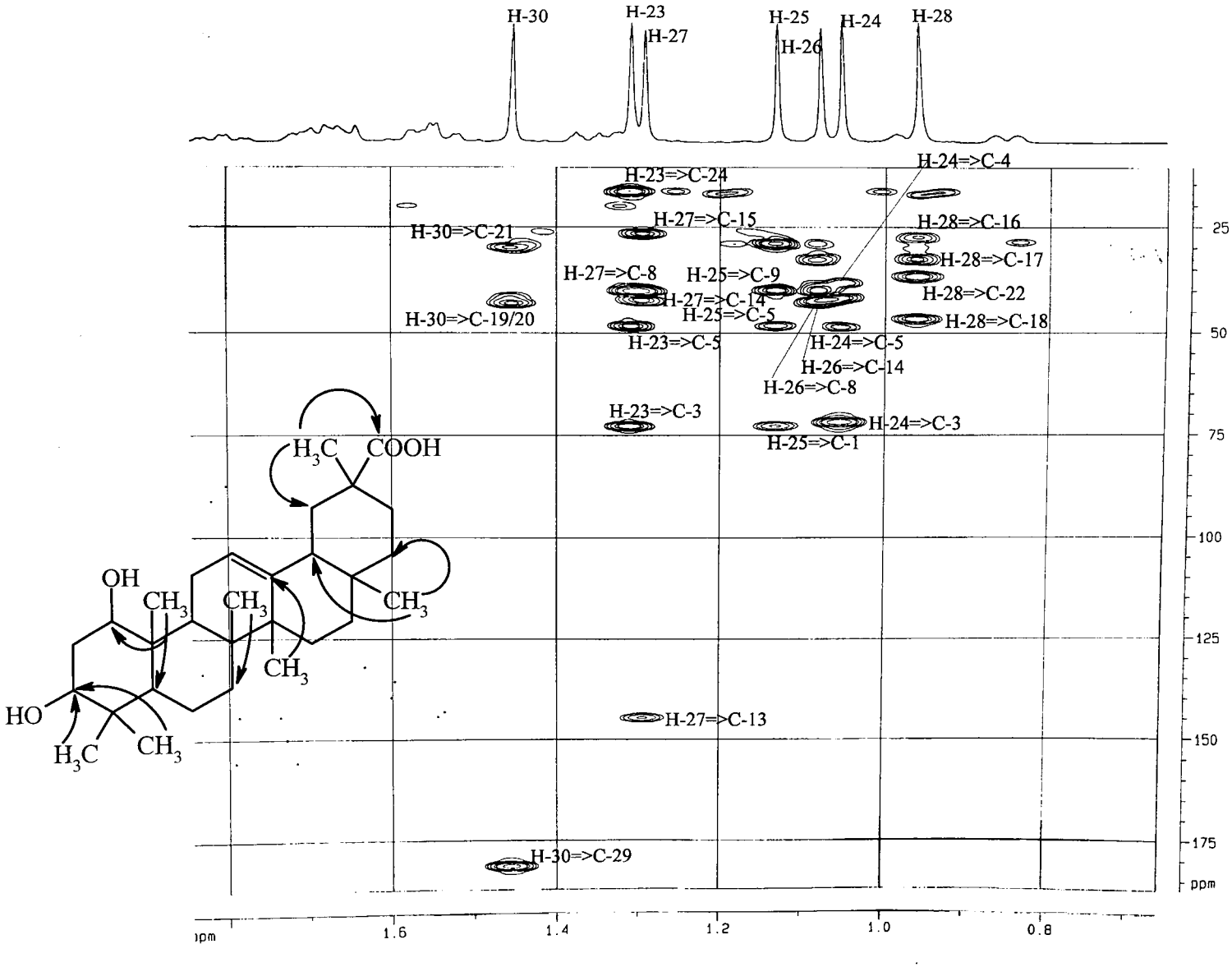
CIM-31 is the prototypical compound expected from *C. imberbe*. This study provides detailed NOESY data for the first time for this compound.



Spectrum 5.39. ^{1}H NMR (500MHz, C_5D_5N*) of CIM-31



Spectrum 5.40 ^{13}C (PENDANT) NMR (125MHz, C_5D_5N*) of CIM-31



Spectrum 5.41: Significant HMBC correlations of CIM-31 used to confirm partial structure

General characterisation of rhamnopyranosides

It was established as discussed above that CIM-31 was the major pentacyclic triterpene in *C. imberbe*. Other fractions obtained from the HPLC work up of both *C. imberbe* and the stem bark of *T. stulhmanii* were characterised as being glycosidic derivatives of this constituent.

The compounds were thought to share a common aglycone because their ^{1}H NMR and ^{13}C NMR spectra were virtually superimposable except in the region between δ_H 4.2 and 6 ppm (δ_C 65-79) where the resonances of the sugar moiety(ies) occur (Agrawal, 1992).

^{1}H-^{1}H COSY is indispensable in the structure elucidation of sugars and it was used in all cases to unravel the bond connectivities which were then confirmed by ^{2}J and ^{3}J coupling correlations obtained from HMBC. The structural reporter groups chosen were the anomeric atoms which normally resonate in the region spanning from δ_H 4-6 in PMR and δ_C 90-112ppm in CMR. Within a typical aldohexopyranosyl ring, the coupling is unidirectional and so it was possible to trace coupling from the anomeric proton, designated R-1 to R-2, R-2 to R-3, R-3 to R-4 etc until the entire sugar was mapped out.

The sugar in this case was based on the rhamnose unit which has its anomeric carbon normally around δ_C 101.5ppm (Seidel, 1999) and assumed to be the L-isomer on biogenetic grounds (Rogers, 1989). In this case modifications appeared largely around the environs of attachment of the sugar. Glycosylation at the carboxylate causes a

downfield shift of the ^{13}C resonance (2-5ppm) of the moiety, an upfield shift (0.5-2ppm) of the adjacent resonance as well as a remarkable shielding of the anomeric carbon (δ_C 93-97ppm) (Agrawal, 1992). This along with HMBC correlations served to confirm the location of the sugar.

The presence of more than one sugar was easily identifiable from the number of anomeric protons available as these are not normally superimposable and also from the number of ^{13}C resonances of the oxymethines δ_C 65-80ppm.

Characterisation of CIM-10/TST-11

CIM-10 was isolated as a white amorphous powder (36.3mg), t_R =22.5min, mp.198°C. LRFABMS showed the presence of a molecular ion, $[M+H]^+$ at *m/z* 677.5 which corresponds to $C_{38}H_{60}O_6$. Other prominent peaks appeared at m/z 658 (41%) $[M-H_2O]^+$, 472 (18%), 453 (80%), 437 (30), 248 (100), 231 (62). The other peaks are for the aglycone and show a similar pattern to that of CIM-31.

The NMR spectra showed resonances in the sugar region but were otherwise quite similar to those of CIM-31 with a few variations, the most significant being around ring A. The resonances for C-3 and C-5 are drastically changed. In imberbic acid, C-3 is deshielded by +4.2ppm relative to that in CIM-10. More importantly C-5 is moved upfield by up to 6.7ppm in CIM-10. The reason is found in the change of C-23 to an O-methylene and also a point of glycosylation. This appears to have the effect of shielding the C-5 and C-3 positions.

CIM-10 was found to be identical to TST-11, a compound isolated by HPLC from *Terminalia stulhmanii*. The NMR data for this compound were obtained in pyridine-*d*5 and appeared to be better resolved. Thus this discussion will now centre on the structure elucidation of TST-11.

The NMR data were suggestive of an acetylrhamnoside. The ^{13}C domain showed two carbonyl signals, one at δ_C 183.2, identical to that for carboxylic acid as seen at C-29 in CIM-31, and another at δ_C 172.9 which appeared to correlate to signals from the sugar in HMBC and thus implied presence of the acetyl function. The position of the sugar was adduced to be C-23 by a comparative analysis of NMR data and observation of the aglycone (CIM-31). It was found that this carbon shows the glycosylation shift. Besides, the anomeric resonance δ_H 5.13 (δ_C 101.8) is hardly changed in its ^{13}C resonance position and this correlates well with similar rhamnosides in previous studies (Yamamoto *et al*, 1991).

The through-bond connectivity mapping by ^1H-^1H COSY is the cornerstone to the elucidation of glycosidic compounds. Thus, the anomeric proton at δ_H 5.13 (δ_C 101.8), H-1' (arbitrarily designated R in some cases), couples to the proton, H-2' at δ_H 4.36 (δ_C 72.7) which couples to H-3' (δ_H 4.60, δ_C 71.0) and so on. See the ^1H-^1H COSY spectrum **5.44**.

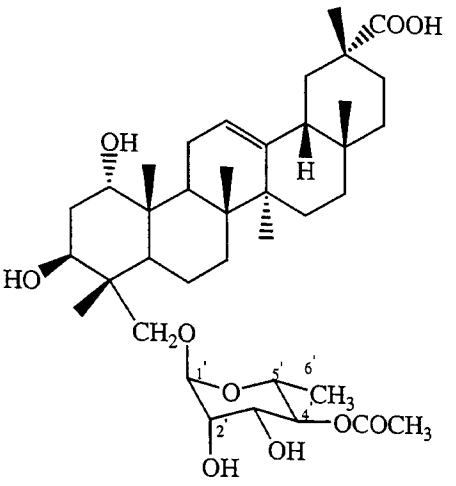


Fig.5.48. Structure of TST-11/ CIM-10

The triplet at δ_H 5.79 δ_C 76.2 J=9.7Hz is for the H-4' proton. It can be concluded that this is the position which is acetylated due to several reasons. The C-4' and attached proton both resonate at lower field than they normally would, while the 5-CH (δ_C 67.6 δ_H 4.27) is unusually shielded (Rippenger, 1981; Powell and York, 1990). Further, HMBC correlations to the carbonyl carbon at δ_C 171.1 are seen for the methyl at δ_H 1.89 (δ_C 21.5) as well as H-4'. The resonances for the acetylated sugar are seen as shown in Table **5.39.**

The structure of the sugar is confirmed by HMBC, which shows the anomeric proton at δ_H 5.13 coupling to C-5' (δ_C 67.6) and to C-3' (δ_C 71.0), and H-2' at δ_H 4.36 couples to C-4' (δ_C 76.2). Also, as already mentioned, the H-4' proton has a correlation to a carbonyl moeity at δ_C 171.1 to which the methyl proton at δ_H 1.89 also correlates. The

^{3}J coupling between the anomeric proton and the oxymethylene C-23 established, irrefutably, the point of glycosylation of the aglycone.

Table.5.37.^{13}C (100MHz) and ^{1}H (400MHz) NMR spectral data for CIM-10 aglycone only

Position	δ_C	δ_H
1	73.0	3.33 (brs)
2	35.1	1.31,*
3	67.6	3.96 (dd, J=4.5, 8)
4	41.2	-
5	41.7	*
6	19.4	1.30, 1.22
7	33.4	1.75, 1.52
8	37.5	-
9	39.7	2.32 (t, J=9.5)
10	43.7	-
11	24.6	1.68, *
12	124.7	5.07 (brs)
13	145.7	-
14	42.4	-
15	27.8	1.54, 0.84
16	28.4	1.88, 0.81
17	33.9	-
18	47.9	1.84,
19	42.3	2.04 (t, J=13.5), 1.30
20	43.9	-
21	30.8	1.58, 1.22
22	36.5	1.14, 1.10
23	71.2	3.32, 3.06 (ABq, J=9)
24	13.1	0.52 (s)
25	17.8	0.81 (s)
26	18.1	0.85 (s)
27	27.2	1.08 (s)
28	29.1	0.69 (s)
29	183.2	-
30	20.2	1.01 (s)

Data obtained in CD_3OD. * designates poorly resolved chemical shifts and/or coupling constants.

Table.5.38.^{13}C (100MHz) and ^{1}H (400MHz) NMR spectral data for TST-11 aglycone only

Position	δ_C	δ_H
1	72.0	3.90 (brs)
2	35.8	2.41, 2.35
3	66.7	4.96 (dd, J=5,7)
4	43.2	-
5	41.5	2.35
6	18.4	1.79, 1.71
7	32.8	1.89, 1.50
8	40.5	-
9	38.9	3.02 (q, J=6, 5.5)
10	43.3	-
11	24.2	2.35, 2.08
12	124.3	5.41 (brs)
13	145.0	-
14	41.8	-
15	27.2	1.89, 1.09
16	27.8	2.16, 0.97
17	33.4	-
18	47.2	2.20
19	42.1	2.53 (t, J=13.8), 1.64
20	43.4	-
21	31.0	2.50, 1.73
22	36.9	1.62, 1.39
23	71.4	3.94, 3.83 (Abq, J=9)
24	13.5	1.09 (s)
25	17.6	1.16 (s)
26	17.9	1.23 (s)
27	26.8	1.37 (s)
28	29.0	0.99 (s)
29	181.8	-
30	20.5	1.47 (s)

TST-11 obtained in C_5D_5N.

Table 5.39.^{13}C (100MHz) and ^{1}H (400MHz) NMR spectral signals for 4-acetylrhamnose (R') for CIM-10 and TST-11 respectively.

	CIM-10		TST-11	
Position	δ_H	δ_C	δ_H	δ_C
1'	4.50 (s),eq	101.9	5.13 (s),eq	101.8
2'	3.71 (s),eq	73.0	4.36 (m),eq	72.7
3'	3.73 (m),eq	72.3	4.60 (dd,3.3;6.3),eq	71.0
4'	4.74 (t, J=9.5),ax	76.1	5.79 (t, J=9.7),ax	76.2
5'	3.58 (m),ax	68.0	4.27 (m),ax	67.6
6'	0.95 (d, J=6)	18.0	1.46 (d, J=4.4)	18.4
4'-OCOC$\underline{H}_3$	1.88 (s)	21.6	1.89 (s)	21.5
4'-O$\underline{C}$OCH$_3$	-	172.9	-	171.1

CIM-10 spectra obtained CD_3OD, and TST-11 obtained in C_5D_5N.

Splitting pattern and coupling constants (**J**, in Hz) are shown in parentheses.

NOESY showed the correlations which were seen in the aglycone – H_3-27/H-9, H-19/H_3-27, H-18/ H_3-30 and H_3-28/H-18. In addition, R-1 (or H-1') correlated to R-2, R-6 to R-4, and R-5 with-R_3-6 for the sugar, and R-1 correlated to H-24/H-23. See Spectrum **5.45.** and Fig.**5.49.**

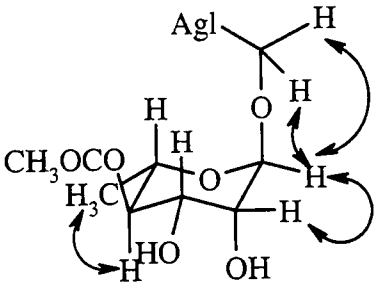
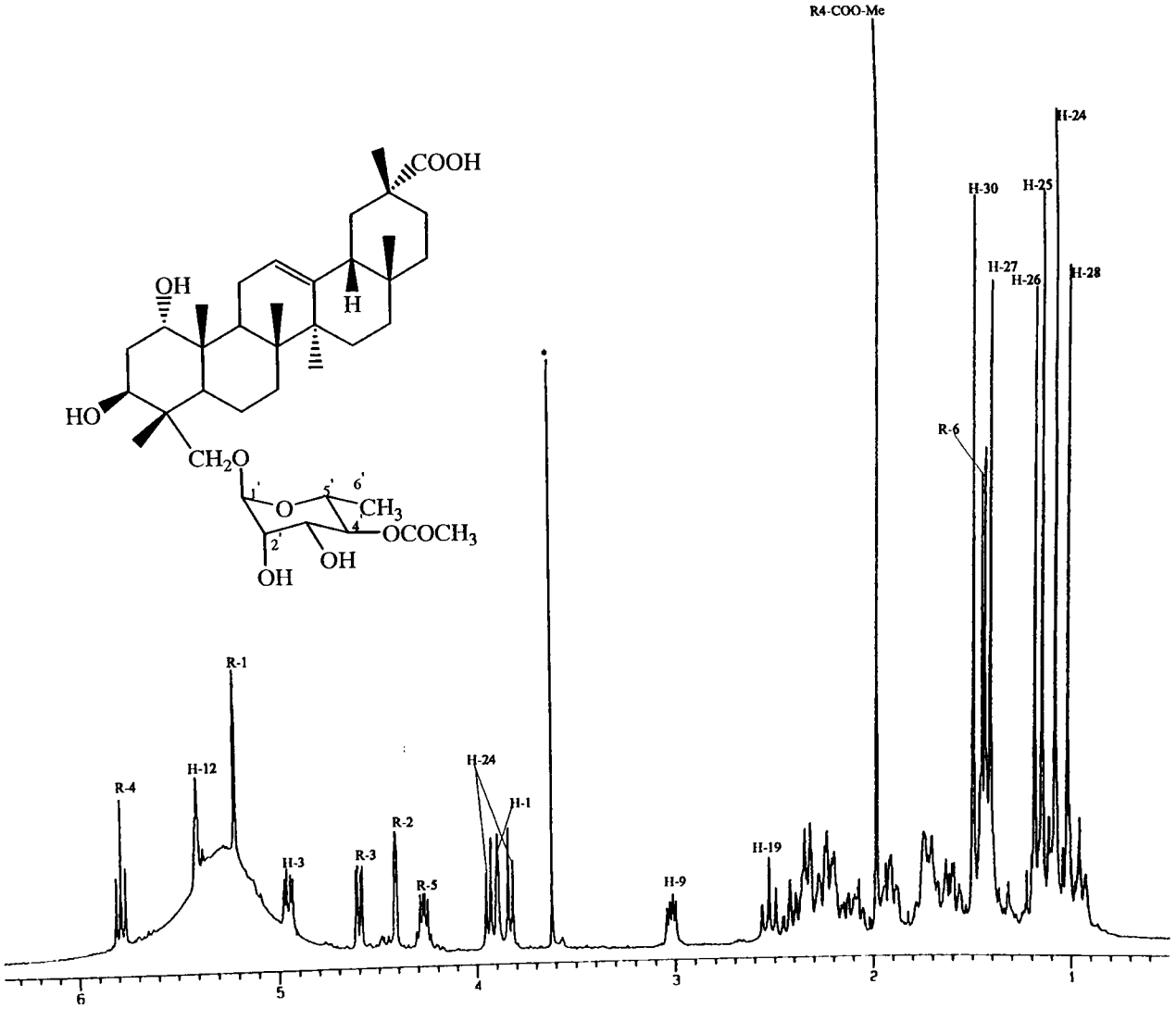


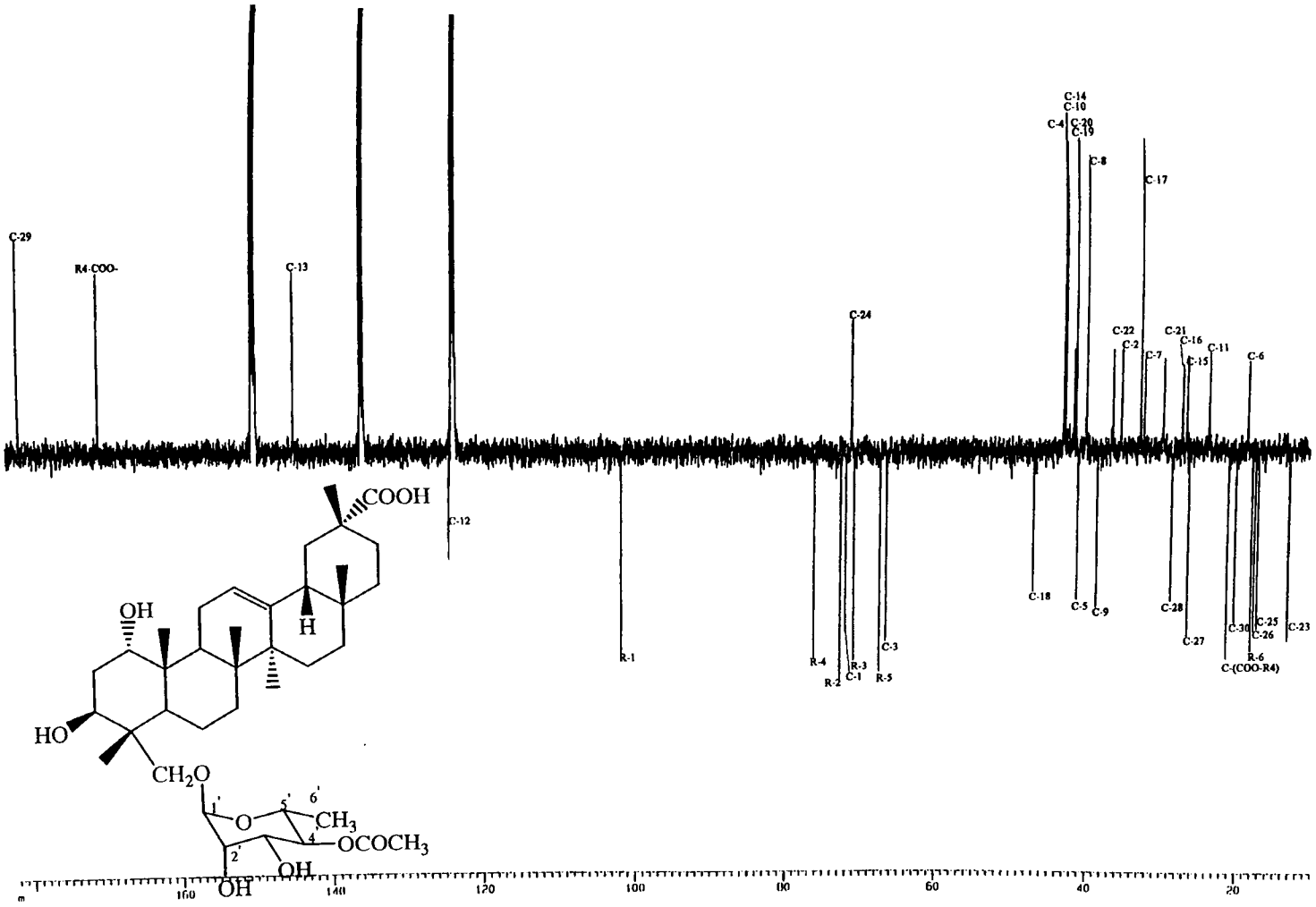
Fig 5.49. Significant NOESY correlations seen in the sugar of TST-11.

TST-11 was identified as 1α, 3β-hydroxyimberbic acid 23-O-α-L-4-acetylrhamnopyranoside (or .1α, 3β-trihydroxy-olean-12-en-29-oic acid 23-O-α-L-4-

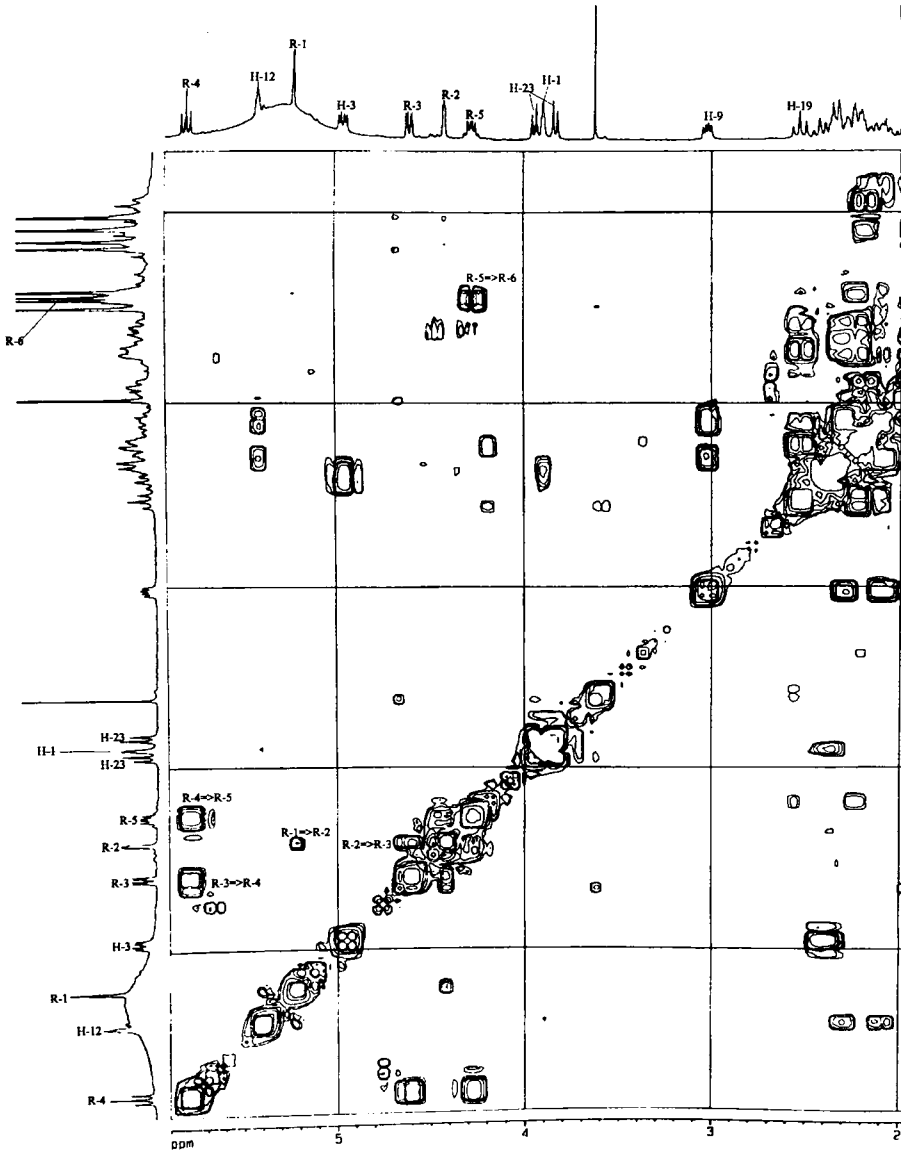
acetylrhamnopyranoside). A search in the literature showed that this compound has been previously isolated from the leaves and fruit of *C. padoides* (Rogers, 1989; Panzini *et al*, 1993). This is the first report of the isolation of this compound from the leaves of *C. imberbe* and the stem bark of *T. stuhlmanii*. It is also the first time that the aglycone is reported in the genus *Terminalia* which has in the past yielded ursane based triterpenes or 28-carboxylated oleananes from the Asian species *T. arjuna* (Panzini *et al* 1993; Kumar and Prabhakar, 1987).



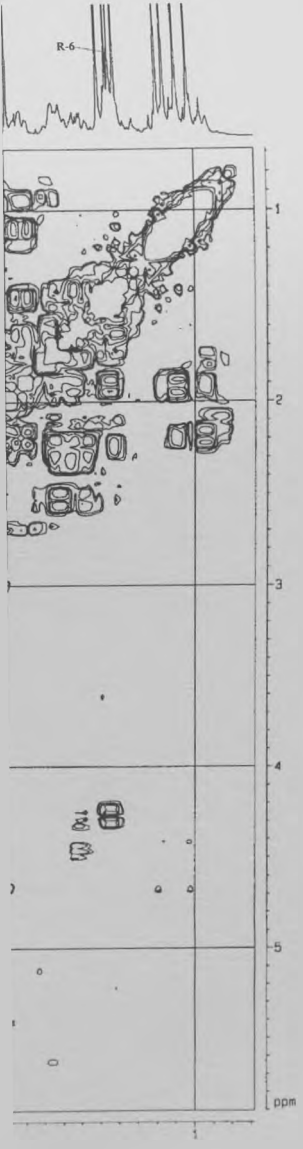
Spectrum 5. 42: ^{1}H NMR (400MHz, C_5D_5N) of TST-11. *residual CD_3OD

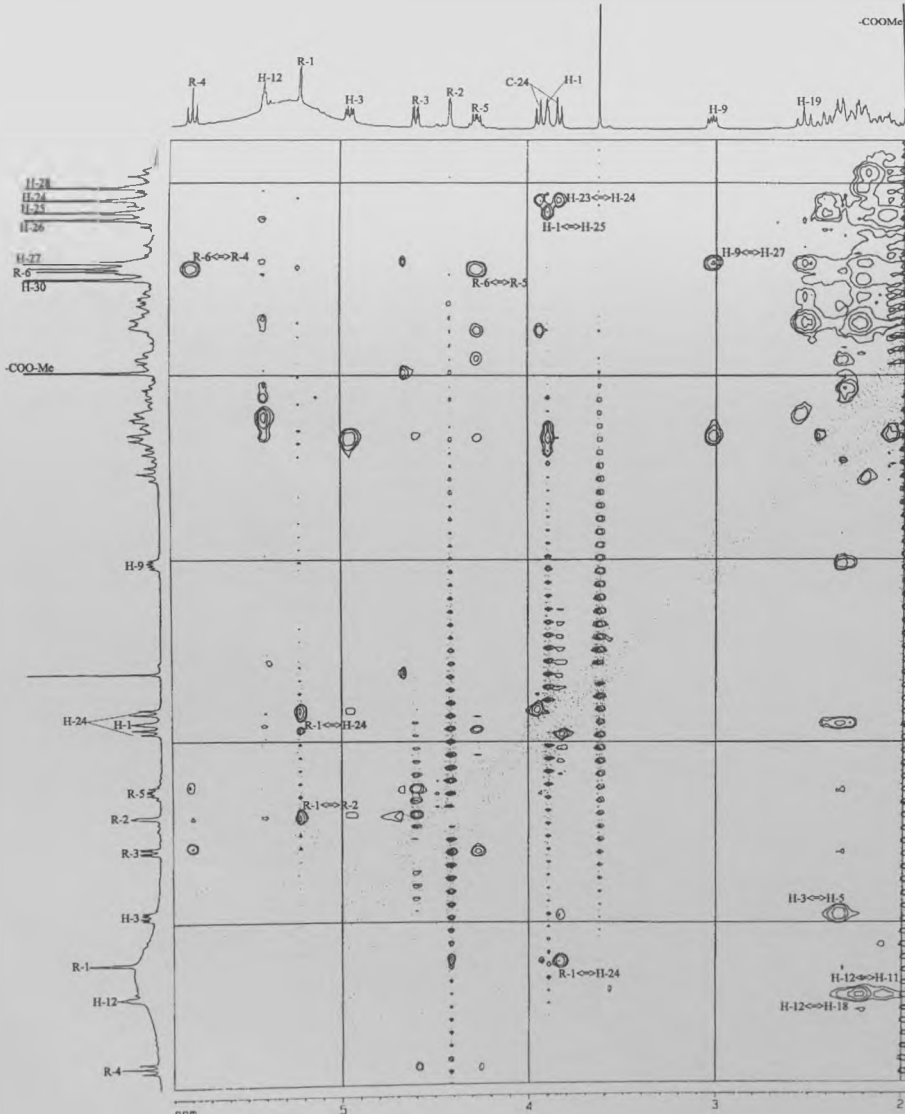


Spectrum 5.43: ^{13}C (J-mod) NMR (100MHz, C_5D_5N) of TST-11.

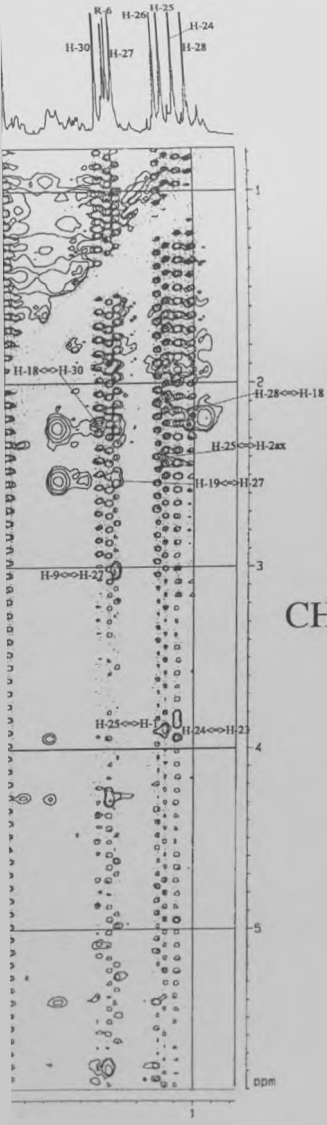


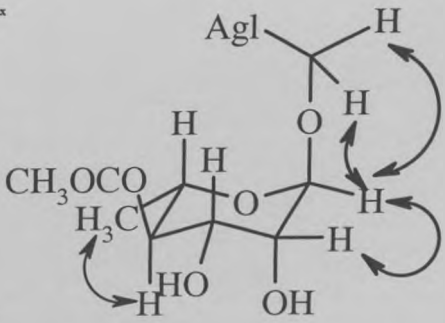
Spectrum 5.44: ^{1}H-^{1}H COSY (400MHz, C_5D_5N) of TST-11


R-6
1
2
3
4
5
ppm
1



Spectrum 5.45: ^{1}H-^{1}H NOESY (400MHz, C_5D_5N) of TST-11.


H-30
H-27
H-26
H-25
H-24
H-28
H-18⇔H-30
H-28⇔H-18
H-25⇔H-2ax
H-19⇔H-27
H-9⇔H-2
H-25⇔H-
H-24⇔H-2
1
2
3
4
5
ppm
1


Agl
H
O
H
H
H
CH3OCO
O
H
H3C
H
HO
H
OH

Characterisation of CIM-11

CIM-11 was obtained as a yellow amorphous powder (25.3mg), t_R = 25.5min, mp. 190°C. IR showed the presence of hydroxy (3450 cm^{-1}), carbonyl ester (1732 cm^{-1}), methyls (1455 cm^{-1}), as well as some peaks also seen in CIM-31. The NMR indicated the presence of resonances similar to CIM-31 and was almost identical to CIM-10. There was a significant deviation however with the presence of two methyl signals at δ_H 2.05 which are part of the acetoxy groups. They are better resolved in the carbon domain at δ_C 21.2 δ_C 21.3 and their corresponding carbonyl resonances at *ca.* δ_C 172. Thus the difference from CIM-10 is that this glycoside is diacetylated.

The LRFABMS showed a pseudomolecular ion $[M+H]^+$ at *m/z* 719 which corresponds to the molecular formula $C_{40}H_{62}O_{11}$. The peak was very small (0.4%) but nevertheless, present. The region between *m/z* 600 and 800 had very small fragments which were difficult to pinpoint. The region below *m/z* 500 was more diagnostic and similar to that seen for CIM-31. Thus fragments for the aglycone were seen at *m/z* 472 (1%) $[C_{30}H_{48}O_4]^+$ and 453 (6%), due to loss of water. The other fragments observed in CIM-31 are likely to appear as well but are not seen because of limitations on the part of the instrument. In addition however, the fragment for the diacetylrhamnose is seen as well at *m/z* 246 (7%) which corresponds to $C_{10}H_{14}O_7$.

Thus CIM-11 was proposed to be a diacetoxy-rhamnoside of hydroxyimberbic acid. ^1H-^1H COSY was used as in the elucidation of TST-11 above to work out the through-bond coupling in the sugar. See Spectrum **5.48**. In this case, both R-3 and R-4 are deshielded to *ca.* δ_H 5ppm implying that they are probably both attached to acetyl

substituents. Further evidence of this comes from the HMBC correlation of the methyls of the acetoxy functions and these protons to common carbonyl signals at *ca* δ_C 172ppm. See Spectrum **5.49**.

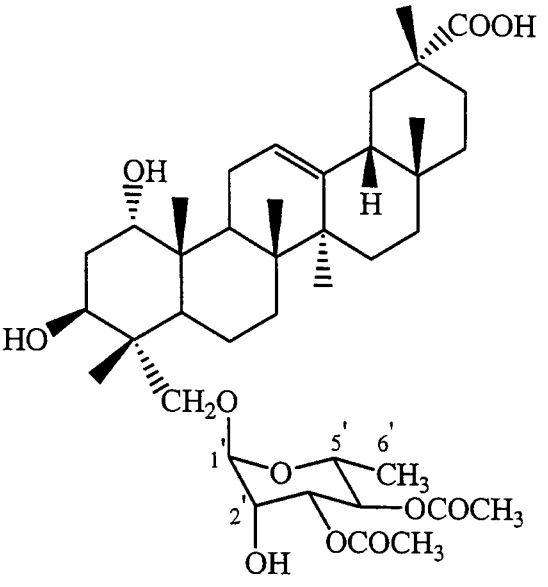


Fig.5.50. Structure of CIM-11 isolated from *C. imberbe*.

Table.5.40.^{13}C (125MHz) and ^{1}H (500MHz) NMR spectral data for CIM-11 rhamnose

Position	δ_H	δ_C
1'	4.63 (s), eq	102.0
2'	3.96 (brs), eq	70.4
3'	5.07 (dd, J=7,3), ax	74.0
4'	5.02 (t, J= 15.5), ax	73.3
5'	3.78 (m), ax	68.1
6'	1.19 (d, J= 6)	18.1
3'-CO<u>C</u>H$_3$	2.05 (s)	21.2
4'-CO<u>C</u>H$_3$	2.05 (s)	21.3
3'-<u>C</u>OCH$_3$	-	172.2
4'-<u>C</u>OCH$_3$	-	172.2

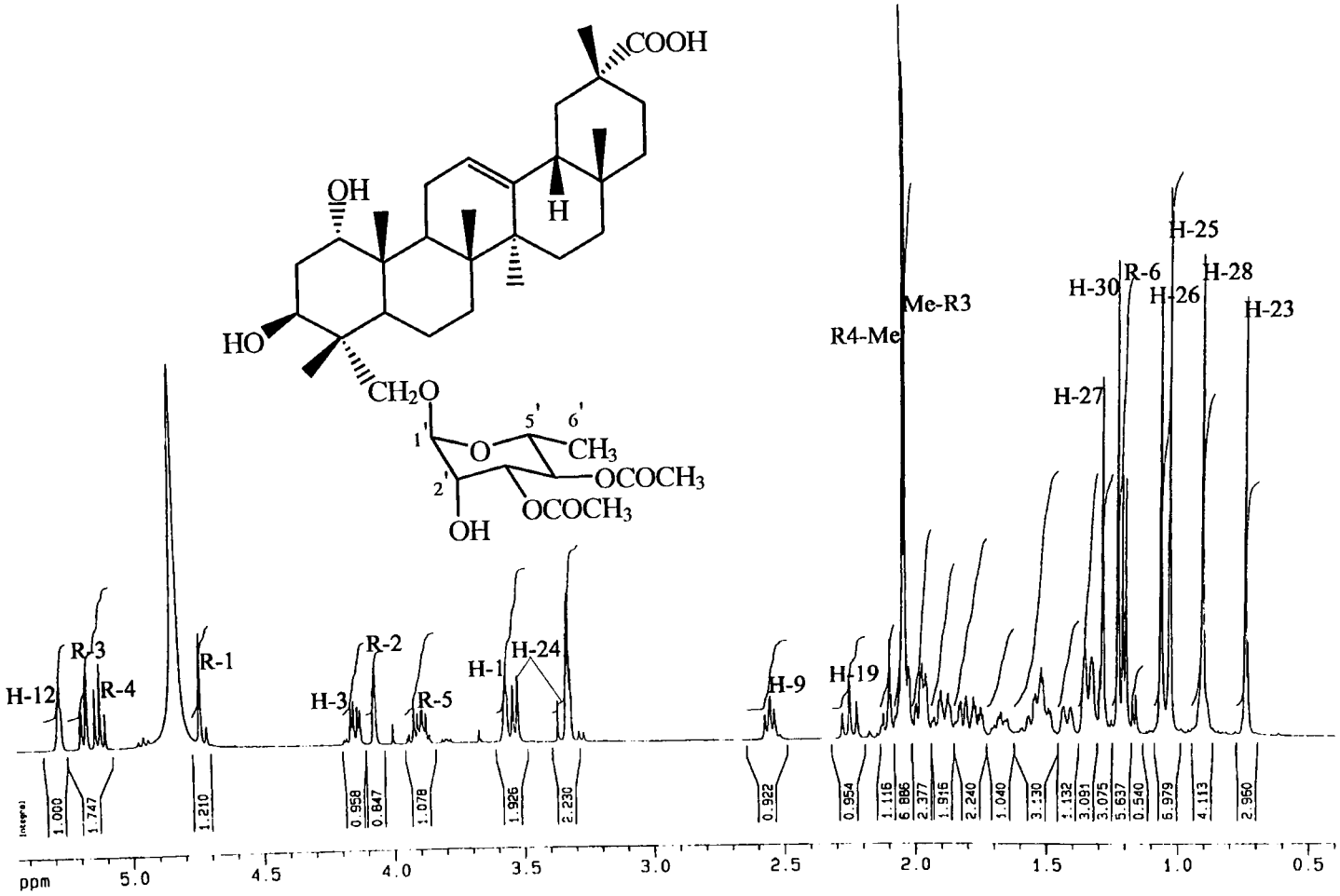
All data obtained in C_5D_5N

Table.5.41.^{13}C (125MHz) and ^{1}H (500MHz) NMR spectral data for CIM-11 aglycone.

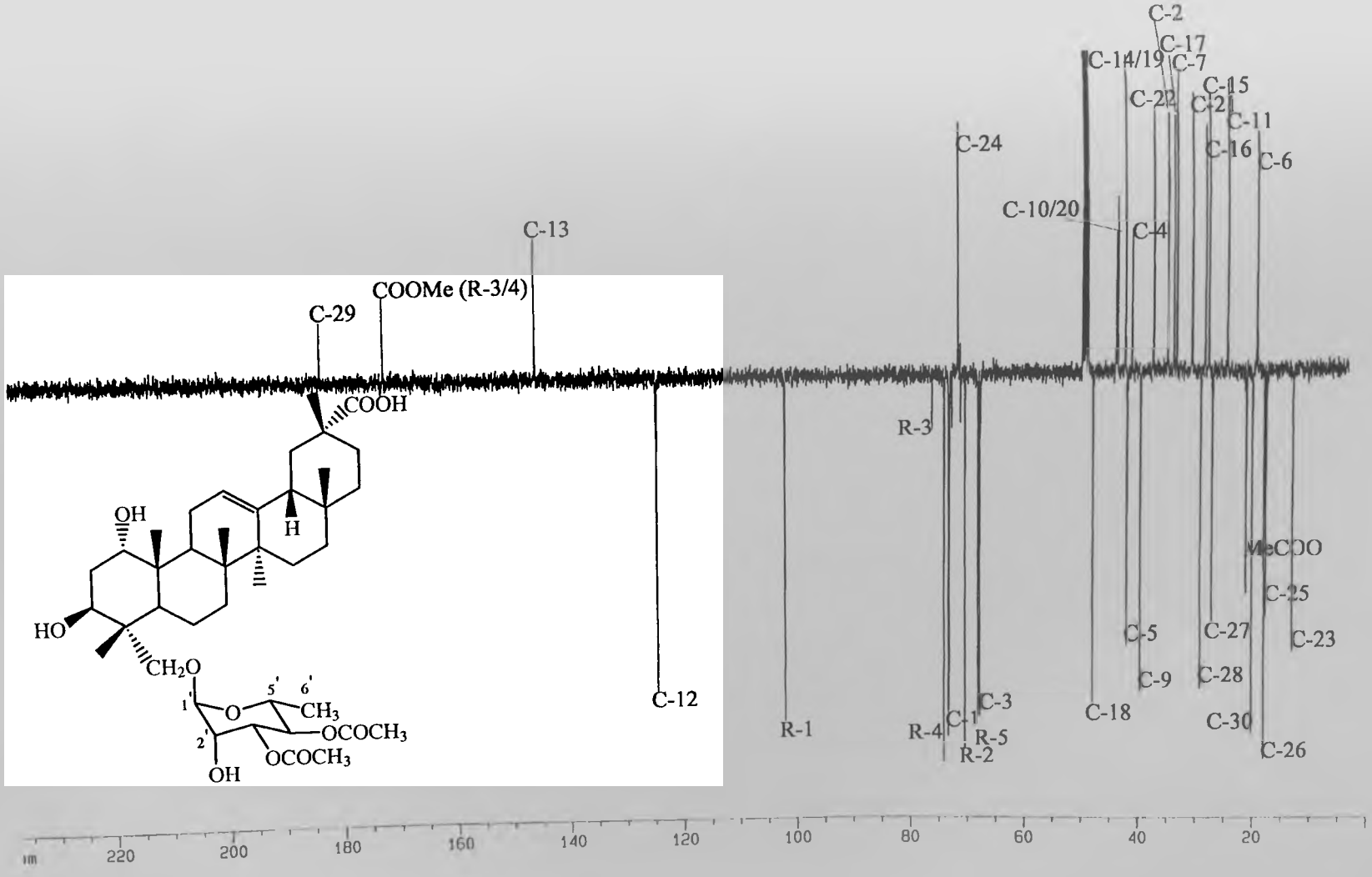
Position	δ_C	δ_H
1	73.1	3.46 (brs)
2	35.0	1.50, 1.85
3	67.8	4.03 (dd, J=8, 4.5)
4	41.3	-
5	42.0	1.71
6	19.5	1.41
7	33.4	1.24, 1.26
8	41.2	-
9	39.6	2.45 (t, J=9.5)
10	43.7	-
11	24.6	1.85
12	124.6	5.17 (brs)
13	145.7	-
14	42.4	-
15	27.8	0.95, 1.70
16	28.5	0.92, 1.99
17	33.9	-
18	48.0	1.94
19	42.4	1.21, 2.14 (t, J=13.5)
20	43.9	-
21	30.8	1.24, 1.72
22	37.5	1.20, 1.43
23	71.7	3.33, 3.53 (Abq J= 9.5)
24	13.2	0.73 (s)
25	17.7	1.02 (s)
26	18.1	1.05 (s)
27	27.1	1.27 (s)
28	29.1	0.89 (s)
29	183.2	-
30	20.2	1.22 (s)

Data obtained in C_5D_5N

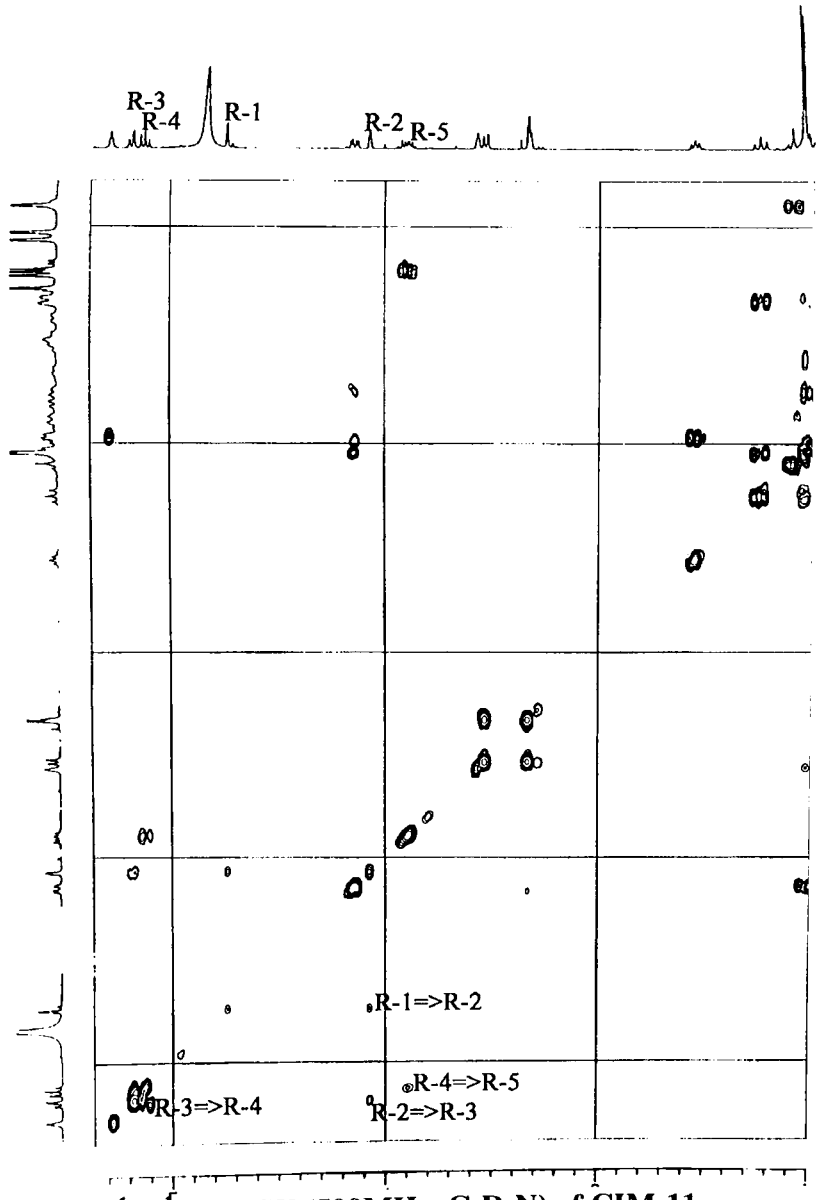
The aglycone is glycosylated at position 23 and this indicated by the changes in ring A as well as other reasons disclosed earlier. In addition there is ^{3}J coupling between the anomeric proton at δ_H 4.74 and the methylene carbon of position 23. Thus CIM-11 was characterised as 1α,3β,23-trihydroxy-olean-12-en-29-oic acid-23-O-α-[3,4-diacetyl]rhamnopyranoside. This is the first report of this compound.



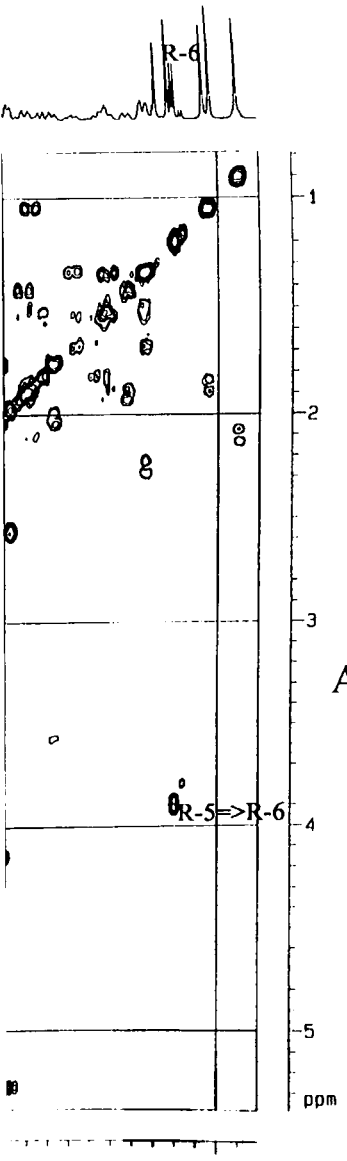
Spectrum 5.46: ^{1}H NMR (500MHz, C_5D_5N) of CIM-11

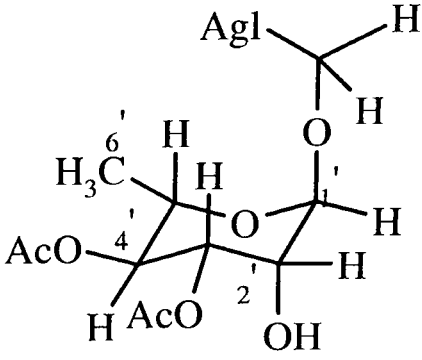


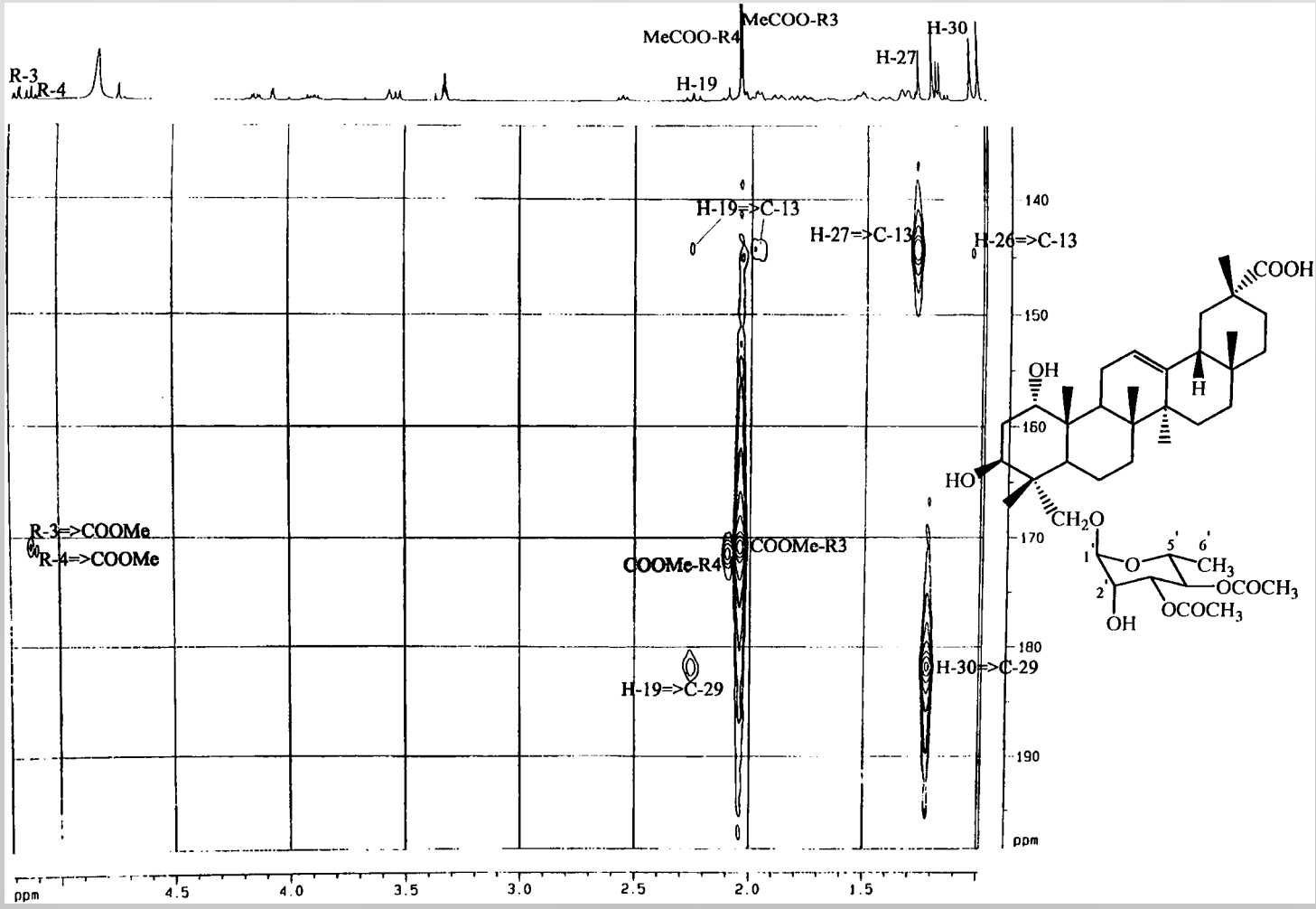
Spectrum 5.47: ^{13}C NMR (125MHz, C_5D_5N) of CIM-11



Spectrum 5.48: ^{1}H-^{1}H COSY (500MHz, C_5D_5N) of CIM-11


R-6
1
2
3
4
5
ppm
R-5=>R-6


Agl
H
H
O
6'
H
H3C
H
1'
O
H
4'
AcO
H
2'
H
AcO
OH



Spectrum 5.49: HMBC correlations (500MHz, C_5D_5N) of CIM-11

Characterisation of CIM-9

CIM-9 was isolated as a yellow powder (47.4mg) by HPLC, t_R = 20min, mp. 178°C. Its IR spectrum was similar to the other compounds discussed earlier in this section showing hydroxyl (*ca.* 3450 cm^{-1}), methyl (*ca* 1455 cm^{-1}), gem-dimethyl (*ca* 1382 cm^{-1}) carbonyl ester (*ca* 1732 cm^{-1}), 1100 cm^{-1}, 964 cm^{-1}, 830 cm^{-1}, 670 cm^{-1} and 550 cm^{-1}.

LRFABMS was diagnostic of an olean-12-ene-based glycoside like to those discussed above. A molecular ion, M^+ peak at *m/z* 864 was found which corresponds to the molecular formula of $C_{46}H_{72}O_{15}$. Small but significant peaks occurred at *m/z* 846 $(M-H_2O]^+$ (0.6%) and 829 $[M-2H_2O]^+$ (0.6%). Further, the most prominent peaks of the sugar and aglycone appear at *m/z* 701 (1.8%) and 683 (2%). It is suggested that these arise from a combination of the dehydrated aglycone (*m/z* = 453) and a diacetylated rhamnose (*m/z* = 246), and the fragment of the aglycone plus sodium (Na^+) (*m/z* = 472+23) and a non-acetylated rhamnose (*m/z* =187). The peak at *m/z* 719 (0.8%) is interesting because it was obtained as $[M+H]^+$ for CIM-11 i.e. the pseudomolecular ion for hydroxy-imberbic acid diacetylrhamnoside discussed earlier.

The NMR spectra are similar to those of the other compounds discussed above with minor differences. H-12 appears as a broad singlet at δ_H 5.14, with H-1, at δ_H 3.52 (brs) and attached to the carbon at δ_C70.4. H-3 attached to C-3 at δ_C 67.7 appears as an double doublet at δ_H 4.14 (J=4.5, 8Hz) while H-19 is, as usual, split into a well-resolved triplet at δ_H 2.25 and an overlapping and hence, indistinguishable multiplet at δ_H 1.78.

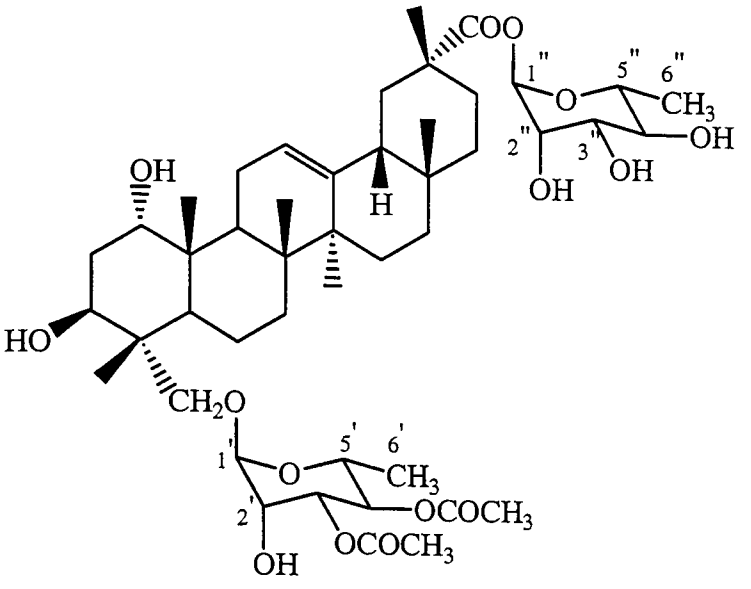


Fig.5.51. CIM-9- the bidesmosidic compound isolated from *C. imberbe*.

An inspection of the sugar region is more informative. It is crowded with what appear to be duplicated multiplets with two broad singlets at δ_H 4.74 and 5.94. For ease of discussion and labelling, the rhamnose units have been arbitrarily called R' and R''.

With the aid of ^1H-^1H COSY, the spin systems of the two sugars were separated as illustrated in Spectrum **5.51**. Using the ^1H-^1H COSY, the proton at δ_H 4.74 was designated (above) as the anomeric proton of rhamnose (R-1') from HMQC and ^{13}C spectra. ^1H-^1H COSY showed that it is linked to R-2' (δ_H 4.07,s/ δ_C 70.7) which is connected to R-3' (5.13, dd, J=8 / 73.1) which in turn is linked to R-4' (5.12, t, J= 8/ 73.0 and then to R-5' (3.88,m / 67.7) and finally the system terminates at R-6' (1.27,s/ 18.6). The acetyl groups whose methyls resonate at δ_H 2.05 (*ca.* δ_C 21) are probably attached to

the R-3'/R-4' carbons because these protons are substantially deshielded. This is confirmed in the HMBC correlations to the acetoxy carbonyl at *ca.* δ_C 172. See Spectrum **5.52**.

Table 5.42.^{13}C (125MHz) and ^{1}H (500MHz) NMR spectral data of CIM-h9

Position	δ_C	δ_H
1	73.0	3.80 (s)
2	35.0	1.56, 1.39
3	67.7	4.14 (dd, J=4.5, 8)
4	41.3	-
5	42.0	1.67
6	19.4	1.56,*
7	33.4	1.56, 1.35
8	41.3	-
9	39.6	2.55 (t, J=9)
10	43.7	-
11	24.6	1.98, 1.97
12	125.0	5.29 brs
13	145.5	-
14	42.4	-
15	27.8	1.76, 1.78
16	28.4	2.08, 2.11
17	34.0	-
18	47.9	2.05
19	42.2	2.25 (t, J=14), 1.39 (*)
20	43.8	-
21	30.5	1.90, 1.47
22	37.3	1.56, 1.39
23	71.6	3.37, 3.30
24	13.2	0.74 (s)
25	17.8	1.03 (s)
26	18.3	1.16 (s)
27	27.1	1.28 (s)
28	29.1	1.03 (s)
29	178.6	-
30	20.1	2.05

All data obtained in CD_3OD. * poor resolution

Table 5.42.^{13}C and ^{1}H NMR spectral data for the sugars of CIM-h9

Position	δ_C	δ_H
1'	102.0	4.74 (s), eq
2'	70.7	4.07 (s), eq
3'	73.1	5.18 (dd, J=10), ax
4'	73.0	5.12 (t, J=10), ax
5'	68.1	3.84 (brm), ax
6'	18.2	1.20 (d, J=5.5),eq
3'-OCO$\underline{C}H_3$	21.3	2.05 (s)
3'-O$\underline{C}OCH_3$	172.3	-
4'-OCO$\underline{C}H_3$	21.4	2.05
4'-O$\underline{C}OCH_3$	172.4	-
1"	95.7	5.94 (s),eq
2"	72.6	3.68 (brs),eq
3"	73.8	3.57 (*),ax
4"	74.0	3.46 (t, J=9.5),ax
5"	73.8	3.67 (m),ax
6"	18.6	1.27 (d,),eq

Data obtained in CD_3OD. * poor resolution.

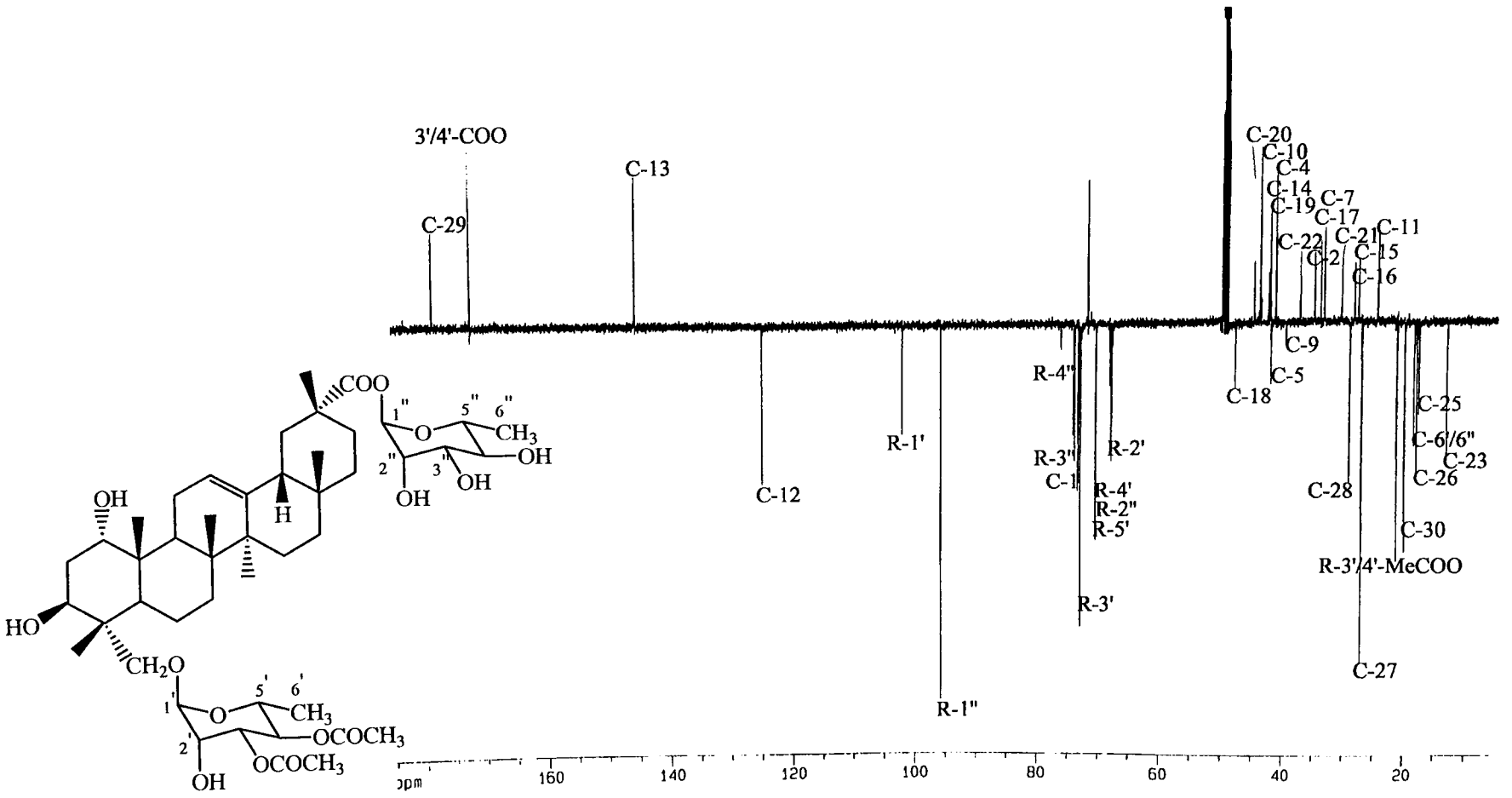
The anomeric proton at δ_H 5.94 (designated R-1") is very highly deshielded and shown by HMQC to be attached to a carbon which is unusually shielded at δ_C 95.7. By using the same scheme as described above, ^{1}H-^{1}H COSY is used to construct the spin system for this sugar. The resonances are similar to those obtained for 'normal' rhamnose (i.e. non-acetylated) (Kizu and Tomimori, 1982; Yamamoto *et al*, 1991; Seidel, 1999).

HMBC data provided information about the point of glycosylation. It is shows that R-1' at δ_H 4.74 couples to the oxymethine at δ_C 71.6, which is the signal for C-23 on the

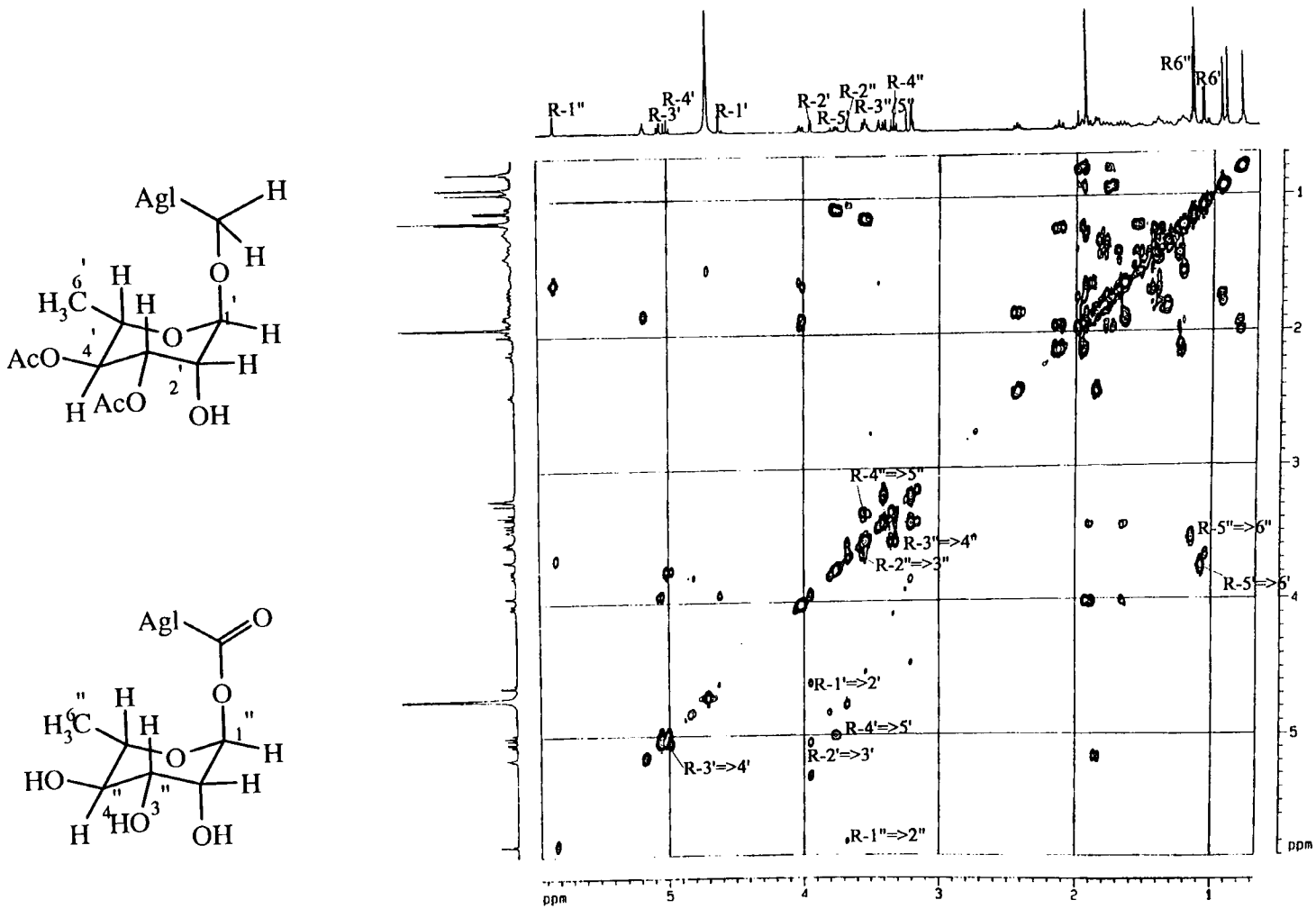
aglycone and has been shown to be the area of preferred glycosylation in the other compounds discussed earlier. The second sugar could be attached to 1 or 3 positions which are hydroxylated, or to the carboxylic group at 29. Panzini *et al* (1991) have previously isolated bidesmosides of hydroxyimberbic acid with O-glycosyl units at C-3 in addition to C-23. The HMBC spectrum in this case, however, showed R-1" with 3-bond correlations to the quaternary carbon at δ_C 178.6 which belongs to the carboxylic acid. It can therefore be concluded that R-1" is attached directly to C-29 to form an ester which is implied by the appearance of a peak in the IR spectrum at *ca.* 1730cm^{-1}. Houghton and Lian (1985) have observed that β-linkage through an ester group such as seen here will give an IR peak at about 1726 cm^{-1} as opposed to the free acid which shows up at *ca.* 1685 cm^{-1}.

The additional glycosylation of the aglycone at C-29 resulted in two fundamental changes to the resonances in this area. The anomeric proton is deshielded (δ_H 5.94) while its carbon becomes shielded (δ_C 95.7) significantly, while C-29 moves upfield (δ_C 178.6) as well by about 3-5ppm in comparison to the free acid. In general other resonances of ring E while not shifting significantly in the carbon domain, show an upfield shift in the proton domain by between 0.2 and 0.5ppm.

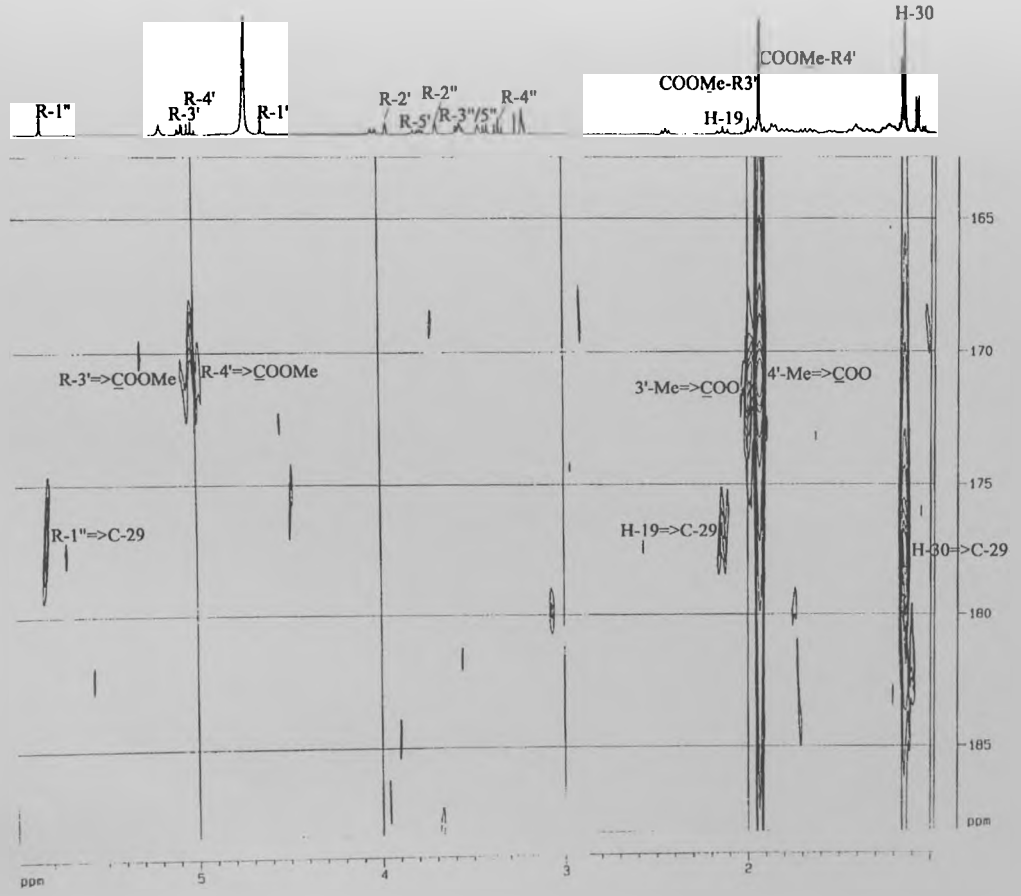
CIM-9 was identified as 1α, 3β, 23-trihydroxy-olean-12-en-29-oate-23-*O*-α-[3,4-diacetylrhamnopyranosyl]-29-*O*-α-rhamnopyranoside. It was given the trivial name, hwesanoside. This is the first time that this compound has been isolated.

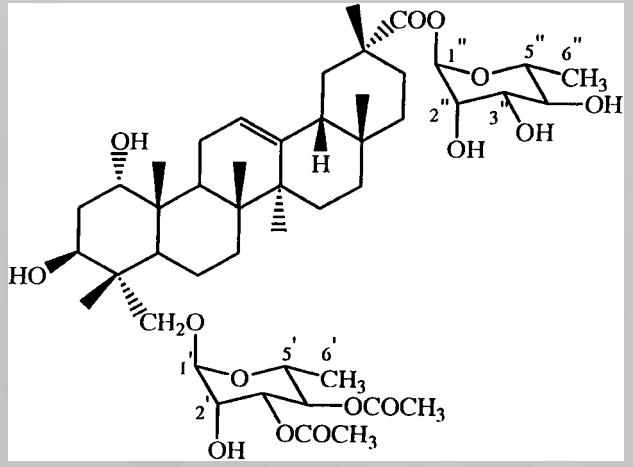


Spectrum 5.50: ^{13}C (PENDANT) NMR (125MHz, CD_3OD) of CIM-9



Spectrum 5.51: ^{1}H-^{1}H COSY (500MHz, CD_3OD) used to elucidate the sugar moieties in CIM-9





Spectrum 5.52: HMBC correlations of the glycosylation resonances of CIM-9

Characterisation of TST-8

TST-8 was isolated as a peach amorphous solid (7.6mg) by HPLC, t_R =16min, mp. 196°C. The FABMS showed pseudomolecular ions of *m/z* 823.2 (6%) $[M+H]^+$ and 846.1 (10%) $[M+H+Na]^+$. These correspond to the molecular formula of $C_{44}H_{70}O_{14}$. The region below *m/z* 500 is similar to that of the other olean-12-ene compounds isolated in this study with fragments at *m/z* 472.2 (60%) for $[C_{30}H_{48}O_4]^+$, 453.2 (100%) for $[C_{30}H_{46}O_3]^+$ and those from the retro-Diels Alder cleavage at *m/z* 248 (34%), 242 (36%) and 236 (50%). This is evidence to suggest that the aglycone is the same as that of the other triterpenes discussed earlier.

NMR shows that the basic structure is of the 1α, 3β-hydroxyimberbic acid aglycone (CIM-31). The 1D-NMR spectra are almost superimposable on that of CIM-10/TST-11, the mono-acetylated rhamnoside. However, they show subtle changes in the resonances of ring A and ring E with shielding of C-1 (-1ppm), C-3 (-3.9ppm) and C-29 (-3.9ppm) and deshielding of C-4 (+3ppm) and C-20 (+1ppm), relative to the aglycone. This implies that there are some modifications around these rings.

The presence of two anomeric protons suggests that this is an olean-12-ene triterpene with two sugar units. One of the sugar units is acetylated in position 4 causing both the proton and carbon in this position to resonate at lowfield (δ_H 5.81 and δ_C 76.1). The two units were disentangled from each other by unravelling the spin systems employing ^{1}H-^{1}H COSY as shown with CIM-11 earlier. The positions of glycosylation were adduced

from the chemical shift positions of the anomeric atoms (Shigenaga *et al*, 1985) and from HMBC correlations.

Table 5.44. ^{13}C and ^{1}H NMR (100 / 400MHz) spectral data for the aglycone of TST-d8

Position	δ_C	δ_H
1	71.7	3.84 (brs)
2	36.5	1.51 (ax), 1.28 (eq)
3	66.7	4.96 (dd, J=5.7)
4	43.0 (q)	-
5	41.5	1.49
6	18.4	1.68
7	33.2	1.88, 1.41
8	40.4 (q)	-
9	38.8	3.01 (dd, J=6.5/5)
10	41.5 (q)	-
11	24.2	2.32, 2.06
12	123	5.35
13	144.7 (q)	-
14	43.0 (q)	-
15	27.2	1.52, 190
16	29.0	2.05, 1.51
17	33.2	-
18	46.8	2.10
19	41.5	2.44 (ax, J=13.5), 2.33 (eq)
20	43.8 (q)	-
21	30.8	*
22	36.9	*
23	71.4	3.95, 3.84 (Abq, J=9)
24	13.5	1.09 (s)
25	17.6	1.14 (s)
26	17.9	1.16 (s)
27	26.7	1.39 (s)
28	28.8	0.98 (s)
29	177.5(q)	-
30	19.9	1.31 (s)

Obtained ion C_5D_5N. *poor resolution

Table 5.45.^{13}C and ^{1}H NMR (100/400MHz) spectral data for the sugars of TST-d8

Position	δC	δH
1'	101.8	5.22 (brs), eq
2'	73.2	4.42 (brm), eq
3'	71.0	4.62 (dd, J= 3.2, 6.4), ax
4'	76.1	5.81 (t, J=9.7), ax
5'	67.6	4.27 (brm), ax
6'	18.4	1.45 (d, J=6.2)
4'-OCOCH3	21.6	2.06 (s)
4'-OCOCH3	171.2	-
1"	96.0	6.79 (brs), eq
2"	71.7	4.54 (m), eq
3"	73.0	4.52 (dd, J=3.3, 5.6), ax
4"	72.7	4.33 (t, *)
5"	74.0	4.31 (brm)
6"	19.1	1.62 (d, J=5.8)

Obtained in C_5D_5N. *poor resolution

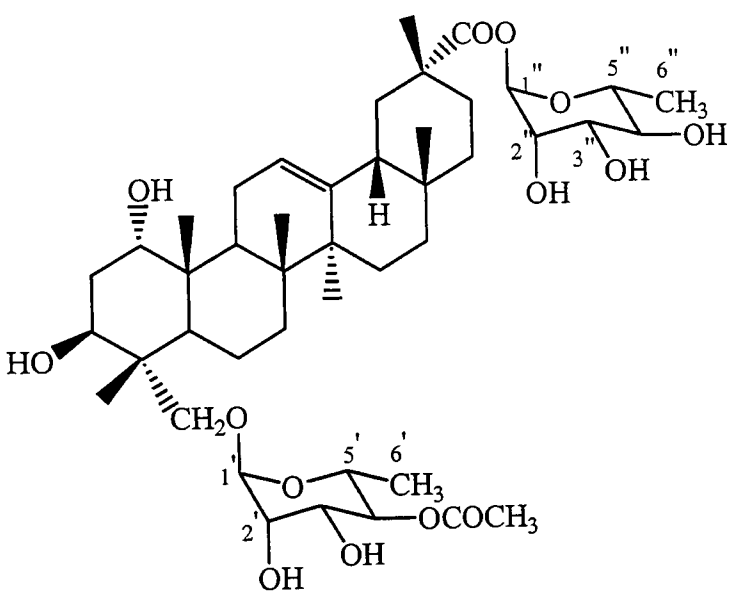
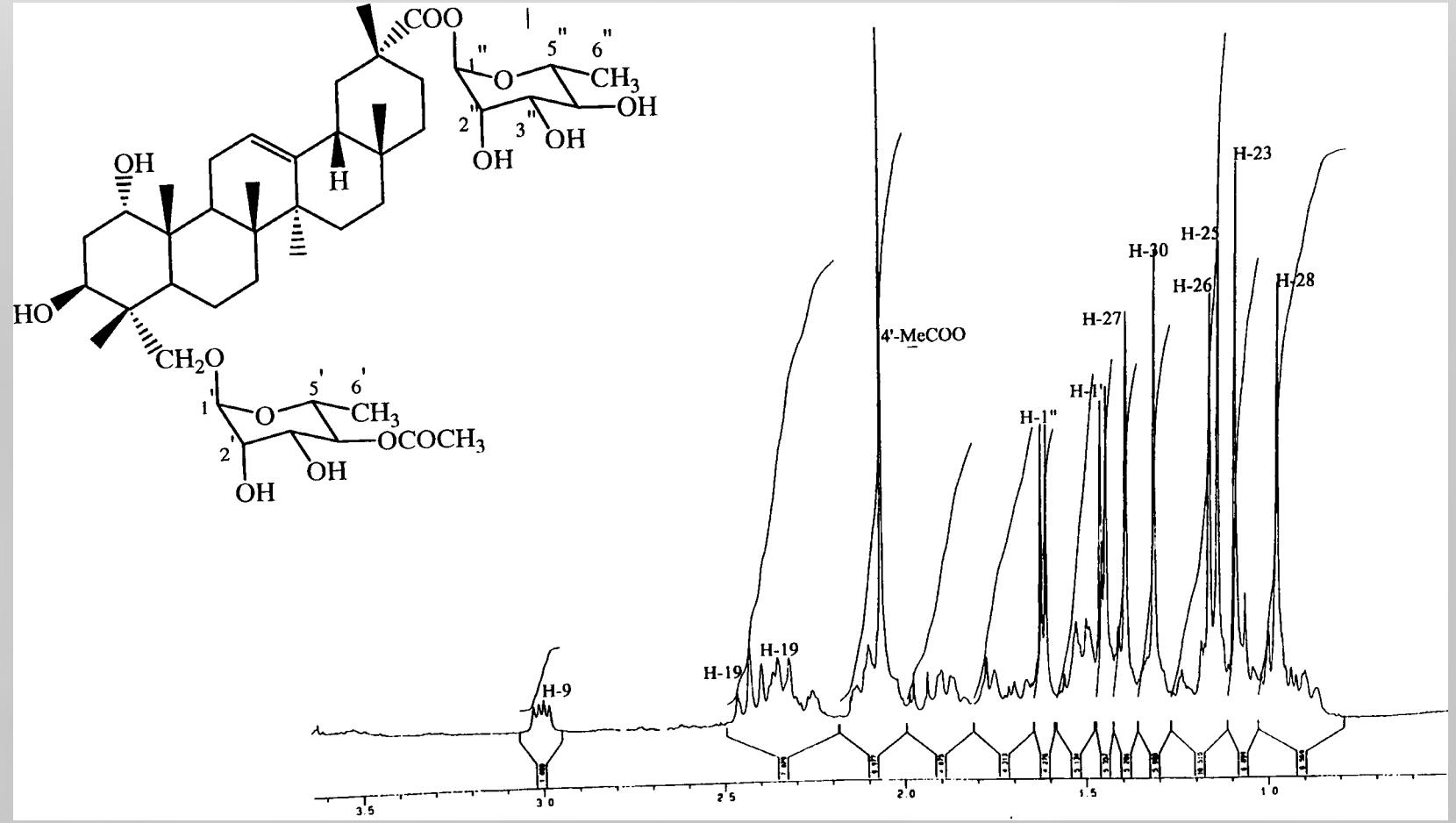


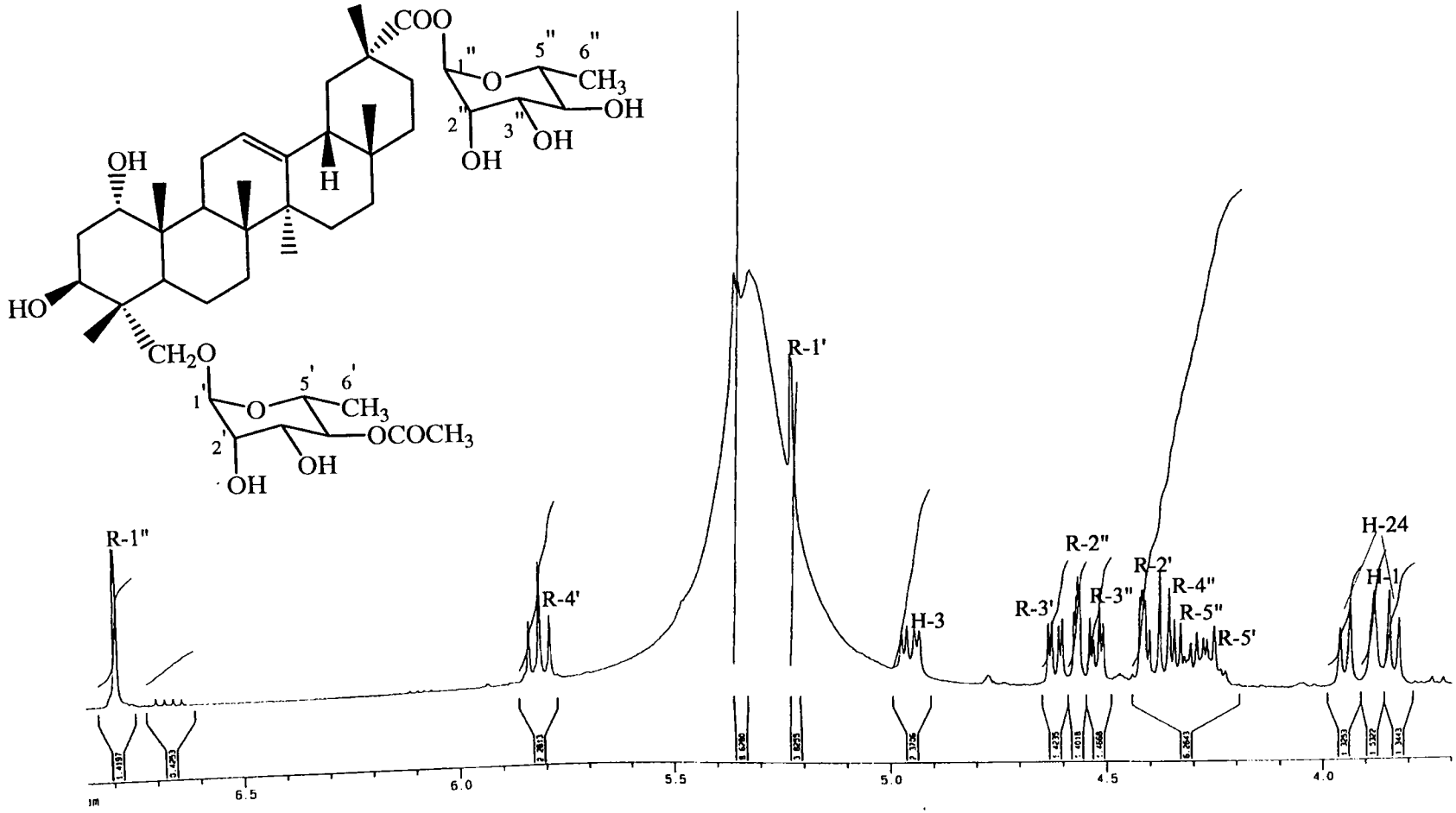
Fig.5.52. TST-8- the bidesmosidic compound isolated from the stem bark of *T. stulhmanii*.

Thus the acetylated rhamnose is attached to C-23 because its anomeric is at δ_H 5.22 (δ_C101.8) while the non-acetylated unit shows a shielding in the ^{13}C domain accompanied by a movement to lower field of the anomeric methine, implying that is attached to the carboxylate unit (Ripperger, 1981). These sites of glycosylation are supported by the ^3J correlations in HMBC between the anomeric proton R-1" (δ_H 6.79) and C-29 (δ_C 177.5), while R-1' (δ_H 5.22) shows correlation with C-23 (δ_C 71.4). The complete elucidation process mimicks that employed for CIM-9 and, for brevity, is not repeated here.

TST-D8 was characterized as 1α,3β,23-trihydroxy-olean-12-en-29-oate-23-*O*-α-[4-acetoxyrhamnopyranosyl]-29-α-rhamnopyranoside. It was given the name sharpleside. This compound is reported here for the first time. Its isolation as well as that of TST-11 is significant as it shows that the trihydroxy-olean-12-en-29-oate aglycone, hitherto associated with species of the genus *Combretum* also exists as a constituent of the genus, *Terminalia*. This establishes a chemotaxonomic link between these two genera that has not been previously reported.



Spectrum 5.53: Upfield section of ^{1}H NMR (500MHz, CD_3OD) of TST-8



Spectrum 5.54: Downfield section of ^{1}H NMR (500MHz, CD_3OD) of TST-8

Summary of the physical properties and spectral data of the triterpenes isolated.

CIM-31 (1α, 3β-hydroxyimberbic acid or **1α, 3β-dihydroxy-olean-12-en-29-oic acid)**

White to colourless crystalline solid. IR ν_{max} KBr cm^{-1}: 3437, 2949, 1697, 1647, 146, 1381, 1225, 1033, 915, 736. Found $[M+H]^+$ 473.30. ($C_{30}H_{48}O_4$ requires 472.3553). HRFABMS *m/z* (rel. int. %): 472.30 (3) 453.40 (19), 436.33 (6), 395 (12), 248.2 (10), 188.9 (100), 129.6 (40), 93.1 (74). ^1H and ^{13}C NMR Table **5.35**; Spectra **5.39-41**.

CIM-10/ TST-11 (1α, 3β-hydroxyimberbic acid-23-*O*-α-L-4-acetylrhamnopyranoside or.**1α, 3β,23-trihydroxy-olean-12-en-29-oate -23-*O*-α-L-4-acetylrhamnopyranoside)**

White amorphous solid. Found $[M+Na]^+$ 701.4294 and $[M+H]^+$ 677.4367. ($C_{38}H_{60}O_{10}$ requires 676.4187). HRFABMS *m/z* (rel. int. %): 661.439 (19), 658.418 (41), 472.0 (18), 453.5 (80), 437.0 (30), 395.5 (50), 248.3 (100), 231.2 (62). ^1H and ^{13}C NMR Tables **5.37** & **5.38**; Spectra **5.42-5.45**.

CIM-11 (1α, 3β-hydroxyimberbic acid-23-*O*-α-L-3,4-diacetylrhamnopyranoside or **1α, 3β,23-trihydroxy-olean-12-en-29-oate-23-*O*-α-L-3,4-acetylrhamnopyranoside)**

Yellow amorphous solid. IR ν_{max} KBr cm^{-1}: 3446, 2944, 1732, 1454, 1378, 1240, 1127, 1075, 820, 603. Found $[M+H]^+$ 719.4. ($C_{40}H_{62}O_{11}$ requires 718.4292). LRFABMS *m/z* (rel. int. %): 719.4 (0.4), 701.1 (1) 683.1 (1), 659 (0.2),

641.2 (0.2), 472.0 (1.5), 453.2 (5.5), 437 (3.5), 395.2 (3.5), 261.1 (2.5), 248 (80) 231.1 (100). ^{1}H and ^{13}C NMR Tables **5.40** & **5.41**; Spectra **5.46-5.49**.

CIM-9 (1α, 3β-hydroxyimberbic acid-23-α-[L-3,4-diacetylrhamnopyranosyl]-29-*O*-α-rhamnopyranoside or **1α, 3β,23-trihydroxy-olean-12-en-29-oate -23-O-α-L-3,4-acetyl-29-dirhamnopyranoside)**

Yellow amorphous solid. IR ν_{max} KBr cm^{-1}: 3446, 2939, 1732, 1455, 1382, 1217, 1047, 965, 799, 603. Found M^{+} 864.4 ($C_{46}H_{72}O_{15}$ requires 864.4871). LRFABMS *m/z* (rel. int. %): 864.4 (0.4), 846.1 (0.6), 828.0 (0.3), 769.1 (0.3), 701.3 (3), 683.2 (3), 599.0 (1), 472.0 (2), 453.1 (9), 436.0 (5.5). ^{1}H and ^{13}C NMR Tables **5.42** & **5.43**; Spectra **5.50-5.52**.

TST-8 (1α, 3β-hydroxyimberbic acid-23-*O* -α-[L-4-acetylrhamnopyranosyl]-29-*O*-α-rhamnopyranoside or **1α, 3β,23-trihydroxy-olean-12-en-29-oate -23-O-α-L-4-acetyl-29-dirhamnopyranoside)**

Peach amorphous solid. Found $[M+H]^{+}$ 823.1. ($C_{44}H_{70}O_{14}$ requires 822.4766). LRFABMS *m/z* (rel. int. %): 823.1 (8), 804.5 (7), 752.6 (8), 677.4 (10), 659.1 (21), 641.2 (21), 472.0 (30), 453.2 (100), 395.3 (60), 335.1 (50), 248.0 (34), 242 (36), 201.1 (65). ^{1}H and ^{13}C NMR Tables **5.43** & **5.44**; Spectra **5.53** & **5.54**.

5.2. POLAR FRACTIONATION

5.2.1. RESULTS FROM "SCAVENGING" METHOD

This aspect of the study involved attempting to isolate polar constituents from the plant material using methods described in Section **4.1.3**. In many ways, this was charting new territory searching for hitherto unknown constituents of Combretaceae of high polarity.

The "scavenging" method was not very successful, yielding only two pure compounds which were neither novel nor biologically active. One of them was isolated in sufficient quantities to merit discussion. The other is based on shikimic acid but possibly mixed making it impossible to completely elucidate its structure. No further work was done on it.

It was very difficult to work with the extracts put through the "scavenging" work-up because they could not be separated by the reverse phase HPLC methods used for the non-polar extracts despite repeated fractionation.

Fig.**5.53** shows the UV output and chromatogram from LC-MS of a failed sample, HF-4. It is the polar extract of the leaves of *C. imberbe* (designated HIE) which was put through the Biotage™ fraction method, and then fractionated further by gradient preparative HPLC using MeOH/H_2O on a reverse phase column. HF-4 was observed to be a single

peak and collected. However, LC-MS, which was used for quality control purposes, showed it to be a mixture of at least three major compounds.

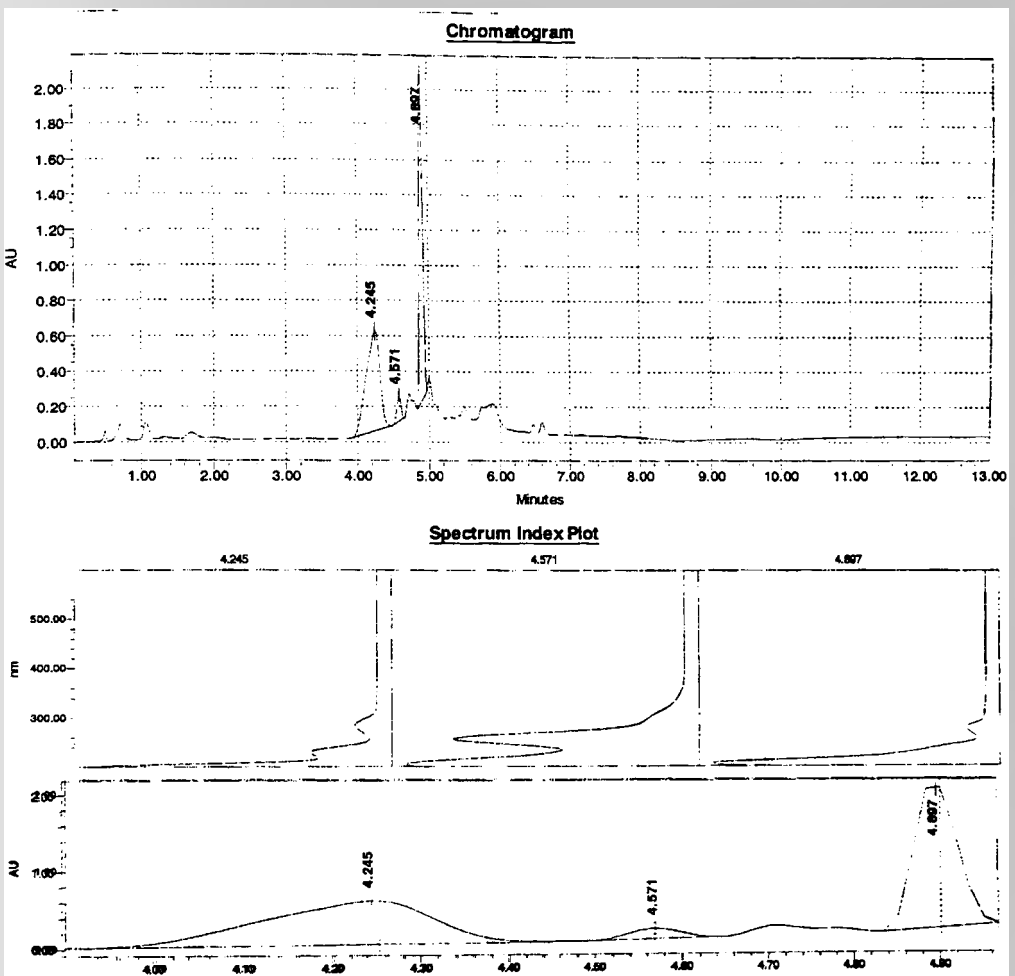


Fig.5.53. Chromatogram and UV profile of HF-4 analysed by LC-MS.

This is only one example of the fate that befell many of the other polar extracts. It clearly demonstrates the short-comings of the HPLC methods developed for non-polar material being applied for the isolation of polar constituents. The limitations and problems are

manifold: selectivity and resolution are poor even when MeOH-H_2O, which are meant to be the most selective combination of the commonly used HPLC solvents, are used as eluents. The constituents all generally tend to co-elute very early in the run i.e. the retention time is too short and could not be increased. Tailing and band-broadening appear to be typical of this type of compounds.

The main reason for these problems is probably the poor interaction between the stationary phase and the polar compounds. A possible solution might lie with running the sample through a normal phase HPLC column, but this too has its own limitations. This was attempted but problems soon arose due to increased back-pressure (leading to erratic pump performance) and the possibility of the stationary phase leaching into the sample. In general, normal phase HPLC appears to be little used in phytochemistry, and there is lack of experience and technical back-up for this technique. However, from the work done in this part of the study, it is important that methods be developed to aid in the routine isolation of polar natural products as the potential for novel discovery in this area is vast. This potential will remain untapped unless higher through-put and cheap technologies are developed.

Characterisation of ^{P}B2-4

^{P}B2-4 was isolated by HPLC fractionation of the unbound fraction from the Dowex-50 Ion Exchange column. FTIR showed the presence of hydroxyl (3434, 1365, 1290, 1260, 1040cm^{-1}), benzene ring (890, 830, 800,710cm^{-1}) with various substitution patterns, an

α,β unsaturated carbonyl (1610 cm^{-1}) and the absence of methoxyl (2850-2810, 1360-1355 cm^{-1}) function.

1D-NMR spectra were suggestive of a flavonoid glycoside. It showed phenolic protons which are *meta*-coupled at δ_H 6.63 and 6.66 (J=2). These appear to be H- 6 and H-8 respectively in ring A of the flavonoid. Ring B is tri-oxygenated with three protons – one *ortho*-coupling at δ_H 7.24 (J=8.4) (δ_C116.7), an *ortho*, *meta* double doublet at δ_H 8.05 (J=2.2, 6.2) (δ_C 123.3) and a deshielded proton showing a small doublet at δ_H 8.40 (J=2.2) (δ_C 118.3). HMQC located these protons as H-5', H-6' and H-2' respectively.

Inspection of the sugar regions showed six carbon signals implying that the sugar in question is probably a hexopyranose unit. ^{1}H-^{1}H COSY-90 was used to track the spin systems in the sugar. The anomeric proton, designated G-1, at δ_H 5.90 (d, J=7.8) is connected to the proton, G-2, at δ_H 4.69 (t, J=9.2) which is linked to the double doublet, G-3, at δ_H 4.20, which, in turn, is joined to the proton, G-4, at δ_H 4.51 (d, J=3). There, the spin system ends. This may be due to the axial/equatorial stereochemistry between the G-4 and G-5 protons. There is a link between the triplet assigned G-5 at δ_H 4.07 (t, J=6) and the two doublet of doublets at δ_H 4.32 and 4.23 (G-6). Thus using ^{1}H-^{1}H COSY, the sugar is shown to be galactose. HMBC shows many ^{2}J (and less ^{3}J) correlations, and this may have to do with the pyranose nature of the ring and it was seen to be quite typical in the

other glycosides isolated in this study. The anomeric proton shows a single HMBC coupling to C-3 (the carbon of aglycone) at *ca.* δ_C 136 confirming this as the position of glycosylation. G-2 proton at δ_H 4.69 correlates to the anomeric carbon at δ_C 106.0 and G-3 (δ_H 4.20) while its carbon is correlated to the G-3 proton (4.20) and G-4 (δ_H 4.51). G-5 proton at δ_H 4.07 correlates to G-4 carbon at δ_C 70.3, G-6 (δ_C 62.4) and G-1 (δ_C 106.0). The sugar is assumed to be β-D-galactose for biogenetic reasons (Bennini *et al*, 1992). NOESY served to confirm the relative stereochemistry of the sugar. It shows a 1,3-diaxial interaction between G-1 and G-3.

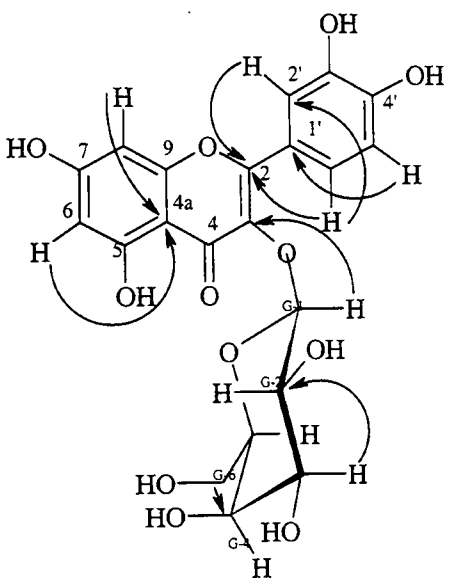


Fig.5.54. Significant HMBC correlation if quercetin 3-galactoside.

LC-MS was used to confirm the identity of the proposed compound and to improve its purity. This was done using a gradient system (MeOH:AcOH 0.1% aq) over 15 minutes and with three known flavonoids (rutin, quercetin, and quecitrin) as controls. The

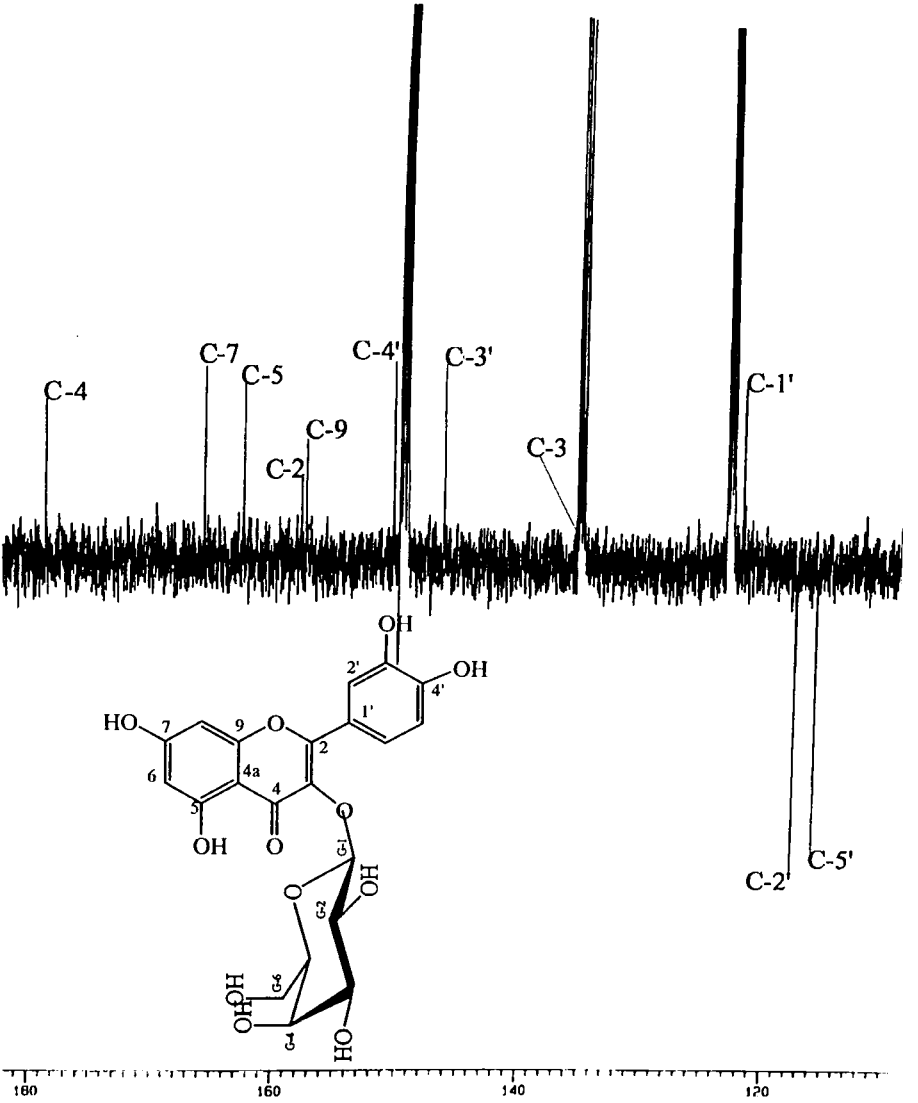
retention time, t_R, of the glycoside was 4.84 min. Mp. 169-73°C The molecular ion, M^+, is *m/z* 464 corresponding to $C_{21}H_{20}O_{12}$. The only other prominent peak appeared at *m/z* 301 (25%) $[M-C_6H_{11}O_5]^+$, this was for the aglycone after the cleavage of the sugar. It was concluded that the aglycone was identical to quercetin since they showed the same retention time.

Table 5.46. ^{1}H (400MHz), ^{13}C (100MHz) NMR and HMBC data for ^{P}B2-4

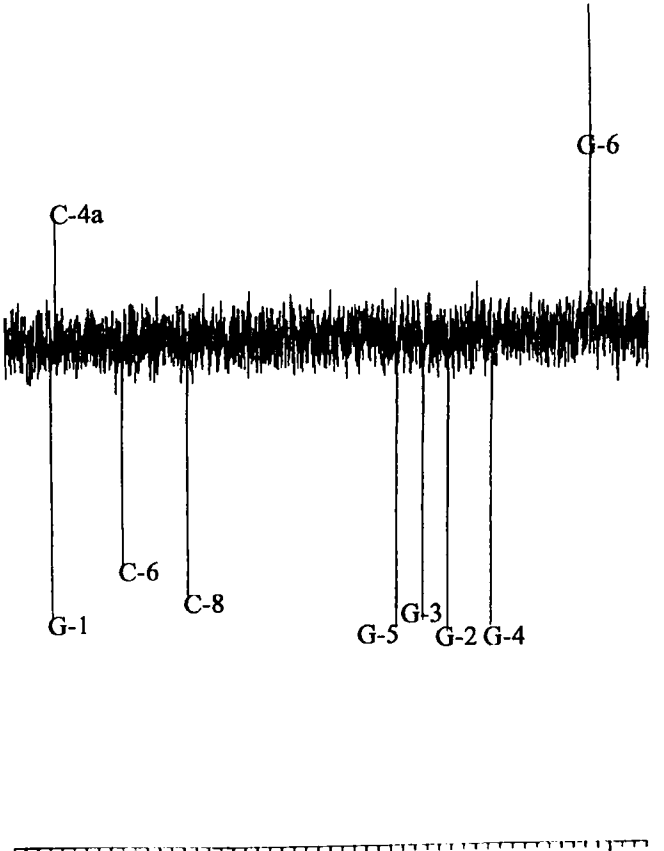
Position	δ_H	δ_C	^2J	^3J
2	-	158.3	-	-
3	-	136.3	-	-
4	-	178.8	-	-
4a	-	105.6	-	-
5	-	163.2	-	-
6	6.63 (d, J=2)	100.3	166.4 (C-7) 163.2 (C-5)	95.0 (C-8), 105.6 (C-4a)
7	-	166.4	-	-
8	6.66 (d, J=2)	95.0	166.4 (C-7)	100.3 (C-6), 158.1 (C-9)
9	-	158.1	-	-
1'	-	122.8	-	-
2'	8.40 (d, J=2.2)	118.3	122.3 (C-1'), 147.2 (C-3')	123.3 (C-6'), 151.2 (C-4'), 158.3 (C-2)
3'	-	147.2	-	-
4'	-	151.2	-	-
5'	7.24 (d, J=8.4)	116.7	123.3 (C-6'), 151.2 (C-4')	147.2 (C-3'), 122.8 (C-1')
6'	8.05 (dd, J=6.2, 2.2)	123.3	122.8 (C-1')	118.3 (C-2')
G1	5.90 (d, J=7.8), ax	106	-	136.3 (C-3)
G2	4.69 (t, J=9.2), ax	73.3	75.9 (G-3) 106.0 (G-1)	-
G3	4.20 (dd, J=6, 3.5), ax	75.9	73.3 (G-2) 70.3 (G-4)	78.1 (G-5)
G4	4.51 (d, J=3), eq	70.3	75.9 (G-3)	73.3 (G-2)
G5	4.07 (t, J=6), ax	78.1	70.3 (G-4) 62.4 (G-6)	106 (G-1)
G6	4.32, 4.23 (dd, J=6,10, each)	62.4	78.1 (G-5)	70.3 (G-4)

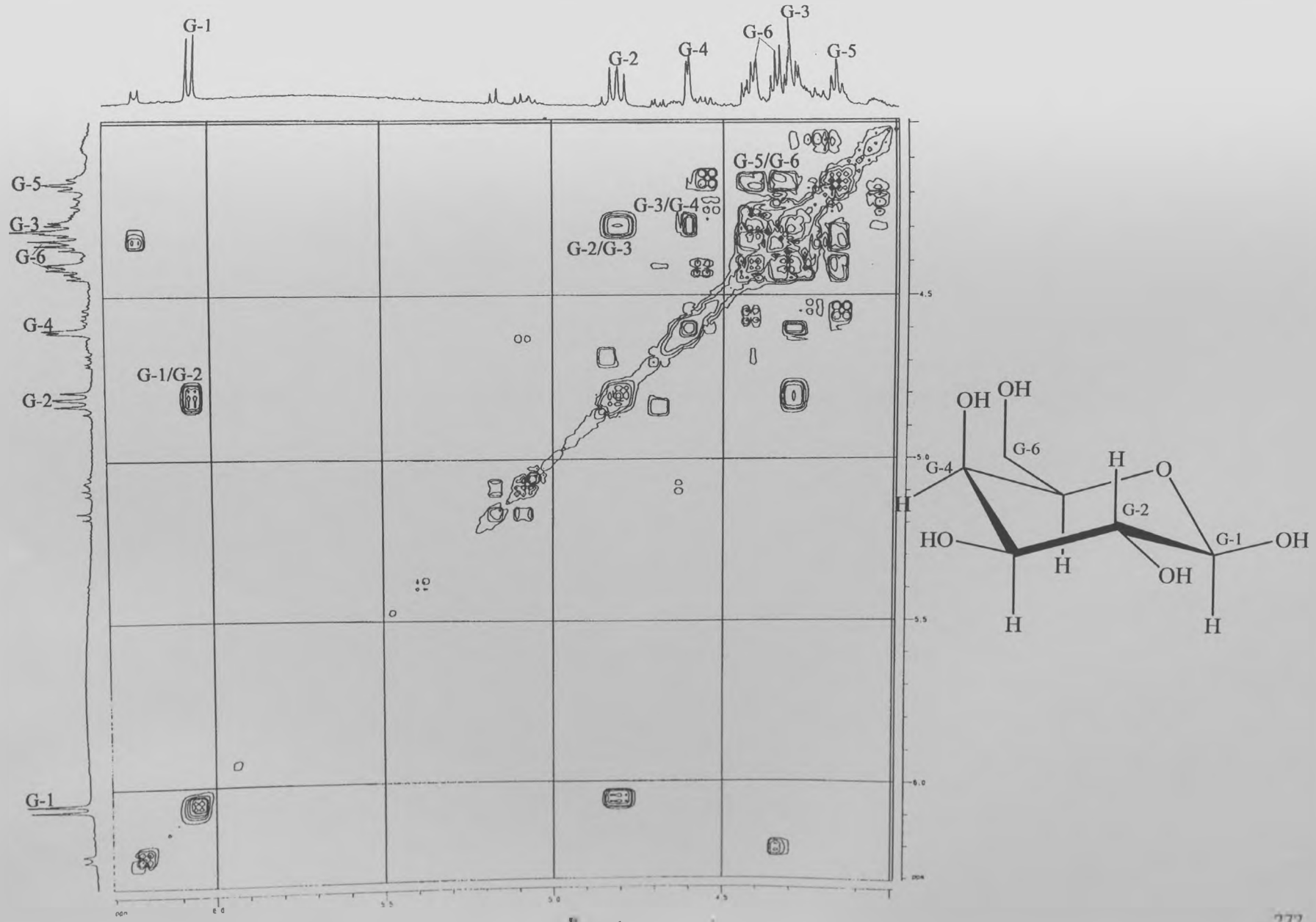
Obtained in C_5D_5N. Coupling constant,**J**, in Hz is in parentheses

^{P}B2-4 was identified as quercetin 3-β-D-galactoside. It has been isolated on many occasions before from a variety of natural sources including *Erica cinerea* (Bennini *et al*, 1992). This is the first time it is reported in *C. apiculatum* and in Combretaceae as far as could be ascertained. Previously 3-O-methylquercetin and 3-O-α-L-rhamnopyranosylquercetrin have been isolated from the South American plant, *C. leprosum* (Facundo *et al*, 1993).

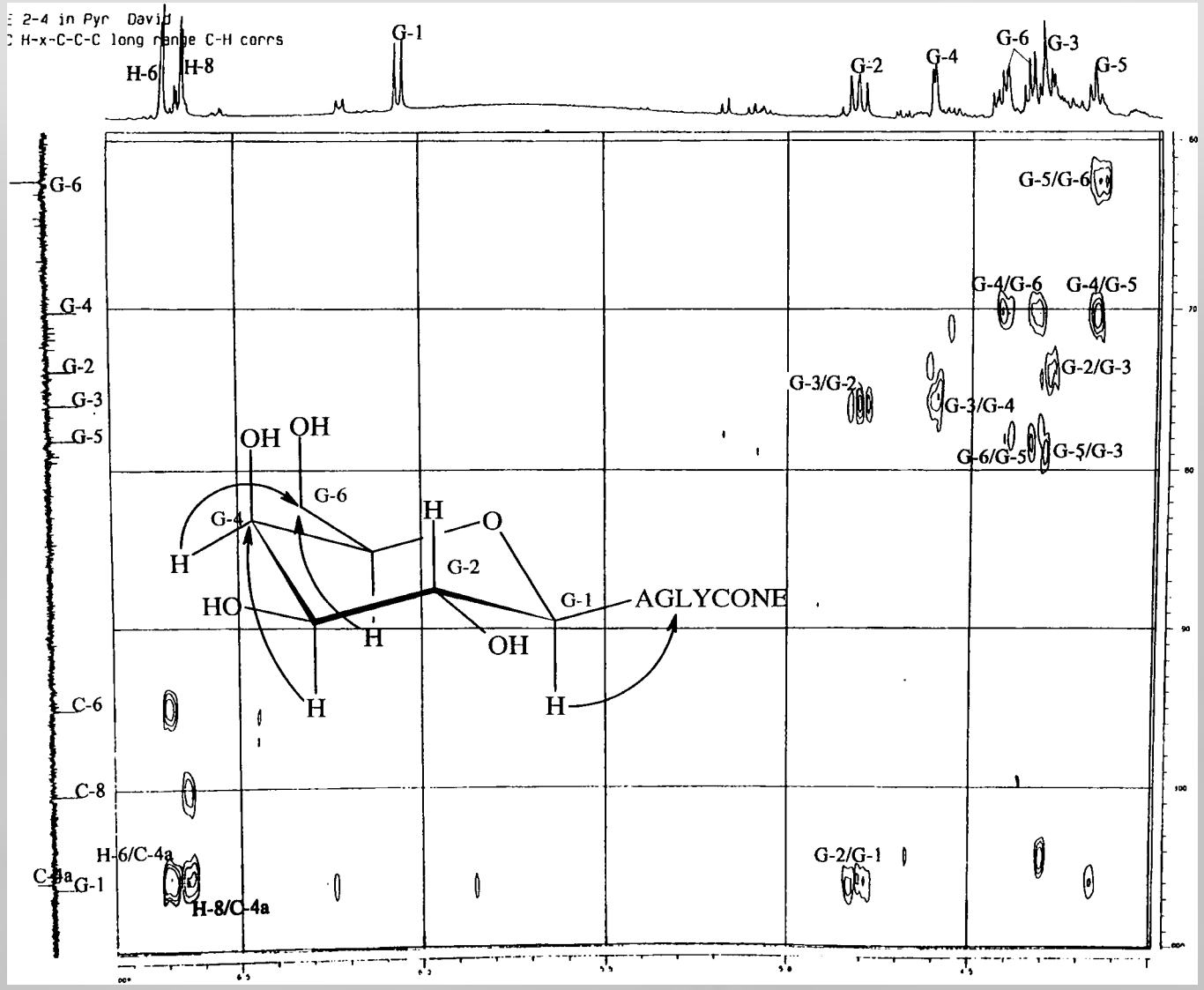


Spectrum 5.55:^{13}C (J-mod) NMR (100MHz, C_5D_5N) of ^{P}B2-4

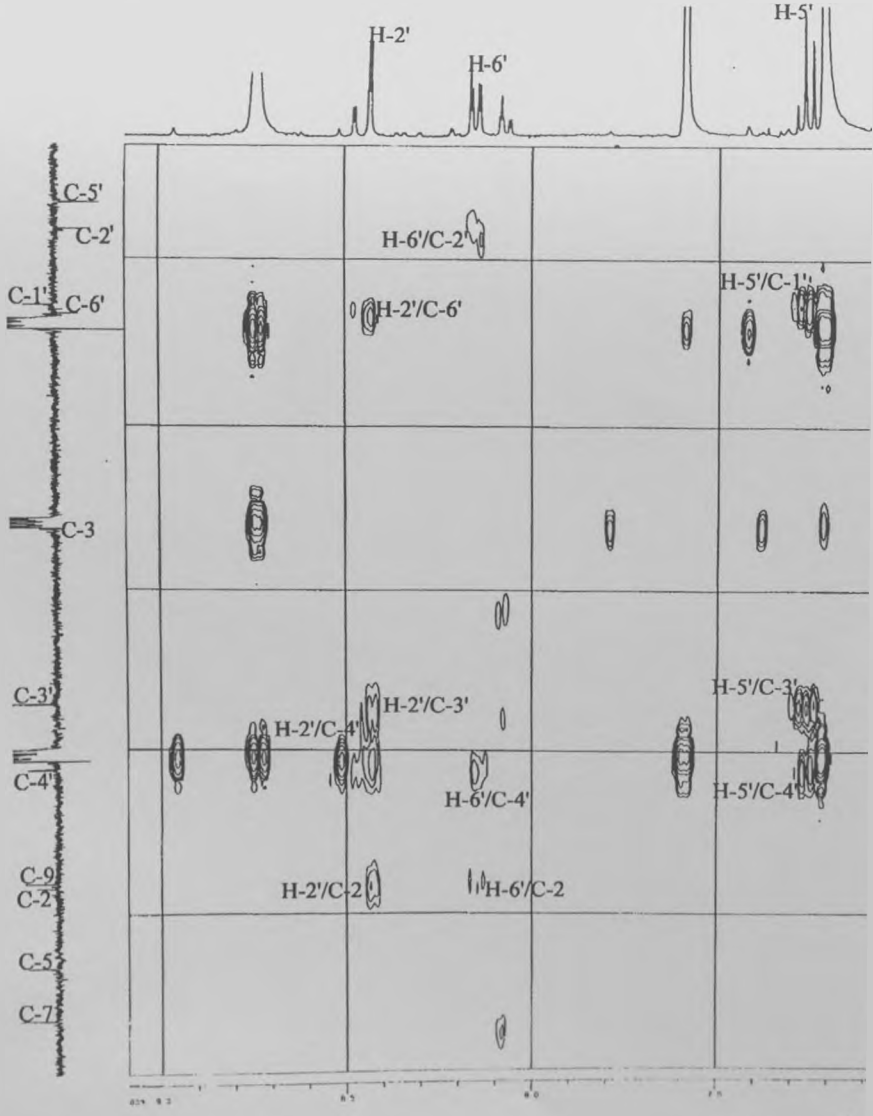

G-6
C-4a
C-6
C-8
G-1
G-5
G-3
G-2
G-4
100
80
60



Spectrum 5.56: ^{1}H-^{1}H COSY (400MHz, C_5D_5N) of ^{n}B2-4



Spectrum 5.57: HMBC correlations of the sugar region of [r]B2-4.



Spectum 5.58:HMBC correlations of the aglycone of [P]B2-4


H-6
H-8
G-1
C-3/G-1
H-8/C-9
H-6/C-5
H-6/C-7
H-8/C-7
120
130
150
160
OH
OH
H
HO
O
OH
HO
HO
HO

5.2.2. RESULTS FROM ION EXCHANGE METHOD

The attempt to isolate polyhydroxy-alkaloids was more successful but severely limited by time. What is discussed below are the preliminary data obtained from this part of the study.

The ion exchange method was designed to isolate polyhydroxy-alkaloidal compounds which tend to occur in very small quantities, and have never been reported in the Combretaceae. However, they have been found to occur in a related family in the order Myrtales to which the Combretaceae belongs (Nash, personal communication, 2000).

High Voltage Electrophoresis (HVE) was used as a preliminary analytical tool. See section.**4.1.3.2.** An analysis of the aqueous extracts of the plants investigated, designated ^{P}AOE, ^{P}BIE, ^{P}CHE, ^{P}HIE, ^{P}DIE, showed the presence of secondary amines (yellow or green after developing), and tertiary amines (weak gray and brown) which were of interest. This was suggestive of the presence of alkaloids. The result from HVE is shown in Fig.**5.55** showing the presence of various amines in the fractions which were worked on. These preliminary data are important as they establish for the first time the presence of polyhydroxy-alkaloids in Combretaceae. No further work to purify these compounds was done due to the constraints of time. Future work should focus on isolating these compounds which are likely to be of phytochemical and pharmacological interest.

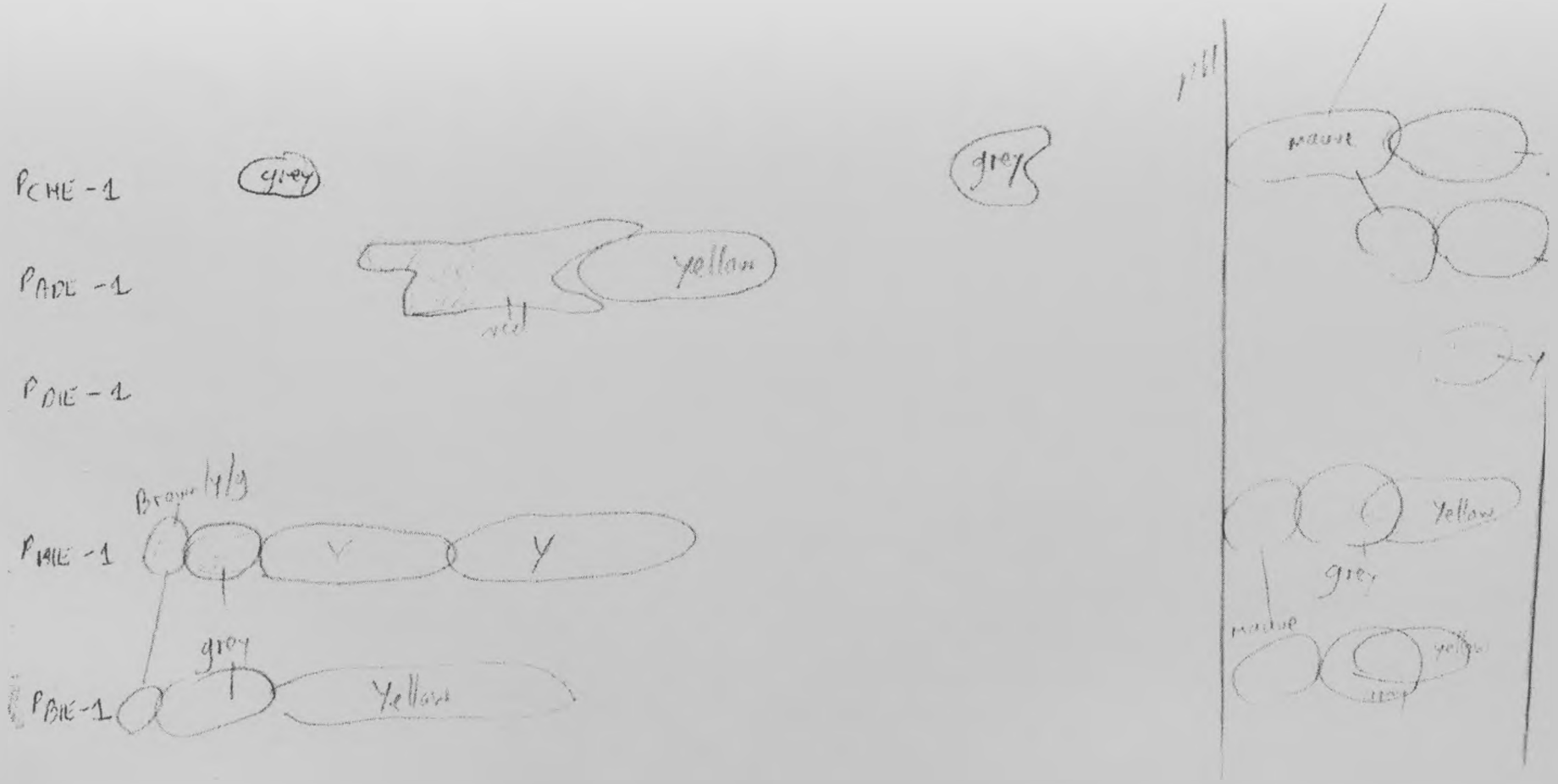


Fig.5.55. High Voltage Electrophoresis (HVE) trace of polar fractions of species of Combretaceae.

CHAPTER SIX

6.0. RESULTS AND DISCUSSION

6.1 BIOLOGICAL ASSAYS

All the compounds reported in Chapter **5** were evaluated for biological activity in search of their chemotherapeutic potential. The results of two antimicrobial assays are reported here. It was not possible to screen for selective anti-tumour activity in our laboratories because of various limitations, not least the vast array of cell types that are required to obtain a statistically valid result. The compounds will be submitted to the National Cancer Institute (NCI) screening programme in the near future for this purpose. The Natural Products Branch (NPB) of the National Cancer Institute's (NCI) Division of Cancer Treatment and Diagnosis have a comprehensive collection of different cancer cells against which potential selective activity can be measured (Cragg *et al*, 1995).

6.1.1. ANTIMICROBIAL ASSAYS

Two different types of assays were used to screen for potential in vitro anti-microbiological activity against a wide number of yeasts, fungi and bacteria using the methods outlined in Section **4.3**.

6.1.1.1. Well Diffusion Assay (WDA)

The results were based on the area in mm^2 of inhibition (zone of inhibition). The final concentration inoculated into the plates was approximately 160µg. Only three of the

isolated compounds showed activity in this assay. B14-7 (3-hydroxy-5,4'-dimethoxybibenzyl) was active against NCYC 2251 (*Williopsis mrakii*) and NCYC 327 (*Candida valida*). See Tables **6.01** and **6.02**. Two triterpene glycosides, CIM-9 (1α, 3β-hydroxyimberbic acid-23-α-[L-3,4-diacetylrhamnopyranosyl]-29-*O*-α-rhamnopyranoside) and TST-8 (1α, 3β-hydroxyimberbic acid-23-*O* -α-[L-4-acetylrhamnopyranosyl]-29-*O*-α-rhamnopyranoside) were active against *Listeria monocytogenes*, a Gram positive bacterial species. See Table **6.03**.

6.1.1.2. Microdilution Assay (MDA)

The second bioassay, the microdilution assay (MDA) was used to find the minimum inhibitory concentrations (MIC) of the compounds, and to also confirm the activity (or lack thereof) seen in the first assay. It did show that the three compounds seen to be active in the Well Diffusion Assay (WDA) are also active in this test. Over and above that, the other compounds were shown to have anti-microbial activity as well. This may be because they were in direct contact with the microbial cells in this case whereas in the WDA their inability to diffuse into the agar may have been a setback. The results of all the compounds tested are presented below. Activity was determined by the change of colour from pink to purple. The last purple-coloured well (compared to the controls) as seen from left to right was considered to give the minimum inhibitory concentration (MIC). An example is given of the plate for *M. fortuitum* culture. The results are presented in Table **6.04**. One obvious disadvantage of this assay has to do with the interference with the result of coloured compounds, so that the cut-off point is not always a clear-cut boundary between purple (inhibited growth)

and pink (full growth). It may also not be possible to distinguish bactericidal agents from bacteriostatic ones.

Table 6.01. Results of B14-7 activity against yeast NCYC 2251 (*Williopsis mrakii*) (mm^2 area of inhibition).

	Plate 1	Plate 2	Mean
+ve control*	557.48	600.86	579.17
B14–7	115.03	111.75	113.39
-ve control	49.73	52.67	51.2

*Pimaricin

Table 6.02. Results of B14-7 activity against yeast NCYC 327 (*Candida valida*) (mm^2 area of inhibition).

	Plate 1	Plate 2	Mean
+ve control*	540.03	510.02	525.03
B14–7	91.38	109.36	100.37
-ve control	63.52	65.84	64.68

*Pimaricin

Table 6.03. Results of B14-7 activity against yeast *Listeria monocytogenes* (IGER culture collection) (mm^2 area of inhibition).

	Plate 1	Plate 2	Mean
+ve control*	631.59	680.08	655.84
CIM-9	233.58	202.77	218.18
TST-8	242.88	218.08	230.48
-ve control	67.88	70.65	69.27

*Chloramphenicol

In this study only the compounds showing activity in the MDA at 100μg/ml and below are tabulated. This assay suggests that the antimicrobial activity is more widespread in the constituents of Combretaceae than the first assay suggests. It also serves to confirm, to some extent, the activity seen with B14-7 and the triterpene glycosides, CIM-9 and TST-9. The former has an MIC of 25μg/ml for both *C. albicans* and *S. aureus*. This is not considered significant.

Table 6.04: Minimum Inhibitory Concentrations (MIC) for isolated compounds (in μg/ml)

Compound	CA	EC	MF	PV	SA	
B69-10	50	100	100	100	100	
B69-10B	6.25	>100	>100	100	12.5	
AO-14	25	>100	>100	100	100	
B69-13	50	>100	>100	100	100	
B27-8	50	>100	100	100	50	
A-68	50	100	50	100	50	
B69-12	25	>100	25	100	25	
CHE-7	50	>100	25	100	25	♦
CHE-10	50	>100	100	100	100	
CHE-12	50	>100	25	100	25	
CHE-17	50	>100	25	100	25	
B14-6	50	>100	100	100	100	♥
B14-7	25	100	100	50	25	
DA-4	100	100	100	50	50	
CIM-31	100	>100	1.56	25	3.13	♠
CIM-10	>100	>100	>100	25	12.5	
CIM-11	50	>100	12.5	25	6.25	
CIM-9	>100	>100	25	12.5	6.25	
TST-8	12.5	>100	25	100	25	
DMSO (ul)	>100	>100	>100	>100	>100	

CA-*Candida albicans*, **EC**-*Escherichia coli*, **MF**-*Mycobacteria fortuitum*, **PV**-*Proteus vulgaris*, **SA**-*Staphylococcus aureus*.

♣Flavonoids,♦Phenanthrenes,♥Dihydrostilbenes,♠Triterpenes

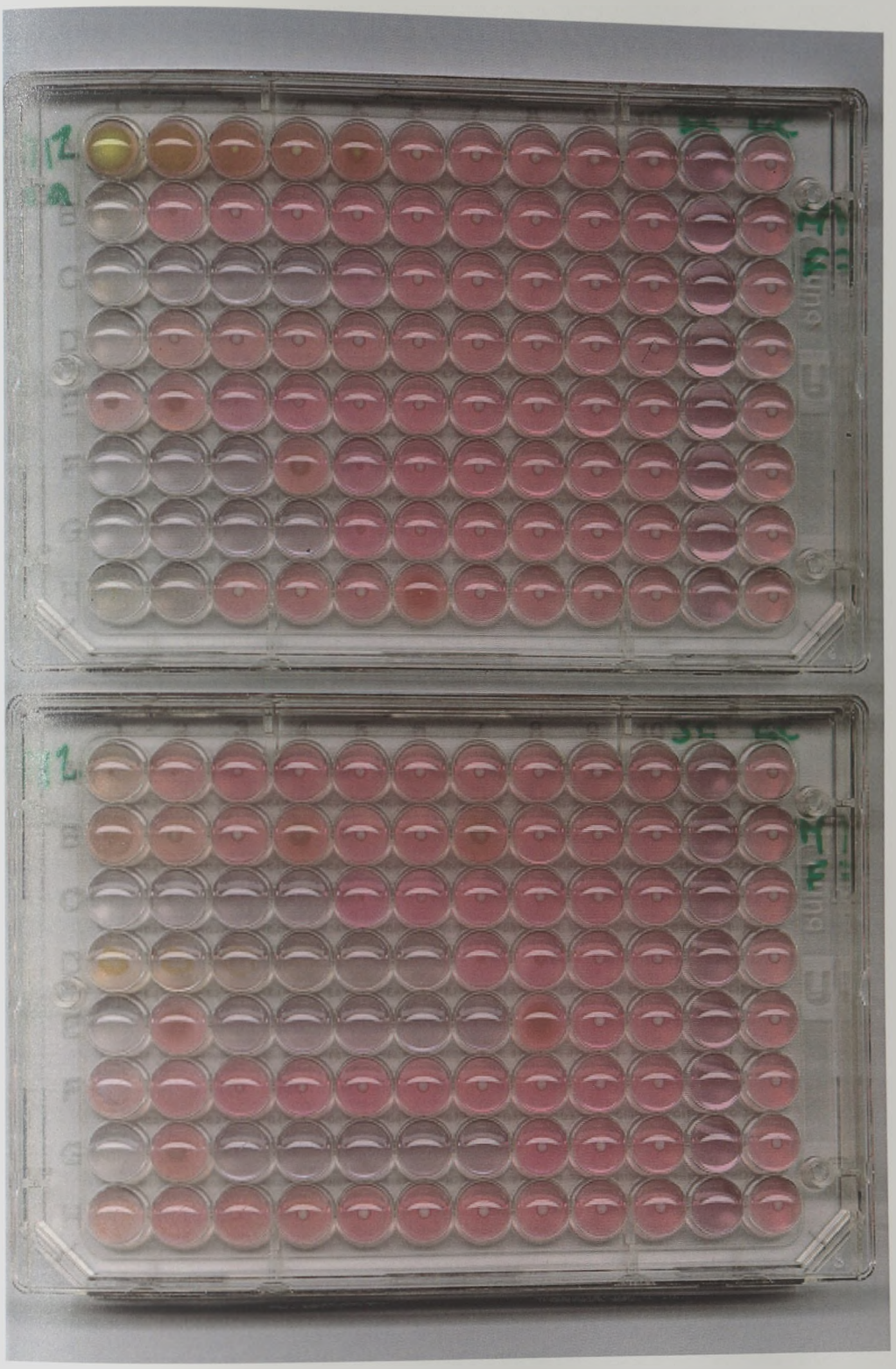


Fig. 6.01. Microdilution plates of M. fortuitum culture and test samples.

CIM-9 has potent inhibitory activity against *P. vulgaris* (12.5μg/ml) and *S. aureus* (6.25μg/ml), while TST-8 acts against *C. albicans* (12.5μg/ml) and *S. aureus* to a lesser extent (25μg/ml). It was the unexpected potency of the other triterpenes, however that was both surprising and interesting. CIM-31, the free aglycone showed activity against *M. fortuitum* at a concentration of 1.56μg/ml and *S. aureus* at 3.13μg/ml. This was the more surprising considering that the Mycobacterium was generally resistant to the other test samples. CIM-10 inhibited *S. aureus* at 12.5μg/ml while CIM-11 inhibited *S. aureus* at 6.25μg/ml and *M. fortuitum* at 12.5μg/ml.

When considering general trends, it was noted that *E. coli* was invariably resistant to these compounds. It may be concluded from this preliminary result that these constituents of Combretaceae are not active against Gram negative bacteria. *S. aureus* on the other hand is the most sensitive, but it shows a gradation of sensitivity. The general trend based on the phytochemistry of the samples is also of interest as it may lead to the prediction of activity based on structure. This will be discussed in greater detail later. In brief, it was observed that the terpenoids are, surprisingly, the most active class here, followed by the phenanthrenes which show a consistent inhibitory action across the spectrum of organisms with the exception of CHE-10 which is four times less active against *M. fortuitum*. However, in general the phenanthrenes are not very active and would have no clinical potential based on these preliminary data. There does not appear to be a pattern linking the flavonoids or the dihydrostilbenes. Only the flavanone, B69-10B, identified as pinocembrin shows significant activity against *C. albicans* (MIC-6.25μg/ml) and *S. aureus* (MIC-12.5μg/ml).

The two assays to screen for *in vitro* anti-microbial activity showed that the majority of compounds isolated in this study possess appreciable selective activity against a broad spectrum of microbiological organisms. At least six of these compounds are possible candidates for further *in vitro* work to evaluate their therapeutic potential in medical, veterinary or agricultural practice.

CHAPTER SEVEN

7.0. GENERAL DISCUSSION

This study has shown the presence of interesting secondary metabolites in members of Combretaceae which have been investigated for the first time, such as *C. albopunctatum* and *T. stulhmanii*. The presence of similar (or related) secondary metabolites within the plants investigated here, and also the commonality with compounds previously isolated is further evidence of their biogenetic relationship and thus justifies (from a chemotaxonomic point of view) their classification as species of the same genus.

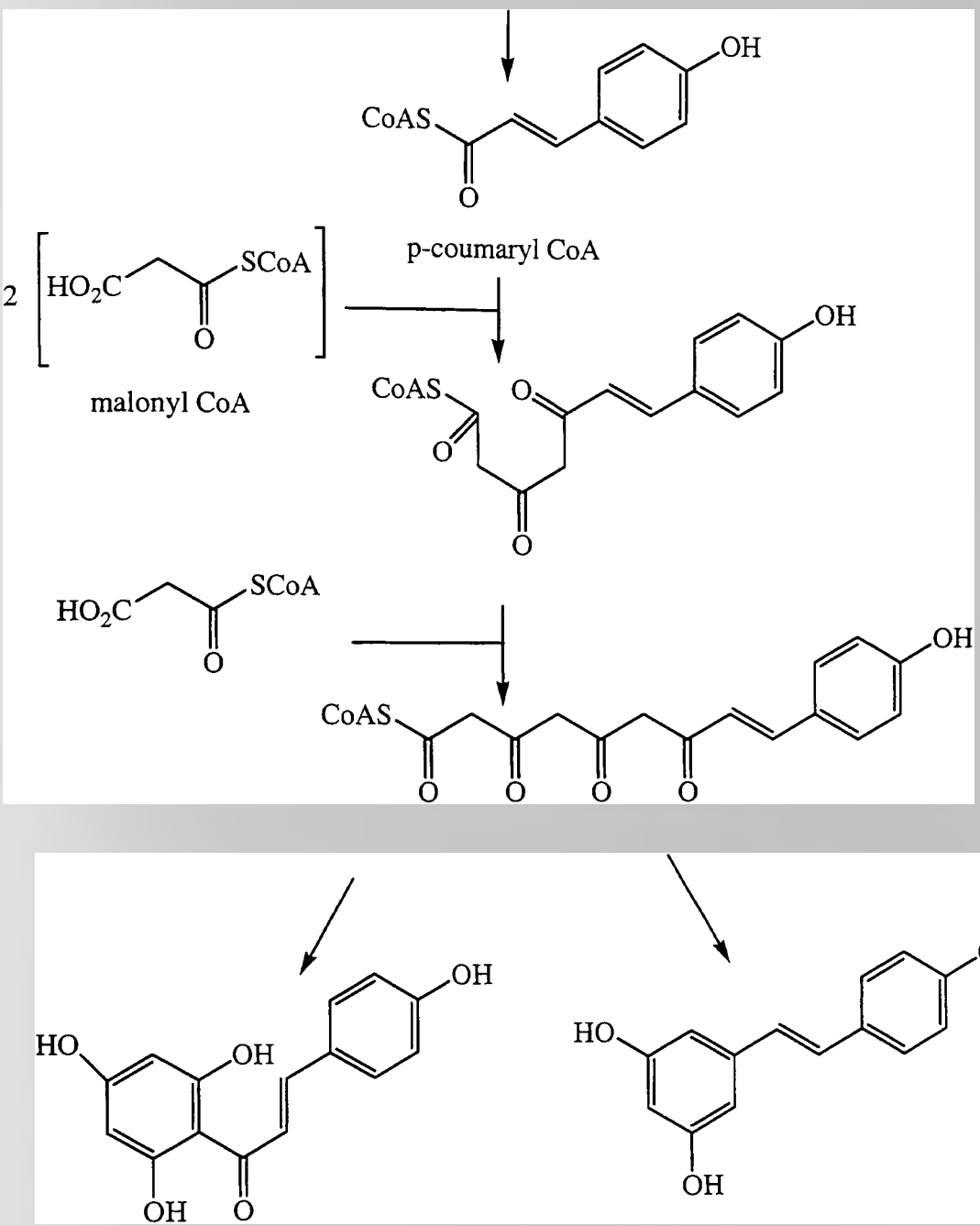
To be able to fully explain the occurrence of similar constituents in the plants investigated and those others belonging to related taxa, it is necessary to go down to the biosynthetic level and examine the manner in which the phenolic compounds as well as the triterpenes arise. The following discussion, while adapted to the niceties of the present study is based on the various hypotheses, speculations and facts collated from various sources including Haslam, 1985; Kindl, 1985; Heller and Forkmann, 1988; Ibrahim, 2000; Nes and Zhou, 2000, Abe *et al*, 1993, among others.

7.1. BIOSYNTHESIS OF PHENOLIC COMPOUNDS

7.1.1. FLAVONOIDS

Flavonoids arise from two biosynthetic pathways – the shikimate pathway which leads to the aromatic ring generally called ring B and its propyl attachment, and the polyketide pathway which gives rise to the second aromatic ring, A. The precursors, a hydroxycinnamoyl-CoA (4-coumaroyl-CoA) and malonyl-CoA are condensed in a reaction involving the enzyme chalcone synthase. Both precursors are products of carbohydrate metabolism. Malonyl CoA comes from the condensation of acetyl CoA and carbon dioxide (CO_2) while 4-coumaroyl CoA arises from the shikimate (arogenate) pathway leading to the formation of L-phenylalanine and L-tyrosine. See Fig. **7.01**.

Chalcone synthase controls the first committed step in flavonoid biosynthesis (O'Hagan, 1991). The resultant chalcone is then transformed by stereospecific enzymes to the flavanone, naringenin. This in turn undergoes various types of reactions such as reduction to give variously derivatized flavanones e.g.AO-14 (alpinetin) or by desaturation reactions, flavones are obtained e.g. B69-10 (chrysin). Any number of reactions appear possible e.g. hydroxylation, glycosylation, isoprenylation etc. The derivatization of flavonoids may depend on other biochemical constituents, for example, an elevated protein content has been found to repress isomerization of chalcones (Kindl, 1985). See Fig.**7.02**.



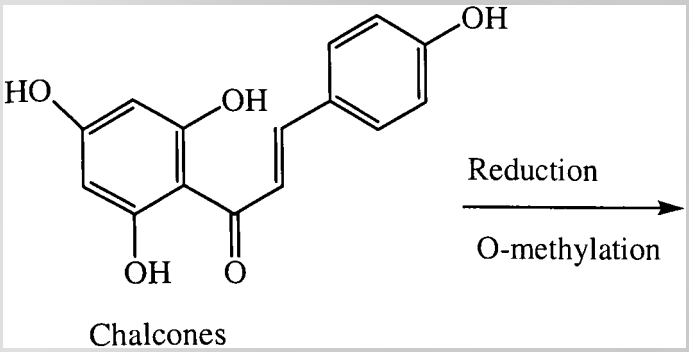
Chalcones

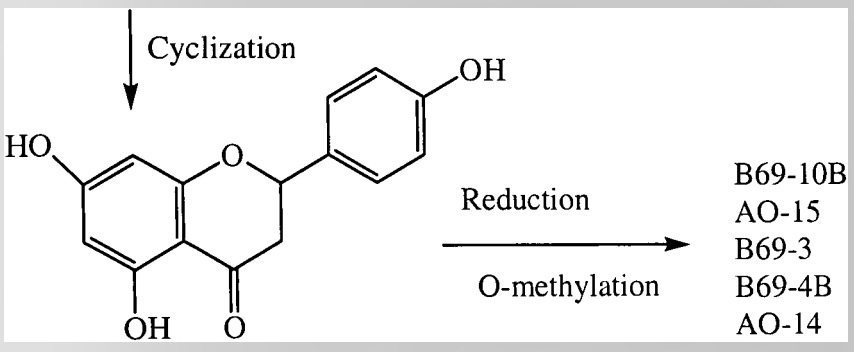
Flavonoids

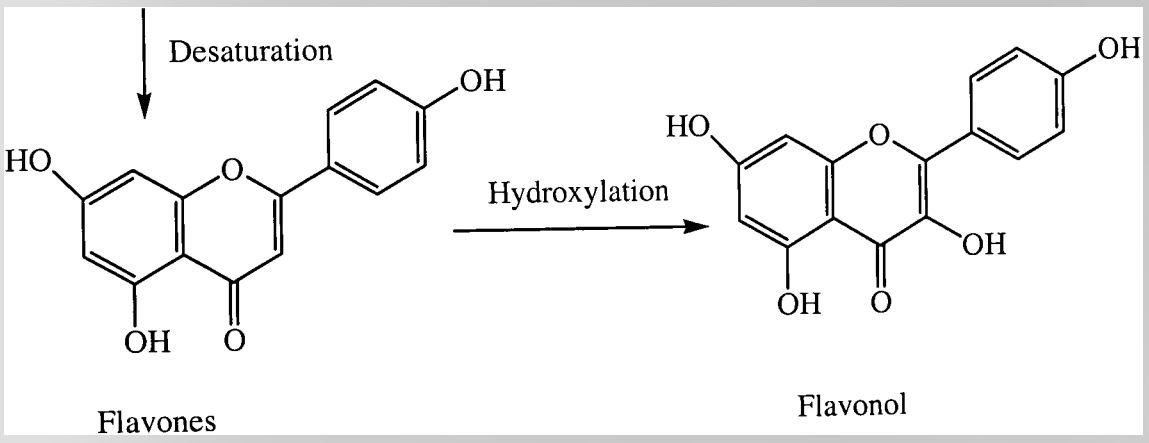
Stilbenes

Bibenzyls
Phenanthrenes etc

Fig.7.01. Phenylpropanoid and acetate metabolism in the biosynthesis of flavonoids







CIM-65

Glycosylation

P.BIE2-4

B69-10
B27-3

Fig. 7.02. The biosynthetic pathway for the formation of flavonoids in the Combretaceae

Haslam (1985) has noted that there are certain 'regularities' in the oxygenation pattern in the aromatic nuclei of plant phenols. In the flavonoids, for example, the oxygen-containing substituents e.g –OH, -OMe, with few exceptions, show the phloroglucinol arrangement in ring A, which is of polyketide origin, and oxygenation in the *para* position of the shikimate-derived ring B. This was true of all the flavonoids isolated in this study.

The isolation of chalcones as well as closely related flavonoids is of interest as it raises certain questions concerning their metabolism. Wollenweber (1981) has been sceptical about the existence of simple chalcones alongside flavanones, showing that, for example, 2',6'-hydroxy-4'-methoxychalcone can be converted to 5-hydroxy-7-methoxyflavanone by application of heat. Previously, von Sauer and Hansel (1967) had shown that 4',6'-hydroxy-2'-methoxychalcone cyclized at 200°C to yield 7-hydroxy-5-methoxyflavanone. What is interesting as well is that Wollenweber, in the same study as cited above, has also shown the possibility of the reverse reactions occurring i.e. flavanones giving rise to the corresponding chalcones in similar circumstances. In this study, some of the flavanones found were characterised as the cyclized derivatives of the chalcones also isolated here e.g. 7-hydroxy-5-methoxyflavanone (alpinetin, B69-4B/AO-14) and alpinetin chalcone (4',6'-dihydro-2'-methoxychalcone, B69-13/AO-18). It is, therefore, feasible that one or the other of these may be an artefact of the extraction process. However it is more likely that the chalcones and flavanones co-exist as they are both intermediates in the biosynthesis of more complex phenolics (Heller and Forkmann, 1988). It will be worth

analysing these plants for these flavonoids under more controlled experimental results if only to establish the proportions of these compounds *in loco*.

It remains difficult to explain the origin of the chalcone dimers. Montaudo *et al* (1974) have suggested that these result from photodimerization. It has not been established if this reaction occurs in the live plant. Bjorn (1996) has stated that flavonoid biosynthesis may arise in response to ultraviolet irradiation, and that the phenylpropanoids play an important role in protection against excessive UV-B, it is possible that the dimers are synthesized for this purpose as well. If so, then they might have been isolated from all the other plants as well. They have been isolated previously from bamboo (*Phyllostachys edulis*) and bamboo grass (*Sasa kurilensis*) and from *Lolium multiflorum* (Tachibana *et al,* 1992; Hartley *et al*, 1988). In both cases they were found to be dihydroxytruxillic acid derivatives resulting from the photodimerization of cinnamates such as *E* –ferulic acid and *trans-p*-coumaric acid. These dimers are said to occur cross-linked as heteroxylan molecules which confer mechanical strength to cell walls. They also limit the plant's digestibility by ruminants.

7.1.1.2. STILBENOIDS

The biosynthetic pathway of the stilbenoids is a branch of the phenylpropanoid metabolic pathway and thus shares common intermediates with the flavonoids as well as other phenolic compounds e.g. lignans and xanthones (O'Hagan, 1991; Haslam, 1985). The branching has been proposed to take place as shown in Fig. **7.01** and **7.03**. More recently

it has been shown to take place much earlier, the 4-coumaroyl condensing with three malonyl-CoA mediated by resveratrol synthase to generate resveratrol, a precursor to the stilbenes.

In this study, five phenanthrenes and five bibenzylic compounds were isolated (dihydrostilbenes) from three different plants. There was co-occurrence of both constituents only in *C. collinum*. In previous studies mainly on the heartwood of *C. hereroense*, *C. apiculatum* and the leaves of *C. caffrum*, different classes of the stilbenoids were invariably isolated together which gave rise to speculation on the biogenetic relationship between these compounds (Letcher and Nhamo, 1972; Pettit *et al*, 1988).

Letcher and Nhamo (1972) proposed that the bibenzylic compounds arose from the phenanthrenes by ring-opening reactions. Pettit *et al* (1988) suggested instead that the biosynthesis, in fact, goes the other way i.e. it proceeds from shikimic acid and tyrosine to the bibenzyls (dihydrostilbenes) initially, styrene (stilbenes) then the dihydrophenanthrenes were formed with the final product being phenanthrenes.

This latter hypothesis appears to be more feasible as the formation (reversible, it is noted) of stilbenes from bibenzylic compounds by dehydrogenation has been demonstrated in the laboratory (Giles and Sargent, 1974; Pettit and Toki, 1999).

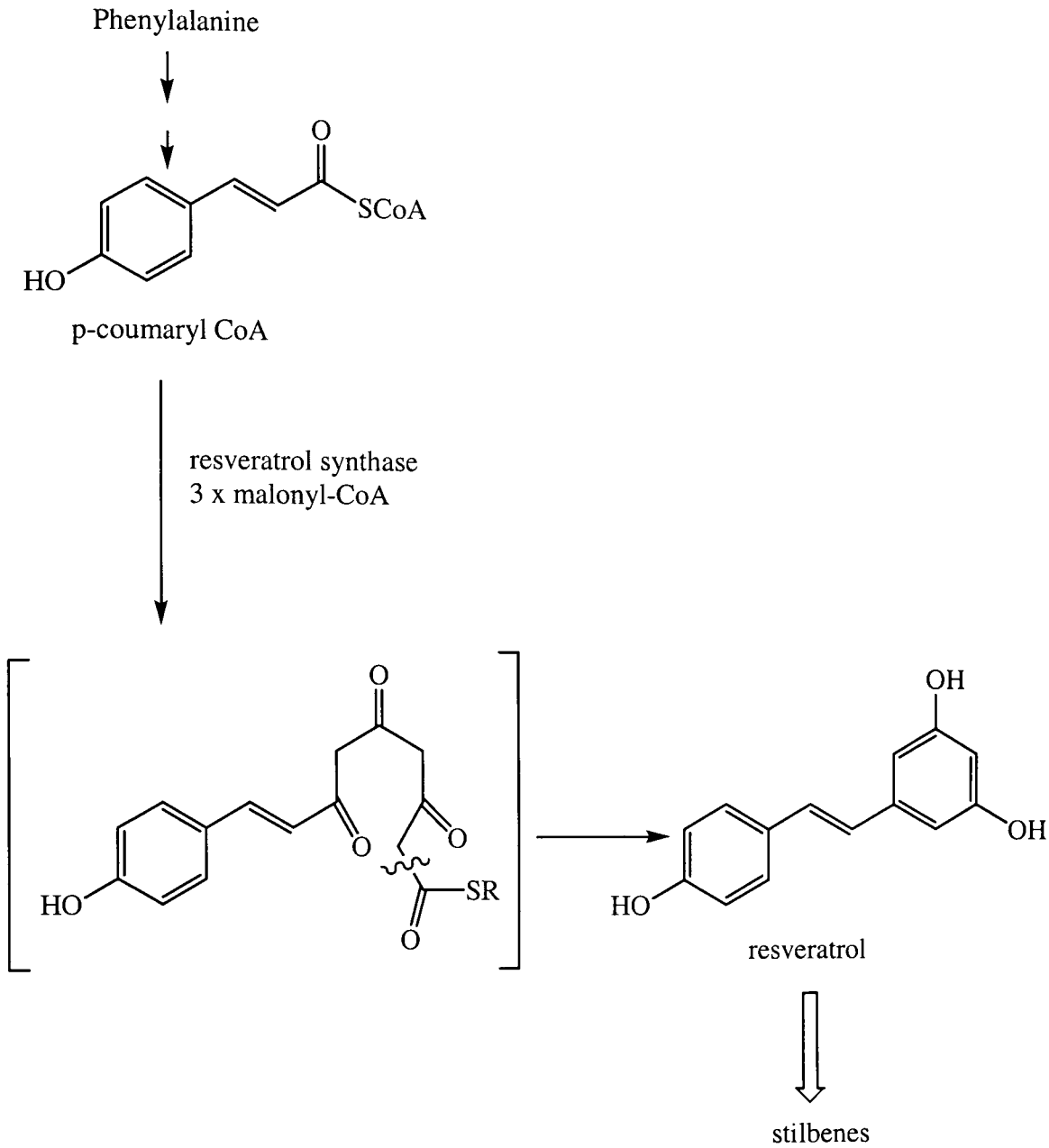


Fig.7.03 The biosynthetic pathway of the stilbenes

The stilbenes that these workers synthesized were then cyclized quite easily in a photochemical reaction to form phenanthrenes. The combretastatins, they concluded, were intermediary products in the synthesis of dihydrophenanthrenes and phenanthrenes. This is probably how the reaction occurs in nature, but it still has to be observed and thoroughly studied. It has been suggested that the enzyme involved is stilbene synthase (STS) which is limited to a number of widely unrelated taxa among them Combretaceae and Orchidaceae (Tropf *et al*, 1995).

Reinecke and Kindl (1994) have shown that the biosynthesis of dihydrophenanthrenes proceeds via bibenzyls and involves dihydro-*m*-coumaroyl-CoA while the synthesis of phenanthrenes is by way of *m*-coumaroyl-CoA with stilbenes as intermediates. They characterised bibenzyl synthase as the enzyme involved in the biosynthesis of stilbenoids in orchids and proposed that the pathways to stilbenes and phenanthrenes on one hand, and dihydrostilbenes (bibenzyls) and dihydrophenanthrenes on the other are biochemically separated and independently controlled.

7.1.2. BIOLOGICAL ACTIVITY

The antimicrobial activity of phenolic compounds has long been recognised, and it is postulated that they are produced in plants to act against phytopathogens (Walker, 1975; Tropf *et al*, 1995). Studies have shown that it is in fact the quinones, oxidative products of phenolic compounds which chelate with amino acids and proteins and convey disease resistance in the plant (Vannini *et al*, 1993). Polyphenoloxidase (PPO), usually higher in

resistant plants than in susceptible plants oxidizes phenolics to quinones which is more toxic to microbes. PPO is the enzyme commonly associated with the browning of fruits e.g. apples and bananas.

Phenolic compounds are thus thought to act either as phytoalexins or phytotoxins or both. Phytoalexins, according to the Theory of Disease Resistance in Plants proposed by Müller and Borger (1940), are compounds produced only in response to pathogenic invasion and are important agents in disease resistance (Kuc, 1972). Phytotoxins (usually phenolic or isoprenoid in nature) on the other hand are allelochemicals regularly synthesized in order to suppress the growth of other plants in the same environment.

Seen in this light, the biological rationale of synthesizing phenolic compounds is easy to understand. These compounds have demonstrated *in vitro* chemotherapeutic activity, which has partly motivated this study. This discussion shall be confined to the studies of the antimicrobial activity of these secondary metabolites.

The flavanones and flavans are most associated with anti-fungal activity but lipophilic flavones and chalcones are known to be active as well and have shown activity against *Cladosporium cucumerinum* (Tomás-Barberán *et al*, 1988). Among the compounds tested was chrysin dimethyl ether which was isolated in this study and coded B69-3. It did not show activity against any of the organisms used in the present study. However, 5,7-dihydroflavanone (pinocembrin coded B69-10B) was active against *Candida albicans*

with an MIC of 6.25µg/ml and *Staphylococcus aureus* (MIC: 12.5µg/ml). This activity has been shown in a previous study against a variety of pathogens (Villanueva *et al*, 1970; Grange and Davey, 1990). It is the constituent to which the antimicrobial activity of honey is attributed. All the other flavonoids showed little activity against the organisms tested.

The stilbenoids have been recognised as phytoalexins as well as constitutive anti-fungal agents for some time (Ward *et al* 1975; Majumder *et al* 1982 and Brinker *et al*, 1991). They are confined, as far as is known, to Orchidaceae, Combretaceae, Dioscoreceae and Horaceae (Miyase and Ueno, 1991). It has been suggested that they inhibit fungal growth via polar inhibition of fungal enzymes e.g. cutinases necessary for the infective agents to establish themselves on the plant (Grayer and Harborne, 1994).

Initial interest in the stilbenoids was ignited by their isolation from the heartwood of various species of (then Rhodesian) Combretaceae. Letcher *et al* (1971, 1972, 1973) were motivated to investigate the phytochemical constituents of the Combretaceae because it is a hardwood much-valued in the furniture industry. They practically stumbled on the phenanthrenes, which invariably co-occurred with the bibenzyls, and concluded that these compounds conferred morbific and anti-termite borer properties. They did not carry out any investigation on biological activity to test this hypothesis.

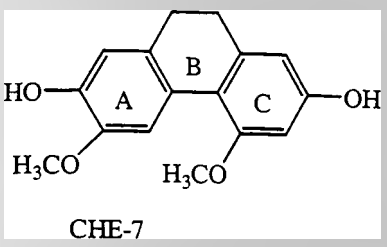
Hydroxylated stilbenes have been found to be constitutive to the heartwood of pine species and other trees and are associated with decay and disease resistance (Hart, 1981). The (*E*)-4- hydroxylated stilbenes and related bibenzyls found in timber from various plants have shown these compounds to be responsible for their durability. Substitution in the 3' position conveyed greater potency against two forms of wood-decaying filamentous fungi, *Gloeophyllum trabeum* and *Poria placenta* (Schultz, 1990). Malan and Swinny (1993) have reported minimum inhibitory concentrations (MIC) of 20µg/ml for some stilbenoids isolated from the heartwood of *C. apiculatum* against *Penicillium expansum*. One of these was isolated in this study from the leaves of the same plant and identified as 4,4'- dihydroxy-3, 5-dimethoxybibenzyl (B27-16B). It showed no significant activity in this study.

B14-7 (3-hydroxy-5, 4'-dimethoxybibenzyl) isolated for the first time from *C. apiculatum* showed potent inhibitory action against yeasts (NCYC 2251 and NCYC 327) in the WDA. In the MDA, it showed moderate activity against *C. albicans* (MIC: 25µg/ml), *P. vulgaris* (50µg/ml) and *S. aureus* (25µg/ml). It was the most active of the bibenzyls isolated. Activity may be explained in terms of possible structure-activity relationships (SAR). B14-7 was unique for at least two reasons- it not only possessed two methoxyl functional groups, but these were each attached to each one of the two aromatic rings. The other compounds had two to four methoxyl substituents, at least two of which were attached to the same ring. The result is loss of planarity in the rings. In addition, B14-7 shows *meta*-substitution in the di-oxygenated ring while the others are 3,4,5-tri-

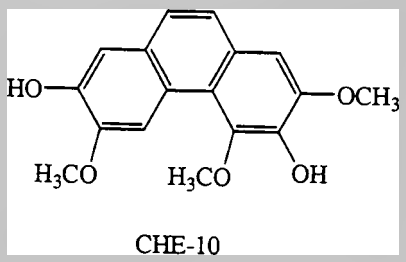
oxygenated. Takagi *et al* (1983) have worked on similar compounds from *Bletilla striata* and have suggested that increased O-methylation reduced anti-microbial activity, whereas the introduction of *p*-hydroxybenzyl enhanced it. Schultz (1990) observed greater activity in compounds with 3'-substitution as stated earlier. From this study, it would appear that the relative positions of the functional groups may be significant.

B14-7 appears to be similar in certain aspects to compounds that have been identified with established phytoalexin activity. For example its oxygenation pattern in ring A is similar to that of piceatannol, a recognized phytoalexin occurring in sugar cane (Brinker *et al*, 1991). Further, it is an analogue of pterostilbene (3,5-dimethoxy-4'-hydroxystilbene) and resveratrol (3,5,4'-trihydroxystilbene) which are phytoalexins in *Pterocarpus santalinus* and the grapevine, respectively (Langcake *et al*, 1979).

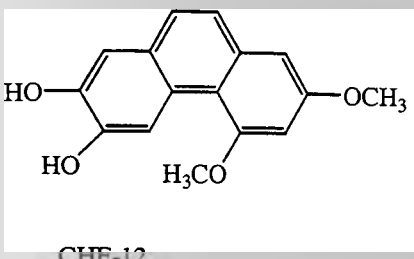
There is little published work involving the phenanthrenes. Letcher and Nhamo (1972) proposed that it may be their presence in the heartwood of Combretaceae which conferred morbific and anti-termite activity. This study demonstrates that these stilbenoids possess some antimicrobial activity. DA-9 (3, 6,7-trimethoxy-9,10-dihydrophenanthrene-2,5-diol) and CHE-10 (3, 5,7-trimethoxyphenanthrene-2,6-diol) were the least active. In fact the former did not show any activity at all in the bioassays. See Fig. **7.04**. The other three phenanthrenes, coded CHE-7, CHE-12, and CHE-17 were all equally active against *C. albicans* (MIC: 50μg/ml), *Mycobacterium fortuitum* (25μg/ml) and *S. aureus* (25μg/ml).



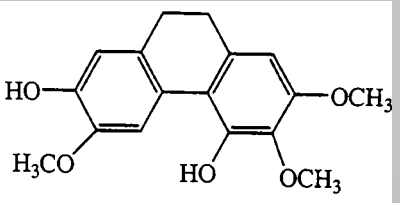
CHE-7



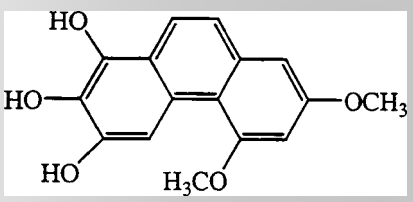
CHE-10



CHE-12



DA-9



CHE-17

Fig.7.04 Phenanthrenes isolated – microbiological activity appears to be determined by substitution in ring C.

The lack of activity by both CHE-10 and DA-9 in the assays performed in this study may be due to the 5,6,7 oxygenation pattern. The relatively more active phenanthrenes have the 5,7 arrangement in ring C. Activity may depend either on this specific substitution pattern or the relative planarity that results from it. Studies on juncusol, a 9,10-dihydrophenanthrene with antimicrobial and cytotoxic activity isolated from *Juncus*

roemerianus have shown that a free phenol is necessary for activity in the phenanthrenes (Boger *et al*, 1985).

For yet to be established reasons, the intermediate metabolites i.e. the stilbenes, and to some extent the bibenzyls, appear to possess more potent biological activity than the end products. It is unclear whether this has to do with the ability of their chemical structure to rotate and twist, while the phenanthrenes are planar and thus, more rigidly fixed. Bibenzyls and to some extent, phenanthrenes have shown activity against Gram positive bacteria and weak anti-fungal activity. (Takagi *et al*, 1983; Malan and Swinny, 1993). Hydrophobicity has been cited as a possible reason for the variation in activity. Increased hydrophobicity leads to increased activity (Hart, 1981). It has been suggested that increased *in vivo* methylation will increase activity because of the concomitant increase in hydrophobicity. It was not possible to investigate this finding in the present study. It is difficult to establish obvious structure activity relationships (SAR) in the stilbenoids with the limited data gathered here. However, it has been said that toxicity is modified by the presence of free hydroxyl groups which are involved in uncoupling at protein binding sites.

7.2.1. BIOSYNTHESIS OF THE TERPENOIDS

The present study resulted in the isolation of six pentacyclic triterpene- based compounds. One of these was the 1α, 3β-dihydroxyolean-12-en-29-oate aglycone and all the others were different glycosidic derivatives of it. Previously this aglycone and its glycosides have been associated with the species of *Combretum*. It is reported here for the first time that this type of structure is also present in the stem bark of the genus *Terminalia* which implies that the biosynthetic pathway may be common to both genera.

The synthesis of all terpenoids is usually via the mevalonic acid pathway and proceeds by the gradual addition of isoprenoid (C_5) units (Samuelsson, 1998). Triterpenes are a class of 30-carbon isoprenoids which possess different degrees of polycyclization – usually tetracyclic or pentacyclic (Nes and Zhou, 2000). Cyclization proceeds from a common precursor, squalene or squalene-2, 3-oxide (oxidosqualene), to a high-energy intermediate. See **Fig.7.05**.

In the formation of the pentacyclic triterpenes such as those isolated in this study, cyclizations via the corresponding "chair-chair-chair" conformation of the (3S)-oxidosqualene with a tetracyclic dammarenyl C-20 cation is the first committed step (Abe *et al*, 1993). Subsequent rearrangements lead to the formation of the pentacyclic oleanyl cation via the baccharenyl and lupenyl cationic intermediates. According to Ruzicka's Biogenetic Isoprene Rule, these reactions employ different enzymes which utilise only small variations of a single catalytic mechanism.

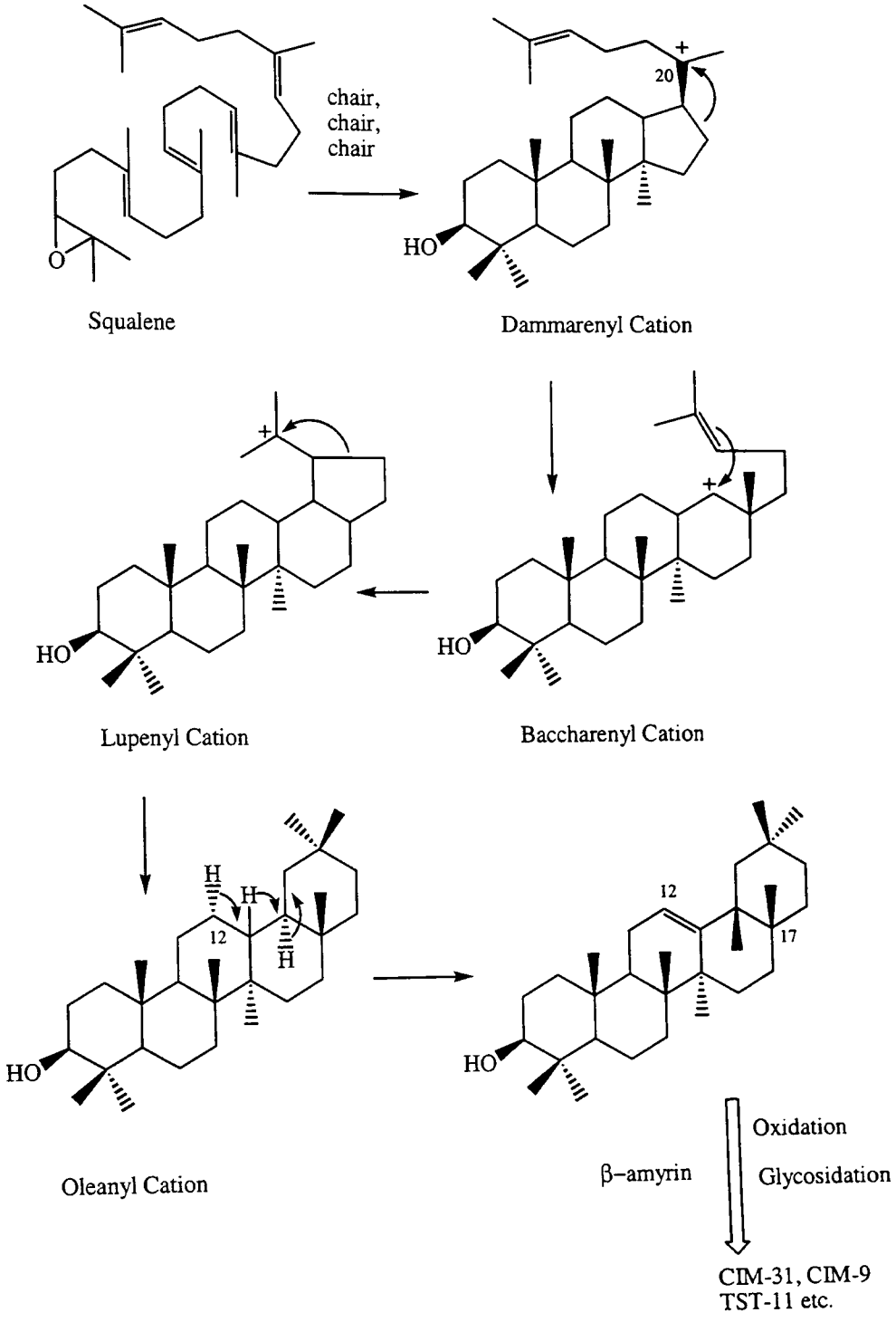


Fig. 7.05.The biosynthetic pathway of pentacyclic triterpenes

The olean-12-enes and urs-12-enes result from a series of 1-2 hydride shifts, elimination of a proton at C-12 and rearrangements during the formation of the D and E-ring systems (Seo *et al*, 1981). The triterpenes isolated in this study were based on the β-amyrin structure, with additional hydroxylation at C-1and oxidation of C-29 to a carboxyl function on ring E in the aglycone.

The majority of triterpenoids isolated here have been in glycosidic form with the sugar, identified as L-rhamnose being acetylated in the 4 (and sometimes 3) position. Only one of them, isolated as 4-acetylrhamnoside of the 1,3-dihydroxy-olean-12-en-29-oic acid (hydroxy-imberbic acid) has been reported before. Two glycosides, one from *C. imberbe* and the other from *T. stulhmanii*, were characterized as occuring in a bidesmosidic form. The sugars are attached to C-23 and to the carbonyl at C-29. It is unclear why these are the preferred points of glycosylation as it might just as well be in the 1,3 position of the aglycone. This must imply the involvement of specific metabolic enzymes.

The isolation of a mixture of aglycones, monodesmosidic and bidesmosidic compounds in this study raises questions concerning the native existence of these constituents. It is possible, for instance, for the aglycones to be mere artefacts of the extraction process and may actually not be found in the plant. Marston and Hostettmann (1985) have found that the extraction process is important in determining what constituents are isolated. They found for example that for bayogenin saponins, aqueous extraction yields more

monodesmosides while methanol extraction yields more of the bidesmosides. In this study a non-polar solvent (DCM) was used.

CIM-31, commonly known as hydroxyimberbic acid appears to be the major constituent of the leaves of *C. imberbe* as it precipitated from various fractions and was also isolated by HPLC. One could argue that were it an artefact, the sugar, once cleaved would also have been isolated or at the very least visualised in HPLC or NMR spectra. Also the fact that it has been isolated previously (Rogers and Subrammony, 1988) using a different method would suggest that it is probably present in the free state in the plant.

7.2.2. BIOLOGICAL ACTIVITY

Triterpenes are the major secondary metabolites of the latex, resins and leaves of many plants and they are believed to be a chemical defence against pathogens, and anti-feedants against herbivores (Mahato and Sen, 1997). They have been extensively investigated for biological activity, but their potential for clinical use has been rather limited, mainly due to their hydrophobicity and hence poor cellular penetration.

The pentacyclic and tetracyclic triterpenes are best known for their action as molluscides particularly in their monodesmosidic form (Msonthi *et al*, 1995; Marston and Hostettmann, 1985). In this regard, *Combretum molle* is best known for its molluscidal constituent, mollic acid which has been recommended for use in rural Africa to control schistosomiasis (Rogers, 1995).

Apart from this work, there are little data on the biological potential of isoprenoid constituents of Combretaceae. Martini and Eloff (1998) have gathered preliminary data to show that crude extracts of *C. erythrophyllum* are active against microbial cultures. As stated earlier, the use of these plants in relieving symptoms that appear to be caused by infective agents e.g. bloody diarrhoea (possibly cholera and dysentery), wounds and conjuctivitis, among others, may be related to their anti-microbial activity. See Section **3.1.2**.

This is the first large study to test pure compounds from the Combretaceae against a large collection of yeasts and bacteria. Two compounds showed very good activity against *Listeria monocytogenes* with the well diffusion assay (WDA). These compounds, coded TST-8 and CIM-9 were isolated from the stem bark of *Terminalia stulhmanii* and the leaves of *Combretum imberbe*, respectively, and their chemical structures were shown to be similar. They are both based on the 1,3-dihydroxy-olean-12-en-29-oate aglycone, and both possess an acetylated rhamnose at C-23 and another rhamnose unit at the C-29 position. The only difference between them appears to be that TST-8 is acetylated at C-4 of the sugar attached to C-23, while CIM-9 is acetylated at both C-3 and C-4 position of this sugar. See Fig. **7.06.**

The biological activity of these compounds may be explained in various ways. The stability of these compounds may be a factor, but the chances are that they are, in fact,

less stable compared to the aglycones and as Marston and Hostettman (1985) have argued, the chances of cleavage are greater due to the extraction process.

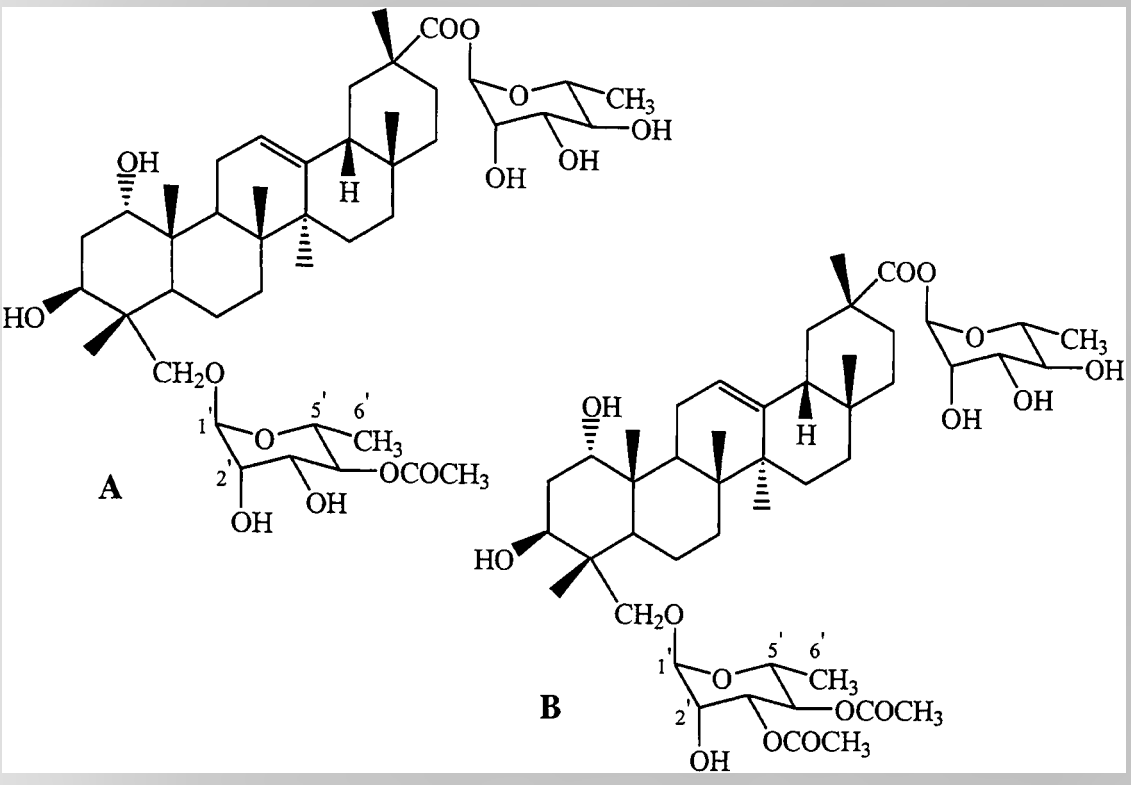


Fig. 7.06: The two glycosides TST-8 (A) and CIM-9 (B) which were active in WDA.

However, it is true to say that their solubility properties are different from the other compounds. Mahato *et al* (1992) have proposed that the main reason why triterpenoids, in spite of their pharmacological potential and ease of isolation, have remained therapeutically unimportant has to do with their poor absorption into eukaryotic tissue which is hydrophilic. Glycosylation will tend to increase water solubility, while acetylation increases hydrophobicity. The inter-play between these opposing effects may

improve overall diffusion of the compound in agar and its subsequent delivery into the microbial cell.

Thus, the activity seen with TST-8 and CIM-9 may have more to do with their ability to penetrate into bacterial cells. If this is indeed true, there would have been a gradation of activity with the mono-glycosylated units showing activity to a small extent, and also these two compounds would have shown activity in the other bacterial cultures. Because only one concentration was being used in the WDA, such activity was not observed.

There is yet another more compelling reason to explain the results in this assay. In general, glycosidic derivatives may show chemotherapeutic activity because of the existence of metabolic pathways and receptors for different sugar units in various body organs (Mahato *et al*, 1992). These pathways, enzymes and receptors differ within certain organs and tissues, and more importantly across different species, enabling some sort of molecular targeting to be employed.

It has been discovered that many naturally-occurring antibiotics contain multifunctional hydrophilic sugars e.g. aldoses, deoxysugars, aminosugars and cyclitols (Kennedy and White, 1983; Kotra *et al*, 2000). It would appear that it is the presence of these sugar units which conveys enzyme inhibitory activity on these compounds. Antibiotics such as the aminoglycosides, for instance, inhibit the biosynthesis of nucleic acids and proteins by binding to particular RNA moieties.

Specificity and toxicity are interlinked in chemotherapy – those agents inhibiting the biosynthesis of DNA, RNA and protein, e.g. cordycepin, are too toxic for clinical use, while those that inhibit protein synthesis, e.g. erythromycin, are safer and more useful (Kennedy and White, 1983). This selectivity and toxicity relationship is important in explaining the pathogen-host relationship in plants, particularly where they produce secondary metabolites that act as phytoalexins to one species deemed phytopathogenic while those organisms that are beneficial remain unharmed.

Watson *et al* (2000) suggested that because rhamnose, for instance, has no role in mammalian activity inhibiting its synthesis is selectively toxic to some microorganisms, particularly the mycobacteria, which are known to possess L-rhamnopyranose units in their cell wall. This then may be the explanation in this instance. It is probably significant that for the compound to have activity against *Listeria* species, which may metabolise similar compounds, it has to be glycosylated at both the C-23 and C-29 positions.

The discovery that in the microdilution assay (MDA) these two compounds are active against *Mycobacterium fortuitum* (MIC 25 μg/ml for both) and *Staphylococcus aureus* (6.25μg/ml for CIM-9 and 25μg/ml for TST-8) is further evidence of their anti-microbial potential. This assay, however, shows some rather confounding results. The aglycone, CIM-31 is shown to be extremely active against *M. fortuitum* and *S. aureus* with MICs of *ca.*1.56μg/ml and 3.13μg/ml respectively, and moderately active against *Proteus vulgaris* (25μg/ml). The monodesmosides, CIM-10/TST-11 and CIM-11 are also active against *P.*

vulgaris and *S. aureus*. In comparison the activity of the bidesmosides is not significantly different.

The MDA appears to be a better predictor of activity because the compounds do not need to diffuse into any matrix such as the agar in the WDA. The major weakness of the WDA is that it assumes that the compounds being tested are agar-soluble and therefore will diffuse through the medium towards the organism (Cole, 1994). This is not valid for non-polar compounds and highly charged molecules. In this study, there appears to have been a false negative for the other terpenoids which are not soluble in agar and probably did not diffuse through to the target cells.

7.3. CONCLUSION

This study has been the single most extensive phytochemical survey of the African Combretaceae to date. Thirty-two compounds were isolated from five different plant species of the twelve different taxa that were collected. The majority of the compounds were isolated from the Combretaceae for the first time, and seven new compounds were discovered and characterised.

Previous work on the African Combretaceae has concentrated on the genus *Combretum* to the exclusion of *Terminalia*, and focussed on *C. caffrum*, *C. molle* and *C. imberbe*. This is probably because they are easier to collect and occur in larger numbers over a wide geographical area.

By extending phytochemical and pharmacological investigations to other related species of *Combretum*, as well as the previously ignored *T. stulhmanii*, this study has been modestly pioneering. It is the first time that a thorough phytochemical investigation has been carried out on *C. collinum* and *C. albopunctatum* and to some extent *C. apiculatum*.

The brief of this project was to investigate the possible occurrence of bioactive secondary metabolites similar to the combretastatins first isolated from *C. caffrum*. This study has shown that such compounds, and/or their biosynthetic derivatives, are present in *C. collinum*, *C. apiculatum* and *C. hereoense*. They were, however, not isolated from *C. albopunctatum*, *C. imberbe* and *T. stulhmanii* which yielded flavonoids in the case of the first plant, and pentacyclic terpenoids in the latter. Why there is a difference in metabolic

products as seen here is not clear and may have to do with the geographical ecology in which the plants are located.

Inevitably, a study such as this one is severely limited by time. It has only managed to scratch the surface and, simultaneously, set the stage for another phase. Further work should have two fundamental motives – first, to gather more phytochemical information on this plant family, and second, to explore further the pharmacological potential of the constituents isolated here. The latter will involve the submission of samples to the NCI Development Therapeutics Program Natural Products Branch (NPB) to screen for anti-tumour activity. The former will be left to future workers with an interest in the phytochemistry, ethnobotany and chemotaxonomy of Southern Africa. It is recommended that they attempt to develop more efficient methods for the fractionation of the polar constituents of Combretaceae increasing the possibility of isolating novel, and possibly unique, phytochemical constituents.

Finally, the chemotherapeutic potential of the Combretaceae has been well established by the pioneering work of Pettit and co-workers during the last two decades and latterly by Fabry *et al* (1996), Sato *et al* (1997) Martini and Eloff (1998). This study suggests that some constituents of this family have potential anti-microbial activity, and might be useful templates for pharmaceutical and/or agrochemical products. It is almost certain however, that pharmaceutical exploitation of the Combretaceae will not directly benefit the indigenous people of the region because of commercial and economic considerations. It is proposed here that work into the possible botanical formulation of standardized crude extracts for use as phytomedicines be seriously considered as an off-shoot of this

study. This would undoubtedly go a long way in improving the health-care of the majority of us.

CHAPTER EIGHT

8.0. BIBLIOGRAPHY

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APPENDIX
SUPPLEMENTARY CRYSTALLOGRAPHIC DATA

Appendix 1.1. Hydrogen Atom Positions and Isotropic Displacement Parameters

Atom	x	y	z	Ueq[Å]
H1	0.75250	0.41660	0.80460	0.0270
H3	0.84450	0.18880	0.91560	0.0300
H2	1.03830	0.25250	0.74430	0.0280
H3	0.67510	1.03440	0.74880	0.0460
H4	1.00530	0.38400	0.88140	0.0300
H7	0.79000	0.44100	0.38030	0.0500
H8	0.88790	0.89210	0.77130	0.0340
H10	0.55120	0.81800	0.69620	0.0320
H12A	1.15630	0.80510	0.74070	0.0610
H12B	1.21370	0.70280	0.81670	0.0610
H12C	1.05640	0.79820	0.84110	0.0610
H13A	0.44980	0.64760	0.71070	0.0480
H13B	0.51260	0.54820	0.64330	0.0480
H13C	0.54150	0.68580	0.61100	0.0480
H17	0.53570	0.35610	0.56090	0.0350
H19	0.97820	0.35180	0.45120	0.0350
H21A	0.36730	0.36220	0.69790	0.0660
H21B	0.34710	0.24730	0.77580	0.0660
H21C	0.41110	0.22440	0.67440	0.0660
H22A	1.20330	0.24180	0.47360	0.0820
H22B	1.28230	0.25910	0.54460	0.0820
H22C	1.16520	0.37270	0.50750	0.0820
H24	1.06470	0.21410	1.01050	0.0430
H25	1.27970	0.08510	1.04530	0.0520
H26	1.41860	-0.07030	0.96090	0.0530
H27	1.34170	-0.09660	0.84180	0.0510
H28	1.12650	0.03250	0.80630	0.0420
H30	0.66130	0.29180	1.00420	0.0370
H31	0.48910	0.38330	1.12160	0.0400
H32	0.51920	0.56210	1.15810	0.0420
H33	0.71850	0.65250	1.07340	0.0480
H34	0.89090	0.56300	0.95520	0.0410

Appendix 1.2 - (An)isotropic Displacement Parameters (Uij)

Atm	U(1,1)	U(2,2)	U(3,3)	U(2,3)	U(1,3)	U(1,2)
O1	0.0266(10)	0.0267(9)	0.0325(10)	-0.0029(7)	-0.0039(8)	-0.0033(8)
O2	0.0314(10)	0.0302(10)	0.0520(12)	-0.0093(8)	-0.0210(9)	-0.0050(8)
O3	0.0370(11)	0.0200(9)	0.0616(13	-0.0112(8)	-0.0203(9)	-0.0009(8)
O4	0.0282(10)	0.0215(9)	0.0399(10	-0.0070(7)	-0.0153(8)	-0.0034(7)
O5	0.0430(11)	0.0234(9)	0.0327(10)	0.0002(8)	-0.0135(8)	-0.0121(8)
O6	0.0254(10)	0.0373(10)	0.0277(10)	-0.0017(7)	-0.0051(8)	-0.0081(8)
O7	0.0355(11)	0.0605(12)	0.0312(11)	0.0145(9)	-0.0142(9)	-0.0228(9)
O8	0.0237(10)	0.0608(12)	0.0264(10	-0.0042(8)	-0.0051(8)	-0.0062(9)
C1	0.0241(13)	0.0210(12)	0.0244(13)	-0.0026(10)	-0.0081(11)	-0.0047(10)
C2	0.0242(13)	0.0209(12)	0.0234(13)	-0.0035(10)	-0.0058(11)	-0.0028(10)
C3	0.0273(14)	0.0228(13)	0.0243(13)	-0.0016(10)	-0.0062(11)	-0.0079(11)
C4	0.0261(14)	0.0229(13)	0.0257(13)	-0.0026(10)	-0.0062(11)	-0.0076(11)
C5	0.0243(14)	0.0264(13)	0.0229(13)	-0.0037(10)	-0.0095(11)	-0.0057(11)
C6	0.0259(14)	0.0190(12)	0.0224(13)	-0.0005(10)	-0.0052(11)	-0.0043(11)
C7	0.0244(14)	0.0265(14)	0.0292(14)	-0.0029(10)	-0.0084(11)	-0.0064(11)
C8	0.0305(15)	0.0224(13)	0.0357(15)	-0.0051(11)	-0.0104(12)	-0.0084(11)
C9	0.0311(15)	0.0212(13)	0.0316(14)	-0.0034(10)	-0.0064(12)	-0.0046(11)
C10	0.0260(14)	0.0243(13)	0.0302(14)	-0.0024(10)	-0.0094(11)	-0.0046(11)
C11	0.0259(14)	0.0218(13)	0.0225(13)	-0.0026(10)	-0.0045(11)	-0.0093(11)
C12	0.0364(17)	0.0435(16)	0.0522(18)	-0.0174(14)	-0.0181(14)	-0.0100(13)
C13	0.0279(15)	0.0308(14)	0.0409(16)	-0.0070(12)	-0.0155(13)	-0.0047(12)
C14	0.0245(14)	0.0195(13)	0.0265(14)	-0.0071(11)	-0.0052(11)	-0.0001(11)
C15	0.0280(14)	0.0213(13)	0.0252(14)	-0.0046(10)	-0.0076(11)	-0.0062(11)
C16	0.0275(14)	0.0198(12)	0.0260(14)	-0.0042(10)	-0.0034(12)	-0.0065(11)
C17	0.0260(14)	0.0320(14)	0.0309(15)	-0.0006(11)	-0.0090(12)	-0.0112(11)
C18	0.0334(16)	0.0312(14)	0.0273(15)	0.0013(11)	-0.0131(12)	-0.0107(12)
C19	0.0262(15)	0.0394(15)	0.0242(14)	-0.0004(11)	-0.0060(12)	-0.0128(12)
C20	0.0238(14)	0.0304(14)	0.0293(15)	-0.0078(11)	-0.0073(12)	-0.0035(11)
C21	0.0291(16)	0.0614(19)	0.0407(17)	-0.0001(14)	-0.0057(13)	-0.0180(14)
C22	0.0258(16)	0.098(3)	0.0364(17)	-0.0043(16)	-0.0039(14)	-0.0140(16)
C23	0.0265(14)	0.0206(13)	0.0277(14)	0.0023(10)	-0.0066(11)	-0.0054(11)
C24	0.0415(17)	0.0346(15)	0.0291(15)	-0.0036(11)	-0.0125(13)	-0.0022(13)
C25	0.0468(18)	0.0430(17)	0.0453(18)	0.0019(14)	-0.0266(15)	-0.0072(15)
C26	0.0346(17)	0.0323(16)	0.062(2)	0.0089(14)	-0.0236(15)	-0.0031(13)
C27	0.0409(18)	0.0289(15)	0.0521(19)	-0.0060(13)	-0.0126(15)	0.0029(13)
C28	0.0346(16)	0.0294(15)	0.0432(17)	-0.0066(12)	-0.0160(13)	-0.0016(12)
C29	0.0268(14)	0.0237(13)	0.0240(13)	-0.0037(10)	-0.0096(11)	-0.0032(11)
C30	0.0336(15)	0.0285(14)	0.0310(15)	-0.0023(11)	-0.0091(12)	-0.0078(12)
C31	0.0267(15)	0.0394(15)	0.0281(15)	-0.0031(12)	-0.0017(12)	-0.0044(12)
C32	0.0311(16)	0.0410(16)	0.0291(15)	-0.0131(12)	-0.0051(12)	0.0011(13)
C33	0.0445(18)	0.0398(16)	0.0403(17)	-0.0191(13)	-0.0065(14)	-0.0097(14)
C34	0.0332(16)	0.0354(15)	0.0361(16)	-0.0107(12)	-0.0048(13)	-0.0109(12)

Appendix 1.3 - Bond Distances (Å). Esd's in parentheses

O1-C5	1.225(3)	C9-C10	1.394(4)
O2-C7	1.365(3)	C10-C11	1.383(3)
O2-C12	1.431(3)	C14-C15	1.486(3)
O3-C9	1.357(3)	C15-C16	1.409(4)
O4-C11	1.370(3)	C15-C20	1.406(4)
O4-C13	1.432(3)	C16-C17	1.380(3)
O5-C14	1.229(3)	C17-C18	1.385(4)
O6-C16	1.365(3)	C18-C19	1.387(4)
O6-C21	1.428(4)	C19-C20	1.383(3)
O7-C18	1.365(3)	C23-C28	1.393(3)
O8-C20	1.364(3)	C23-C24	1.382(4)
O8-C22	1.432(3)	C24-C25	1.384(4)
O3-H3	0.8399	C25-C26	1.371(3)
O7-H7	0.8402	C26-C27	1.375(4)
O1S-C1S	1.36(4)	C27-C28	1.387(4)
C1-C2	.546(3)	C29-C34	1.397(3)
C1-C4	1.586(3)	C29-C30	1.391(4)
C1-C5	1.504(3)	C30-C31	1.385(3)
C2-C3	1.539(3)	C31-C32	1.377(3)
C2-C14	1.506(4)	C32 -C33	1.376(4)
C3-C23	1.502(4)	C33-C34	1.384(4)
C3-C4	1.557(3)	C1-H1	0.9994
C4-C29	1.504(3)	C2-H2	1.0009
C5-C6	1.482(3)	C3 -H3	0.9995
C6-C7	1.409(3)	C4-H4	0.9999
C6-C11	1.399(4)	C8-H8	0.9500
C7-C8	1.379(3)	C10-H10	0.9493
C8-C9	1.393(4)	C12-H12A	0.9793
C12-H12B	0.9794	C22-H22A	0.9804
C12-H12C	0.9800	C24-H24	0.9508
C13-H13B	0.9802	C25-H25	0.9498
C13-H13C	0.9800	C26-H26	0.9500
C13-H13A	0.9801	C27-H27	0.9494
C17-H17	0.9497	C28-H28	0.9502
C19-H19	0.9500	C30-H30	0.9496
C21-H21A	0.9798	C31-H31	0.9497
C21-H21C	0.9810	C32-H32	0.9497
C21-H21B	0.9807	C33-H33	0.9498
C22-H22B	0.9795	C34-H34	0.9507
C22-H22C	0.9795	C1S-C2S	1.39(4)