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List of abbreviations

APAD: 3-acetylpyrimidine adenine dinucleotide

^{13}C-NMR: carbon-nuclear magnetic resonance

COSY: correlated spectroscopy

DEPT: distortionless enhancement by polarization transfer

DHFR: dihydrofolate reductase

DHODase: dihydroorotate dehydrogenase

DHPS: dihydropteroate synthase

DMSO: dimethylsulfoxide

DPP: dimethylallyl pyrophosphate

EDTA: ethylenediaminotetra-acetic acid

FPIX: ferriprotoporphyrin IX

FV: food vacuole

FVM: food vacuole membrane

HEPES: N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid

HMBC: heteronuclear multiple bond correlation

HMQC: heteronuclear multiple quantum correlation

^{1}H-NMR: proton-nuclear magnetic resonance

IPP: isopentenyl pyrophosphate

IR: infra red

LD_{50}: 50% lethal dose

MP: melting point

MS: mass spectroscopy

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

NBT: nitroblue tetrazolium

NHE: Na^+ /H^+ exchanger

NMR: nuclear magnetic resonance

NOESY: nuclear overhauser effect spectroscopy

PBS: phosphate buffer saline

PES: phenazine ethosulphate

PF: potentiating factor

PHA: phytohemagglutinin

SERCA: sarco/endoplasmic reticulum Ca^{2+}-ATPase

SP: sulphadoxine-pyrimethamine

TLC: thin layer chromatography

TMS: tetramethylsilane

TRIS: N-tris (hydroxymethyl) aminomethane

UV: ultra violet

Chapter 1

Introduction

1 Introduction

1.1 Secondary compounds of plants

Plants produce a large and diverse array of organic compounds. Of these the primary metabolites are needed for normal function and growth, while some compounds appear to have no direct function in growth and development. These substances are known as secondary metabolites, secondary products, or natural products. Unlike primary metabolites, such as chlorophyll, amino acids, nucleotides, simple carbohydrates or membrane lipids, secondary metabolites have no generally recognized roles in the processes of photosynthesis, respiration, solute transport, translocation, nutrient assimilation, and differentiation. Secondary metabolites also differ from primary metabolites in having a restricted distribution in the plant kingdom. That is, particular secondary metabolites are often found in only one plant species or a taxonomically related group of species, whereas the basic primary metabolites are found throughout the plant kingdom (Taiz & Zeiger 1998).

Plant secondary metabolites can be divided into three chemically distinct groups: terpenes, phenolics, and nitrogen-containing compounds (Taiz & Zeiger 1998). Figure 1.1 shows in a simplified form the pathways involved in the biosynthesis of the main secondary metabolite groups and their interconnections with primary metabolism. Terpenes are lipids synthesized from acetyl CoA or from basic intermediates of glycolysis. Phenolic compounds are aromatic substances formed via the shikimic acid pathway or the malonic acid pathway. The nitrogen-containing secondary products, such as alkaloids, are biosynthesised primarily from amino acids.

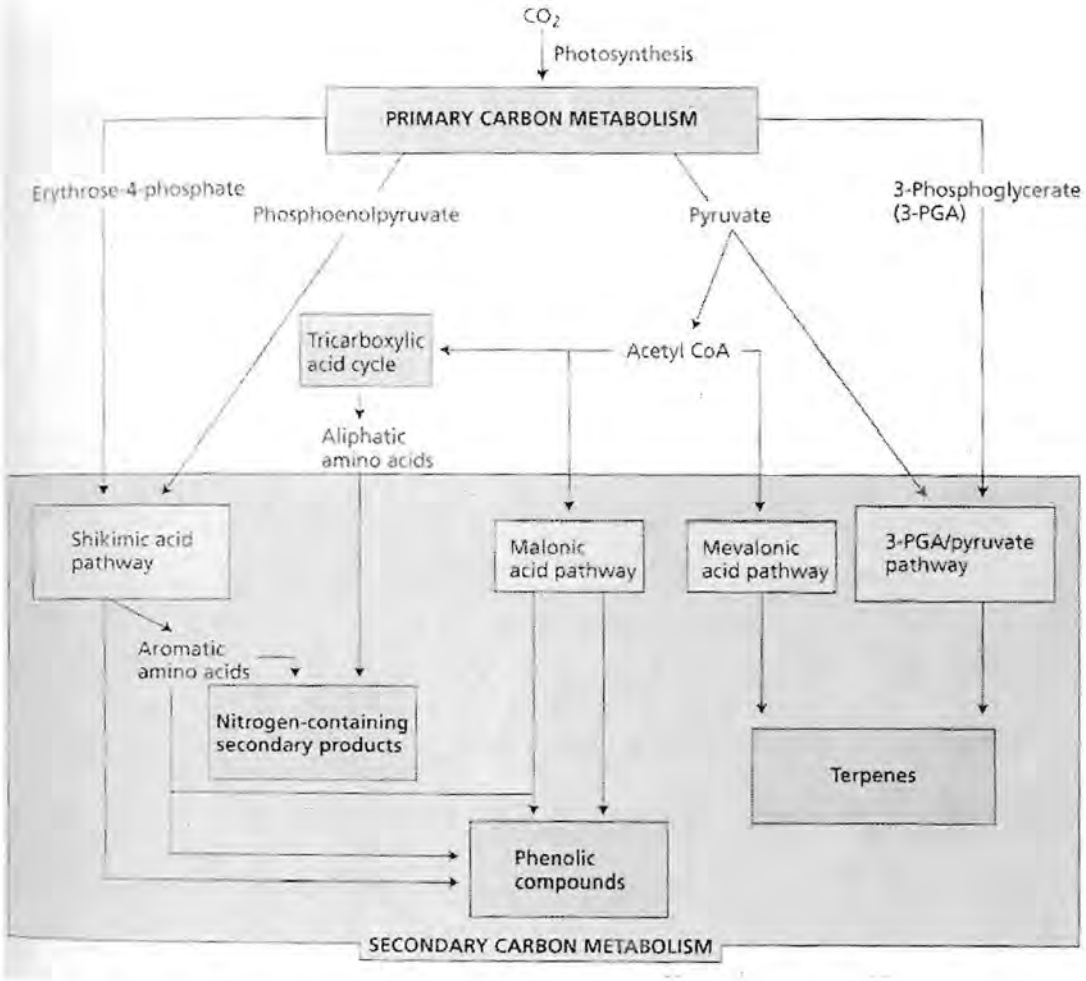
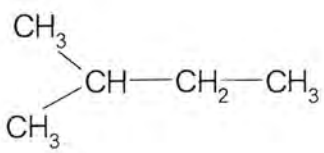


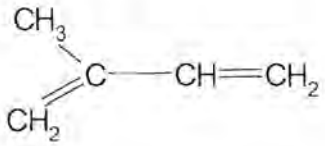
Figure 1.1: The link between primary and secondary plant metabolism (Taiz & Zeiger 1998).

1.1.1 The terpenoids

The terpenes, or terpenoids, constitute the largest class of secondary products. The diverse substances of this class are generally insoluble in water and re-united by their common biosynthetic origin from acetyl CoA or glycolytic intermediates. All terpenes are derived from the union of 5-carbon elements that have the branched carbon skeleton of isopentane:



The basic, structural elements of terpenes are sometimes called isoprene units because terpenes can decompose at high temperatures to give isoprene:



Thus, all terpenes are occasionally referred to as isoprenoids.

Terpenes are classified by the number of five-carbon units they contain, although because of extensive metabolic modifications it is sometimes difficult to pick out the original five-carbon residues. Ten-carbon terpenes, which contain two C_5 units, are called monoterpenes; 15-carbon terpenes (three C_5 units) are sesquiterpenes; and 20-carbon terpenes (four C_5 units) are diterpenes. Larger terpenes include triterpenes (30 carbons), tetraterpenes (40 carbons), and polyterpenoids ($[C_5]_n$ carbons where $n > 10$) (Hopkins & Hüner 2001).

Terpenes are biosynthesised from primary metabolites in at least two different ways (Taiz & Zeiger 1998). In the well-studied mevalonic acid pathway, three molecules of acetyl CoA are joined together stepwise to form mevalonic acid (Figure 1.2). This key six-carbon intermediate is then pyrophosphorylated, decarboxylated, and dehydrated to yield isopentenyl pyrophosphate (IPP). IPP is the activated five-carbon building block of terpenes. Recently it was discovered that IPP could also be formed from intermediates of glycolysis or the photosynthetic carbon reduction cycle via a separate pathway that operates in chloroplasts and other plastids (Lichtenthaler *et al.* 1997) (Figure 1.1). IPP and dimethylallyl pyrophosphate (DPP), are the activated five-carbon building blocks of terpene biosynthesis that join together to form larger molecules (Figure 1.3).

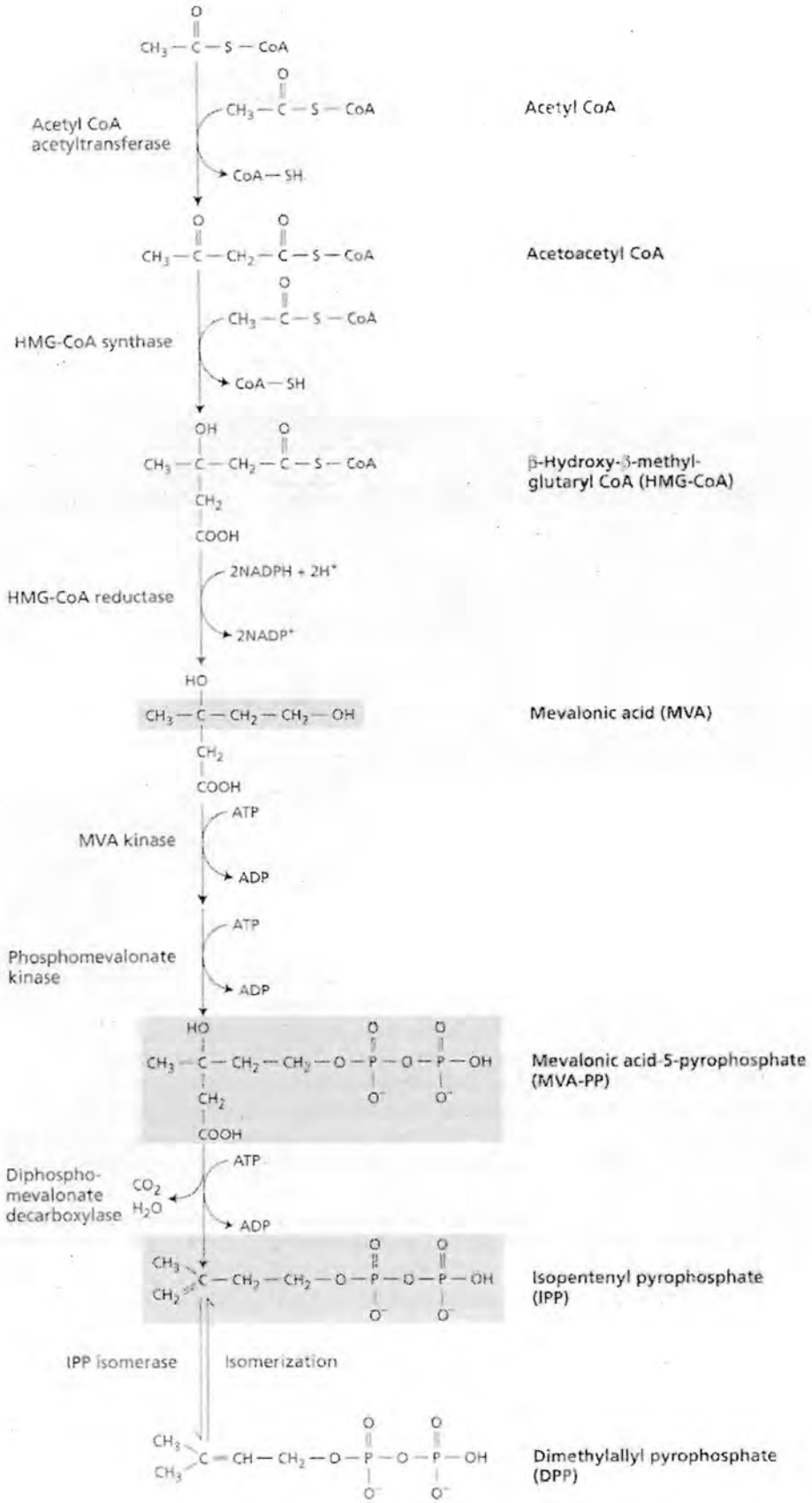


Figure 1.2: The mevalonic acid pathway (Taiz & Zeiger 1998).

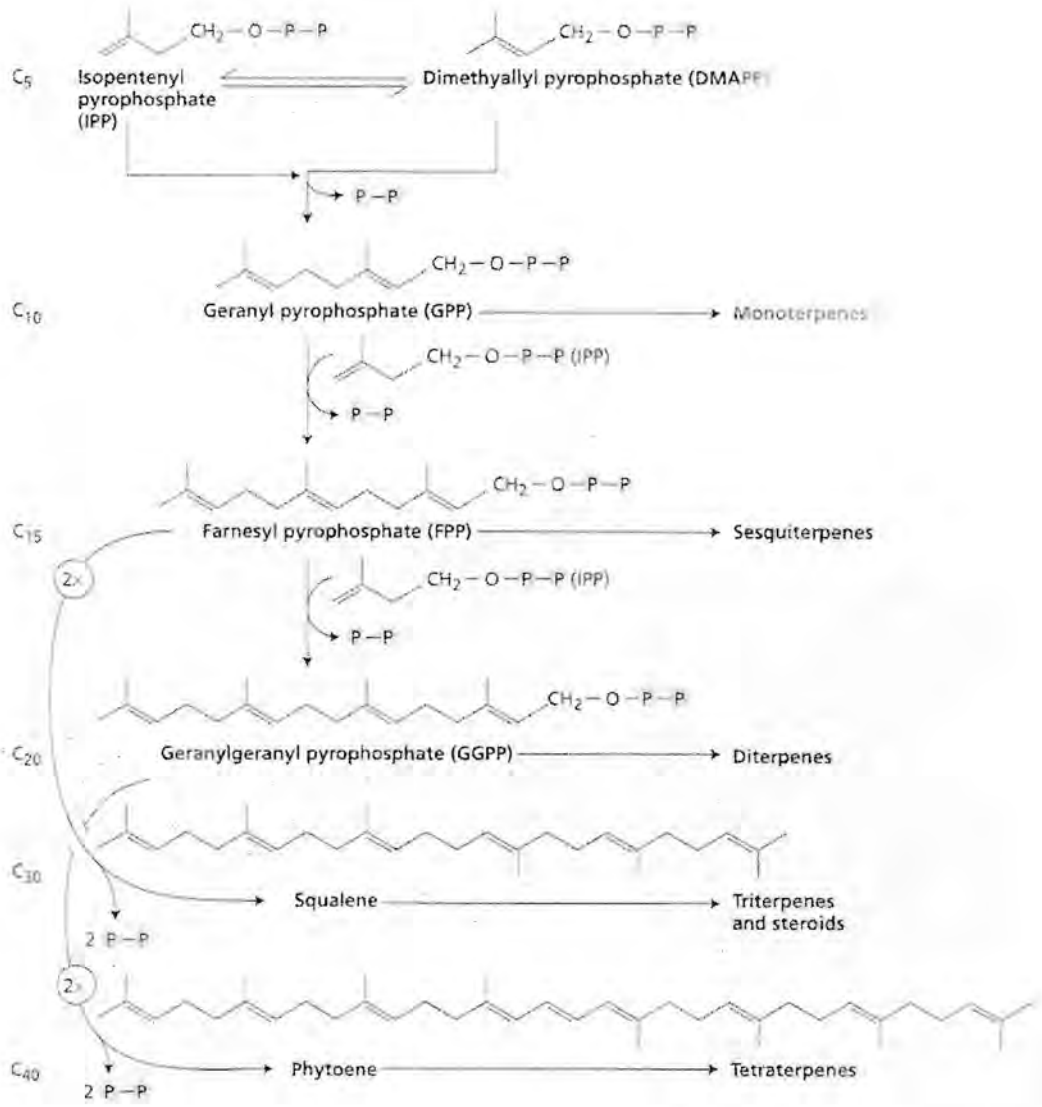


Figure 1.3: The major subclasses of terpenes (Taiz & Zeiger 1998).

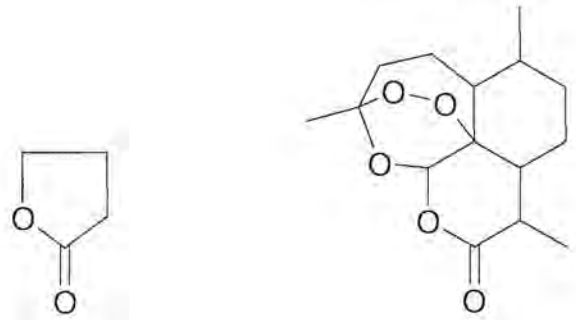
1.1.1.1 Monoterpenes

Many monoterpenes and their derivatives are important agents of insect toxicity. For example, the monoterpene esters called pyrethroids that occur in the leaves and flowers of *Chrysanthemum* spp. show very striking insecticidal activity (Hopkins & Hüner 2001). Both natural and synthetic pyrethroids are popular ingredients in commercial insecticides because of their low persistence in the environment and their negligible toxicity to mammals. Many

plants contain mixtures of volatile monoterpenes, called essential oils that lend a characteristic odour to their foliage. Peppermint and lemon are examples of plants that contain essential oils. They have well-known insect repellent properties and are frequently found in glandular hairs that project outward from the epidermis (Lea & Leagood 1999).

1.1.1.2 Sesquiterpenes

Among the many sesquiterpenes known to be antiherbivore agents are the sesquiterpene lactones found in the glandular hairs of members of the composite family, such as the sunflower. These compounds are characterized by a five or six membered lactone ring, a cyclic ester (Figure 1.4a). The antimalarial drug artemisinin has been isolated from *Artemisia annua* (Figure 1.4b) (Ridley 2002).



a) **b)**

Figure 1.4: a) Lactone ring, b) Artemisinin

1.1.1.3 Diterpenes

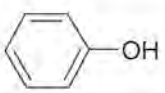
Many diterpenes have been shown to be toxins and feeding deterrents to herbivores. Plants from the Euphorbiaceae family produce diterpene esters of phorbol and other compounds that are skin irritants and internal toxins to mammals. Currently, phorbol-type diterpenes are of great interest as model tumor promoters in studies of carcinogenesis in animals (Vogg *et al.* 1999). Another diterpene, taxol, from the Pacific yew (*Taxus brevifolia*) is a powerful new anticancer drug (Phillipson 2001).

1.1.1.4 Triterpenes

The triterpenes comprise a variety of structurally diverse compounds, including steroids, many of which have been modified to have fewer than 30 carbon atoms. Several steroid alcohols (sterols) are important components of plant cell membranes, while other steroids are defensive secondary products. Several high-molecular-weight polyterpenes occur in plants. Rubber, the best known of these, is a polymer containing 1500 to 15 000 isopentenyl units and is found in numerous plants, but the most commercially important one is the rubber tree, *Hevea brasiliensis* (Taiz & Zeiger 1998).

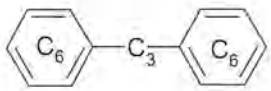
1.1.2 Phenolic compounds

Plants produce a large variety of secondary compounds that contain a phenol group, a hydroxyl functional group on an aromatic ring.



These substances are classified as phenolic compounds. Plant phenolics are a chemically heterogeneous group: Some are soluble only in organic solvents, some are water-soluble carboxylic acids and glycosides, and others are large, insoluble polymers. In keeping with their chemical diversity, phenolics play a variety of roles in the plant. Many serve as defence compounds against herbivores and pathogens. Others function in mechanical support, in attracting pollinators and fruit dispersers, in absorbing harmful ultraviolet radiation, or in reducing the growth of nearby competing plants (Lea & Leegood 1999).

One of the largest classes of plant phenolics is the flavonoids. The basic carbon skeleton of a flavonoid contains 15 carbons in a;



arrangement, with two aromatic rings connected by a three-carbon bridge (Figure 1.5). This structure results from two separate biosynthetic pathways (Figure 1.6) (Taiz & Zeiger 1998). The bridge and one aromatic ring (ring B) constitute a phenylpropanoid unit biosynthesised from phenylalanine, itself a product of the shikimic acid pathway (Figure 1.7). The six carbons of the other aromatic ring (ring A) originate from the condensation of three acetate units via the malonic acid pathway. The fusion of these two parts involves the stepwise condensation of a phenylpropanoid, para-coumaroyl CoA, with three malonyl CoA residues (each of which donates two carbon atoms) in a reaction catalysed by chalcone synthase (Figure 1.8).

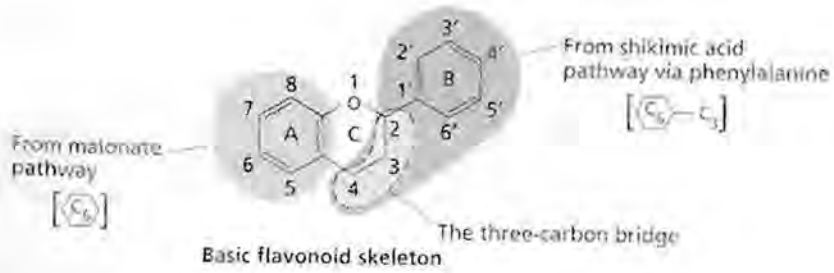


Figure 1.5: Basic flavonoid carbon skeleton (Taiz & Zeiger 1998).

Flavonoids are classified into different groups based primarily on the degree of oxidation of the three-carbon bridge (Figure 1.5 and 1.8). The basic flavonoid carbon skeleton may have numerous substituents. Hydroxyl groups are usually present at positions 4, 5, and 7, but they may also be found at other positions. Sugars are very commonly bound

to flavonoids as well; in fact, the majority of flavonoids exist naturally as glycosides. Whereas both hydroxyl groups and sugars increase the water solubility of flavonoids, other substituents, such as methyl ethers or modified isopentyl units, make flavonoids lipophilic (hydrophobic) (Taiz & Zeiger 1998).

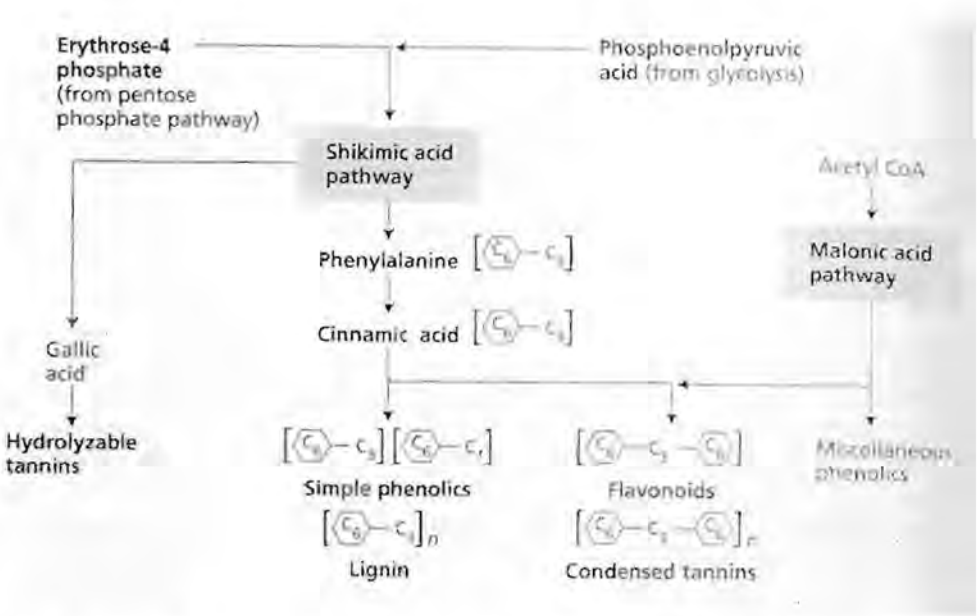


Figure 1.6: Pathways involved in flavonoid biosynthesis (Taiz & Zeiger 1998).

1.1.3 Nitrogen-containing compounds

A large variety of plant secondary metabolites have nitrogen in their structure. Included in this category are well-known antiherbivore defences such as alkaloids and cyanogenic glycosides, which are of considerable interest because of their toxicity to humans and their medicinal properties. Most nitrogenous secondary metabolites are biosynthesised from common amino acids. Nitrogen-containing secondary metabolites include alkaloids, cyanogenic glycosides, glucosinolates, and nonprotein amino acids (Lea & Leagood 1999).

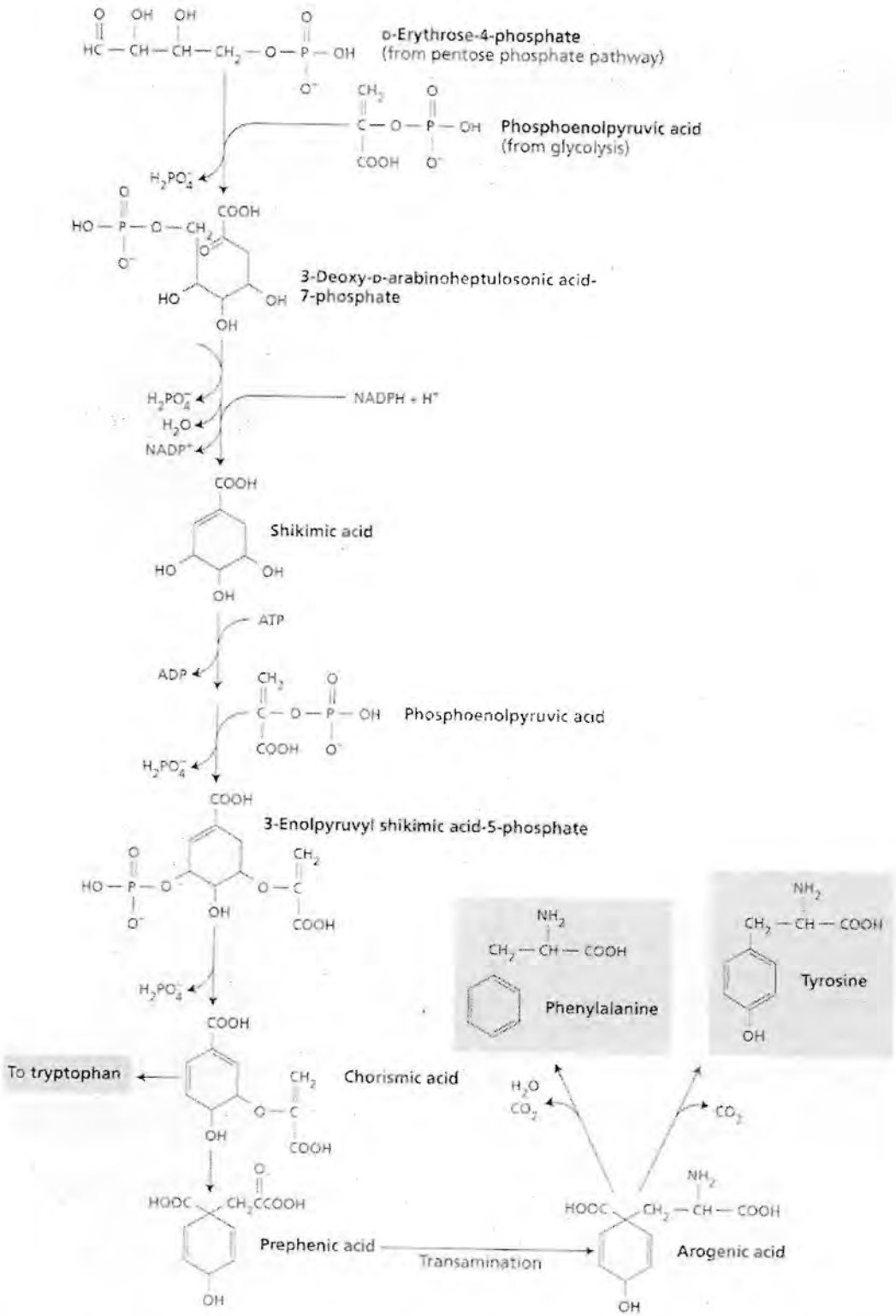


Figure 1.7: Shikimic acid pathway; synthesis of phenylalanine (Taiz & Zeiger 1998).

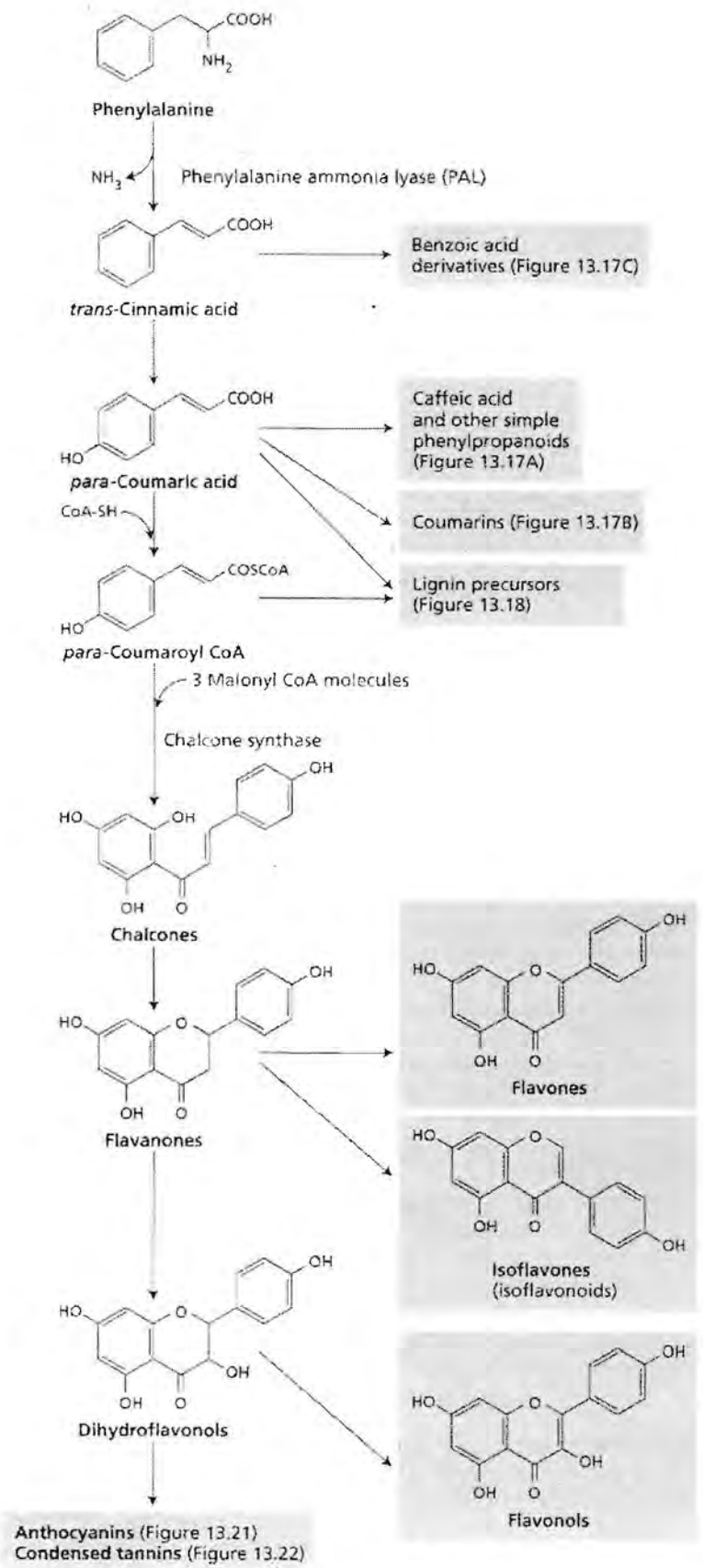
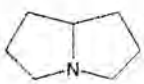
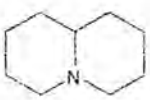
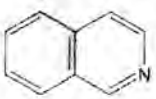
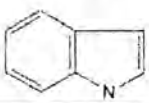


Figure 1.8: Biosynthesis of flavonoids (Taiz & Zeiger 1998).

1.1.3.1 Alkaloids

The alkaloids are a large family of nitrogen-containing compounds found in about 20% of species of vascular plants. They are known for striking pharmacological effects on vertebrate animals. Nearly all alkaloids are toxic to humans when taken in sufficient quantities, although when taken at lower doses many are useful pharmacological agents. Alkaloids are usually synthesised from one of a few common amino acids, in particular, aspartic acid, lysine, tyrosine and tryptophan (Table 1.1) (Taiz & Zeiger 1998).

Table 1.1: Major types of alkaloids, their amino acid precursors and well-known examples of each type (Taiz & Zeiger 1998).

Alkaloid class	Structure	Biosynthetic precursor	Examples
Pyrrolidine		Ornithine	Nicotine
Tropane		Ornithine	Atropine, cocaine
Piperidine		Lysine (or acetate)	Coniine
Pyrrolizidine		Ornithine	Retrorsine
Quinolizidine		Lysine	Lupinine
Isoquinoline		Tyrosine	Codeine, morphine
Indole		Tryptophan	Psilocybin, reserpine, strychnine

1.1.3.2 Cyanogenic glycosides and glucosinolates

Various nitrogenous compounds other than alkaloids are found in plants. Two groups of these substances, cyanogenic glycosides and glucosinolates, are not in themselves toxic but are readily broken down to give off volatile poisons when the plant is crushed. Cyanogenic glycosides release the well-known poisonous gas hydrogen cyanide while glucosinolates release mustard smelling volatiles (Lea & Leagood 1999).

1.1.3.3 Nonprotein amino acids

Many plants contain nonprotein amino acids that are not incorporated into proteins, but are present instead in the free form and act as protective substances. Nonprotein amino acids exert their toxicity by blocking the synthesis or uptake of protein amino acids or can be mistakenly incorporated into proteins (Lea & Leagood 1999).

1.2 Malaria

Malaria remains the most important parasitic disease of humans, effecting populations of tropical and subtropical areas worldwide, as well as an increasing number of travelers to these areas. Four species of *Plasmodium* infects man, *P. vivax, P. malariae, P. ovale* and *P. falciparum*. Of these *P. falciparum* is the most dangerous, as it often leads to death (Wernsdorfer & Payne 1991). The disease affects almost half of the world's population in many countries and kills nearly two million people per year, but the major burden is carried by African nations, where 90% of all deaths from *P. falciparum* occur (Wernsdorfer & Payne 1991). The high levels of morbidity and transmission place considerable strain on public health services and economic infrastructure. In the absence of effective vaccines,

management of the disease has largely depended upon chemotherapy and chemoprophylaxis (Hyde 2002). Of the various antimalarial drugs available the aminoquinoline, chloroquine, was for several decades the agent of choice, as it was safe, effective and cheap. Parasite resistance to this drug was first observed in Thailand in 1957 and then on the border of Columbia and Venezuela in 1959. By the late 1970's it had spread to East Africa and by the mid 1980's had become a major problem in several areas of the continent (Wernsdorfer & Payne 1991). An increasing number of countries have been forced to adopt another class of drug, the antifolates, as the first-line alternative to chloroquine. The most widely used combination of this type consists of pyrimethamine and sulfadoxine, known as Fansidar or SP, which is also cheap and, until recently, was effective against the chloroquine-resistant parasites found in Africa. However resistance to this formulation, long established in parts of South-East Asia and South America (Wernsdorfer 1994), now threatens to leave Africa with no treatment affordable on a mass scale (Figure 1.9). Further combination of antifolates with newer drugs such as the artemisinin derivatives, or the development of alternative combinations, may be the only way to limit the pace of parasite resistance to chemotherapy. For example, the antifolate prodrug proguanil has now been formulated together with a new type of inhibitor, atovaquone, to yield Malarone, recently listed for clinical use (Hyde 2002).

In the treatment of malaria, plants have been the most important source of antimalarial drugs. Even before the cause of the disease was known, local people in South America used to treat it with the bark from *Cinchona* spp. Europeans collected the bark and seeds and this remedy became the standard treatment for malaria for many centuries. Eventually the active component, quinine was isolated some 300 years later and this structure became the basis for synthetic analogues like chloroquine. Recently success with

artemisinin, a new class of antimalarial drug once again stimulated research into plants as sources of antimalarial drugs (Figure 1.10).

Figure 1.9: Antimalarial drug resistance in the world (Ridley 2002).

Research in plant medicines is a very popular field of study today. Many exciting new drugs have been isolated from plant resources in the last few decades. These and the well-known plant-derived drugs have played such an inestimable role in the alleviation of human suffering, that the research into plant medicines should continue (Farnsworth 1990). Today people seek more natural ways of preventing and treating disease. In rural areas many people still rely on traditional medicine for their daily health requirements and plants form the most important part of these medicines. South Africa with a wealth of unexplored plants and a history of traditional plant use, is an ideal place to search for new drugs.

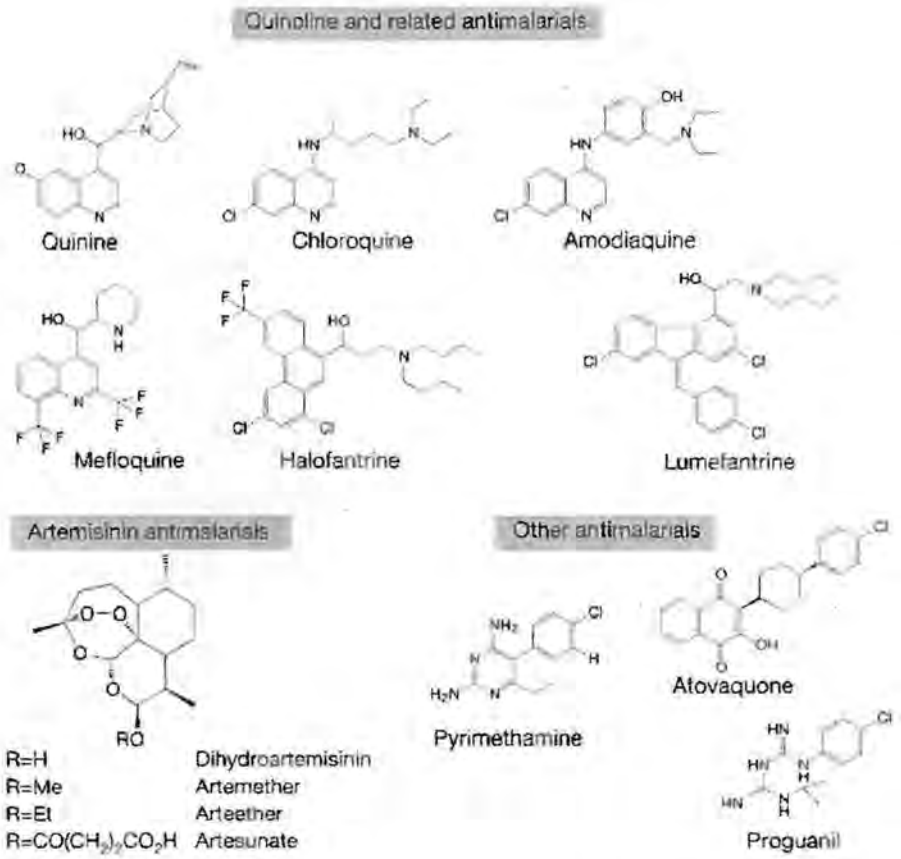


Figure 1.10: Structures of the most widely used antimalarial drugs (Ridley 2002).

1.3 Croton steenkampianus

A previous study on the antiplasmodial activity of South African plants yielded good results on the extract level (Prozesky *et al.* 2001). Several species of the genus *Croton* (Euphorbiaceae) showed excellent antiplasmodial activity in that study. Of the species tested, *C. steenkampianus* Gestner had the best activity in the leaves and was selected for isolation of active principles in this study.

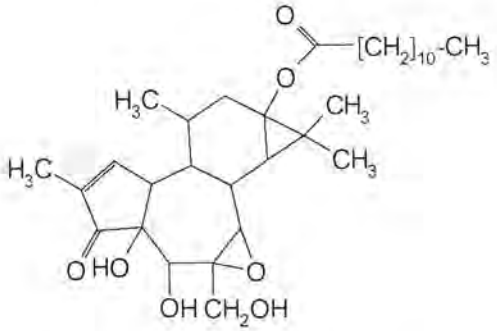
The members of the family Euphorbiaceae are mostly monoecious herbs, shrubs, and trees, sometimes succulent and cactus-like, comprising one of the largest families of plants with about 300 genera and 7500 species that are further characterized by the frequent

occurrence of milky sap (Leistner 2000). Species in the family have a variety of uses and commercial products include rubber (*Hevea*), tung oil (*Aleurites*), castor oil (*Ricinus*), and cassava (*Manohot*) as well as many ornamentals (Leistner 2000).

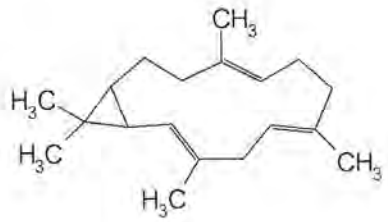
Medicinally the family is used for a wide variety of ailments despite reports of many species being poisonous. South African species are used for ailments like snakebite, chest complaints, headaches, stomach complaints, malaria, sterility, eye complaints, pain, sinusitis, warts and respiratory complaints (Pooley 1993). Chemically the family is diverse with diterpenes well represented (Figure 1.11) (Silva 2002; Halaweish *et al.* 2002).

The genus *Croton* is found all over the world in tropical and subtropical regions. They are mostly shrubs or small trees (Pooley 1993). Various medicinal uses are reported all over the world and many species are used to treat malaria. Of the South African species only one, *Croton megalobotrys* is used for malaria. Other uses of South African species include the treatment of fever, coughs, bleeding gums, rheumatism, chest complaints and indigestion (Pooley 1993). Chemically the genus contains very diverse compound types including alkaloids, flavonoids and triterpenes. Many structurally diverse diterpenes have been isolated from the genus (Figure 1.12).

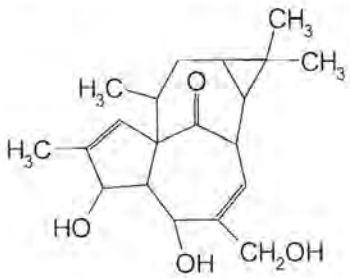
Croton steenkampianus is a shrub to small tree (1.5 - 4 m), found on margins of sand forest and thicket in the eastern parts of South Africa and further north into Africa (Figure 1.13). The main stem is much branched from the base, with smoothish gray bark. Its leaves are large, heart shaped, gray olive green above, white beneath, with a pointed tip (Figure 1.14) (Pooley 1993).



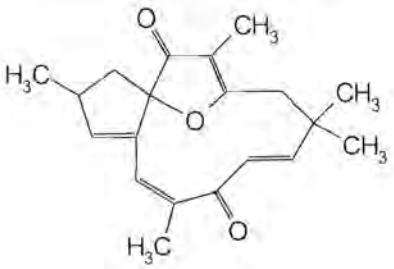
Baliospermin: Tigliane diterpene



Casbene: Casbane diterpene

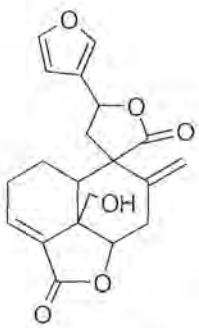


Ingenol: Ingenane diterpene

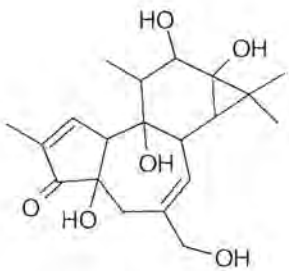


Jatrophone: Jatrophane A diterpene

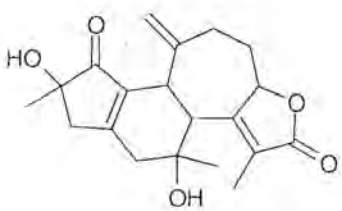
Figure 1.11: Some diterpenes isolated from the Euphorbiaceae (Harborne & Baxter 1993).



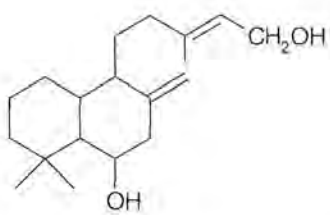
Plaunol B: Clerodane diterpene



Phorbol: Tigliane diterpene



Crotofolin A: Jatropholane diterpene



Crotonadiol: Labdane diterpene

Figure 1.12: Diterpenes from *Croton spp.* (Harborne & Baxter 1993).

Chemically nothing specific is known about the species, other than the general characteristics of the genus. Medicinally very little is known from the literature, the only mention is the use of steam from the fresh leaves to relieve aches (Pooley 1993).

Figure 1.13: Distribution of *Croton steenkampianus* in South Africa (Pooley 1993).

Figure 1.14: *Croton steenkampianus* leaves.

1.4 Objectives

- Isolation of active antiplasmodial compounds from *C. steenkampianus*
- *In vitro* testing of pure compounds for antiplasmodial activity
- Cytotoxicity testing of active compounds
- Chloroquine resistance reversal effects of isolated compound(s)
- Mechanism of action of isolated compounds

1.5 Scope of the thesis

The bio-guided fractionation (antibacterial and antiplasmodial activity) of the leaf extract from *C. steenkampianus* and the isolation and identification of some of the active compounds is described in Chapter 2. Two flavonoids, two new diterpenes with a newly described skeleton, and a triterpene were identified. Their antiplasmodial activity is also described in this chapter. Chapter 3 describes the cytotoxicity of the extract and selected isolated compounds as determined by the lymphocyte cytotoxicity test. Chapter 4 reports on the chloroquine potentiating/reversal effects of the isolated compounds. Chapter 5 deals with the mode of action of the new diterpene, crotrene A, while chapter 6 consists of a general discussion and conclusions.

1.6 Hypothesis

Many *Croton* spp. are used traditionally to treat malaria and it is likely that *C. steenkampianus*, which is not used to treat this disease, also has antiplasmodial activity.

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Chapter 2

Bio-guided fractionation of extract and antiplasmodial activity of isolated compounds

2 Bio-guided fractionation of extract and antiplasmodial activity of isolated compounds

2.1 Introduction

The incidence of malaria, the most important parasitic disease of humans, is increasing in the tropical world. This is directly attributed to the increase and spread of resistant parasites. Since the 1950's we have relied largely on chloroquine, a safe, inexpensive, widely available and once highly effective treatment (Wernsdorfer & Payne 1991). Unfortunately resistance to chloroquine now occurs throughout the tropical world, and resistance to its successor sulphadoxine-pyrimethamine (SP) has followed rapidly. The situation would have been even more critical were it not for the discovery of another plant derived antimalarial drug, artemisinin and its derivatives. The artemisinins are the most powerful and efficient drugs ever discovered for the treatment of malaria. They have a novel mode of action, therefore resistance is unlikely to occur, have few side effects and can be used prophylactically and therapeutically in all forms of malaria (Jansen 2002). With no effective vaccine expected in the near future and total eradication of the vector a near impossible task, the development of effective novel drugs remains a priority. The success of artemisinin has stimulated the continued investigation of plants as sources of novel antimalarial drugs. The search for antimalarial drugs from plants involves the screening of crude extracts against the malaria parasite and thereafter isolation of the active components. Before 1976 this was done only *in vivo*, making it a very expensive and difficult procedure, but since the development of the method for continuous *in vitro* culturing of human malaria parasites (Trager & Jensen 1976), extracts and compounds could be tested *in vitro*, making antimalarial testing much more efficient.

Although *in vitro* antiplasmodial testing is a lot easier than *in vivo* methods, bio-guided isolation of antiplasmodial compounds using *in vitro* methods still remains a long and difficult process. In previous assays on the antiplasmodial activity of fractions, an antibacterial method was investigated to simplify this process. Isolation of antibacterial compounds can be simplified dramatically by direct bioassay on thin layer chromatography (TLC) (Cordell 1995). This approach was repeated with the most active antiplasmodial fractions, with the hypothesis that there might be a correlation between the antibacterial and antiplasmodial activity. Various antibiotics have shown antiplasmodial activity, for example doxycycline and tetracycline. Their mode of action is linked to the plastid, a prokaryotic structure found in *Plasmodium* (Gleeson 2000). Screening for antibacterial activity therefore targets compounds with possible activity against the plastid in *Plasmodium*.

2.3 Materials and Methods

2.2.1 Collection of plant material

Leaves of *Croton steenkampianus* were collected in northern KwaZulu-Natal and various field trips were undertaken to collect plant material. A voucher specimen is preserved in the HGWJ Schweickerdt herbarium at the University of Pretoria (PRU no: 92520).

2.2.2 Methods

2.2.2.1 Preparation of extract

Leaves were collected and left at room temperature for two weeks to dry. Crushed leaves (twigs removed) were placed in a container with acetone (0.2 kg leaves/litre solvent) and macerated with an ultra-turrax. The container was closed and left for a week at room temperature before the extract was filtered under vacuum and dried with a rotary evaporator (Büchi) at a temperature of 40°C. Samples of extracts for antiplasmodial testing were dissolved in DMSO at a stock concentration of 20 mg/ml and stored at 4°C.

2.2.2.2 *In vitro* culturing of malaria parasites

The South African isolate (RB1, chloroquine resistant) of the malaria parasite *Plasmodium falciparum*, was used in the fractionation bioassays (Stoltz 1992). For continuous *in vitro* culturing a slightly modified version of the Trager and Jensen method was employed (Trager & Jensen 1976; Hoppe 1993). The wash medium consisted of 10.4 g RPMI 1640 L-glutamine, 5.94 g HEPES buffer, 4.0 g D-glucose, 44 mg hypoxanthine, 5% sodium hydrogen carbonate and 4 mg of gentamycin dissolved in 900 ml deionised sterile water. For use as culture medium, this wash medium was supplemented with 5% Albumax II (Generous gift of Prof. P. Smith, Department of Pharmacology, University of Cape Town).

The malaria-isolates were stored in liquid nitrogen for long-term storage. The cryotube with the isolate (about 5% parasitemia) to be thawed was removed from the liquid nitrogen and quickly thawed in a water bath at 37°C. The contents of the cryotube were transferred under sterile conditions to a 10 ml centrifuge tube (~1 ml) and 0.2 ml sterile 12% NaCl was slowly added to the thawed culture and mixed well (~10-20 sec.). To this 1.8 ml of a sterile 1.6% NaCl solution was then slowly added, mixed well (~10-20 sec). The solution was then centrifuged (2500g, 5min) and the supernatant was removed. The parasite

culture was then suspended in 10 ml of culture medium, in a 75 ml culture flask (Sterilin). The culture, consisting of parasites and culture medium was then further supplemented with fresh, uninfected human erythrocytes with an O^+ blood group.

Erythrocytes for maintenance of the culture were obtained from whole blood which was centrifuged in a Hermle Z 320 bench centrifuge at 500 g for 5 minutes. The plasma portion as well as the leukocyte buffy coat was removed. The erythrocytes were then suspended in the wash medium and centrifuged at 500 g for 5 minutes. The supernatant was removed and the procedure repeated three times. Washed erythrocytes were then stored in 10 ml wash medium at 4°C, for up to 2 weeks.

The culture's hematocrit was adjusted to about 5% by adding washed erythrocytes to the parasite-culture medium. The culture flask was then filled with a special gas mixture consisting of 5% oxygen, 5% carbon dioxide and 90% nitrogen before being incubated at a constant temperature of 37°C. Culture medium was exchanged daily while the hematocrit was continually maintained at 5% by adding fresh cells at least every 2-3 days.

2.2.2.2.1 Giemsa-stained thin blood smear preparation

A drop of parasite culture was placed on a microscope slide close to the frosted edge. This drop was then smeared across the length of the slide with a second slide held at an angle and allowed to dry. Methanol (analytical grade) was used to fixate the blood smear and allowed to stand for ca. 1 minute, after which the methanol was removed by decanting. The DNA intercalator Giemsa (Sigma) was used to stain the parasite DNA (Wright 1984). Giemsa stain was formulated in glycerol and methanol as indicated by the supplier. A phosphate

buffer, containing 9.5 g/l sodium dihydrogen phosphate and 9.2 g/l disodium hydrogen phosphate at a pH of between 6.8 and 7.2, was used to dilute the Giemsa solution. Two drops of Giemsa solution were added for every 1 ml of phosphate buffer. The slide was covered with this solution for ca. 5 minutes and then allowed to air dry. A drop of microscope immersion oil was placed on the slide and it was viewed under the oil immersion 100x objective of a Nikon light microscope.

2.2.2.2.2 *In vitro* synchronisation of malaria parasites

The method described by Vernes *et al.* (1984) and modified by Hoppe (1993) was used for the *in vitro* synchronisation of malaria parasites. Synchronisations were performed on malaria cultures consisting of ca. 80% ring-phase parasites. The cultures were transferred from the culture flasks to 50 ml centrifuge tubes, centrifuged, at 500 g for 5 minutes and the supernatant removed. The pellet volume was about 0.5 ml when 10 ml of a parasite culture (5% hematocrit) was centrifuged. Hereafter 4 ml of a 15% D-sorbitol solution was added to every 0.5 ml of parasite pellet. After careful mixing, by tube inversion, the solution was incubated at 37°C for 5 minutes, where after, 8 ml of a 0.1% D-glucose solution was added per 0.5 ml of parasite pellet, followed by mixing by tube inversion. The parasite solution was again incubated at 37°C for 5 minutes, centrifuged at 500 g for 5 minutes and the supernatant containing lysed erythrocytes, was removed. The pelleted ring-phase-infected and uninfected erythrocytes were resuspended in 10 ml of culture medium and returned to the culture flasks. The hematocrit was adjusted to about 5% by adding washed erythrocytes, filled with the special gas mixture as described in 2.2.2.2 and returned to the incubator. This procedure was repeated until the cultures consisted of about 95% ring-phase parasites.

2.2.2.2.3 Preparation of microculture plates

Microculture plates were prepared in the same way for all the antiplasmodial bioassays. Final concentrations of extract/compounds were made by appropriate dilutions of stock solutions with culture medium. 20 µl extract/compound at different concentrations were added to the flat-bottomed wells of a 96-well microculture plate (Flow), as well as 80 µl of a 5% hematocrit of 0.5 - 1.0% parasitised cells (95-100% rings) in supplemented RPMI 1640 medium. Microcultures were incubated for 48 hours in a modular incubation chamber (Flow) at 37°C in a gas mixture of 5% oxygen, 5% carbon dioxide and 90% nitrogen for assessment of antiplasmodial activity. After 24 hours, 50 µl of medium from each well was removed and replaced by 10 µl of extract/compound and 40 µl of fresh culture medium.

2.2.2.2.4 Malstat method

To determine the activity of extracts against *P. falciparum* in a preliminary *in vitro* assay, a slightly modified version of parasite lactate dehydrogenase assay was used (Makler *et al.* 1993). The experiment was done in triplicate at 1% parasitemia and 5% hematocrit and plates prepared as described in 2.2.2.2.3. After 48h, a duplicate 96-well plate was prepared by adding 100 µl Malstat reagent [133 ml Triton X-100, 1.33 g lactate, 0.44 g TRIS buffer and 44 mg 3-acetylpyrimidine adenine dinucleotide (APAD) made up to 200 ml] to each well together with 25 µl developing dye solution [160 mg nitroblue tetrazolium (NBT) and 8 mg phenazine ethosulphate (PES) to 100 ml Millipore water] and 10 µl from the incubated plate. The duplicate plate was incubated for 20 min in the dark and read with an ELISA plate reader at 620 nm.

2.2.2.2.5 Strains used for flow cytometric antiplasmodial tests

The South African isolates (RB1, chloroquine resistant and FAB9, chloroquine sensitive) of the malaria parasite *P. falciparum*, were used for determination of IC_{50} values (Stoltz 1992). For continuous *in vitro* culturing the methods describe in 2.2.2.2 were followed, using Flow cytometry as assay method. Two independent experiments were done in triplicate to determine the antiplasmodial activity.

2.2.2.2.6 Flow cytometric analysis

To determine the activity of extracts against *P. falciparum* in an accurate *in vitro* assay, the flow cytometric method of (Schulze *et al.* 1997) was used. Samples of cultures with extracts or compounds, as well as controls were stained using thiazole orange. The flow cytometer was programmed to have three electronic gates, each of which counts the erythrocytes of a different fluorescence intensity. All uninfected erythrocytes were counted in gate 1, which covers the region near zero fluorescence intensity. Gate 2 counts ring-infected erythrocytes, which have a fluorescence intensity lower than that of the later-phase parasites and gate 3 counted trophozoite- and schizont-phase infected erythrocytes, which showed the highest fluorescence intensity. The percentage of parasites present in the ring-phase or later phases could then be determined.

2.2.2.2.6.1 Flow cytometric analysis of fixed parasite cultures

Parasites in the 96-well plates were pre-fixed by adding fixing solution in a 1:1 ratio after which they were incubated at 4°C for at least 18 hours. The fixing solution consisted of 10% formaldehyde and 4% D-glucose formulated in a Tris-saline buffer (10 mM Tris, 150 mM sodium chloride and 10 mM sodium azide). The final pH was adjusted to 7.3 using sodium hydroxide. The adjustment of the pH was important in preventing lysis of the erythrocytes. After incubation at 4°C for 18 hours or longer, 50 µl of fixed parasite culture was added to 1 ml phosphate buffer saline (PBS) containing 0.25 µg thiazole orange, in plastic tubes (Corning). This amount of thiazole orange was used to ensure that there was sufficient DNA intercalating dye available, even at higher parasitemias. The parasite-PBS-thiazole orange solution was mixed carefully, by inverting the tube 2-3 times, and incubated at ambient temperature, in the dark, for 1 hour. The samples were then placed on ice to stop further staining of the parasite DNA prior to flow cytometric analysis. A volume of 200 µl of prepared parasite sample was analysed by the flow cytometer and a total of 100 000 erythrocytes were counted in each sample (Prozesky *et al.* 2001).

2.2.2.3 Antibacterial testing

The extract as well as the most active antiplasmodial fractions (7-9 and 11) was tested for antibacterial activity by direct bioautography on TLC plates. The extract and fractions (± 5 µl) were applied to silica gel 60 plates (Merck) and developed in chloroform: ethyl acetate (6:1). The TLC plate was observed under ultra violet (UV) light (254 and 366 nm) after

development, left overnight for the solvent to evaporate completely and sprayed with the bacterial suspension prepared as described below.

Staphylococcus aureus was collected from the Department of Microbiology and Plant Pathology, University of Pretoria. The bacteria were maintained on nutrient agar slant and were recovered for testing by growth in nutrient broth (No.2, Biolab) for 24 hours at 37°C. The culture was then centrifuged at 3000 rpm for 20 minutes. The supernatant was discarded and the sedimented bacteria resuspended in fresh nutrient broth to an absorbance of 0.84 at 560 nm with a spectrophotometer (Lund & Lyon 1975). The bacterium suspension was sprayed with a fine spray onto the TLC plates. These plates were then dried for a few minutes until they appeared translucent and incubated at 25°C for 24 hours in humid conditions. The plates were then sprayed with an aqueous solution of 2.0 mg/ml *p*-iodonitrotetrazolium violet (Sigma) and reincubated at 25°C for 3 hours. Any inhibition of bacterial growth could clearly be seen as white spots on a red background.

2.2.2.4 Isolation and identification of compounds

A 10 cm diameter glass column (30 cm length, 5 cm diameter) was filled with 500 g of silica gel 60 (Merck) (column A). The extract sample was dissolved in a minimal amount of solvent and mixed with silica gel. This mixture was air dried and applied to the column. The column was developed with a solvent gradient of hexane: ethyl acetate in a 100:0 to 0:100 ratio (50 ml fractions collected). Fractions containing the same compounds as determined by TLC on silicagel 60 (Merck), were combined and each of the pooled fractions concentrated to dryness under reduced pressure. Fractions were spotted on a TLC plate and then developed. TLC plates were viewed under UV light (254 and 366 nm) after

development and also dipped in vanillin reagent (15 g vanillin, 500 ml ethanol and 10 ml concentrated 98% sulfuric acid) and heated to detect compounds not absorbing UV light. Each pooled fraction was tested for antiplasmodial activity and most active fractions further purified. Figure 2.1 shows the isolation procedure.

Fraction A6 crystallized and crystals were collected for identification. Triterpene 1 was identified by mass spectroscopy (MS) after comparison, to MS-data from a database and to a standard on TLC.

Combined chlorophyll fractions (fraction A9) collected from silica gel column A had good antiplasmodial activity. For easier isolation of the active compounds from fraction A9, the chlorophyll was removed with activated charcoal. Granular charcoal was powdered with an IKA dry mill and added to the fraction in ethyl acetate and shaken. Enough charcoal was added to remove all the green colour from the fraction. The fraction was then filtered and the charcoal repeatedly rinsed with ethyl acetate to wash the non-aromatic compounds from the charcoal. The collected liquid was dried under reduced pressure and used for further isolation. A 2.5 cm diameter glass column (30 cm long) was filled with 100g of silica gel (Column B). The extract sample was dissolved in a minimal amount of solvent and mixed with silica gel. This mixture was air dried and applied to the column. A solvent gradient of chloroform: methanol was applied to the column in 99:1 to 98:2 ratios (5 ml fractions collected). Fractions containing the same compounds as determined by TLC, were combined and each of the pooled fractions concentrated to dryness under reduced pressure. Fractions were spotted on a TLC plate and then developed. TLC plates were viewed under UV light (254 and 366 nm) after development and also dipped in vanillin reagent

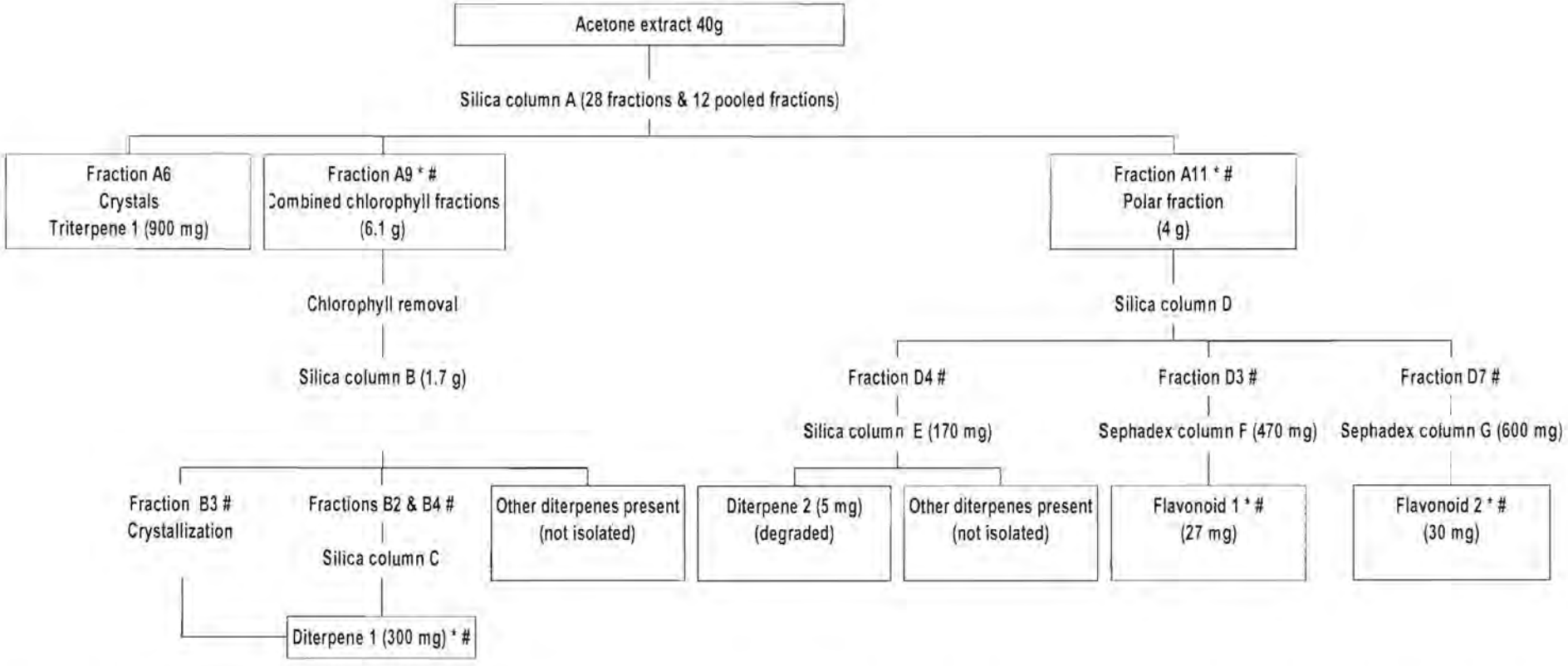


Figure 2.1: Schematic presentation of isolation steps followed. * antiplasmodial guided fractionation. # antibacterial guided fractionation.

and heated to detect compounds not absorbing UV light. Fraction B3 from silica column B crystallized after solvent evaporation and a compound (diterpene 1) was isolated.

Fractions B2 and B4 were combined and further purified on a silica gel column (column C). A 2.5 cm diameter glass column (30 cm long) was filled with 100 g of silica gel. The silica gel was deactivated with 10 ml of water by mixing it with the silica gel and left to dry. The extract sample was dissolved in a minimal amount of solvent and mixed with silica gel. This mixture was air dried and applied to the column. A solvent gradient of Hexane: Ethyl acetate 8:2 was applied to the column (10 ml fractions collected). Fractions containing the same compounds as determined by TLC, were combined and each of the pooled fractions concentrated to dryness under reduced pressure. Fraction C8 contained crystals of diterpene 1 after evaporation of the solvent. Other fractions contained a mixture of compounds. Diterpene 1 was identified with MS, infra red (IR), proton-nuclear magnetic resonance (^{1}H-NMR), carbon-nuclear magnetic resonance (^{13}C-NMR), nuclear overhauser effect spectroscopy (NOSEY), heteronuclear multiple bond correlation (HMBC), distortionless enhancement by polarization transfer (DEPT) 135, correlated spectroscopy (COSY) 45, heteronuclear multiple quantum correlation (HMQC) and X-ray crystallography. Nuclear magnetic resonance (NMR) data were obtained at 300 MHz on a Bruker ARX 300 NMR spectrometer using $CDCl_3$ as solvent with tetramethylsilane (TMS) as internal standard. X-ray crystallography data were obtained at room temperature on an Enraf Nonius CAD 4 automated single crystal X-ray diffractometer.

Fraction A11 (polar fraction) was further purified with a silica gel column (column D). A 2.5 cm diameter glass column (30 cm long) was filled with 100 g of silica gel. The

extract sample was dissolved in a minimal amount of solvent and mixed with silica gel. This mixture was air dried and applied to the column. A solvent gradient of hexane: ethyl acetate was applied to the column in 8:2 to 6:4 ratios (20 ml fractions collected). Fractions containing the same compounds as determined by TLC, were combined and each of the pooled fractions concentrated to dryness under reduced pressure. Fractions were spotted on a TLC plate and then developed. TLC plates were viewed under UV light (254 and 366 nm) after development and also dipped in vanillin reagent and heated to detect compounds not absorbing UV light.

Fraction D3 and D7 were further purified by Sephadex column chromatography (columns F & G). The glass columns (50 cm length, 1.25 cm diameter) were loaded with Sephadex LH-20 (Sigma) by means of slurry packing with methanol as eluent. The columns were left to settle for at least an hour before fraction D3 or D7 were added. Fractions (5 ml) were collected and flavonoid 1 and 2 isolated and identified with ^{1}H-NMR spectra compared to published data (Mabry *et al.* 1970). NMR data were obtained at 300 MHz on a Bruker ARX 300 NMR spectrometer using $CDCl_3$ as solvent with TMS as internal standard.

Fraction D4 was further purified on silica column E. A 2.5 cm diameter glass column (30cm long) was filled with 100 g of silica gel. The extract sample was dissolved in a minimal amount of solvent and mixed with silica gel. This mixture was air dried and applied to the column. A solvent mixture of hexane: ethyl acetate was applied to the column in a 8:2 ratio. Fractions (5 ml) were collected and diterpene 2 isolated and identified with ^{1}H-NMR (at 300 MHz on a Bruker ARX 300 NMR spectrometer using $CDCl_3$ as solvent with TMS as internal standard) spectra and in comparison with data obtained from diterpene 1.

2.3 Results & Discussion

2.3.1 Fractions

From the first silica gel column 12 pooled fractions were collected and tested against the malaria parasite with the Malstat method. A typical result of this method is shown in Figure 2.2. Of the fractions tested four showed good antiplasmodial activity (Table 2.1). Fractions A1-5 and A12 had weak antiplasmodial activity, A6 had moderate antiplasmodial activity, while fractions A7-9 and A11 had good activity with a higher than 50% inhibition at 10 μg/ml. Fraction A9 and A11 were further purified by bio-guided fractionation. For this an antibacterial method on TLC provided an easier way of isolation.

Figure 2.2: A typical result obtained with the Malstat method. Dark coloured wells indicate normal parasite growth. Light coloured wells indicate little or no parasite growth. a) Inactive fraction, b) Active fraction c) Positive control (chloroquine) d) Negative control (culture medium).

Results obtained from the antibacterial testing correlated with the most active antiplasmodial fractions and this method was used in further purification of fraction A9 and A11. Purified fractions showed some antibacterial activity, but not significantly (Figures 2.3-2.5). Isolated compounds showed no antibacterial activity. The antibacterial activity

observed in some fractions in possibly due to other compounds not isolated or synergistic effects. Although isolated compounds had no significant antibacterial activity, using antibacterial bio-guided fractionation simplified the isolation process of antiplasmodial compounds.

Table 2.1: Antiplasmodial activity (Resistant strain RB1) as obtained with the Malstat method against pooled fractions A1-12 collected from silica column A.

Fraction number	% Inhibition at 10 μg/ml
A1-5, 12	20 (±3.8)[a]
A6	35 (±5.7)
A7, 8	50 (±7.3)
A9, 11	65 (±8.5)

[a] **standard deviation**

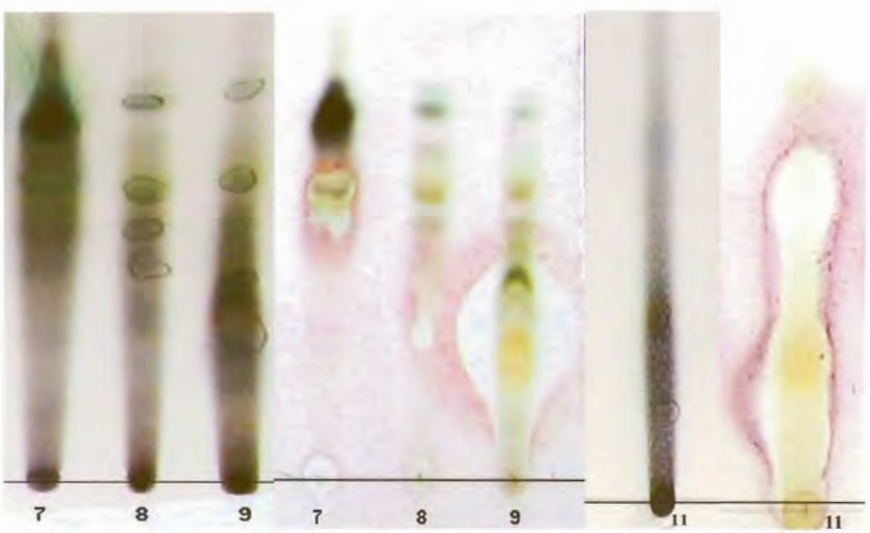


Figure 2.3: Fractions from silica column A tested for antibacterial activity. a and b) TLC plates developed with hexane:ethyl acetate (2:1). c and d) Plates developed with hexane:ethyl acetate (1:1). b and d) Antibacterial activity. White zones indicate activity.

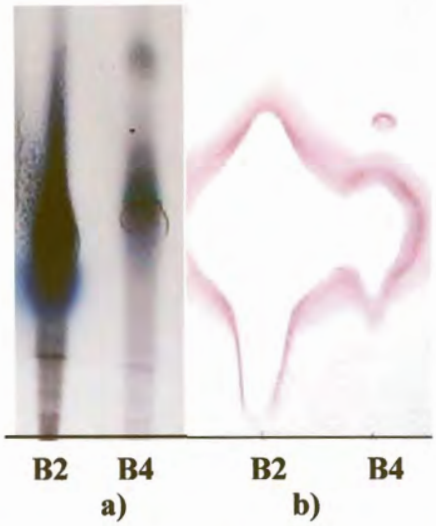


Figure 2.4: Purified fractions of diterpene 1. a) TLC plate sprayed with Vanillin reagent. Solvent hexane: ethyl acetate (2:1). b) Antibacterial activity indicated by white zones.

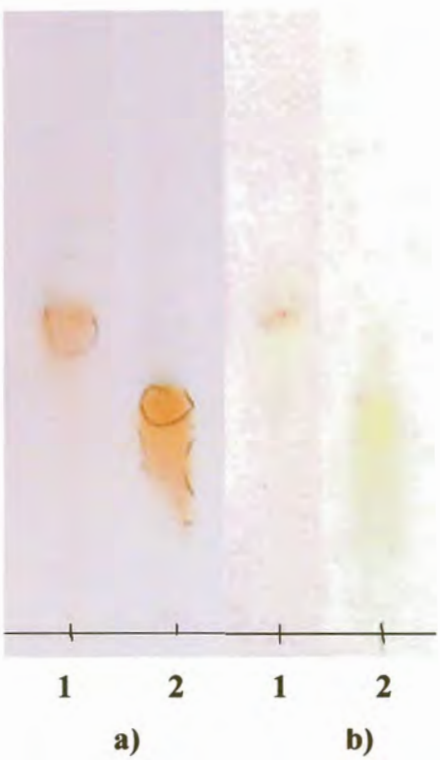


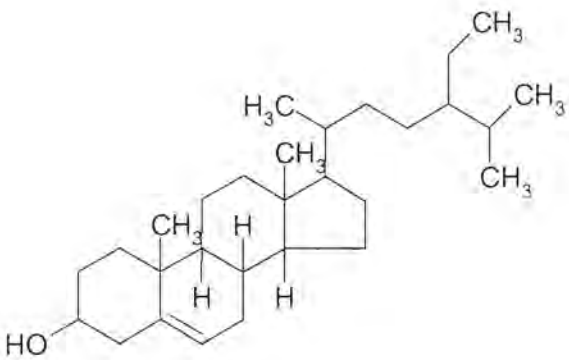
Figure 2.5: Two flavonoids isolated. a) TLC plate sprayed with Vanillin reagent. Solvent hexane: ethyl acetate (2:1) 1= Flavonoid 1, 2 = Flavonoid 2 b) Antibacterial activity (not significant).

2.3.2 Isolated compounds

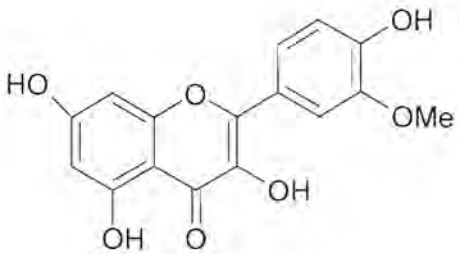
Structures of the isolated compounds are shown in figure 2.6.

2.3.2.1 Triterpene

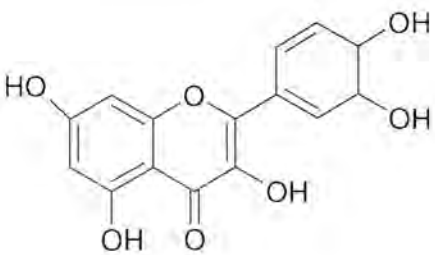
Fraction A6 crystallized and was identified as β-sitosterol with MS when compared to database MS-data and spotted on TLC with a β-sitosterol standard. (Figures 2.7-2.8).



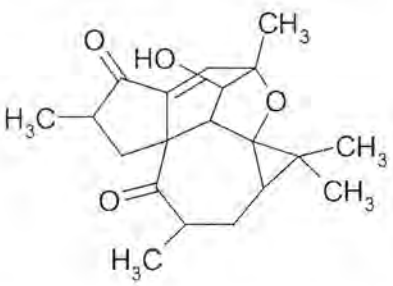
Triterpene 1: β-sitosterol



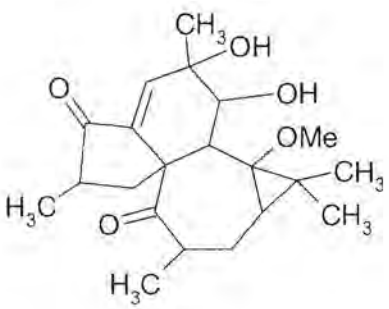
Flavonoid 1: Tamarixetin



Flavonoid 2: Quercetin



Diterpene 1: 6a,14b-epoxy-7b-hydroxy-4-crotrene-3,10-dione (Crotrene A)



Diterpene 2: 6a,7b-hydroxy-14b-methoxy-4-crotrene-3,10-dione (Crotrene B)

Figure 2.6: Structures of the isolated compounds.

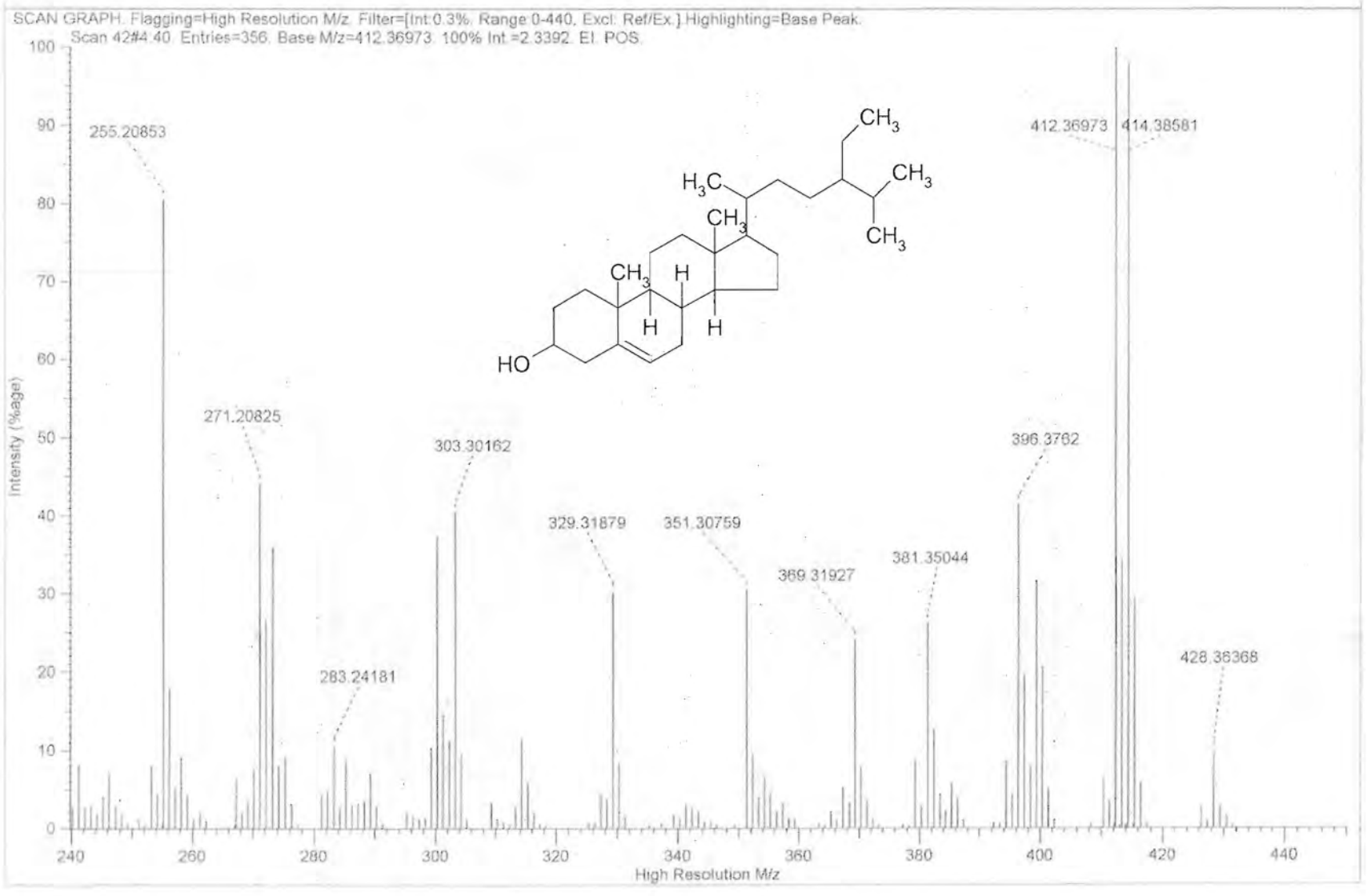


Figure 2.7: MS data from triterpene 1.

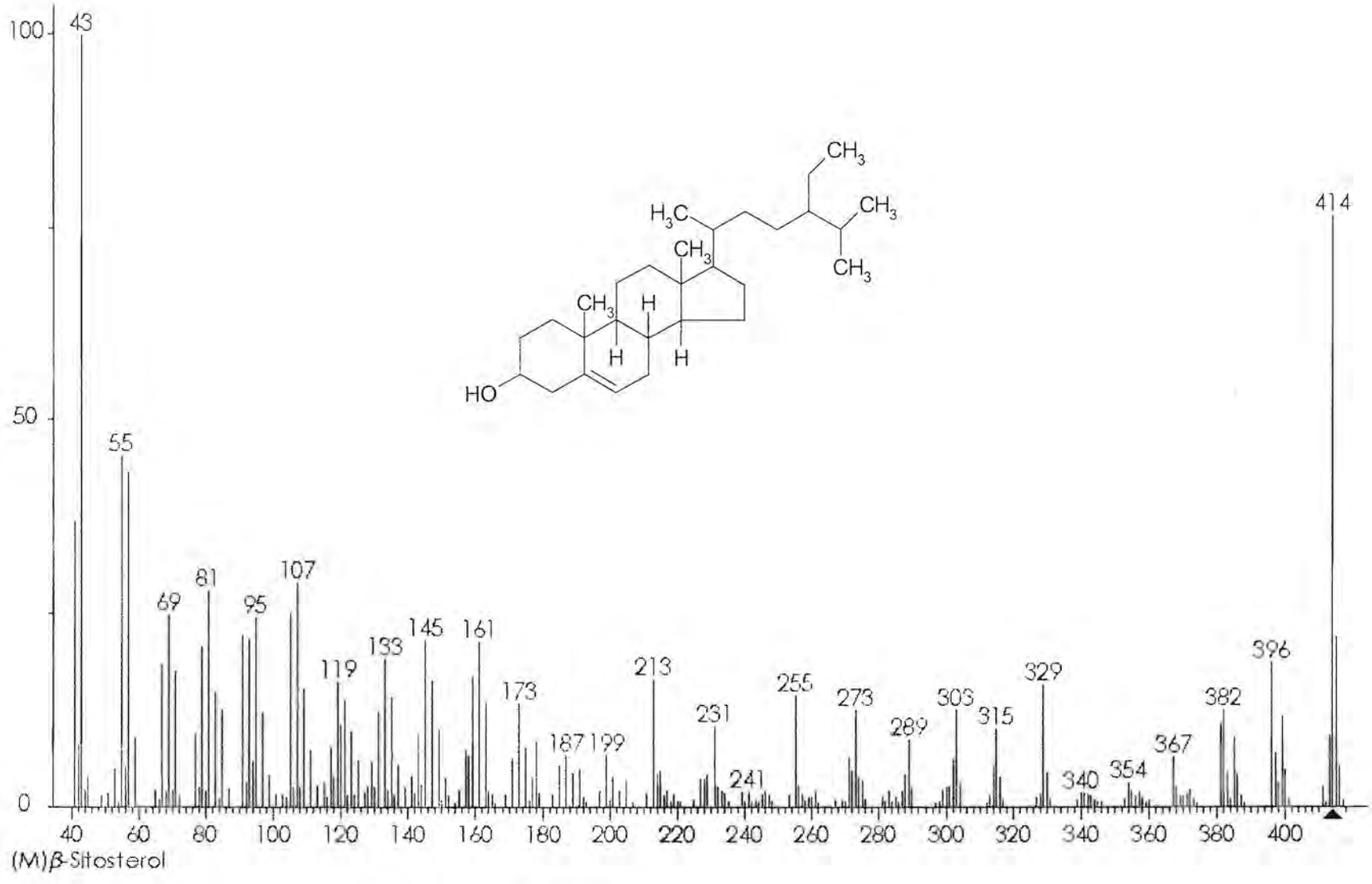


Figure 2.8: MS data of β-sitosterol from the MS-database.

2.3.2.2 Flavonoids

Flavonoid 1 was obtained as yellow crystals and identified as tamarixetin with ^{1}H-NMR spectra compared to published data (Figure 2.9, 2.10).

Flavonoid 2 was obtained as yellow crystals and identified as quercetin with ^{1}H-NMR spectra compared to published data (Figure 2.11, 2.12). This is a well-known flavonoid widely distributed throughout the plant kingdom.

2.3.2.3 Diterpenes

Diterpene 1 was obtained as colourless crystals melting point (mp.) 195°C. MS of diterpene 1 showed $[M]^{+}$ at m/z 330 (Figure 2.13). IR showed the following peaks: 3330, 2915, 2880, 2820, 2670, 1660, 1605, 1424,1355, 1290, 1240, 1190, 1125, 1090, 1055, 1010, 950, 920, 890, 855, 809. ^{1}H and ^{13}C NMR data (Figures 2.14, 2.15) of diterpene 1 indicated the presence of five methyl groups 1.42 s (δ_C 21.7), 1.01s (δ_C 20.6), 1.06s (δ_C 16.9), 1.03d (J=3.1 δ_C 16.4) and 1.05d (J=3.1 δ_C 14.8), two ketonic groups at δ_C 205.1s, 212.7s, one olefinic proton at δ_H 6.55 s (δ_C 134.3), in addition to two methylene groups at δ_H 1.96 dd, 2.19 dd (δ_C 35.9 t), 1.66 ddd, 1.84 dd (δ_C 29.6), one proton at 4.21 d (J=3.7, δ_C 79.3) and one proton at 0.68 dd (J=2.3, 10.0, δ_C 25.4) characteristic of a cyclopropyl group (Table 2.2).

The most important information was obtained from HMBC cross peaks (Figure 2.16); the methyl group at 1.05 showed HMBC correlations with δ_C 205.1 and 35.9 and the ethylene protons at δ_H 1.96, 2.19 showed correlations with δ_C 62.5, 205.1, 212.7, and 143.7, and correlations between dH 6.55 and dC 205.1, 62.5; the band 1424 in IR indicated the

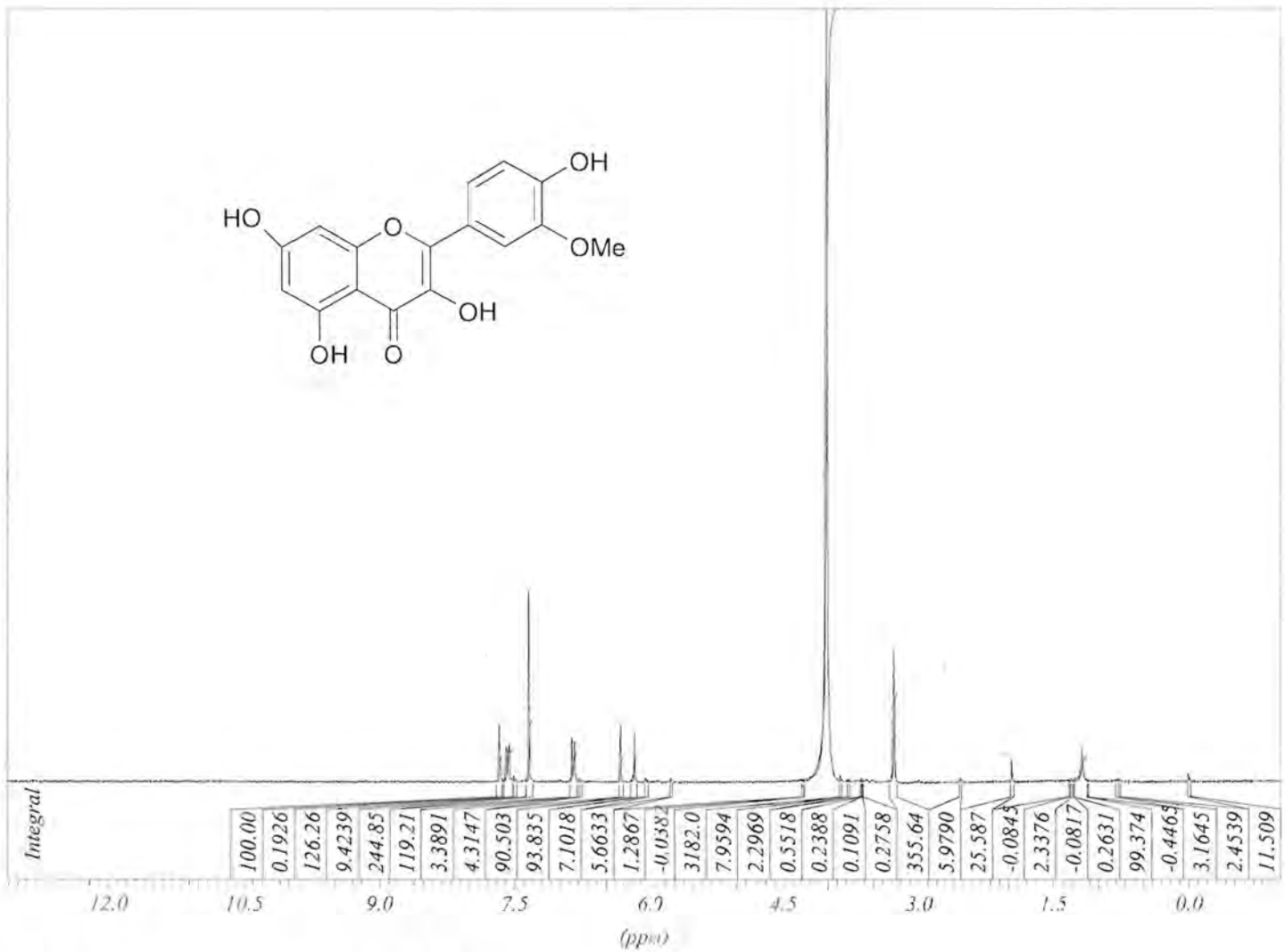


Figure 2.9: ^{1}H-NMR spectra of flavonoid 1.

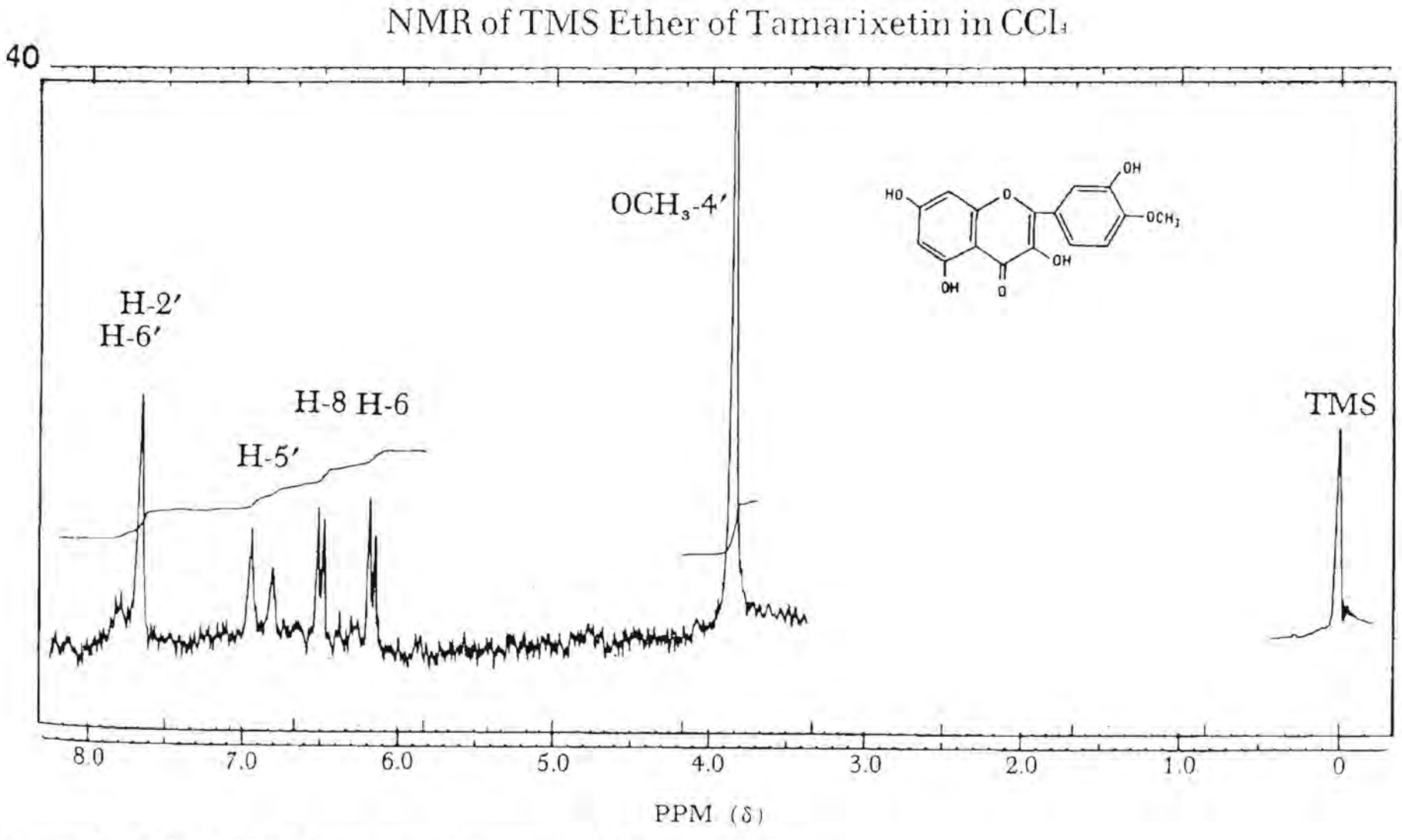


Figure 2.10: Published ^{1}H-NMR spectra of tamarixetin (Mabry *et al* 1970).

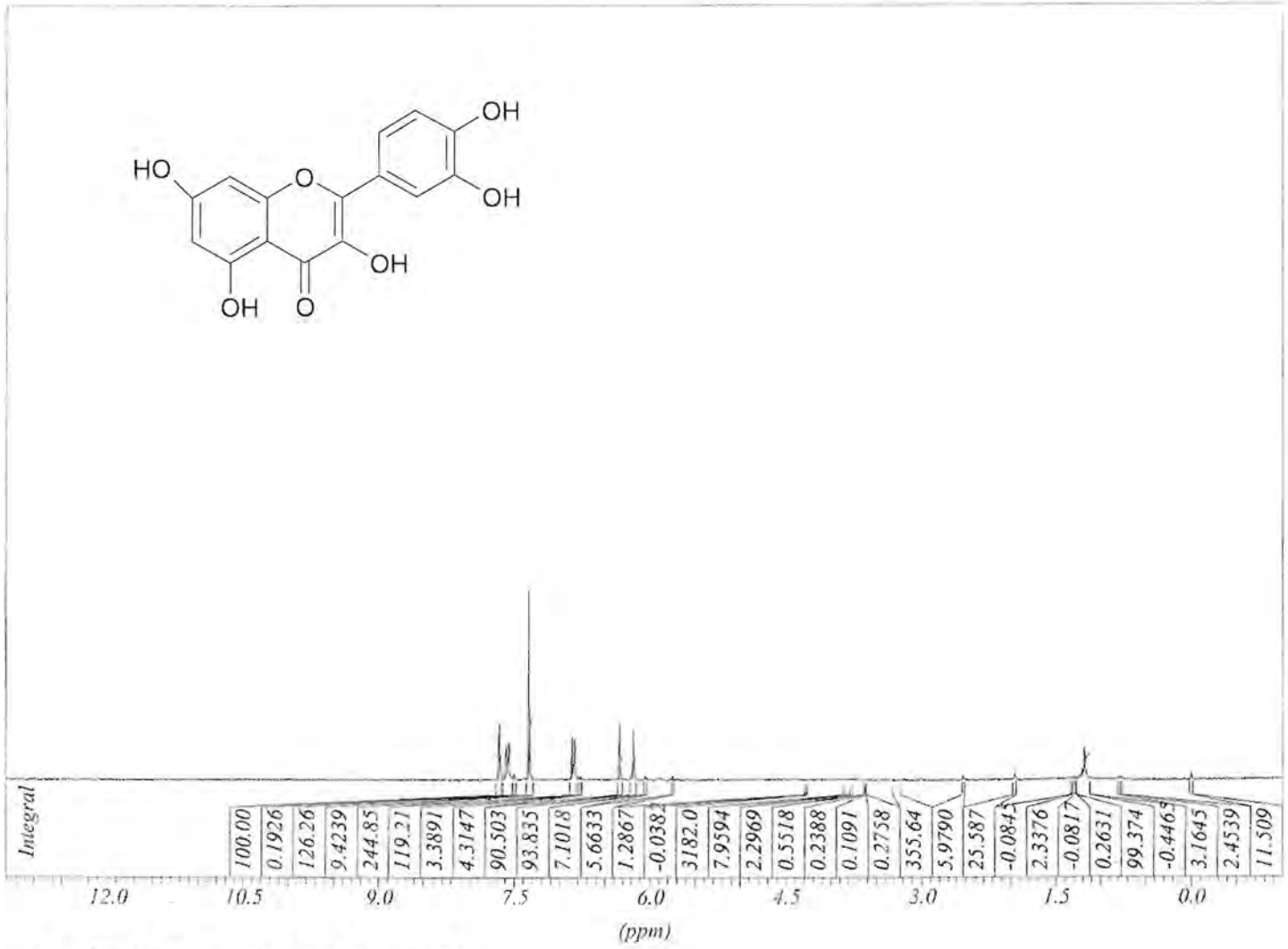


Figure 2.11: ^{1}**H-NMR spectra of flavonoid 2.**

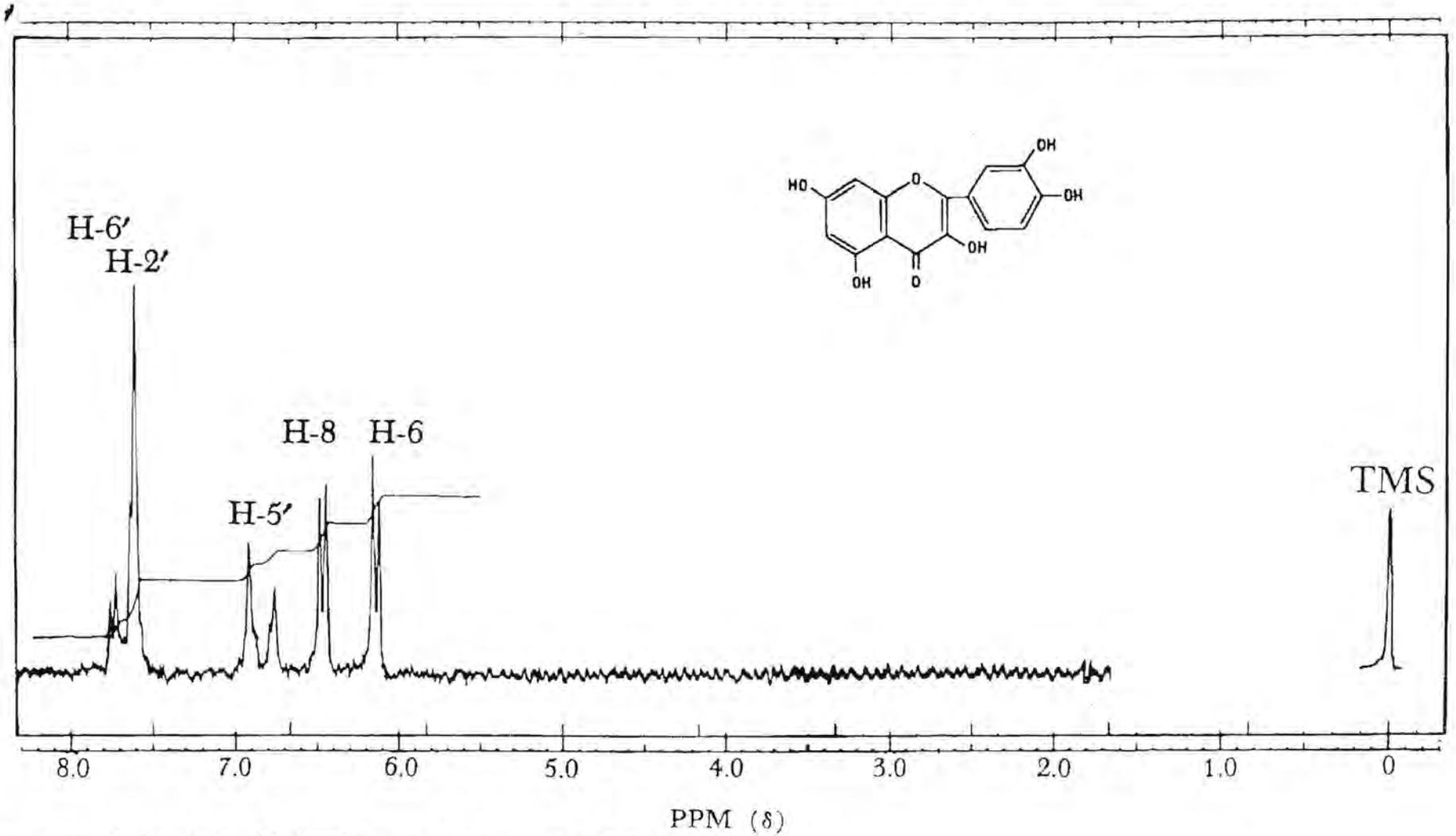


Figure 2.12: Published ^{1}H-NMR spectra of quercetin (Mabry *et al* 1970).

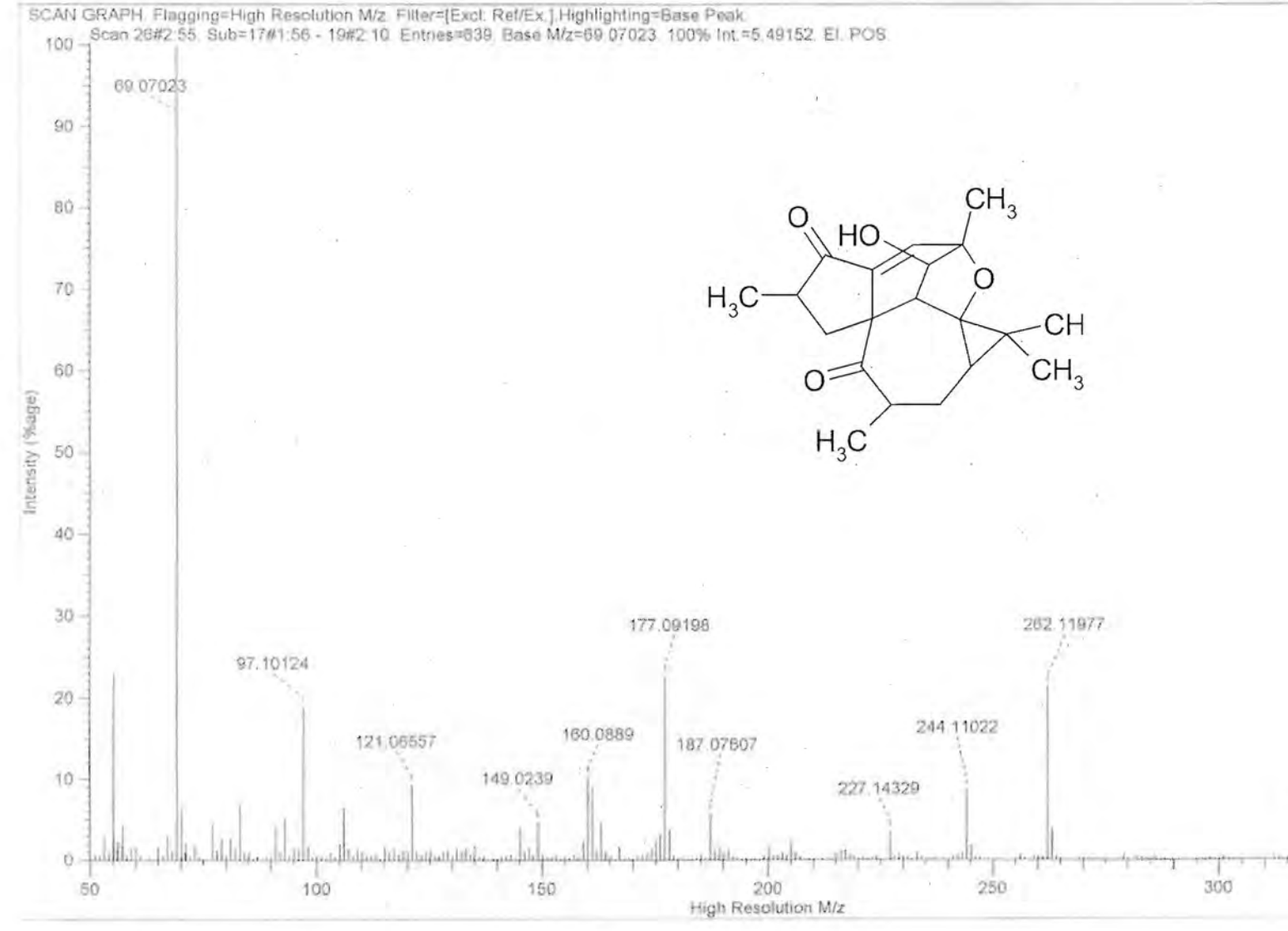


Figure 2.13: MS data from diterpene 1.

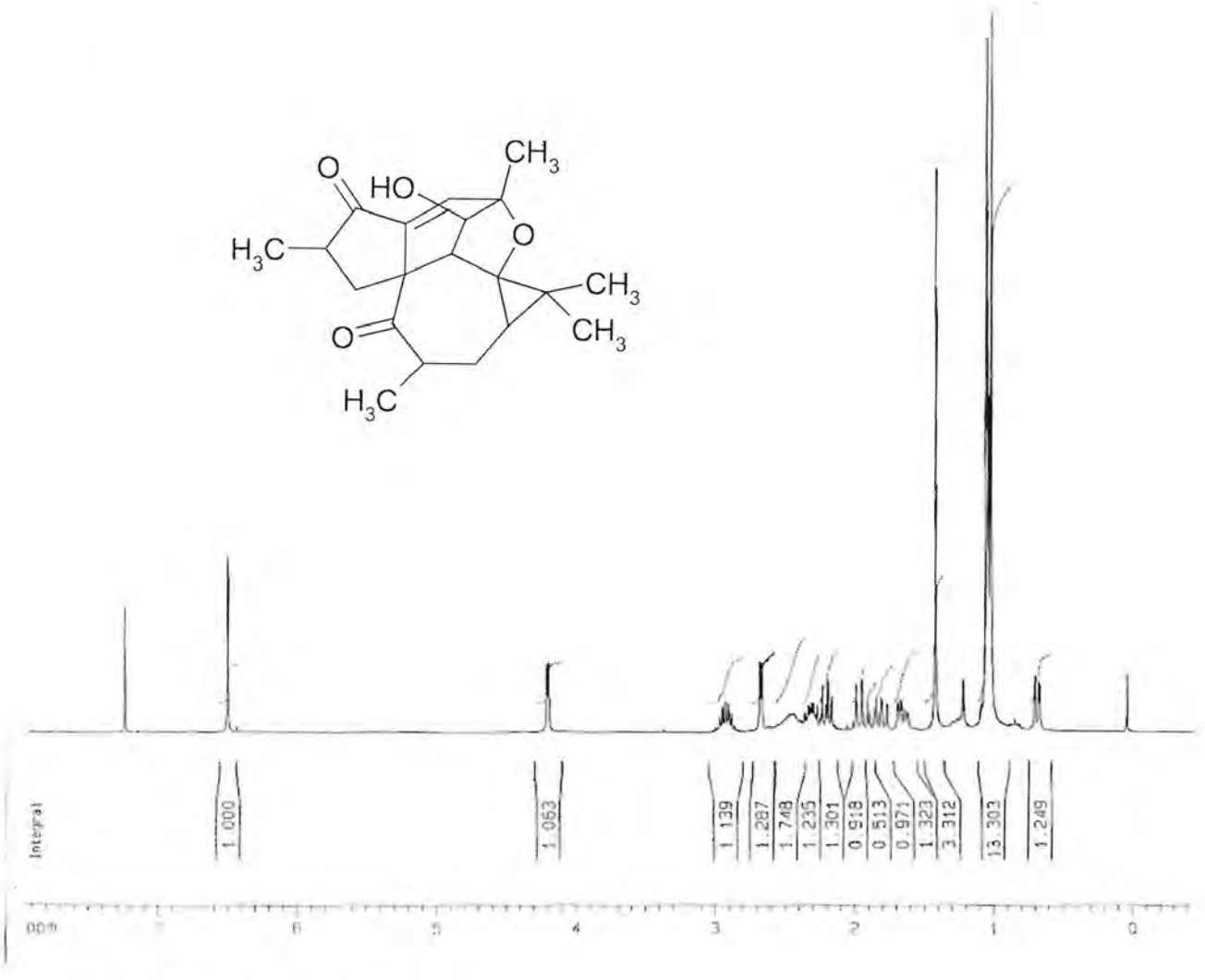


Figure 2.14: ^{1}H-NMR spectra of diterpene 1.

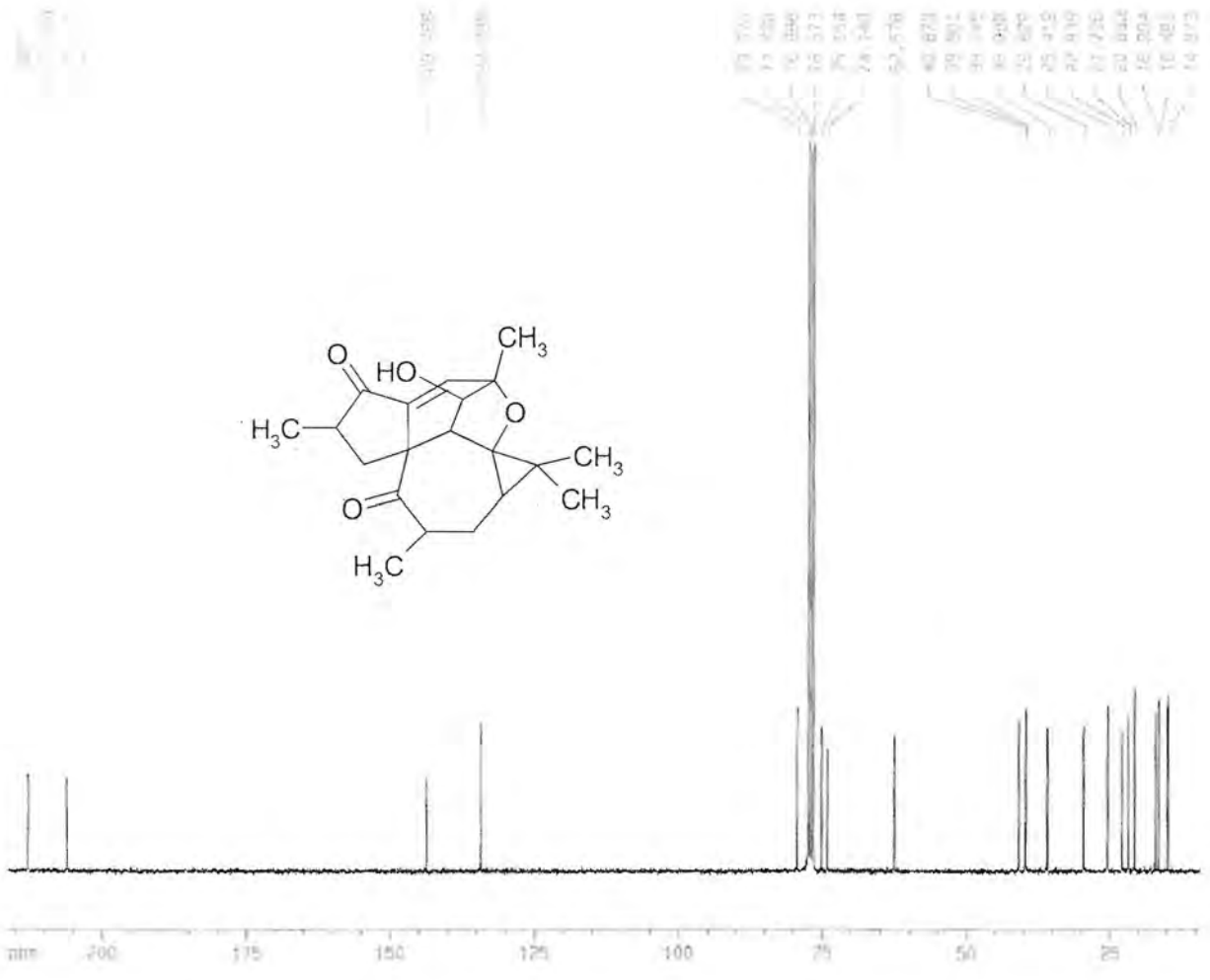


Figure 2.15: ^{13}C-NMR spectra of diterpene 1.

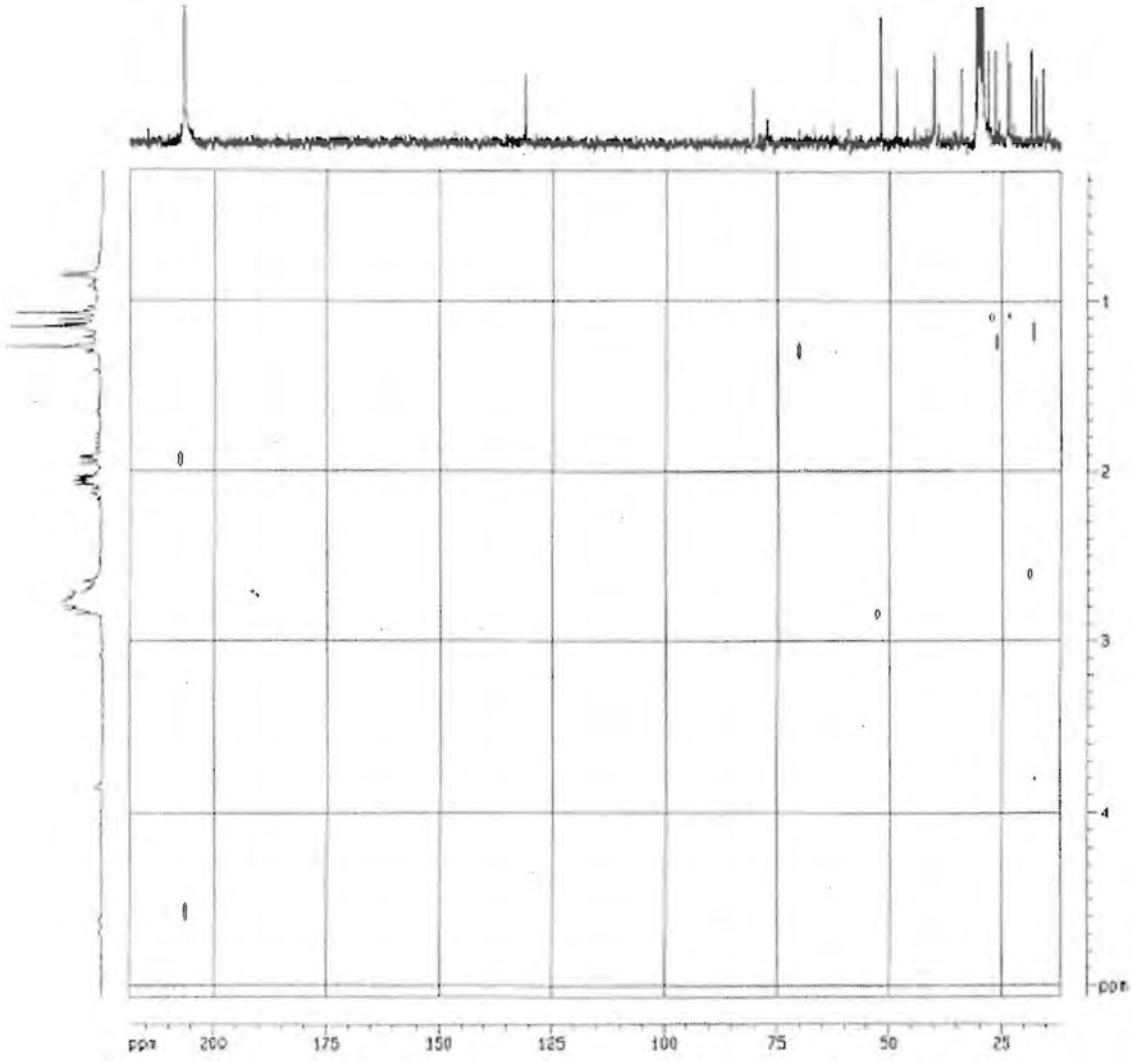


Figure 2.16: HMBC spectra of diterpene 1.

presence of αβ-unsaturated ketone. These data clearly confirm the existence of the partial structure (Figure 2.17).

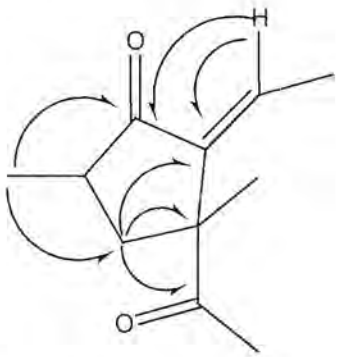


Figure 2.17: HMBC correlations of partial structure.

Table 2.2: ^{1}H-NMR and ^{13}C-NMR data from diterpene 1.

No.	Carbon*	Proton
1	35.9t	1.96dd, 2.19dd, to be measured
2	39.7d	2.3m
3	205.1s	
4	143.7s	
5	134.3d	6.55s
6	75.1s	
7	79.3d	4.21d, 3.7
8	40.8d	6.55s
9	62.5s	
10	212.7s	
11	39.8d	2.92septet, 6.3
12	29.6t	1.66ddd, 14.2, 7.0, 2.3, 1.84ddd, to be measured
13	25.4d	CH 0.68dd, 10.0, 2.3
14	74.1s	
15	22.8s	
16	20.6q	CH3 1.01s
17	16.9q	CH3 1.06s
18	16.4q	CH3 1.03d, 4.1
19	14.8q	CH3 1.05d, 4.1
20	21.7q	CH3 1.42s

*The multiplicity was determined from the DEPT experiment.

The other structural parts were confirmed from the other HMBC correlations as follows: the methyl group at 1.03 showed correlations with δ_C 62.5, 212.7, 29.6 in addition to the correlations δ_H 0.68/ δ_C 74.7, 39.8, 20.6, 21.7; δ_H 6.55/δ_C 134.9, 75.1, 63.3, 21.7. All the foregoing information in addition to the other data of HMBC, DEPT 135, COSY 45, and HMQC (Figures 2.19 - 2.21) confirm the plane structure given in Figure 2.18.

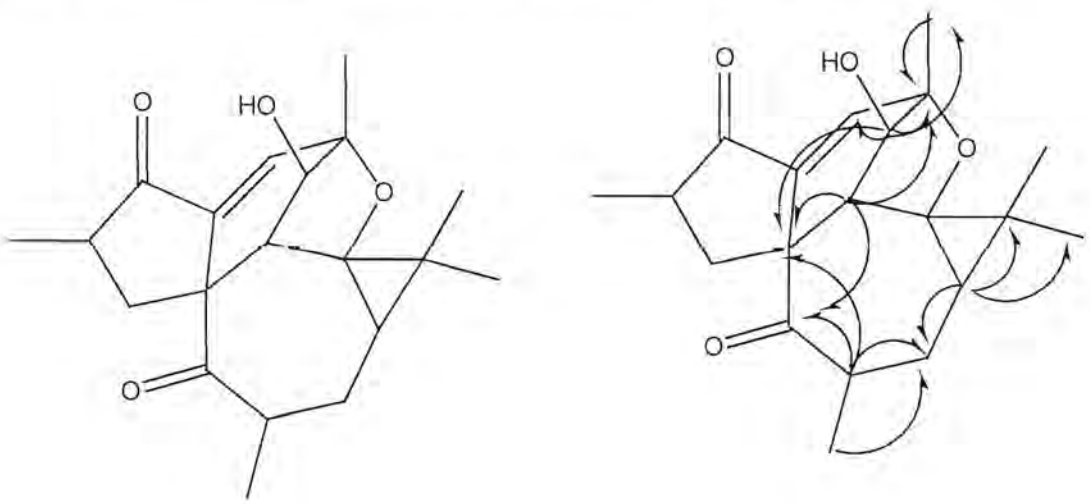


Figure 2.18: Plane structure and HMBC correlations H—C.

The relative configuration of the asymmetric centres in diterpene 1 were established from NOESY correlations (Figure 2.22, 2.23) in which correlations were found between methyl-20, H-8, H-6, H-5, H-11, H-3 (δ_H 2.19) and H-12 (δ_H 1.66) and between H-13 and H-12, and finally between H-3 (δ_H 1.96) and H-2. These data indicate that the groups; methyl-20, H-8, H-6, H-5, H-11 H-3 (δ_H 2.19) and H-12 (δ_H 1.66) are on one side and the groups H-13, H-12, H-3 (δ_H 1.96) and H-2 are on the other side.

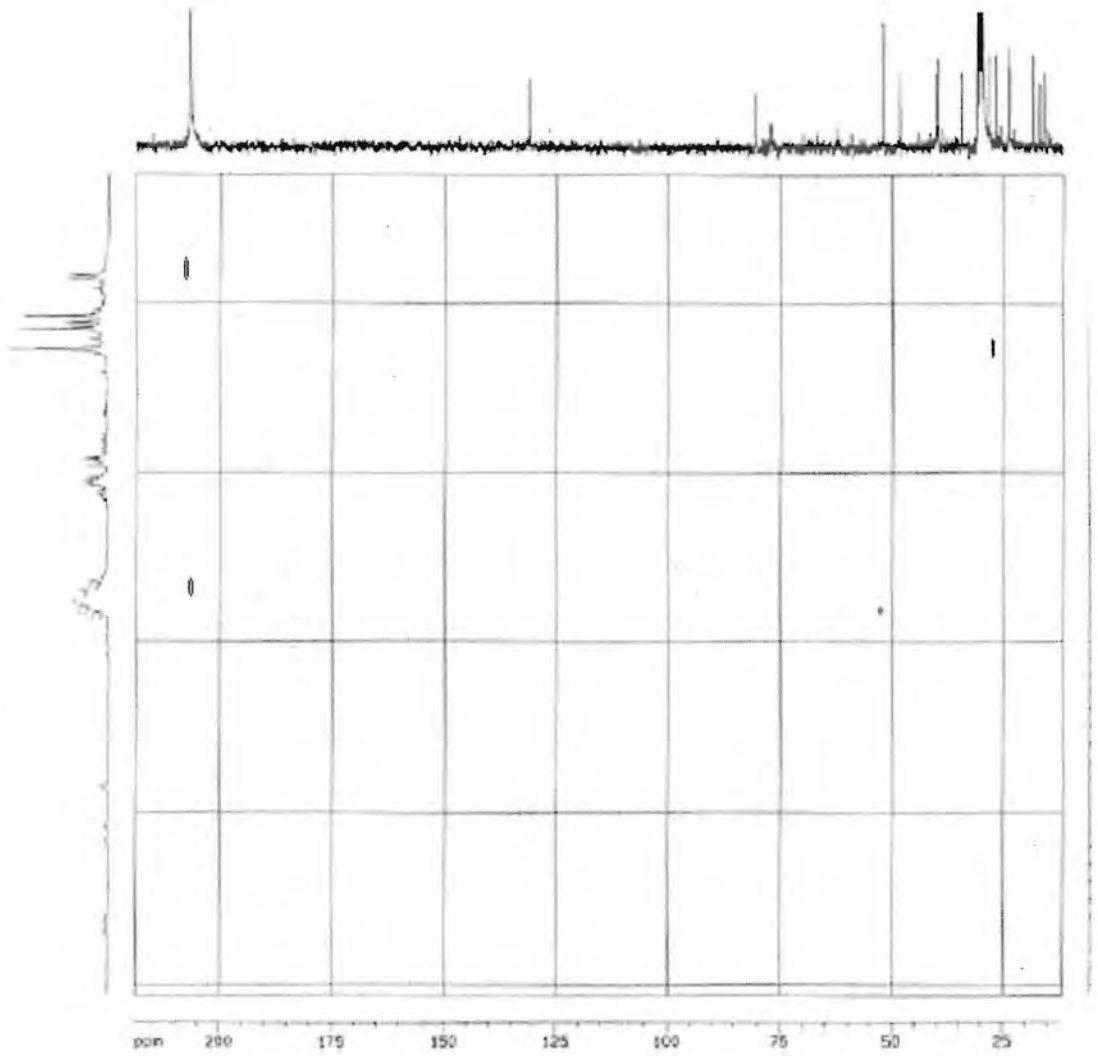


Figure 2.19: HMQC spectra of diterpene 1.

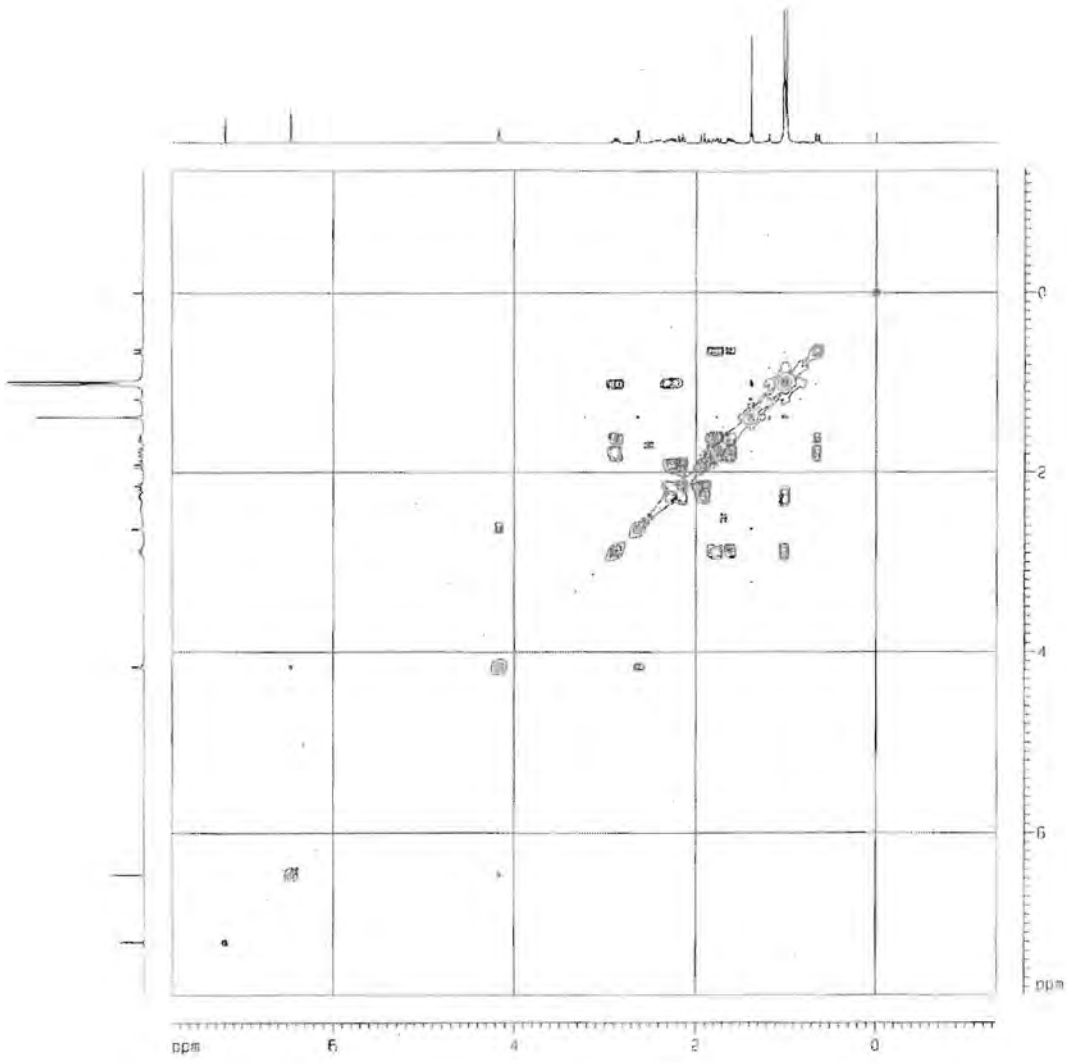


Figure 2.20: COSY 45 spectra of diterpene 1.

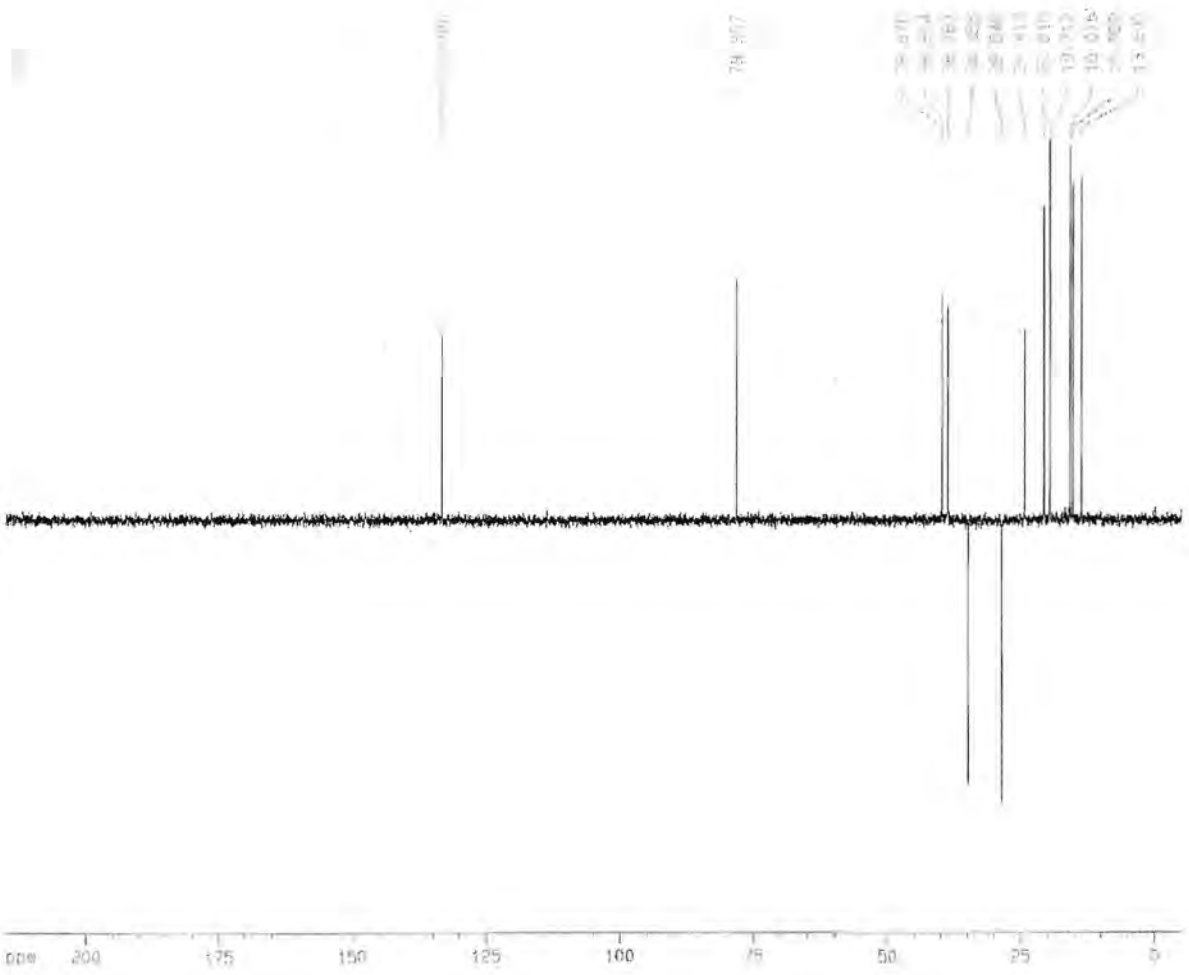


Figure 2.21: Dept 135 spectra of diterpene 1.

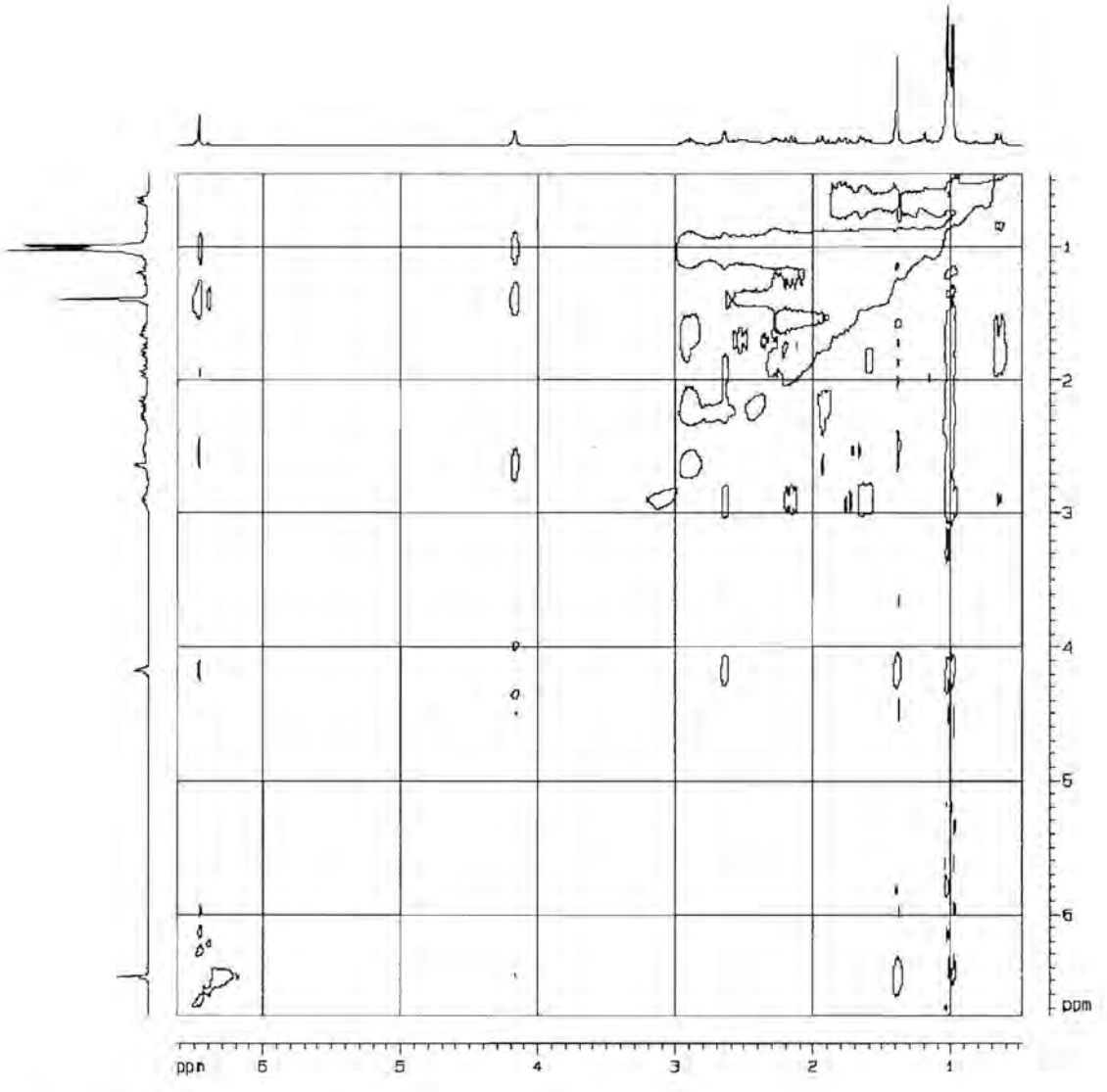


Figure 2.22: NOESY spectra of diterpene 1.

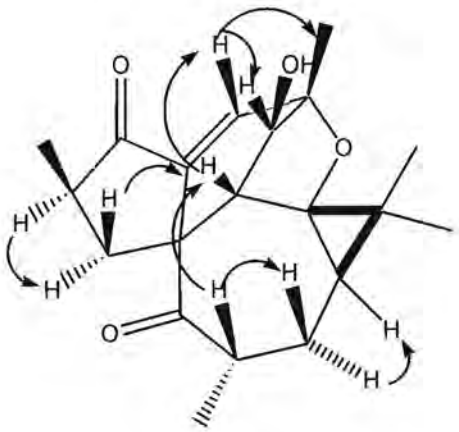


Figure 2.23: NOESY correlations of diterpene 1.

Finally the absolute configurations were established by X-ray analysis, which confirmed all the foregoing interpretations of data (Figure 2.24, Appendix 1).

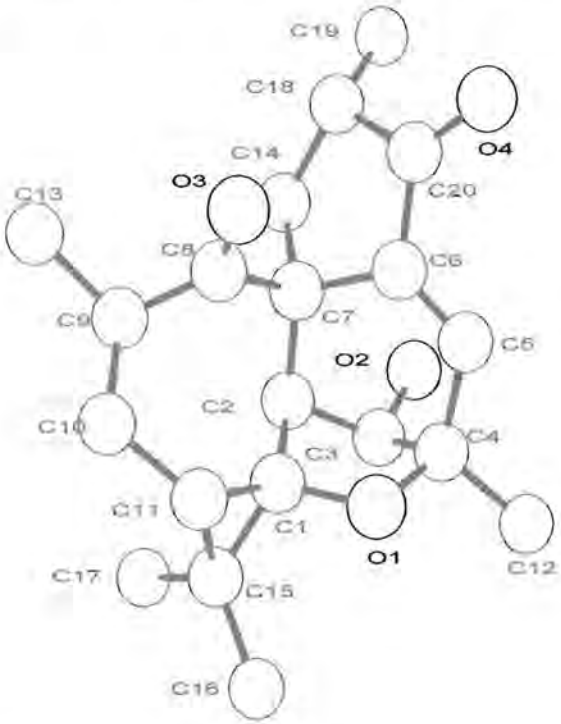


Figure 2.24: X-ray structure of diterpene 1.

Diterpene 2 was obtained as yellowish crystals and identified with ^{1}H-NMR (Figure 2.25) and compared to data obtained with diterpene 1. From this data a closely

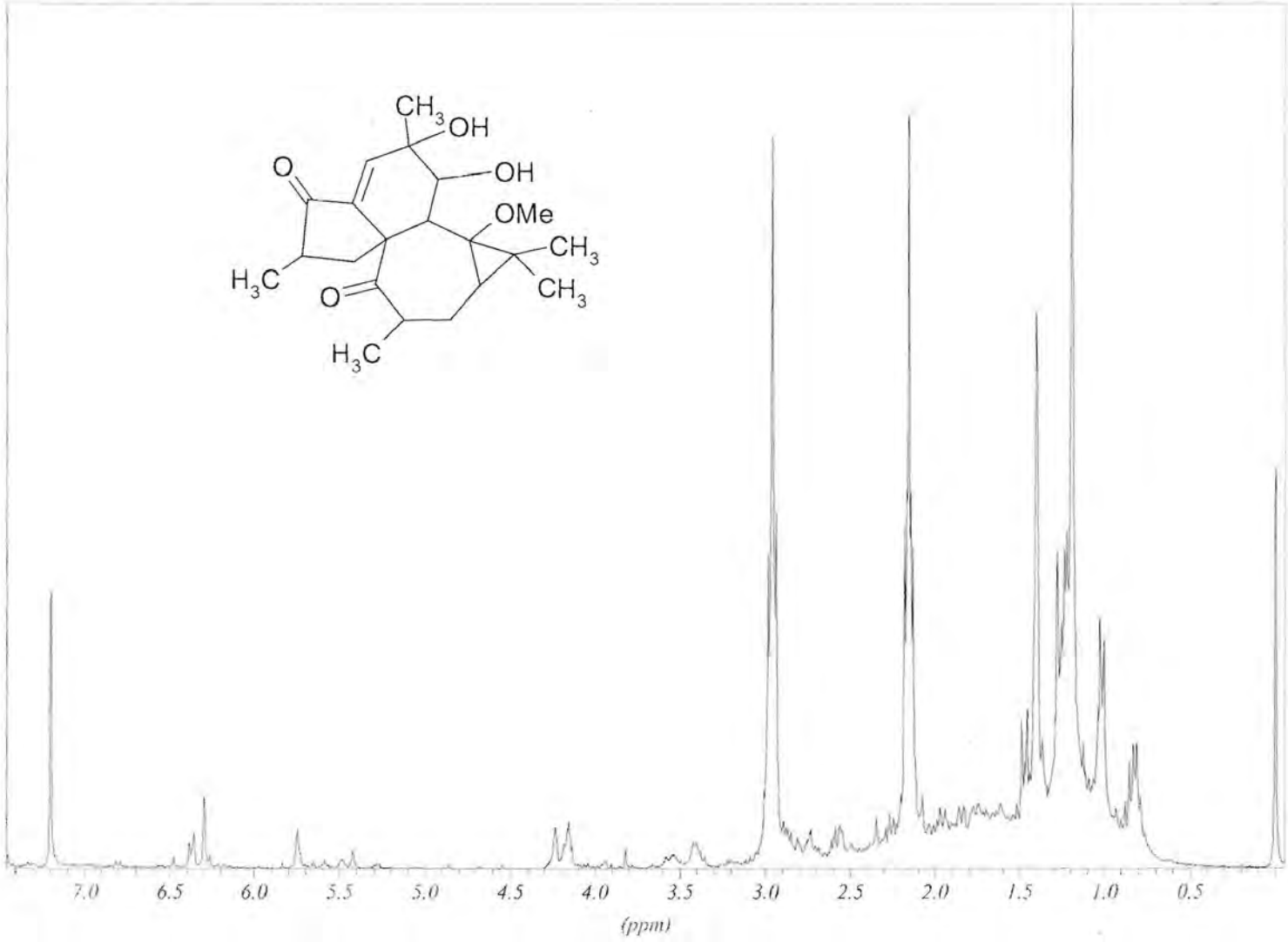


Figure 2.25: ^{1}H-NMR of diterpene 2.

related structure to crotrene A was identified (crotrene B). Crotrene B was not very stable and possibly degraded to form the more stable crotrene A (Figure 2.26).

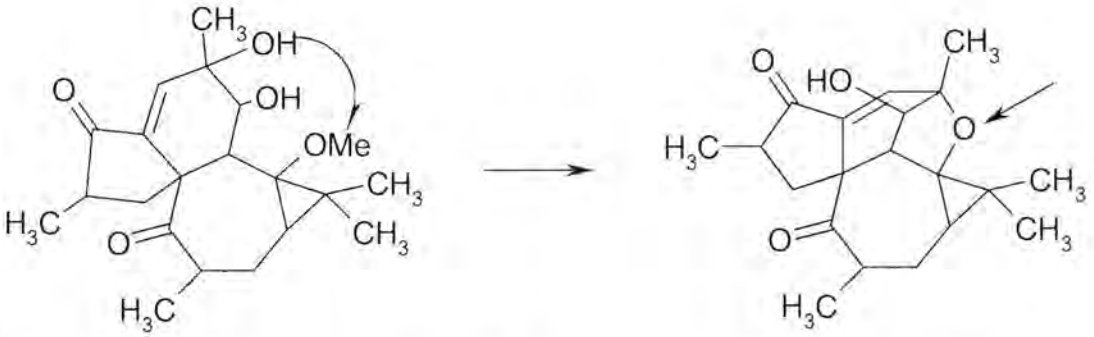
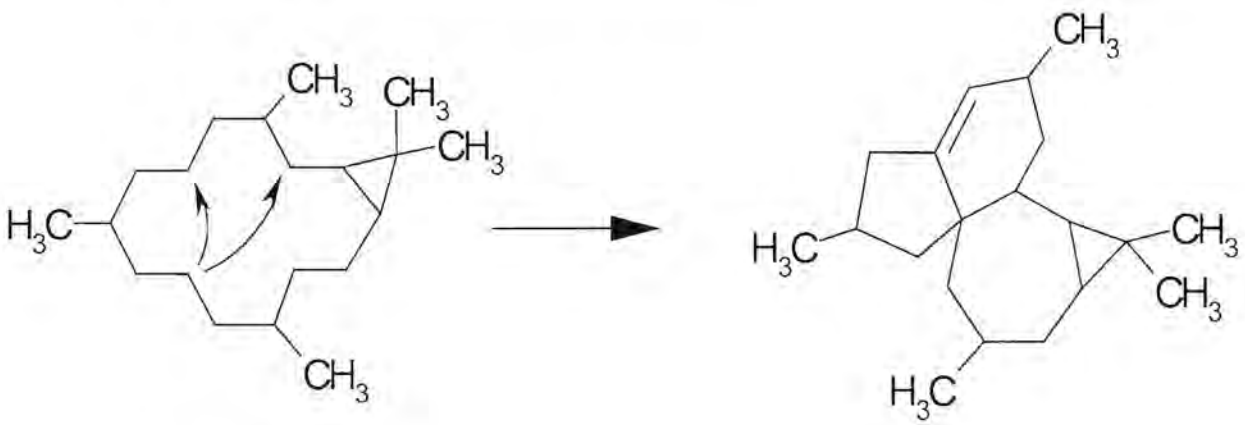


Figure 2.26: Possible way in which crotrene B degraded to the more stable crotrene A.

From the data it was established that diterpene 1 and 2 had a new skeleton. It is possibly synthesized from a casbene skeleton (Figure 2.27) via 10/6 and 10/3-cyclization (Figure 2.28) to form the new skeleton named crotrane.



casbene 10/6 and 10/3-cyclization crotrane

Figure 2.28: Possible formation of crotrane type skeleton.

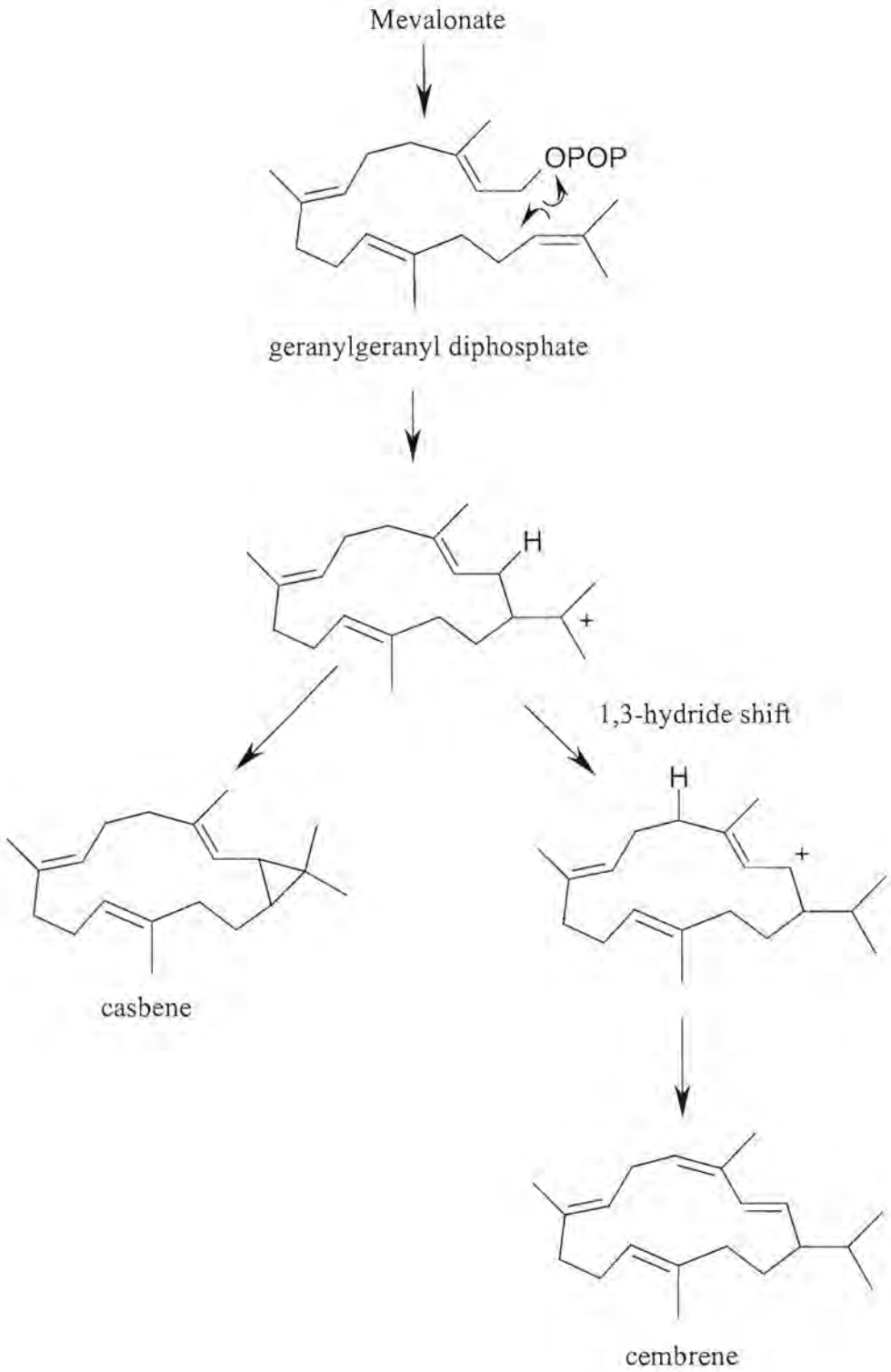


Figure 2.27: Biosynthesis of casbene type skeleton (Gershenzon & Croteau 1993).

2.3.3 Antiplasmodial activity of isolated compounds

Antiplasmodial activity of the isolated compounds is shown in Table 2.3. Crotrene B was not tested as insufficient material was obtained and it was unstable.

Table 2.3: Antiplasmodial activity of the isolated compounds as determined with the flow cytometric test.

Compound	Sensitive strain (FAB 9)		Resistant strain (RB1)	
	IC_{50} µg/ml	IC_{50} µM	IC_{50} µg/ml	IC_{50} µM
β-sitosterol	32.5 (±18.7)[a]	78.5 (±44.3)	36.4 (±20.6)	87.9 (±51.2)
Tamarixetin	35.3 (±6.8)	110.3 (±16.3)	91.2 (±17.3)	285.1 (± 43.2)
Quercetin	2.8 (±0.7)	9.27 (±2.6)	3.9 (±0.3)	12.91 (±1.2)
Crotrene A	0.323 (±0.042)	0.979 (±0.093)	0.301 (±0.035)	0.912 (±0.102)
Chloroquine	0.010 (±0.003)	0.031 (±0.008)	0.095 (±0.013)	0.298 (±0.025)
Acetone extract	26.2 (±5.6)	-	20.1 (±4.8)	-

[a] **Standard deviation**

Antiplasmodial activity of the isolated compounds ranged from weak to excellent. The highest activity was obtained with crotrene A against both strains, while the lowest activity was obtained with tamarixetin. The antiplasmodial activity of quercetin is well-known and the values obtained in this study are comparable with published data (4.8 µg/ml) (Khalid *et al.* 1986). The antiplasmodial properties of tamarixetin were not found in literature. The results of the two very similar flavonoids differed dramatically, with quercetin being 12.5 and 23.3 times more active against the FAB9 and RB1 strains respectively. β-sitosterol was not very soluble and this is probably reflected in the high standard deviation against both strains.

The most exciting results were obtained with crotrene A. It had very high activity against both strains with IC_{50} values of 323 ng/ml and 301 ng/ml against the sensitive and resistant strain respectively. One of the important properties of a good antimalarial drug is the ability to have constant properties against most parasite strains. Against the strains tested in this study, crotrene A displayed such properties and further tests on more strains will be important in future studies. Given the excellent antiplasmodial activity of crotrene A and the possibility of even better results when this drug is tested *in vivo*, as various other compounds have shown higher activity *in vivo* than *in vitro* (Gessler *et al.* 1995), its potential as an antimalarial drug needs further investigation.

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Chapter 3

Cytotoxicity of extract and compounds

3 Cytotoxicity of extract and compounds

3.1 Introduction

The determination of cytotoxicity of medicinal compounds forms an integral part of their development and use as safe medicines. Human and animal cell culture systems can provide rapid evaluation of cytotoxicity of new substances. In many instances, experience to date shows that cell culture data is as good as or better than traditional animal LD_{50} studies in terms of predicting toxicity to humans (Barile *et al.* 1994). Compounds which appear safe with cell culture tests should be tested on animals, in cases where substantial human exposure is involved, to evaluate for possible metabolic or trans-placental effects. Nevertheless, cell cultures provide a rapid, inexpensive and accurate method for preliminary evaluation of toxicity of compounds (Barile *et al.* 1994).

Various methods exist for the *in vitro* determination of cytotoxicity. A very basic method used is the brine shrimp method (Carballo *et al.* 2002). This method is not very accurate and is sometimes also used for determination of bioactivity. A more popular method is the use of mammalian cell lines in the determination of cytotoxicity. Various cell lines are used eg. primate- and human cell lines. In this study human lymphocytes were used to determine the effect of the isolated compounds on cell proliferation. Cells were actively stimulated and serial dilutions from the isolated compounds added. The inhibition on cell proliferation was then measured and calculated relative to a control.

3.2 Materials & Methods

3.2.1 Material

Croton steenkampianus acetone extract and pure compounds obtained as described in chapter 2, were dissolved in DMSO or water at a concentration of 20 mg/ml or 10 mg/ml respectively. From these stock solutions serial dilutions were made for experiments.

3.2.2 Methods

The principle of this method is a metabolic assay based on the reactivity of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) with viable cells (Mossman 1983). Microtitre plates with human lymphocytes (Pretoria Biomedical Research Center) were used for testing the plant extract and compounds for cytotoxicity.

Lymphocytes were prepared in the following way: 30 ml of human heparinized blood was loaded onto 15 ml histopaque 1077 (Sigma Aldrich) and centrifuged for 25 minutes at 1800 rpm at room temperature. The top plasma layer was removed and the lymphocyte/monocyte layer (about 12 ml) transferred to sterile 50 ml tubes and filled up with sterile RPMI-1640 medium (Sterilab). The tubes were centrifuged for 15 minutes at 1000 rpm to remove the contaminating platelets. The supernatant was discarded, the tubes filled with RPMI medium and centrifuged for 10 minutes at 1000 rpm. The supernatant was discarded again, filled with cold ammonium chloride [8.3 g ammonium chloride (NH_4Cl) (Merck), 1 g sodium bicarbonate ($NaHCO_3$) (Merck), 74 mg EDTA (Merck) and 1000 ml distilled water] and left to stand for about 10 minutes on ice to lyse contaminating red blood cells. Tubes were centrifuged for 10 minutes at 1000 rpm, the supernatant discarded and

filled up with RPMI medium and again centrifuged for 10 minutes at 1000 rpm. The supernatant was discarded again and cells re-suspended in 1 ml RPMI+, consisting of RPMI, supplemented with 1% of a penicillin/streptomycin mixture (Sterilab) and 10% heat inactivated fetal calf serum (Sterilab). Cells were diluted in RPMI+ for the experiments. To remove the monocytes the lymphocyte suspension was resuspended in 15 ml RPMI+ in a 75 cm^2 tissue culture flask and incubated at 37°C in 5% CO_2 for 1 – 1.5 hours to allow for the adherence of the monocytes. The flask was then agitated gently and the lymphocyte suspension decanted into 15ml tubes and washed with RPMI+ for 10 minutes at 1000 rpm. The supernatant was discarded and lymphocytes resuspended in 1ml RPMI+ and diluted.

To each well of a sterile round-bottomed 96-well culture plate, 100 µl RPMI+ and 60 µl lymphocytes ($2x10^6$/ml) were added and the plate incubated for 60 minutes in a 37°C/CO_2 incubator. After this incubation, 20µl of the test compound/extract was added together with 20µl of phytohemagglutinin (PHA) and incubated for 3 days at 37°C in a CO_2 incubator in a closed container with water. For negative controls 20 µl of RPMI+ was added with the 20µl of PHA. Solvent controls were also included. Serial dilutions of the test compounds were made and 7 concentrations were included.

After 3 days the cells were assayed with MTT to determine their viability. 20 µl of a MTT solution [200 mg MTT Stain (Sigma) dissolved in 40 ml PBS (5 mg/ml), (BBL Microbiology systems USA), sterilized with 0.2 µm pore size filter and stored at 5°C in the dark], was added to each well and the plate re-incubated for 3.5 – 4 hrs at 37°C in a 5% CO_2 incubator. The plate was then centrifuged at 2000 rpm for 10 minutes, the supernatant carefully removed and the pellets washed with 150 µl PBS. 100 µl DMSO was added and

shaken gently for ± 1 hour on a shaker and measured spectrophotometrically at a wavelength of 540 nm.

3.3 Results and discussion

Results from the cytotoxicity test are shown in Table 3.1.

Table 3.1: *In vitro* cytotoxicity of *C. steenkampianus* extract and compounds tested on human lymphocytes

Substance	**ID_{50} µg/ml**
Extract	>45 (±8.3)[a]
Tamarixetin	>250+ (±18.2)
Quercetin	>50 (±6.89)
Crotrene A	>16.61 (±1.37)
Chloroquine	>25 (±3.96)

[a] **standard deviation**

The highest cytotoxicity value was obtained with crotrene A, while the lowest value was obtained with tamarixetin. These values were obtained over 72h on stimulated lymphocytes.

The therapeutic index is expressed as the antiplasmodial activity (flow cytometric test) to cytotoxicity ratio (Figure 3.1). Due to different experimental times these indexes are only estimates and should be higher when the same experimental duration is applied. The therapeutic indexes were between 2.75 and 55.18. It has been proposed that a good therapeutic index value should be > 1000, as for example in quinine (Likhitwitayawuid *et al.*

1993). The best therapeutic index was obtained with crotrene A with a value >55.18. This is not as high as the proposed index for a good remedy, but shows enough selectivity to have potential as a drug. The important factor would be its therapeutic index in an animal model and this would be the next step in the determination of the potential of this drug as a marketable entity. Various drugs have exhibited moderate toxicity *in vitro* but showed no significant toxicity *in vivo*. Given the good antiplasmodial properties of crotrene A, further toxicological studies should clear up its potential as a marketable antimalarial drug. Quercetin and tamarixetin had low therapeutic indexes.

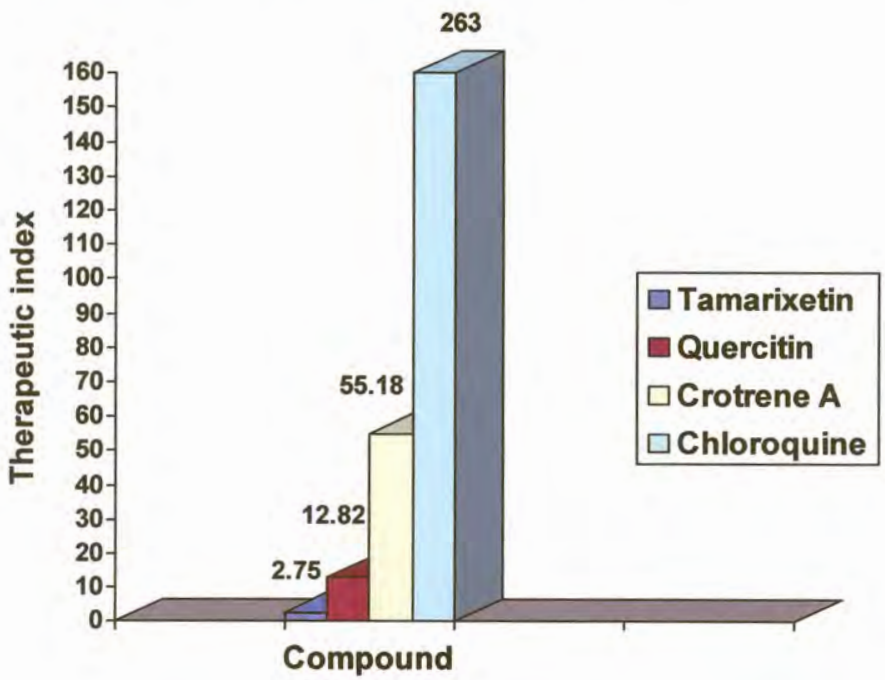

Figure 3.1: Therapeutic index of tested compounds.

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Chapter 4

Chloroquine reversal /synergistic effects of isolated compounds

4 Chloroquine reversal /synergistic effects of isolated compounds

4.1 Introduction

Current difficulties with the effective, safe, and affordable treatment of *Plasmodium falciparum* malaria worldwide are due largely to the loss of efficacy of chloroquine, once the mainstay of control and treatment programs. Yet in many parts of the world it remains a drug of choice for the treatment of malaria because of its low cost and availability and because it provides some clinical benefit even in the face of drug resistance. Among the other antimalarial drugs and drug combinations, in most endemic countries, economic reality dictates that the only affordable alternative to chloroquine is Fansidar, an antifolate drug combination, itself subject to rapidly spreading resistance (Winstanley *et al.* 2002).

Efforts have been made to understand the basis of chloroquine resistance in the hope of obtaining information valuable in devising cheap and effective ways of making chloroquine as useful as it used to be. Early laboratory observations pointed to the fact that resistant parasites accumulated significantly less chloroquine than did sensitive parasites (Verdier *et al.* 1985), a situation analogous to how some cancer cells acquire multidrug resistance through over expression of an ATP-binding cassette. This impaired chloroquine uptake has been correlated with mutations of the *Pfmdr1*, *Pfcg2* and *Pfcrt* genes (Le Bras & Durand 2003). Notably, the drug resistance of these cancer cells was reversible *in vitro* by a range of "chemosensitizers", of which verapamil was the first to be identified (Tsuruo *et al.* 1981). Two types of actions are distinguished: The first is the reversal of chloroquine resistance, where the compound only has a marked effect on resistant parasites, and therefore

lowers the chloroquine IC_{50} in resistant parasites but has little or no effect on sensitive parasites. This action is therefore assumed to have to do with the resistance mechanism only. The second is the sensitization of chloroquine to the parasites, where the effect of chloroquine is enhanced in both resistant and sensitive strains (IC_{50} lower against all strains).

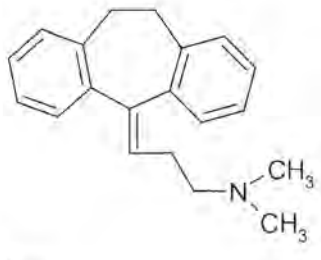
A better understanding of the mechanism of chloroquine uptake in *P. falciparum* would be a significant step forward since it should explain the specificity of the drug and may also offer clues to the basis of resistance. The specificity of antimalarial activity of chloroquine stems from the potential of malaria parasites to accumulate more chloroquine than any other type of eukaryotic cell. Chloroquine resistant parasites of some species, including *P. falciparum*, have evolved ways to reduce the extent of chloroquine accumulation. Chloroquine undoubtedly penetrates the parasite by passive diffusion as observed in other eukaryotic cells (MacIntyre & Cutler 1993). Chloroquine is a diprotic weak base and, as such, undoubtedly accumulates to some extent inside the acidic compartments of the parasite by a proton trapping mechanism (Yayon *et al.* 1985). This mechanism may account for a considerable concentration of chloroquine inside cells (Geary *et al.* 1986). However, it probably does not account for the full extent of chloroquine accumulation by *P. falciparum*, which is notably greater than chloroquine accumulation by other eukaryotic cells with large acidic compartments (MacIntyre and Cutler 1993). Pioneering studies by Fitch in the 1970's demonstrated that chloroquine uptake into *P. falciparum* is saturable, energy-dependent, and possibly inhibited by various compounds (Fitch 1970; Fitch *et al.* 1974). These studies suggest that malaria parasites possess an additional chloroquine concentrating mechanism acting in combination with passive diffusion and proton trapping.

Two putative chloroquine concentrating mechanisms are currently under consideration: active chloroquine import (Warhurst 1986) mediated by the parasite Na^+ /H^+ exchanger (NHE) (Sanchez *et al.* 1997; Wünsch *et al.* 1998); and intracellular binding of chloroquine to ferriprotoporphyrin IX (FPIX) (Chou *et al.* 1980; Bray *et al.* 1998). Experimental data presented by Bray *et al.* (1999) concluded that saturable chloroquine uptake is driven by its binding to FPIX. Chloroquine's antimalarial action is caused by the formation of a drug–FPIX complex accumulating in the parasite upon exposure to chloroquine (Chou *et al.* 1980; Fitch 1983; Balsubramanian *et al.* 1984; Sullivan *et al.* 1996; Ginsburg *et al.* 1998). In a process unique to the malaria parasite, the FPIX released during proteolysis of hemoglobin is polymerized into an inert crystalline substance called hemozoin (Francis *et al.* 1997). Chloroquine inhibits this polymerization process, causing a buildup of free FPIX and/or chloroquine–FPIX complex that may ultimately kill the parasite (Slater 1993; Dorn *et al.* 1995). Chloroquine resistant parasites have a reduced apparent affinity for chloroquine–FPIX binding compared with chloroquine sensitive parasites and Bray *et al.* (1999) suggested that chloroquine resistance stems from an alteration in the local environment of FPIX generation in acid vesicles. They found that a wide range of lysosomotropic compounds mimics the effects of verapamil. This could indicate the inhibition of a drug transporter similar to P-glycoprotein. However, since many of these compounds are not known to interact with P-glycoprotein, they suggested an alternative mode of resistance reversal. The concentrations required to reverse resistance produced no alkalinisation of the parasite cytosol but might be expected to produce a significant alkalinisation of lysosomes and endosomes (Millot *et al.* 1998).

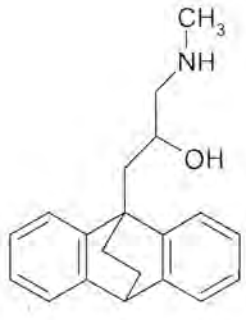
There is evidence in the literature that hemoglobin digestion begins in hemoglobin delivery vesicles, before they fuse with the food vacuole (Slomianny & Prensier 1990). To

protect the parasite, the resistance mechanism must be operational throughout the endocytic pathway. The intracellular localization of chloroquine resistance gene CG2 throughout the endocytic pathway is certainly consistent with this hypothesis (Su *et al.* 1997). It is therefore believed that chloroquine resistance results from a selective change in vesicular function within relevant hemoglobin processing acidic compartments. This reduces the affinity of chloroquine–FPIX binding, which can be reversed by lysosomotropic agents. CG2 and related proteins could potentially alter the binding of chloroquine to FPIX by directly binding to FPIX or by altering vesicular pH or buffering capacity.

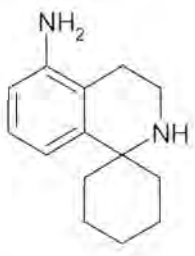
Although the exact mechanism of this reversal effect in the parasite is still unclear, the end result is promising and various other chemicals have been identified to reverse resistance *in vitro* (Figure 4.1).



Amitriptyline



Oxaprotiline

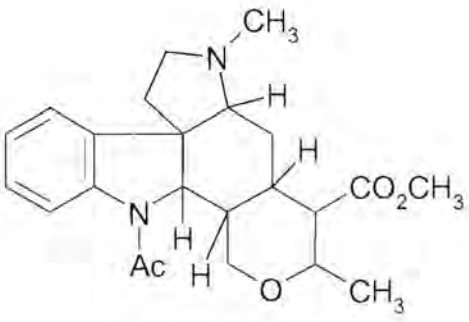


Nomifensine

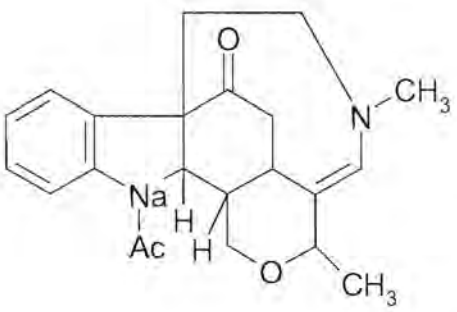
Citalopram

Figure 4.1: Chemicals used to reverse chloroquine resistance *in vitro* (Taylor *et al.* 2000).

The resistance reversal effect of natural products has also come under the spotlight. Local people in Madagascar used the extracts from local plants in combination with low doses of chloroquine successfully to treat malaria. Investigation of these plants lead to the isolation of various alkaloids of which strychnobrasiline and malagashanine were the major active components (Figure 4.2) (Rafatro *et al.* 2000). Both these compounds reversed chloroquine resistance *in vitro*, while malagashanine was also active *in vivo*. Apart from this work, very little is known about natural products that could potentiate chloroquine action, and thus be candidates for useful biochemical tools, although relevant information exists in the traditional uses of herbal remedies (Rafatro *et al.* 2000). In this study three of the isolated compounds (chapter 2) were evaluated for their chloroquine reversal potential: tamarixetin, quercetin and crotrene A.



Malagashanine



Strychnobrasiline

Figure 4.2: Compounds isolated from *Strychnos spp.* reversing chloroquine resistance (Rafatro *et al.* 2000).

4.2 Methods

4.2.1 Chloroquine resistance reversal

To determine the chloroquine resistance reversal effect of compounds with chloroquine, the effect relative to the drug alone and in combination is determined. Synchronized cultures of two *P. falciparum* strains were used, RB1, a chloroquine resistant strain and FAB9, a chloroquine sensitive strain as described in section 2.2.2.2. *In vitro* antiplasmodial tests were done with the flow cytometric method as described in section 2.2.2.2.6. Isolated test compounds were dissolved in DMSO at 20 mg/ml and stock solutions made up from these. Serial dilutions were made from the stock solutions to make up final concentrations. Each compound was applied in serial dilutions and was tested in triplicate to assess intrinsic antiplasmodial activity. The IC_{50}'s of the isolated compounds and chloroquine were determined from two independent experiments and the isolated compounds added at concentrations lower than the IC_{50}'s of the drugs alone. Synergistic effects will be present if the IC_{50} is lower than with the drugs alone. A potency factor was also introduced to determine the relative effect of each drug. The potency factor equals 2, <2, or >2 for additive, antagonistic, or synergistic effects of compounds on chloroquine inhibition respectively (Frederich *et al. 2001*). Three compounds were tested for their ability to reverse chloroquine resistance, quercetin, tamarixetin and crotrene A.

4.2.2 Cytotoxicity test

To determine the possible synergistic cytotoxic effects of compounds the lymphocyte cytotoxicity test as described in section 3.2 was used. Cytotoxicity of the compounds alone and in combination with chloroquine was determined.

4.3 Results and Discussion

Antiplasmodial activity of the compounds varied from weak, moderate to excellent. Their effects as reversal agents showed interesting results. Tamarixetin (potentiation) and crotrene A had reversal effects while quercetin had antagonistic effects (Table 4.1).

The most potent activity was observed with crotrene A. It was able to restore the sensitivity of chloroquine to levels comparable to the values obtained with the sensitive strain. Concentrations of crotrene A needed was very low with a potentiating factor of 12.6. The cytotoxicity of this combination was also excellent with no synergistic cytotoxicity observed. The therapeutic index was between 664.4 and 1328.8 depending on the concentration used.

The compounds tested displayed very interesting properties when combined with chloroquine. Two of the compounds with very similar chemistry (quercetin and tamarixetin) had very different results. Quercetin had the better antiplasmodial activity, but had antagonistic properties with chloroquine, while tamarixetin with basically no antiplasmodial activity had very good results when combined with chloroquine lowering the chloroquine IC_{50} to levels of the sensitive strains. This effect was even more with the sensitive strain, lowering the IC_{50} to levels far below the IC_{50} of the sensitive strain. This effect has been described as potentiation, where the parasites are made more sensitive to the uptake of chloroquine. The ratio of this effect is constant with the difference between the two strains when tamarixetin is used alone.

Table 4.1: Results obtained with the three test compounds

Compound	Antiplasmodial activity (IC_{50} µg/ml)		Cytotoxicity (ID_{50} µg/ml)	Therapeutic index RB1	Potentiating factor[d] RB1	Therapeutic index FAB9	Potentiating factor FAB9
	RB1 (Resistant)	FAB9 (Sensitive)					
Tamarixetin (T)	91.2 (±17.3)[a]	35.3 (±6.8)	>250+ (±18.2)	>2.75	-[e]	>7.14	-
Quercetin (Q)	3.9 (±0.3)	2.8 (±0.7)	>50 (±6.89)	>12.82	-	>17.85	-
Crotrene A (D)	0.301 (±0.035)	0.323 (±0.042)	>16.61 (±1.47)	>55.18	-	>51.42	-
Chloroquine (C)	0.095 (±0.013)	0.010 (±0.003)	>25 (±3.96)	>263	-	>2500	-
T+C 25µg/ml[a]	0.014[c] (±0.002)	0.00061 (±0.00006)	-	-	6.4	-	16.1
T+C 12.5µg/ml[b]	0.016[c] (±0.004)	0.0011 (±0.00007)	-	-	-	-	-
Q+C 1.25µg/ml[b]	0.110[c] (±0.016)	0.0117 (±0.004)	-	-	0.86	-	0.85
Q+C 0.625µg/ml[b]	0.129[c] (±0.013)	0.0142 (±0.006)	-	-	-	-	-
D+C 25ng/ml[b]	0.008[c] (±0.002)	0.005 (±0.0014)	>15.98 (±1.3)	>664.4	12.6	-	2
D+C 12.5ng/ml[b]	0.015[c] (±0.003)	0.006 (±0.0011)	>15.98 (±1.3)	>1328.8	6.3	-	1.67

[a] Standard deviation [b] Concentration of each compound added to give chloroquine IC_{50} values. [c] Chloroquine IC_{50} values. [d] Potentiating factor (PF) determines the type of effect present: synergistic if PF > 2, additive if PF = 2 and antagonistic if PF < 2. [e] Not determined.

The best and most exciting result was obtained with crotrene A. It was able to lower the IC_{50} of chloroquine to sensitive levels when very small amounts of crotrene A added. This effect was also limited to the resistant strain with no, synergistic effects present against the sensitive strain. This holds true for the term chloroquine resistance reversal. Although the properties of an ideal chloroquine resistance modulator have not yet been elucidated, it is clear that a potential resistance modulator must exhibit good activity at concentrations that are nontoxic and pharmacologically achievable. In this regard crotrene A holds a lot of promise and further studies on this compound are planned.

It has been shown that chemosensitizers are able to increase the chloroquine uptake into the parasite when combined with chloroquine (Taylor *et al.* 2000), whether this is the same mechanism for the tested compounds is a matter of speculation. Several essential components are believed to play a role in resistance reversal. These include the presence of planar (benzene) groups, a secondary or tertiary nitrogen, and a cationic charge. Lipophilicity is also considered important. In the case of the compounds tested, lipophilicity seems to play a role, although none of the compounds contain nitrogen.

4.4 References

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Chapter 5

Mode of action of crotrene A, a new diterpene isolated from *C. steenkampianus*

5 Mode of action of crotrene A, a new diterpene isolated from *C. steenkampianus*

5.1 Introduction

Most of the antimalarial drugs available currently have been in use for decades, but their use is now severely limited by the emergence and spread of drug resistance, primarily in *Plasmodium falciparum*, the malaria parasite that causes a severe form of the disease and most of the disease burden (Olliaro *et al.* 1996). Despite years of use, little is known about the mode of action and mechanisms of resistance to most of the antimalarial drugs available today. Whilst this has not been perceived as a major problem when such drugs performed satisfactory, it is becoming painfully evident that this inadequate knowledge is severely limiting our ability to devise a course of action. We need to know more about sites of parasite vulnerability and the basic mechanisms through which drugs act and resistance is generated in order not only to generate new drugs with novel mechanisms of action, but also to make better use of the drugs that we have (White 1998). Several classes of drugs are used in the treatment of malaria and they target different stages of the malaria life cycle (Tracey & Webster 1996), although the majority of them act on the intra-erythrocytic phases of development of the malaria parasite (Figure 5.1). See Figure 1.10 for chemical structures.

5.1.1 Nucleic acid inhibitors

5.1.1.1 Folate antagonists

Some of the most widely used antimalarial drugs belong to the folate antagonist class.

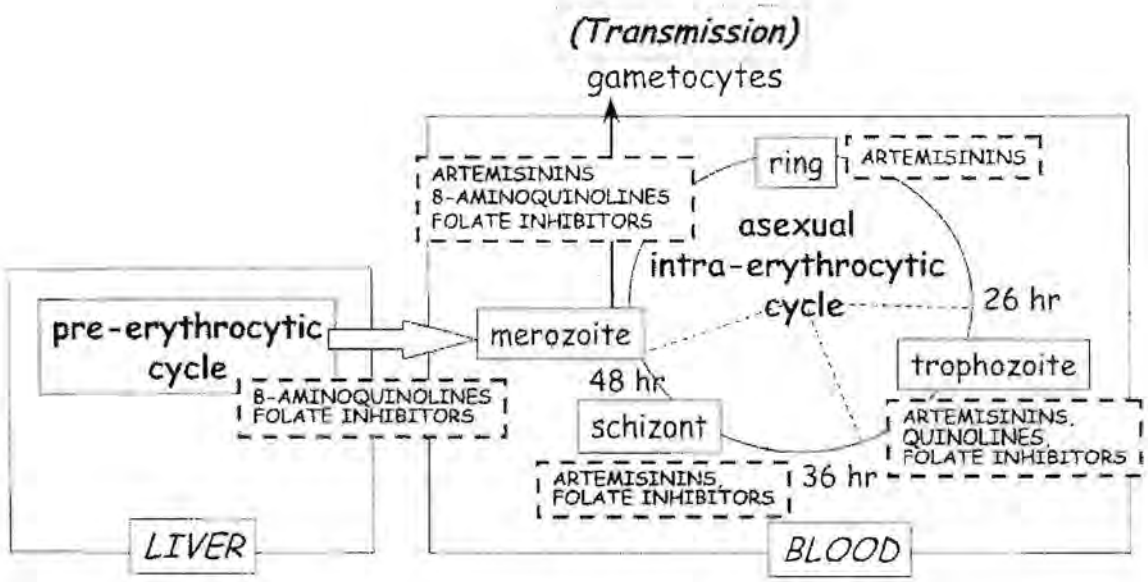

Figure 5.1: Plasmodium life cycle with phases targeted by antimalarial drugs (Olliaro *et al.* 1996).

Inhibition of enzymes of the folate pathway results in decreased pyrimidine synthesis, hence, reduced DNA, serine, and methionine formation. Activity is exerted at all growing stages of the asexual erythrocytic cycle and on young gametocytes. Traditionally, antifolates are classified into two classes (Figure 5.2):

(1) Type-1 antifolates (sulfonamides and sulfones) mimic p-aminobenzoic acid (PABA). They prevent the formation of dihydropteroate from hydroxymethyldihydropterin (strictly speaking, the pyrophosphate derivative) catalised by dihydropteroate synthase (DHPS) by competing for the active site of DHPS (a bifunctional enzyme in plasmodia).

(2) Type-2 antifolates (pyrimethamine, biguanides and triazine metabolites, quinazolines) inhibit dihydrofolate reductase (DHFR, also a bifunctional enzyme in plasmodia).

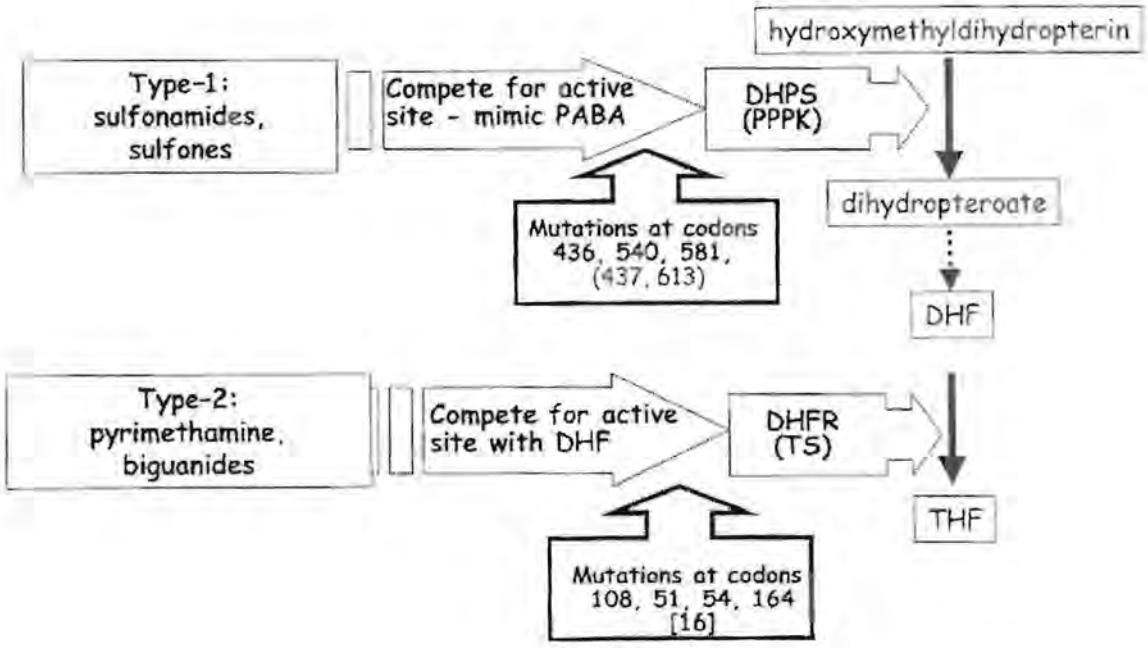

Figure 5.2: Targets of and mutations conferring resistance to antifolate drugs (Olliaro *et al.* 1996).

5.1.1.2 Atovaquone

Atovaquone, a hydroxynaphthoquinone (Figure 1.10), is used for both the treatment and prevention of malaria in a fixed combination with proguanil. Whilst known to act primarily on mitochondrial functions, its mode of action and synergy with proguanil are not completely understood. One proposed site for atovaquone's activity is dihydroorotate dehydrogenase (DHODase), a critical enzyme in electron transport. Inhibition of DHODase blocks pyrimidine synthesis (Vaidya *et al.* 1993; Hudson 1993). Atovaquone was also shown to inhibit the respiratory chain of malarial mitochondria at the cytochrome bc1 complex by mimicking the natural substrate, ubiquinone. This inhibits mitochondrial electron transfer from ubiquinol to cytochrome c by depolarising malarial mitochondria (Fry & Pudney 1992). Specificity was ascribed to unique structural features of the parasite cytochrome b (Vaidya *et al.* 1993).

5.1.2 Blood schizontocydes

Blood schizontocydes are drugs that act on intra-erythrocytic (asexual and partly also sexual) parasites. Their (primary) target is believed to be the parasite food vacuole (FV). They include quinoline-containing drugs and the artemisinin-type compounds. A recent article by Eckstein-Ludwig *et al.* 2003, proposes the inhibition of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), outside the food vacuole for the artemisinin-type compounds. A schematic representation of putative mechanisms of action can be found in Figure 5.3.

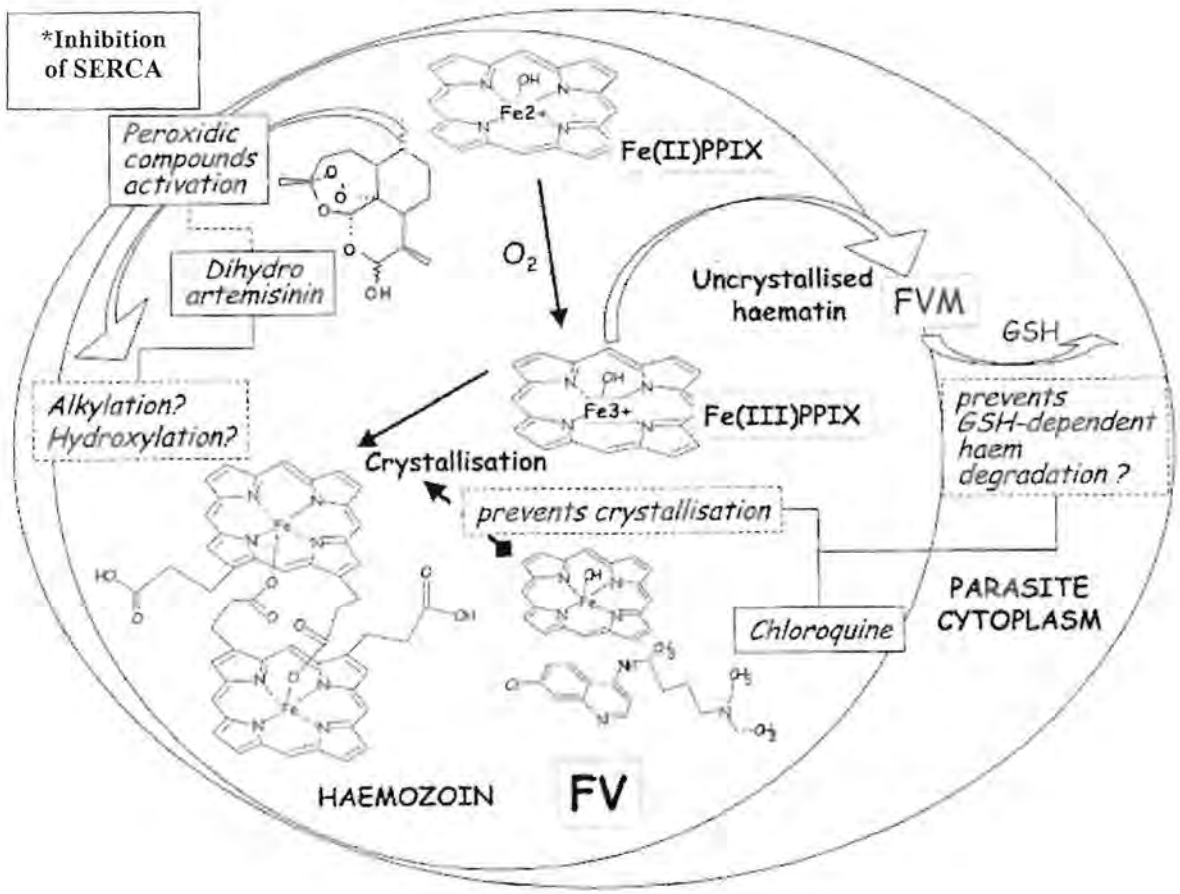

Figure 5.3: Events in the parasite food vaucole (FV) targeted by quinoline and peroxidic antimalarials. FV membrane (FVM) (Olliaro *et al.* 1996).
*** Latest proposed mode of action of artemisinin-type compounds suggests inhibition of SERCA outside food vacuole (Eckstein-Ludwig *et al.* 2003).**

5.1.2.1 Quinoline-containing drugs

The quinoline-containing drug class includes some of the most common antimalarial drugs: (1) type-1 drugs (the 4-aminoquinolines chloroquine and Mannich-base amodiaquine, pyronaridine) and (2) type-2 drugs (the aryl-amino alcohols quinine and quinidine, mefloquine, halofantrine). The two groups differ in that type-1 drugs are weak bases, diprotonated and hydrophilic at neutral pH, whereas type-2 drugs are weaker bases and lipid soluble at neutral pH. In addition, the two groups appear to interact differently with their putative target (Warhurst, 1987) and show an inverse relationship with respect to parasite sensitivities. The commonly accepted hypothesis is that quinoline-containing drugs act primarily on haem disposal, a process whereby intra-erythrocytic-stage malaria parasites detoxify haem in the FV.

5.1.2.2 Artemisinin-type compounds

The artemisinin-type compounds in current use are either the naturally extracted artemisinin itself or the semi-synthetic derivatives (dihydroartemisinin, artesunate, artemether, arteether). They achieve higher reduction rates of parasitaemia per cycle than any other drug known to date (White 1998), and are being used for the treatment of uncomplicated and severe forms of malaria. All members of this drug group have activity throughout the phases of the asexual intra-erythrocytic schizogonic cycle, and also act on young gametocytes. The mechanism of action is incompletely understood, but one hypothesis is that reductive cleavage of the intact peroxide by ferroheme ferrous-protoporphyrin IX (Fe(II)PPIX) generates C-centred radicals, which, in turn, would alkylate biomolecules, leading to the death of the parasite. Recently a new mode of action has been proposed by Eckstein-Ludwig *et al.* 2003. It is now believed that the artemisinin-type compounds inhibit the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) orthologue (PfATP6) of *P. falciparum*

outside the food vacuole. Artemisinins produce carbon-centred free radicals in the presence of catalytic quantities of Fe^{2+} with the selective targeting of PfATP6 by activated artemisinins.

It is clear that understanding of the mode of action of potential antimalarial drugs is an essential step in their development progress. With this in mind crotrene A was subjected to a basic test to determine at what stage of the parasite cycle its greatest effect was.

5.2 Methods

To get a better understanding of the possible mode of action of crotrene A, it was added at different times to a synchronized parasite culture. Cultures were maintained as described in section 2.2.2.2. Fixed concentrations of the drug were added at the ring- and late trophozoite/schizont phases and incubated for 48 hours at 37°C. Microscope slides were made at 24h intervals. The different effects of the drug were determined by a comparison to the control where no drug was added.

5.3 Results & Discussion

Results obtained are presented in Table 5.1.

Table 5.1: Results obtained by adding crotrene A at different stages to the parasite culture.

Starting phase	24h crotrene A	24h control	48h crotrene A	48h control
Rings	Small trophozoites	Full trophozoites	Small trophozoites	rings
Late trophs	rings	rings	Full trophozoites	Rings/small trophozoites

Results obtained showed a dramatic effect of the drug between the ring and trophozoite phase. Parasite development was completely halted. Trophozoites were small and did not develop further even after 48h. When the drug was added to the culture at a later stage, there was also a reduction in parasitaemia, but it was not nearly as marked as in the earlier trophozoite phase. Controls developed normally.

The proportion of the parasite biomass killed by a drug within a given time is determined by a) the importance of the drug/target interaction to parasite biochemical processes and b) the proportion of the parasite biomass that is susceptible at any given stage of the lifecycle. It has been argued that for drugs that kill efficiently (i.e. they have a large log-kill rate) resistance selection would not be favored as the size of the parasite population exposed to sub-therapeutic concentrations of drug is proportionally small compared to drugs with poorer efficiency to kill (Winstanley *et al.* 2002). This proposition appears to hold if we consider the artemisinins (which have the greatest log-kill rate) and the 4-aminoquinolines compared to the antifolates (which have a relatively poor log-kill rate).

Results obtained from crotrene A suggest that this drug has its greatest effect in the stage between rings and trophozoites, preventing parasite development. The only other drugs effective at this early stage in the parasite cycle are the artemisinins, and they are to date the most powerful and effective antimalarial drugs developed, reducing the parasite biomass dramatically very quickly. In this regard this new compound holds a lot of promise and further studies on the mechanism is very important.

5.4 References

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Chapter 6

General discussion and conclusions

6 General discussion and conclusions

6.1 Introduction

Mortality and morbidity from *Plasmodium falciparum* malaria in Africa remain immense problems. It seems likely that they are about to be exacerbated by antimalarial drug resistance. Although effective new drugs are available, there are too few examples, and they are too expensive. Furthermore, the difficulties of effecting change in national antimalarial drug policy (and implementing that policy) cannot be underestimated. To hold our ground against malaria there will need to be a concerted collaboration between scientists (ranging from molecular biologists to public health specialists), the pharmaceutical industry and malaria control program personnel. This collaboration will require that each of these 'groups' try to understand the difficulties faced by their colleagues in other disciplines.

Plants have been a very important source of antimalarial drugs and are likely to be an important source in the future. South Africa, with a wealth of unexplored natural resources is an ideal place to search for new drugs. A previous study on South African plants yielded some highly active extracts and chemical investigation of these extracts could lead to promising new antimalarial drugs. In this study *Croton steenkampianus* was selected for isolation of active principles.

6.2 Bio-guided fractionation of extract and antiplasmodial activity of isolated compounds

Bio-guided fractionation of the acetone extract from *C. steenkampianus* was done with antiplasmodial and antibacterial bioassays. A good correlation between the most active antiplasmodial fractions and the most active antibacterial fractions made isolation of compounds easier. Active antiplasmodial compounds were isolated by silica column chromatography and Sephadex column chromatography. Good activity was found in three fractions. From these fractions five compounds were isolated: 2 flavonoids, a triterpene and two new diterpenes with a newly described skeleton. Compounds were identified with NMR, MS, and X-ray crystallography. Antiplasmodial activity of the compounds varied from weak to excellent, with crotrene A having excellent activity. Two closely related flavonoids, quercetin and tamarixetin had very different antiplasmodial activity. Tamarixetin had weak activity with an IC_{50} of 91 μg/ml against a resistant strain RB1, while quercetin had good activity against this strain with an IC_{50} of 3.9. Crotrene A had excellent antiplasmodial activity with an IC_{50} of 0.301 μg/ml against RB1 comparable to the IC_{50} of chloroquine against this strain of 0.095 μg/ml. Crotrene A has promising antiplasmodial properties and further investigation into its potential as an antimalarial drug is planned.

6.3 Cytotoxicity of extract and compounds

Cytotoxicity determination of potential medicinal compounds is an important step in their development as medicines. The most popular method used is animal or human cell lines *in vitro*. In this study human lymphocytes were used to determine the cytotoxicity of the

compounds. Three compounds and the extract were tested. Results obtained varied from poor to moderate with ID_{50}'s between >16.61 µg/ml and >250 µg/ml. Therapeutic indexes ranged from poor to moderate. The best result was obtained with crotrene A having good selectivity with a therapeutic value of >55.18. Although this is not as high as the value of quinine, the important factor will be its *in vivo* therapeutic index. Further studies to determine more accurate cytotoxicity values against other cell lines will be conducted.

6.4 Chloroquine reversal / synergistic effects of isolated compounds

Chloroquine, the most important success of malaria chemotherapy, has been widely used for the treatment of malaria from the 1940's to the present day, because it exhibits many advantages over the existing antimalarials, namely rapid onset of action, good tolerability, limited host toxicity, low cost, and versatility for both prophylactic and curative uses. On the other hand, extensive chloroquine monotherapy has been one of the main reasons behind the spread of resistance to this drug. It is this resistance that has proven to be the driving force behind research devoted to the understanding of the mechanism of action of chloroquine as well as the process of resistance and its reversal. Although decisive advances have been made, these topics are still a matter of debate.

Compounds tested for their reversal potential showed interesting results. Two closely related flavonoids, tamarixetin and quercetin had potentiating and antagonistic effects respectively, while crotrene A had strong reversal effects. Two of the compounds were able to lower chloroquine IC_{50} to levels below the chloroquine resistance threshold of 33 ng/ml. Although both these compounds had this effect, crotrene A had a much better

therapeutic index and the potential of this compound as a useful *in vivo* drug will be investigated further.

6.5 Mode of action of crotrene A

Although most antimalarial drugs have been in use for decades, very little is known about the mode of action of most of these drugs. With the rapid spread of resistance a better understanding of the mode of action of these drugs could help in devising ways to slow the spread of resistance. Mode of action studies of new potential antimalarial drug candidates is therefore an important step in their development. A basic test to determine the possible mode of action of crotrene A showed that it had its greatest affect on the ring phase, preventing further parasite development. The only other drugs acting this early in the parasite lifecycle are the artemisinins, able to reduce the parasitaemia dramatically in a short period. Crotrene A therefore holds a lot of promise and further detailed mode of action studies are planned.

The importance of plants as novel sources of antimalarial drugs have been proven throughout the centuries. Quinine and artemisinin came from the Americas and the Far East, with no significant leads from Africa. Given the rich ethnomedical culture and diverse plant sources of the continent it should only be a matter of time before a lead drug from African plant sources will be discovered. Results obtained in this study with crotrene A show a lot of promise and further studies on this novel compound could lead to the development of the first successful antimalarial drug from Africa.

Chapter 7

Summary

Antiplasmodial- and chloroquine resistance reversal properties of a new diterpene from *Croton steenkampianus*

by

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Promotor: Prof JJM Meyer

Co-Promotor: Prof AI Louw

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Doctor of Philosophiae

Malaria remains the most serious and deadly parasitic disease, affecting millions of people mostly in the poorest countries in the world. With no vaccine likely in the foreseeable future, drugs remain the best means of controlling the disease. Plants have provided most of the antimalarial drugs so far and it is likely that more antimalarial drugs will be discovered in this way. A previous study on South African plants yielded very good results on the extract level. In this study *Croton steenkampianus* leaf extract was selected for isolation of active principles.

Bio-guided fractionation of the extract was done on silica column chromatography and Sephadex column chromatography. Five compounds, two flavonoids, a triterpene and two new diterpenes, with a novel skeleton were isolated. Compounds were identified with NMR, MS and X-ray crystallography. Antiplasmodial activity of the compounds varied from moderate to excellent, with crotrene A having excellent activity. Further studies on the

antimalarial potential of this compound are planned.

Cytotoxicity of compounds and extract were determined against human lymphocytes. Results obtained had an ID_{50} between >16.61 μg/ml and >250 μg/ml. The therapeutic indexes were between 2.75 and 55.18, showing poor to moderate selectivity towards *Plasmodium*. Crotrene A had the best therapeutic index and more detailed studies on its cytotoxicity are necessary.

Resistance to antimalarial drugs is a major problem in effective treatment of the disease. One way of overcoming this problem is combination drugs working synergistically. Chloroquine the most affordable antimalarial drug was combined with the isolated compounds. Two compounds showed synergistic activity with crotrene A having excellent activity, completely reversing chloroquine resistance. This combination of drugs showed no synergistic cytotoxic effects and its potential as a drug will be further investigated.

The mode of action of antimalarial drugs can provide useful information about the long term potential and the likelihood of resistance development. Crotrene A was subjected to a basic test to determine a possible mode of action. Results showed a marked effect in the early phase of development (rings). The results suggest a very potent mode of action able to reduce the amount of parasites quickly and this holds promise for further development of this compound.

Chapter 8

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Appendix 1: X-ray data of crotrene A

Cell data: a= 9.769, b=10.228, c=10.100 A, α=90, β=112.37, γ=90°

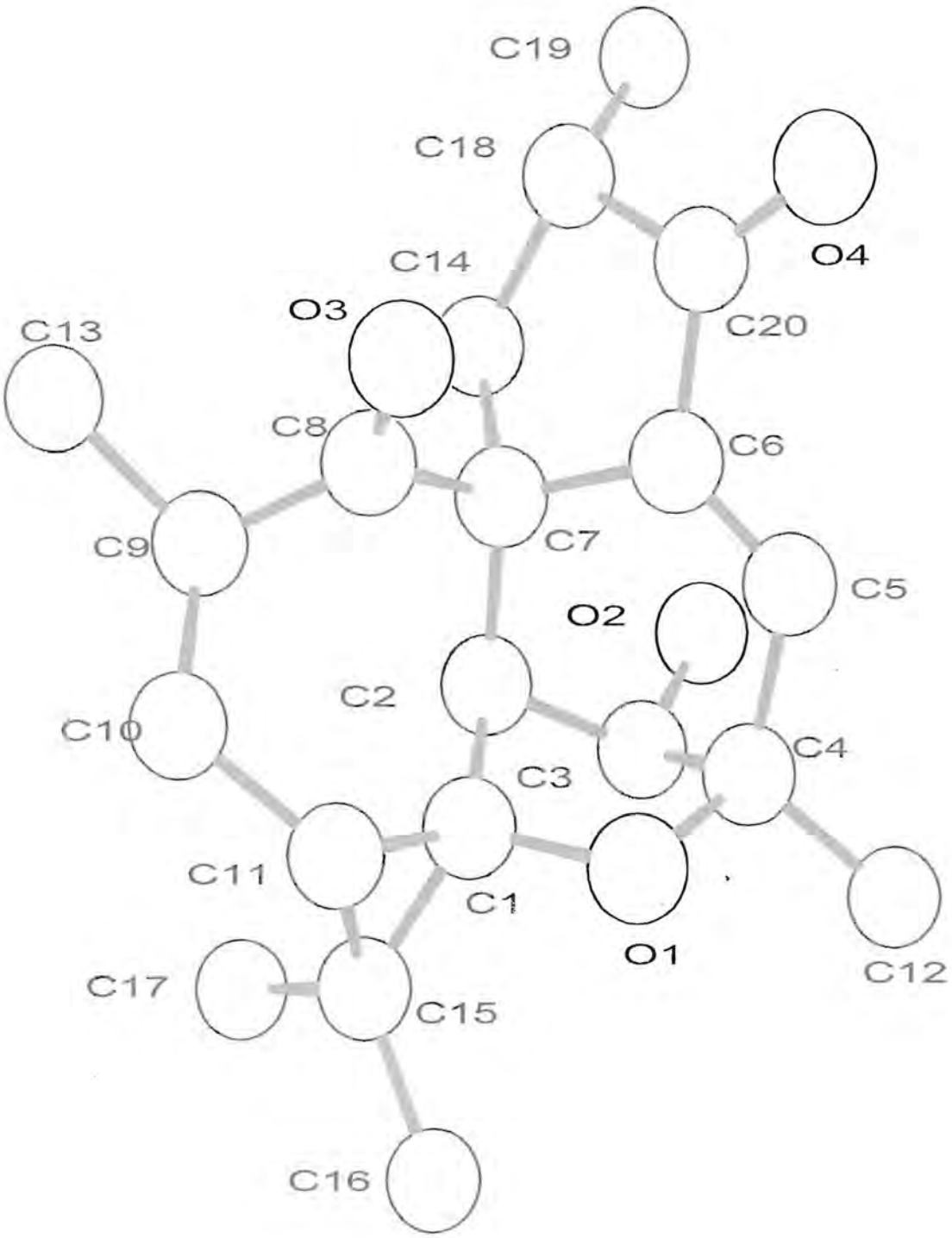

Table A1: Fractional atomic coordinates of the non-hydrogen atoms ($x10^4$) and equivalent thermal factors ($x\ 10^3$ Å^2) for diterpene 1.

Atom	x/a	y/b	z/c	U_{eq}
O(1)	1306(7)	6598	6841(8)	58(2)
C(1)	304(10)	7446(13)	7194(12)	43(3)
C(2)	1251(9)	8218(13)	8449(11)	36(2)
C(3)	2592(11)	8363(14)	8016(11)	49(3)
C(4)	2818(12)	6973(16)	7671(12)	52(3)
C(5)	3420(11)	6290(14)	9090(11)	46(3)
C(6)	2869(10)	6497(13)	10036(12)	40(3)
C(7)	1649(11)	7501(13)	9851(10)	39(3)
C(8)	256(12)	6858(13)	9899(12)	41(3)
C(9)	-1172(10)	7552(14)	9310(12)	47(3)
C(10	-2062(11)	7099(12)	7825(11)	48(3)
C(11)	-1184(11)	6887(14)	6874(11)	53(3)
C(12)	3726(12)	6835(17)	6724(14)	79(4)
O(2)	3873(8)	8949(11)	9121(8)	66(2)
O(3)	368(9)	5784(11)	10472(9)	70(3)
C(13)	-2036(14)	7419(16)	10311(14)	75(4)
C(14)	2290(12)	8262(13)	11246(10)	49(3)
C(15)	-949(14)	7878(15)	5944(14)	64(4)
C(16)	-1033(13)	7567(17)	4447(12)	81(4)
C(17)	-1558(14)	9226(16)	6002(13)	82(4)
C(18)	3195(13)	7301(14)	12440(12)	53(3)
C(19)	4602(14)	7811(15)	13645(12)	78(4)
C(20)	3606(12)	6266(15)	11598(13)	59(3)
O(4)	4494(10)	5350(12)	12157(10)	92(3)

Table A2: Bond lengths (Å) for diterpene 1.

Atoms	Distance	Atoms	Distance
O(1)-C(1)	1.451(11)	O(1)-C(4)	1.445(12)
C(1)-C(2)	1.481(13)	C(1)-C(11)	1.479(13)
C(1)-C(15)	1.45(2)	C(2)-C(3)	1.538(12)
C(2)-C(7)	1.507(12)	C(3)-C(4)	1.50(2)
C(3)-O(2)	1.452(11)	C(4)-C(5)	1.499(14)
C(4)-C(12)	1.539(13)	C(5)-C(6)	1.281(13)
C(6)-C(7)	1.530(12)	C(6)-C(20)	1.483(13)
C(7)-C(8)	1.529(13)	C(7)-C(14)	1.521(13)
C(8)-C(9)	1.474(14)	C(8)-O(3)	1.227(11)
C(9)-C(10)	1.492(13)	C(9)-C(13)	1.550(13)
C(10)-C(11)	1.528(13)	C(11)-C(15)	1.46(2)
C(14)-C(18)	1.545(13)	C(15)-C(16)	1.516(14)
C(15)-C(17)	1.51(2)	C(18)-C(19)	1.54(2)
C(18)-C(20)	1.51(2)	C(20)-O(4)	1.255(13)

Table A3: Bond angles (°) for diterpene 1.

Atoms	Angle	Atoms	Angle
C(1)-O(1)-C(4)	109.6(8)	O(1)-C(1)-C(2)	105.6(7)
O(1)-C(1)-C(11)	114.4(9)	C(2)-C(1)-C(11)	129.5(10)
O(1)-C(1)-C(15)	113.1(10)	C(2)-C(1)-C(15)	128.9(10)
C(11)-C(1)-C(15)	59.7(7)	C(1)-C(2)-C(3)	97.5(8)
C(1)-C(2)-C(7)	113.5(9)	C(3)-C(2)-C(7)	111.8(8)
C(2)-C(3)-C(4)	101.0(9)	C(2)-C(3)-O(2)	113.5(9)
C(4)-C(3)-O(2)	114.2(10)	O(1)-C(4)-C(3)	100.3(9)
O(1)-C(4)-C(5)	112.0(9)	C(3)-C(4)-C(5)	105.0(9)
O(1)-C(4)-C(12)	108.6(8)	C(3)-C(4)-C(12)	113.7(10)
C(5)-C(4)-C(12)	116.2(10)	C(4)-C(5)-C(6)	120.8(10)
C(5)-C(6)-C(7)	123.3(10)	C(5)-C(6)-C(20)	126.5(10)
C(7)-C(6)-C(20)	106.1(9)	C(2)-C(7)-C(6)	110.6(8)
C(2)-C(7)-C(8)	109.0(8)	C(6)-C(7)-C(8)	111.6(9)
C(2)-C(7)-C(14)	119.3(9)	C(6)-C(7)-C(14)	101.7(8)
C(8)-C(7)-C(14)	104.4(8)	C(7)-C(8)-C(9)	119.8(9)
C(7)-C(8)-O(3)	118.7(10)	C(9)-C(8)-O(3)	121.5(10)
C(8)-C(9)-C(10)	110.9(9)	C(8)-C(9)-C(13)	111.2(10)
C(10)-C(9)-C(13)	112.0(9)	C(9)-C(10)-C(11)	115.1(9)
C(1)-C(11)-C(10)	123.2(10)	C(1)-C(11)-C(15)	59.3(7)
C(10)-C(11)-C(15)	124.7(10)	C(7)-C(14)-C(18)	108.0(9)
C(1)-C(15)-C(11)	61.1(8)	C(1)-C(15)-C(16)	120.7(11)
C(11)-C(15)-C(16)	122.3(12)	C(1)-C(15)-C(17)	117.2(11)
C(11)-C(15)-C(17)	116.6(10)	C(16)-C(15)-C(17)	110.9(11)
C(14)-C(18)-C(19)	118.4(10)	C(14)-C(18)-C(20)	101.7(9)
C(19)-C(18)-C(20)	109.6(10)	C(6)-C(20)-C(18)	111.3(10)
C(6)-C(20)-O(4)	124.7(12)	C(18)-C(20)-O(4)	124.0(11)

Table A4: Anisotropic thermal parameters (x10^3 Å^2) for diterpene 1

Atom	U(11)	U(22)	U(33)	U(23)	U(13)	U(12)
O(1)	35(4)	56(6)	75(5)	-32(5)	13(4)	-10(4)
C(1)	23(5)	35(6)	71(8)	6(6)	17(5)	4(5)
C(2)	19(5)	37(6)	50(7)	14(6)	12(5)	2(5)
C(3)	36(6)	76(9)	35(6)	5(7)	14(5)	-7(7)
C(4)	42(6)	71(9)	45(7)	-18(7)	21(6)	2(7)
C(5)	51(7)	38(7)	51(7)	-8(6)	23(6)	7(6)
C(6)	35(6)	35(7)	54(7)	-2(6)	19(5)	3(6)
C(7)	48(7)	27(6)	43(6)	-13(6)	17(5)	-1(6)
C(8)	47(7)	18(6)	65(8)	-9(6)	28(6)	-9(5)
C(9)	35(6)	34(6)	80(8)	3(7)	31(6)	3(6)
C(10)	55(7)	20(6)	76(9)	-5(6)	33(6)	-10(5)
C(11)	44(7)	47(8)	65(8)	14(7)	16(6)	-1(7)
C(12)	52(8)	87(11)	112(11)	-26(10)	45(8)	-10(9)
O(2)	60(5)	72(6)	63(6)	-20(5)	21(4)	-24(5)
O(3)	67(6)	34(5)	117(8)	18(6)	43(6)	-2(5)
C(13)	72(9)	60(9)	109(12)	21(9)	52(8)	21(8)
C(14)	57(7)	38(7)	42(7)	3(6)	8(6)	-5(7)
C(15)	65(9)	48(9)	81(10)	-2(7)	31(8)	2(7)
C(16)	79(10)	114(13)	54(8)	0(9)	28(8)	-3(10)
C(17)	87(11)	89(13)	68(9)	19(10)	28(8)	24(10)
C(18)	73(8)	45(8)	50(7)	-6(6)	33(6)	7(7)
C(19)	89(11)	70(11)	59(9)	-8(8)	10(8)	-13(9)
C(20)	47(7)	65(9)	68(9)	17(8)	25(7)	11(8)
O(4)	84(7)	103(9)	92(7)	26(6)	36(6)	54(7)